IW 7545714

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

September 09, 2015

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS OF:

APPLICATION NUMBER: ***12/094,173***
FILING DATE: ***May 19, 2008***
PATENT NUMBER: ***9,006,224***
ISSUE DATE: ***April 14, 2015***

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

M. Tarver

M. TARVER
Certifying Officer

34678.US

DECLARATION AND POWER OF ATTORNEY FOR UNITED STATES PATENT APPLICATION

☒ Original ☐ Supplemental ☐ Substitute

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, and

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if more than one name is listed below) of the subject matter which is claimed and for which a United States patent is sought on the invention entitled

Neuroendocrine tumor treatment

the specification of which:

☐ is attached hereto.

☐ was filed on _____ (day/month/year) as Application No. _____

and, if this box (☐) contains an *

☐ was amended on _____ (day/month/year)

☒ was filed as Patent Cooperation Treaty international Application No.

PCT/EP2006/068656 on **20.11.2006** (day/month/year)

and, if this box (☐) contains an *

☐ entered the national stage in the United States and was accorded Application No.

_____

and, if this box (☐) contains an *

☐ was amended, subsequent to entry into the national stage, on _____ (day/month/year)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) specifically referred to above and, if this application was filed as a Patent Cooperation Treaty International application, by any amendments made during the international stage (including any made under Patent Cooperation Treaty Rule 91, Article 19 and Article 34).

I acknowledge my duty to disclose information which is material to patentability as defined in 37 C.F.R. 1.56, including, for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or Patent Cooperation Treaty international filing date of the continuation-in-part application.

I hereby claim the benefit under 35 U.S.C. 119(a)-(d) or (f) or 365(b) of any foreign application(s) for patent, inventor's certificate or plant breeder's right certificate listed below and under 35 U.S.C. 365(a) of any Patent Cooperation Treaty international application(s) designating at least one country other than the United States listed below and have also listed below any foreign application(s) for patent, inventor's certificate or plant breeder's right certificate and Patent Cooperation Treaty international application(s) designating at least one country other than the United States for the same subject matter and having a filing date before that of the application the priority of which is claimed for that subject matter:

COUNTRY/REGION (OR P.C.T.)	APPLICATION No.	FILING DATE (day/month/year)	PRIORITY CLAIMED	
Great Britain	**0523658.3**	**21.11.2005**	☒ Yes	☐ No
Great Britain	**0601082.1**	**19.01.2006**	☒ Yes	☐ No
Great Britain	**0602747.8**	**10.02.2006**	☒ Yes	☐ No
Great Britain	**0607942.0**	**21.04.2006**	☒ Yes	☐ No
Great Britain	**0609272.0**	**10.05.2006**	☒ Yes	☐ No
Great Britain	**0609912.1**	**18.05.2006**	☒ Yes	☐ No
European Procedure	**06120660.3**	**14.09.2006**	☒ Yes	☐ No

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below:

APPLICATION NO.	FILING DATE (day/month/year)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s) listed below and under 35 U.S.C. 365(c) of any Patent Cooperation Treaty international application(s) designating the United States listed below:

United States Application No.	United States Filing Date (day/month/year)	Status (Pending, Abandoned or U.S. Patent No.)	International Application No.	and Filing Date (day/month/year)

I hereby appoint all of the registered practitioners associated with Customer No. 001095, respectively and individually, as my attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

If this box (☐) contains an x ☒, I hereby authorize the registered practitioners associated with Customer No. 001095 and any others acting on my behalf to take any action relating to this application based on communications from Corporate Intellectual Property of Novartis International AG, Basle, Switzerland, or an affiliate thereof or a successor thereto, without direct communication from me.

Please send all correspondence relating to this application to the address associated with Customer No. 001095.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full name of sole or first joint inventor	**Peter Wayne MARKS**
Inventor's signature	Peter Wayne Marks Date 4/12/2006 (day/month/year)
Residence	**Woodbridge, CT 06525-1913, US**
Citizenship	**USA**
Post Office Address	**145 Rimmon Road Woodbridge, CT 06525-1913 US**
Full name of second joint inventor, if any	**David LEBWOHL**
Inventor's signature	David Lebwohl Date 20 DEC 2006 (day/month/year)
Residence	**Madison, New Jersey 07940 US**
Citizenship	**USA**
Post Office Address	**55 Pomeroy Road Madison, New Jersey 07940 US**

IMPORTANT: Before this declaration is signed, the patent application (the specification, the claims and this declaration) must be read and understood by each person signing it, and no changes may be made in the application after this declaration has been signed.

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Express Mail Label Number _____ Date of Deposit _____

Form PTO-1390-MOD (REV 10-96) U. S. Department of Commerce Patent and Trademark Office

ATTORNEY'S DOCKET NUMBER 34678-US-PCT

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP2006/068656	20 November 2006 (20.11.06)	21 November 2005 (21.11.05)

TITLE OF INVENTION
NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS

APPLICANT(S) FOR DO/EO/US
MARKS ET AL.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau. (See Form PCT/IB/308)
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C.371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An executed Declaration and Power of Attorney (original or copy) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included.

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ An Application Data Sheet under 37 CFR 1.76.

15. ☐ A substitute specification.

16. ☐ A change of power of attorney and/or address letter.

17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 37 CFR 1.821-1.825.

18. ☐ A second copy of the published International Application under 35 U.S.C. 154(d)(4).

19. ☐ A second copy of the English language translation of the International application under 35 U.S.C. 154(d)(4).

20. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	PCT/EP2006/068656	34678-US-PCT

The following fees are submitted:	CALCULATIONS PTO USE ONLY
21. ☒ Basic national fee. $310	
22. Examination Fee ☐ If International preliminary examination report was prepared by USPTO and all claims satisfy provisions of PCT Article 33(1)-(4) . . . . . $ ☒ All other situations. $210	
23. Search fee ☐ If Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority. . . . . $ ☒ If International Search Report was prepared and provided to the Office. . . $410 ☐ All other situations. . . . . $	
TOTAL OF 21, 22 AND 23 =	$ 930

Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing or computer program listing filed in an electronic medium). The fee is **$260** for each additional 50 sheets of paper or fraction thereof.

Total Sheets	Extra sheets		Number of each additional 50 or fraction thereof (round **up** to a whole number)	RATE	
40 - 100 =	0	/50 =	0	X $ 260	$

Surcharge of **$130** for furnishing the oath of declaration later than ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). $

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total claims	12	- 20 =	0	X $ 50	$
Independent claims	7	- 3 =	4	X $ 210	$ 840
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ $ 370	$
			TOTAL OF ABOVE CALCULATIONS =		$ **1,770**
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					$
			SUBTOTAL =		$ **1,770**
Processing fee of **$130** for furnishing the English translation later than ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	$
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			TOTAL FEES ENCLOSED =		$ **1,770**
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 19-0134 in the name of Novartis in the amount of $1,770 to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0134 in the name of Novartis.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to the address associated with Customer No. 001095, which is currently:

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080

Gregory D. Houghton
Attorney for Applicants
Reg. No. 47,666
(862) 778-2614

FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10

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Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					$
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				Amount to be: refunded	$
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a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

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c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0134 in the name of Novartis.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to the address associated with Customer No. 001095, which is currently:

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080

Gregory D. Houghton
Attorney for Applicants
Reg. No. 47,666
(862) 778-2614

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF

MARKS ET AL.

INTERNATIONAL APPLICATION NO: PCT/EP2006/068656

FILED: 20 NOVEMBER 2006

U.S. APPLICATION NO: Not yet known

35 USC §371 DATE: Herewith

FOR: NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Sir:

This paper is being filed within three months of the date of entry of the national stage as set forth in 37 C.F.R. §1.491 of the international application. Therefore, no fees are required. If a fee is deemed to be required, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 19-0134.

In accordance with 37 C.F.R. §1.56, applicants wish to call the Examiner's attention to the references cited on the attached form(s) PTO-1449.

The listed references were cited in the international stage search report. Since these references are of record in the instant PCT application PCT/EP2006/068656, copies are not enclosed herewith.

The Examiner is requested to consider the foregoing information in relation to this application and indicate that each reference was considered by returning a copy of the initialed PTO 1449 form(s).

Respectfully submitted,

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080
(862) 778-2614

Gregory C. Houghton
Attorney for Applicants
Reg. No. 47,666

Date:

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/31 — A1

(11) International Publication Number: **WO 97/47317**

(43) International Publication Date: 18 December 1997 (18.12.97)

(21) International Application Number: PCT/EP97/03036

(22) International Filing Date: 11 June 1997 (11.06.97)

(30) Priority Data:

9612171.0	11 June 1996 (11.06.96)	GB
9619310.7	16 September 1996 (16.09.96)	GB

(71) Applicant *(for all designated States except US)*: NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: WECKBECKER, Gisbert [DE/CH]; Loeliring 31, CH-4105 Biel-Benken (CH).

(74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent- und Markenabteilung, Klybeckstrasse 141, CH-4002 Basel (CH).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: COMBINATION OF A SOMATOSTATIN ANALOGUE AND A RAPAMYCIN

(57) Abstract

A combination of a compound of the somatostatin class and a rapamycin macrolide is useful for the prevention or treatment of cell hyperproliferation.

FOR THE PURPOSES OF INFORMATION ONLY

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COMBINATION OF A SOMATOSTATIN ANALOGUE AND A RAPAMYCIN

The present invention relates to a pharmaceutical combination and its use in the treatment of disorders associated with excess benign and malignant cell proliferation, e.g. tumors or intimal cell proliferation.

There is a continuing need for the development of drugs having increased effectiveness in inhibiting or slowing down undesired cell proliferation, particularly in the cancer field and in vasculopathies.

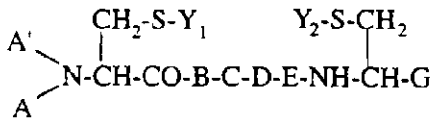
Accordingly, there is provided a pharmaceutical combination comprising a compound of the somatostatin class, and a rapamycin macrolide.

The somatostatin class is a known class of small peptides comprising the naturally occurring somatostatin-14 and analogues having somatostatin related activity, e.g. as disclosed by A.S. Dutta in Small Peptides, Vol.19, Elsevier (1993). By "somatostatin analogue" as used herein is meant any straight-chain or cyclic polypeptide having a structure based on that of the naturally occurring somatostatin-14 wherein one or more amino acid units have been omitted and/or replaced by one or more other amino radical(s) and/or wherein one or more functional groups have been replaced by one or more other functional groups and/or one or more groups have been replaced by one or several other isosteric groups. In general, the term covers all modified derivatives of the native somatostatin-14 which exhibit a somatostatin related activity, e.g. they bind to at least one somatostatin receptor (hSST-1, hSST-2, hSST-3, hSST-4 or hSST-5), preferably in the nMolar range, more preferably to at least the hSST-2 receptor in the nMolar range.

Cyclic, bridge cyclic and straight-chain somatostatin analogues or derivatives are known and have been described together with processes for their production e.g. in US Patent Specifications 4,310,518 and 4,235,886, in European Patent Specifications EP-A-1295; 23,192; 29,310; 29,579; 30,920; 31,303; 63,308; 70,021; 83,305; 215,171; 203,031; 214,872; 143,307; 298,732; 277,419; 389,180; 395,417; 450,480A2; in Belgian Patent Specification BE-A-900,089; and in WO 91/09056; WO 97/01579; WO 97/14715,

the contents thereof, in particular with respect to the compounds, being incorporated herein by reference.

Preferred somatostatin analogues are e. g. compounds of formula I



(I)

wherein

A is C_{1-12}alkyl, C_{7-10}phenylalkyl or a group of formula RCO-,

whereby

i) R is hydrogen, C_{1-11}alkyl, phenyl or C_{7-10}phenylalkyl, or

ii) RCO- is

a) a D-phenylalanine residue optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy; or

b) the residue of a natural or a synthetic α-amino-acid other than defined under a) above, or of a corresponding D-amino acid, or

c) a dipeptide residue in which the individual amino acid residues are the same or different and are selected from those defined under a) and/or b) above,

the α-amino group of amino acid residues a) and b) and the N-terminal amino group of dipeptide residues c) being optionally mono- or di-C_{1-12}alkylated or substituted by C_{1-8}alkanoyl;

A' is hydrogen or C_{1-3}alkyl,

Y_1 and Y_2 represent together a direct bond or each of Y_1 and Y_2 is hydrogen

B is -Phe- optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and /or

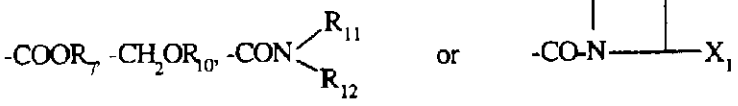
C_{1-3}alkoxy (including pentafluoroalanine), naphthylalanine or pyridylalanine,

C is (L)-Trp- or (D)-Trp- optionally α-N-methylated and optionally benzene-ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy,

D is Lys, 4-aminocyclohexylAla or 4-aminocyclohexylGly

E is Thr, Ser, Val, Tyr, Ile, Leu or an aminobutyric or aminoisobutyric acid residue

G is a group of formula



wherein

R_7 is hydrogen or C_{1-3}alkyl,

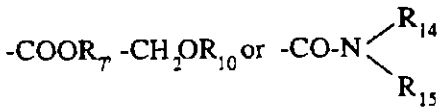
R_{10} is hydrogen or the residue of a physiologically acceptable, physiologically hydrolysable ester, e.g. formyl, C_{2-12}alkylcarbonyl, benzoyl,

R_{11} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenyl-alkyl,

R_{12} is hydrogen, C_{1-3}alkyl or a group of formula $-CH(R_{13})-X_1$,

R_{13} is CH_2OH, $-(CH_2)_2-OH$, $-(CH_2)_3-OH$, $-CH(CH_3)OH$, isobutyl, butyl, benzyl, naphthyl-methyl or indol-3-yl-methyl, and

X_1 is a group of formula



wherein

R_7 and R_{10} have the meanings given above,

R_{14} is hydrogen or C_{1-3}alkyl and

R_{15} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenylalkyl, and

R_{16} is hydrogen or hydroxy,

with the proviso that

when R_{12} is $-CH(R_{13})-X_1$ then R_{11} is hydrogen or methyl,

wherein the residues B, D and E have the L-configuration, and the residues in the 2- and 7-position each independently have the (L)- or (D)- configuration,

in free form or in pharmaceutically acceptable salt or complex form.

Individual compounds of formula I suitable in accordance with the present invention are the following somatostatin analogues:

a. (D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr-ol
   also known as octreotide

b. (D)Phe-Cys-Tyr-(D)Trp-Lys-Val-Cys-ThrNH_2

c. (D)Phe-Cys-Tyr-(D)Trp-Lys-Val-Cys-TrpNH_2
   also known as vapreotide

d. (D)Trp-Cys-Phe-(D)Trp-Lys-Thr-Cys-ThrNH_2

e. (D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-ThrNH_2

f. 3-(2-(Naphthyl)-(D)Ala-Cys-Tyr-(D)Trp-Lys-Val-Cys-ThrNH_2
   also known as lanreotide

g. (D)Phe-Cys-Tyr-(D)Trp-Lys-Val-Cys-β-Nal-NH_2

h. 3-(2-naphthyl)-Ala-Cys-Tyr-(D)Trp-Lys-Val-Cys-β-Nal-NH_2

i. (D)Phe-Cys-β-Nal-(D)Trp-Lys-Val-Cys-Thr-NH_2

j. (D)Phe-Cys-Tyr-(D)Trp-Lys-Leu-Cys-Thr-NH_2

k. (D)Phe-Cys-Tyr-(D)Trp-Lys-Cys-Thr-NH_2.

A preferred compound of formula I is octreotide.

Compounds of formula I may exist e.g. in free form, salt form or in the form of complexes thereof. Acid addition salts may be formed with e.g. organic acids, polymeric acids and inorganic acids. Such acid addition salt forms include e.g. the hydrochlorides and acetates. Complexes are e.g. formed from compounds of the invention on addition of inorganic substances, e.g. inorganic salts or hydroxides such as Ca- and Zn-salts, and/or an addition of polymeric organic substances.

Further somatostatin analogues suitable for use in accordance with the present invention are:

cyclo [-Asn-Phe-Phe-DTrp-Lys-Thr-Phe-Gaba-],

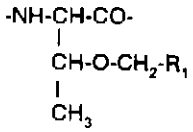
cyclo(Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser), and

(D)Nal-Glu-Tyr-(D)Trp-Lys-Val-Lys-Thr-NH_2

According to an alternatively preferred embodiment of the invention, the somatostatin component of the combination is a somatostatin analogue comprising the amino acid sequence of formula (II)

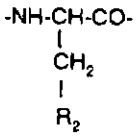
-(D/L)Trp-Lys-X_2-X_3- (II)

wherein X_2 is a radical of formula (a) or (b)



(a)

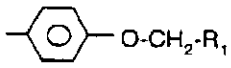
or



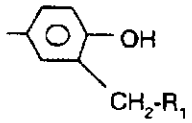
(b)

wherein R_1 is optionally substituted phenyl,

R_2 is $-Z_1-CH_2-R_1$, $-CH_2-CO-O-CH_2-R_1$,



or



wherein Z_1 is O or S,

and

X_3 is an α-amino acid having an aromatic residue on the C_α side chain, or an amino acid unit selected from Dab, Dpr, Dpm, His,(Bzl)HyPro, thienyl-Ala, cyclohexyl-Ala and t.-butyl-Ala,

the residue Lys of said sequence corresponding to the residue Lys^9 of the native somatostatin-14.

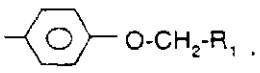
Such somatostatin analogues are e.g. disclosed in WO/ 97/01579, the contents thereof, in particular with respect to the specifically exemplified compounds, being

incorporated herein by reference.

Preferably the sequence of formula II as defined above corresponds to the residues at positions 8 through 11 of the somatostatin-14. More preferably the somatostatin analogue as disclosed above comprises a hexapeptide unit, the residues at positions 3 through 6 of said hexapeptide unit comprising the sequence of formula II. More particularly the hexapeptide unit is cyclic, e.g. having a direct peptide linkage between the α-carbonyl group of the residue at position 6 and the α-amino group of the residue at position 1.

While Lys, X_2 and X_3 in the sequence of formula II have the L-configuration, Trp may have the D- or L-configuration, preferably the D-configuration.

X_2 is preferably a residue of formula (a) or (b), R_2 being preferably $-Z_1-CH_2-R_1$ or



When X_3 comprises an aromatic residue on the C_α side chain, it may suitably be a natural or unnatural α-amino acid, e.g. Phe, Tyr, Trp, Nal, Pal, benzothienyl-Ala, Tic and thyronin, preferably Phe or Nal, more preferably Phe. X_3 is preferably an α-amino acid bearing an aromatic residue on the C_α side chain.

When R_1 is substituted phenyl, it may suitably be substituted by halogen, methyl, ethyl, methoxy or ethoxy e.g. in ortho and/or para. More preferably R_1 is unsubstituted phenyl. Z_1 is preferably O.

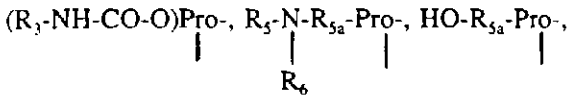
Representative somatostatin analogues comprising a residue of formula II are e.g compounds of formula (III)

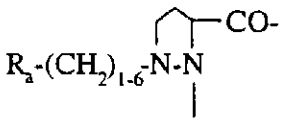
$$\underset{1}{\text{cyclo[A}} - \underset{2}{ZZ_a} - \underset{3}{\text{Trp}} - \underset{4}{\text{Lys}} - \underset{5}{X_2} - \underset{6}{X_3}] \qquad \text{(II)}$$

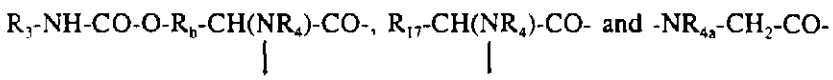
wherein

X_2 and X_3 are as defined above,

A_1 is a divalent residue selected from Pro,







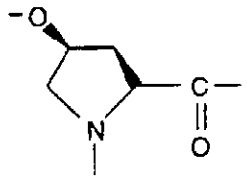
wherein R_3 is NR_8R_9-C_{2-6}alkylene, guanidino-C_{2-6}alkylene or C_{2-6}alkylene-COOH, R_{3a} is H, C_{1-4}alkyl or has independently one of the significances given for R_3, R_{3b} is H or C_{1-4}alkyl, R_a is OH or NR_5R_6, R_b is $-(CH_2)_{1-3}-$ or $-CH(CH_3)-$, R_4 is H or CH_3, R_{4a} is optionally ring-substituted benzyl, each of R_5 and R_6 independently is H, C_{1-4}alkyl, ω-amino-C_{1-4}alkylene, ω-hydroxy-C_{1-4}alkylene or acyl, R_{5a} is a direct bond or C_{1-6}alkylene, each of R_8 and R_9 independently is H, C_{1-4}alkyl, ω-hydroxy-C_{2-4}alkylene, acyl or CH_2OH-$(CHOH)_c$-CH_2- wherein c is 0, 1, 2, 3 or 4, or R_8 and R_9 form together with the nitrogen atom to which they are attached a heterocyclic group which may comprise a further heteroatom, and R_{17} is optionally ring-substituted benzyl, $-(CH_2)_{1-3}$-OH, CH_3-CH(OH)- or $-(CH_2)_{1-5}-NR_5R_6$, and

ZZ_a is a natural or unnatural α-amino acid unit.

ZZ_a may have the D- or L-configuration. When ZZ_a is a natural or unnatural α-amino acid unit, it may suitably be e.g. Thr, Ser, Ala, Val, Ile, Leu, Nle, His, Arg, Lys, Nal, Pal,

Tyr, Trp, optionally ring-substituted Phe or N$^{\alpha}$-benzyl-Gly. When ZZ_a is Phe, the benzene ring thereof may be substituted by e.g. NH_2, NO_2, CH_3, OCH_3 or halogen, preferably in para position. When ZZ_a is Phe, the benzene ring thereof is preferably unsubstituted.

When A_1 comprises a Pro amino acid residue, any substituent present on the proline ring, e.g. R_3-NH-CO-O- etc., is preferably in position 4. Such substituted proline residue may exist in the cis form, e.g.



as well as in the trans form. The present invention covers each geometric isomer individually as well as mixtures thereof.

When A_1 is (NR_8R_9-C_{2-6}alkylene-NH-CO-O)Pro- where NR_8R_9 forms a heterocyclic group, such group may be aromatic or saturated and may comprise one nitrogen or one nitrogen and a second heteroatom selected from nitrogen and oxygen. Preferably the heterocyclic group is e.g. pyridyl or morpholino. C_{2-6}Alkylene in this residue is preferably -CH_2-CH_2-.

Any acyl as R_5, R_6, R_8 and R_9 in A_1 may be e.g. R_{18}CO- wherein R_{18} is H, C_{1-4}alkyl, C_{2-4}alkenyl, C_{3-6}cycloalkyl or benzyl, preferably methyl or ethyl. When R_{4a} or R_{17} in A_1 is ring-substituted benzyl, the benzene ring may be substituted as indicated above for ZZ_a.

A preferred group of compounds of formula III are such wherein A_1 is free of a lateral -NH-CO-O- moiety. A further group of preferred compounds of formula III are such wherein A_1 comprises a basic lateral radical, e.g. a R_3-NH-CO-O- or R_5-N(R_6)-R_{5a}-

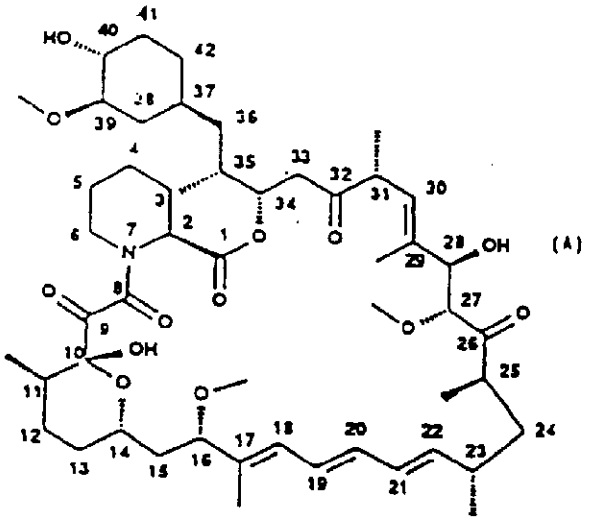
moiety.

A still further group of preferred compounds of formula III are such wherein the N-terminal amino acid comprises a substituted Pro, particularly 4-substituted Pro, e.g. compounds of formula III wherein A_1 is 4-substituted Pro.

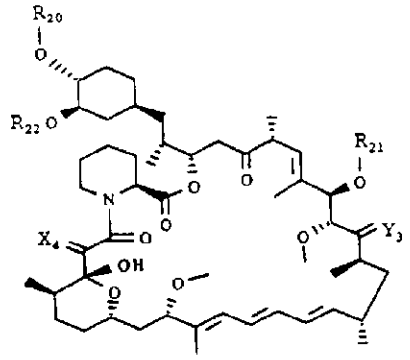
Preferably A_1 is 4-(R_3-NH-CO-O)Pro.

Examples of somatostatin analogues comprising a residue of formula II include e.g. cyclo[4-(NH_2-C_2H_4-NH-CO-O-)Pro-Phe-DTrp-Lys-Ser(Benzyl)-Phe].

The term "macrolide" as used herein, refers to a macrocyclic lactone, for example a compound having a 12-membered or larger lactone ring. Of particular interest are the "lactam macrolides", i.e. macrocyclic compounds having a lactam (amide) bond in the macrocycle in addition to a lactone (ester) bond, for example rapamycin and its numerous derivatives and analogues. Rapamycin is an immunosuppressive lactam macrolide that is produced by Streptomyces hygroscopicus, and having the structure depicted in Formula A:



See, e.g., McAlpine, J.B., et al., J. Antibiotics (1991) 44: 688; Schreiber, S.L., et al., J. Am. Chem. Soc. (1991) 113: 7433; US Patent No. 3 929 992. One group of rapamycin derivatives are 40-0-substituted derivatives of rapamycin having the structure of Formula IV:



IV

wherein

X_4 is (H,H) or O;

Y_3 is (H,OH) or O;

R_{20} and R_{21} are independently selected from H, alkyl, arylalkyl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxycarbonylalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylaminoalkyl, acylaminoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, dialkyl-dioxolanylalkyl, di(alkoxycarbonyl)-triazolyl-alkyl and hydroxyalkoxy-alkyl; wherein "alk-" or "alkyl" refers to C_{1-6}alkyl, branched or linear, preferably C_{1-3}alkyl,; "aryl" is phenyl or tolyl; and acyl is a radical derived from a carboxylic acid; and

R_{22} is methyl or R_{22} and R_{20} together form C_{2-6}alkyl;

provided that R_{20} and R_{21} are not both H; and hydroxyalkoxyalkyl is other than hydroxyalkoxymethyl.

Such compounds are disclosed in WO 94/09010 the contents of which, in particular with respect to the specifically exemplified compounds, are incorporated herein by reference.

A preferred compound is e.g. 40-O-(2-hydroxy)ethyl-rapamycin (referred thereafter as Compound B).

Further preferred rapamycin derivatives are e.g. those disclosed in WO 96/41807, the contents thereof, in particular with respect to the specifically exemplified compounds of formula I disclosed therein, being incorporated herein by reference. Particularly preferred are 32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-40-O-(2-hydroxyethyl)-rapamycin, 16-O-pent-2-ynyl-32-(S)-dihydro-rapamycin and 16-O-pent-2-ynyl-32-(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin.

Further rapamycin derivatives are known, e.g. carboxylic acid esters such as disclosed in WO 92/05179, amide esters such as disclosed in US 5 118 677, carbamates such as described in US 5 118 678, fluorinated esters such as disclosed in US 5 100 883, acetals, e.g. in US 5 151 413, silyl ethers, e.g. in US 5 120 842, arylsulfonates and sulfamates, e.g. in US 5 177 203, derivatives wherein the methoxy group at the position 16 is replaced with alkynyloxy, e.g. in WO 95/16691 and further derivatives such as disclosed in WO 93/11130, WO 94/02136, WO 94/02385 and WO 95/14023, all incorporated herein by reference.

Rapamycin and above mentioned derivatives have been shown to have potent immunosuppressant properties. Rapamycin has also been shown to inhibit smooth muscle cell proliferation and to inhibit cancer growth.

Somatostatin analogues, e.g. octreotide, vapreotide and lanreotide, have been disclosed i.a. to inhibit growth hormone secretion and to have an inhibiting effect on malignant tumor growth, e.g. in breast cancer. Octreotide and lanreotide have also been disclosed to inhibit smooth muscle cell proliferation.

In accordance with the invention, it has now surprisingly been found that a combination of 2 active ingredients believed to act on basically different mechanisms such as a somatostatin analogue and rapamycin or a derivative thereof, can be combined and synergistically inhibit cell hyperproliferation.

In accordance with the particular findings of the present invention, there is provided in a first aspect:

1. Use of a compound of the somatostatin class, in free form or in pharmaceutically acceptable salt form, for manufacturing a pharmaceutical composition for use in synergistically effective amounts in the prevention or treatment of cell hyperproliferation in combination with a rapamycin macrolide, e.g. for the manufacture of a kit as disclosed hereinafter.

2. Use of a compound of the somatostatin class, in free form or in pharmaceutically acceptable salt form, in combination in synergistically effective amounts with a rapamycin macrolide for the prevention or treatment of cell hyperproliferation.

3. A method for preventing or treating cell hyperproliferation in a subject in need of such treatment which comprises administering to such subject a synergistically effective amount of a compound of the somatostatin class in free form or in pharmaceutically acceptable salt form, and a rapamycin macrolide.

4. A kit or package for the treatment or prevention of cell hyperproliferation, said kit or package including a pharmaceutical composition comprising a compound of the somatostatin class in free form or in pharmaceutically acceptable salt form, and a

pharmaceutical composition comprising a rapamycin macrolide. The kit or package may also contain instructions to use the pharmaceutical compositions in accordance with the present invention.

According to the invention, the combination of a compound of the somatostatin class and a rapamycin macrolide is indicated for the prevention or treatment of malignant tumor growth, e.g. breast, lung, GEP tumors, pituitary adenomas, lymphomas, etc., for the prevention or treatment of proliferative vascular diseases, e.g. biologically or mechanically induced vascular injury causing intimal thickening, e.g. restenosis, atherosclerosis, vascular occlusion, injury following percutaneous transluminal coronary angioplasty, vascular surgery or transplantation surgery, transplant vasculopathies, for example chronic rejection of various tissues and organs such as heart, kidney, pancreas, lung, liver, bowel, trachea and combined heart-lung.

The combination is particularly indicated for preventing intimal smooth muscle cell hyperplasia, restenosis and vascular occlusion in a mammal.

Utility of the combination in the treatment of disorders and diseases as hereinbefore specified, may be demonstrated for example in accordance with the method hereinafter described.

A. In vitro Assay

AR42J cell cultures are propagated in DMEM supplemented with 10 % fetal calf serum (FCS) at 5 % CO_2. Cells are grown in the absence of antibiotics or antifungal agents. Subconfluent AR42J cells growing in DMEM and supplemented with 10 % FCS are trypsinized, diluted in DMEM + 2.5 % FCS and seeded in uncoated 96-well plates (5'000 to 10'000 cells per well in 180 µl). After a 48-hr incubation period (Day O), the number of cells in a separate control plate is determined both by counting cells in a Coulter counter and by the sulforhodamine B (SRB) staining assay. The cells are then exposed either to the somatostatin analogue alone, e.g. octreotide, or to rapamycin or a derivative thereof alone or to a combination of the somatostatin analogue and rapamycin

or its derivative up to 5 days at various concentrations. Total drug exposure lasts for up to 5 days following the first addition and SRB analysis as described above is performed e.g. on day 2 and day 5. Growth is determined as difference in absorbance (OD) between day 0 and day x values (= delta OD). Calculations are made based on the fractional product method of Webb (Valeriote and Lin, 1975; Cory and Carter, 1986; Berenbaum, J. Theor. Biol. 114: 413-431, 1985) and the method by Chou and Talalay (Adv. Enz. Regul. 22: 27-55, 1984). If the measured cell growth (% of control) is < to the calculated cell growth, this shows evidence for a synergistic effect. Under these conditions a combination of a somatostatin analogue at a concentration of from 10^{-10} to 10^{-6} M with a rapamycin macrolide thereof at a concentration of from 1 to 1000 nM significantly inhibits the growth of the tumor cells.

In this assay, the following results are obtained with octreotide alone, Compound B alone and a combination of octreotide and Compound B. The synergy according to the Webb Method is confirmed by using the Chou-Talalay Method.

	Cell Growth (% of CONTROL)			
	Concentration (nM)	Cell Growth (ΔOD) (%)	Observed (%)	Calculated (Webb Method) (%)
Control		664 ± 9	100	
Octreotide	1.2	397 ± 16	59.8	
Compound B	12.0	420 ± 12	63.3	
Octreotide + Compound B	1.2 + 12.0	103 ± 5	15.6	37.9

B. In Vivo Assay

The AR42J (AR4-2J) rat pancreatic tumor cell line is derived from an azaserine-induced exocrine pancreatic tumor (Jessop and Hay, 1980). It was obtained from ATCC.

Cultures are propagated in DMEM supplemented with 10% fetal calf serum (FCS) at 5% CO_2. Cells are grown in the absence of antibiotics or antifungal agents. Female nude mice (nu/nu Balbc-A from Iffa Credo, Lyon, France) weighing 19-22 g, are kept in groups of 5 animals in macrolon cages (type III, 16 x 22 x 11 cm). The cages are placed in ventilated cabinets (Iffa Credo) that are maintained at 24 ± 1° C. The animals have free access to drinking water and a pathogen-free rodent diet (Diet A, Kliba, Basel, Switzerland). To initiate tumors from cultured cells, AR42J cells are trypsinized and $10x10^6$ tumor cells (in 0.2 ml) are injected subcutaneously (s.c.) into both flanks of nude mice. When tumors have reached a volume of 0.03 cm^3, animals are randomized into control and treatment groups. Control animals receive placebo. Animals are treated as indicated below for 3 weeks with single agents or the drug combination. The somatostatin analogue is given as a single injection of a slow release form at 30 mg/kg s.c.. The size of the tumors is determined with a caliper. To calculate the tumor volume in ml the equation "volume (ellipsoid) = length x depth x height x 0.52" was used.

Results

After 4 weeks the following tumor size were determined.

(Please note that values in the control group correspond to 3 week values, since animals were killed afterwards for tumors became excessively large.)

Treatment	**Volume** mm^3	**SE**
Control	4020	579
A) Compound B, 5 mg/kg p.o.	3685	263
B) Rapamycin, 5 mg/kg p.o.	2748	325
C) Octreotide pamoate (biodegradable, sustained release formulation), 30 mg/kg, single inj.	2205	339

Compound B + octreotide (C)	130	75
Rapamycin + octreotide (C)	106	44

C. Clinical trial

Patients are included who have breast cancer as evidenced by histological biopsy (glandular analysis - EOA). They present a metastatic illness and/or loco-regional localisation which is measurable and evaluable. If desired, patients may be included who are resistant to other treatment to conventional therapy such as surgery, radiotherapy, other chemotherapy and/or hormone therapy.

The patients present at least one target, on X-ray analysis, which is measurable or evaluable such as a primitive metastatic tumour which is cutaneous or sub-cutaneous. It may be gangliar or visceral. Preferably the patients have lesions which have progressed within the month preceding the trial and have an estimated survival time of at least 3 months.

The rapamycin macrolide, e.g rapamycin or compound B is administered orally. The treatment is for at least 3 months or until complete remission. The response may be followed by conventional methodology, e.g. according to IUCC response criteria, e.g. progression, stabilization, partial or complete remission.

The somatostatin analogue, e.g. octreotide, is administered parenterally, e.g. subcutaneous, particularly in a continuous subcutaneous way by means of a portable syringe pump (infusion pump).

According to the invention, the somatostatin analogue and the rapamycin macrolide are preferably administered in the form of a pharmaceutical composition. Rapamycin and its derivatives, e.g. Compound B, may be administered by any conventional route, in particular enterally, e.g. orally, e.g. in the form of tablets, capsules, drink solutions, emulsions or microemulsion preconcentrates, nasally, pulmonary (by inhalation),

parenterally, e.g. in the form of injectable solutions or suspensions, or topically. Rapamycin and its derivatives are preferably administered per os and the somatostatin analogue is preferably administered parenterally, e.g by infusion. The somatostatin analogue may also be administered in a slow release form, e.g. as disclosed in UK Patent Specification 2,265,311B. The administration of each component of the combination may take place either separately, simultaneously or sequentially, e.g. rapamycin or Compound B may be administered at first followed later, e.g. 8 to 24 hours later, by the somatostatin analogue.

The amount of each component administered is determined taking into account various factors such as the etiology and severity of the disease, and the patient's condition. Rapamycin or its derivatives may conveniently be administered at doses which are in the range used in immunosuppressive applications such as prevention and treatment of graft vs. host disease, transplant rejection or autoimmune diseases e.g. at a daily dosage from about 0.5 to 500 mg as a single dose or in divided doses. Such doses may also be given intermittently, for example, every other day or every third day. The somatostatin analogue may be administered, e.g. subcutaneously, in a dosage range of about 100 µg to 10 mg per day as a single dose or in divided doses. Thus octreotide may be administered at a dose of from 0.2 mg to 10 mg twice or three times daily. When administered as a slow release form, such formulation may comprise the somatostatin peptide in a concentration from 2.0 to 10% by weight. The release period of such a formulation may be from 1 week to about 2 months. The combination of the somatostatin analogue with rapamycin or its derivative allows to maximize the antiproliferative effect.

The invention contemplates that the active ingredients discussed herein may be utilized in combination with pharmaceutically acceptable diluents and carriers.

Formulation Examples:

A. Somatostatin Formulations:

1. Ampoules

Octreotide	0.5 mg
Mannitol	45.0 mg
Lactic acid (88%)	3.4 mg
Sodium hydrogeno-carbonate	to pH 4.2
Water (inject.grade)	to 1 ml
Carbon dioxide	q.s.

2. Biodegradable sustained release formulation:

Octreotide Acetate	4.65 %	(by weight)
Poly(DL-lactide-co-glycolide)	78.35 %	
Sterile Mannitol	17 %	

Vehicle:	Carboxymethylcellulose	0.5 %	(by weight)
	Mannitol	0.6 %	
	Water for injection	98.9 %	

B. Rapamycin (or derivative thereof) formulation: e.g. capsules

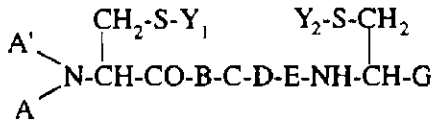
Ethanol	20.0 mg
1,2-propylene glycol	81.0mg
Refined oil	121.5mg
Cremophor RH40	202.5mg
Rapamycin or Compound B	20.0mg
Total	500 mg

CLAIMS

1. Use of a compound of the somatostatin class, in free form or in pharmaceutically acceptable salt form, for manufacturing a pharmaceutical composition for use in synergistically effective amounts in the prevention or treatment of cell hyperproliferation in combination with a rapamycin macrolide.

2. Use of a compound of the somatostatin class, in free form or in pharmaceutically acceptable salt form, in combination in synergistically effective amounts with a rapamycin macrolide for the prevention or treatment of cell hyperproliferation.

3. Use according to claim 1 or 2, wherein the compound of the somatostatin class is a compound of formula I



(I)

wherein

A is C_{1-12}alkyl, C_{7-10}phenylalkyl or a group of formula RCO-,

whereby

i) R is hydrogen, C_{1-11}alkyl, phenyl or C_{7-10}phenylalkyl, or

ii) RCO- is

a) a D-phenylalanine residue optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy; or

b) the residue of a natural or a synthetic α-amino-acid other than defined under a) above, or of a corresponding D-amino acid, or

c) a dipeptide residue in which the individual amino acid residues are the same or different and are selected from those defined under a) and/or b) above,

the α-amino group of amino acid residues a) and b) and the N-terminal amino group of dipeptide residues c) being optionally mono- or di-C_{1-12}alkylated or substituted by C_{1-8}alkanoyl;

A' is hydrogen or C_{1-3}alkyl,

Y_1 and Y_2 represent together a direct bond or each of Y_1 and Y_2 is hydrogen

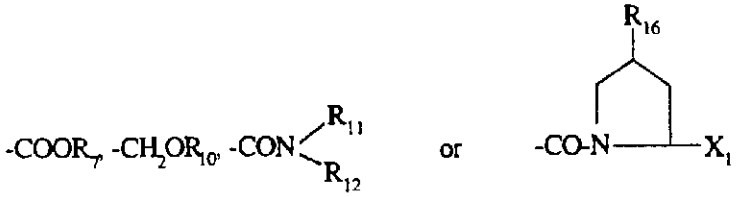
B is -Phe- optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and /or C_{1-3}alkoxy (including pentafluoroalanine), naphthylalanine or pyridylalanine,

C is (L)-Trp- or (D)-Trp- optionally α-N-methylated and optionally benzene-ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy,

D is Lys, 4-aminocyclohexylAla or 4-aminocyclohexylGly

E is Thr, Ser, Val, Tyr, Ile, Leu or an aminobutyric or aminoisobutyric acid residue

G is a group of formula



wherein

R_7 is hydrogen or C_{1-3}alkyl,

R_{10} is hydrogen or the residue of a physiologically acceptable, physiologically hydrolysable ester,

R_{11} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenyl-alkyl,

R_{12} is hydrogen, C_{1-3}alkyl or a group of formula $-CH(R_{13})-X_1$,

R_{13} is CH_2OH, $-(CH_2)_2-OH$, $-(CH_2)_3-OH$, $-CH(CH_3)OH$, isobutyl, butyl, benzyl, naphthyl-methyl or indol-3-yl-methyl, and

X_1 is a group of formula

$$-COOR_7, -CH_2OR_{10} \text{ or } -CO-N\begin{matrix}R_{14}\\R_{15}\end{matrix}$$

wherein

R_7 and R_{10} have the meanings given above,

R_{14} is hydrogen or C_{1-3}alkyl and

R_{15} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenylalkyl, and

R_{16} is hydrogen or hydroxy,

with the proviso that

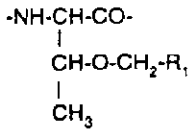
when R_{12} is $-CH(R_{13})-X_1$ then R_{11} is hydrogen or methyl.

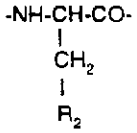
wherein the residues B, D and E have the L-configuration, and the residues in the 2- and 7-position each independently have the (L)- or (D)- configuration

or a somatostatin analogue comprising the amino acid sequence of formula II

$$-(D/L)Trp-Lys-X_2-X_3- \qquad (II)$$

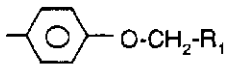
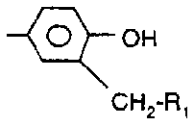
wherein X_2 is a radical of formula (a) or (b)

or  (a)

 (b)

wherein R_1 is optionally substituted phenyl,

R_2 is $-Z_1-CH_2-R_1$, $-CH_2-CO-O-CH_2-R_1$,

 or 

wherein Z_1 is O or S,

and

X_3 is an α-amino acid having an aromatic residue on the C_α side chain, or an amino acid unit selected from Dab, Dpr, Dpm, His,(Bzl)HyPro, thienyl-Ala, cyclohexyl-Ala and t.-butyl-Ala,

the residue Lys of said sequence corresponding to the residue Lys^9 of the native somatostatin-14,

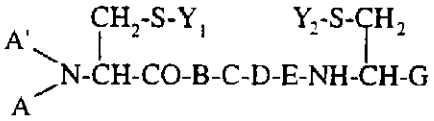
in free form or in pharmaceutically acceptable salt form.

4. Use according to claim 3, wherein the compound of the somatostatin class is octreotide, lanreotide or vapreotide.

5. A method for preventing or treating cell hyperproliferation in a subject in need of such treatment which comprises administering to such subject a synergistically effective amount of a compound of the somatostatin class in free form or in pharmaceutically acceptable salt form, and a rapamycin macrolide.

6. A kit or package for the treatment or prevention of cell hyperproliferation, said kit or package including a pharmaceutical composition comprising a compound of the somatostatin class in free form or in pharmaceutically acceptable salt form, and a pharmaceutical composition comprising a rapamycin macrolide, together with instructions for use.

7. A kit or package according to claim 6, wherein the compound of the somatostatin class is a compound of formula I



(I)

wherein

A is C_{1-12}alkyl, C_{7-10}phenylalkyl or a group of formula RCO-,

whereby

i) R is hydrogen, C_{1-11}alkyl, phenyl or C_{7-10}phenylalkyl, or

ii) RCO- is

a) a D-phenylalanine residue optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy; or

b) the residue of a natural or a synthetic α-amino-acid other than defined under a) above, or of a corresponding D-amino acid, or

c) a dipeptide residue in which the individual amino acid residues are the same or different and are selected from those defined under a) and/or b) above,

the α-amino group of amino acid residues a) and b) and the N-terminal amino group of dipeptide residues c) being optionally mono- or di-C_{1-12}alkylated or substituted by C_{1-8}alkanoyl;

A' is hydrogen or C_{1-3}alkyl,

Y_1 and Y_2 represent together a direct bond or each of Y_1 and Y_2 is hydrogen

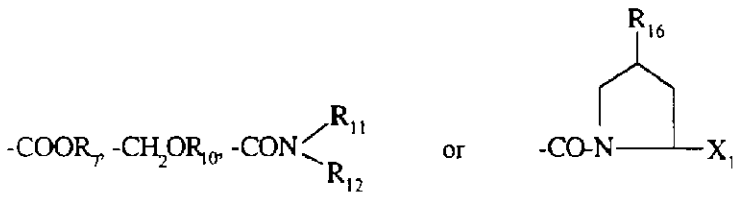
B is -Phe- optionally ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and /or C_{1-3}alkoxy (including pentafluoroalanine), naphthylalanine or pyridylalanine,

C is (L)-Trp- or (D)-Trp- optionally α-N-methylated and optionally benzene-ring-substituted by halogen, NO_2, NH_2, OH, C_{1-3}alkyl and/or C_{1-3}alkoxy,

D is Lys, 4-aminocyclohexylAla or 4-aminocyclohexylGly

E is Thr, Ser, Val, Tyr, Ile, Leu or an aminobutyric or aminoisobutyric acid residue

G is a group of formula



wherein

R_7 is hydrogen or C_{1-3}alkyl,

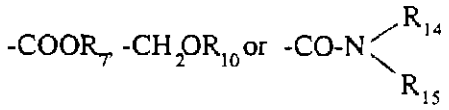
R_{10} is hydrogen or the residue of a physiologically acceptable, physiologically hydrolysable ester,

R_{11} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenyl-alkyl,

R_{12} is hydrogen, C_{1-3}alkyl or a group of formula $-CH(R_{13})-X_1$,

R_{13} is CH_2OH, $-(CH_2)_2-OH$, $-(CH_2)_3-OH$, $-CH(CH_3)OH$, isobutyl, butyl, benzyl, naphtyl-methyl or indol-3-yl-methyl, and

X_1 is a group of formula



wherein

R_7 and R_{10} have the meanings given above,

R_{14} is hydrogen or C_{1-3}alkyl and

R_{15} is hydrogen, C_{1-3}alkyl, phenyl or C_{7-10}phenylalkyl, and

R_{16} is hydrogen or hydroxy,

with the proviso that

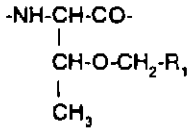
when R_{12} is $-CH(R_{13})-X_1$ then R_{11} is hydrogen or methyl,

wherein the residues B, D and E have the L-configuration, and the residues in the 2- and 7-position each independently have the (L)- or (D)- configuration

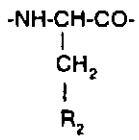
or a somatostatin analogue comprising the amino acid sequence of formula II

$$-(D/L)Trp-Lys-X_2-X_3- \qquad (II)$$

wherein X_2 is a radical of formula (a) or (b)



(a)

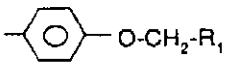


(b)

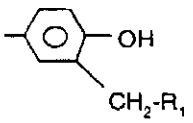
or

wherein R_1 is optionally substituted phenyl,

R_2 is $-Z_1-CH_2-R_1$, $-CH_2-CO-O-CH_2-R_1$,



or



wherein Z_1 is O or S,

and

X_3 is an α-amino acid having an aromatic residue on the C_α side chain, or an amino acid unit selected from Dab, Dpr, Dpm, His,(Bzl)HyPro, thienyl-Ala, cyclohexyl-Ala and t.-butyl-Ala,

the residue Lys of said sequence corresponding to the residue Lys^9 of the native somatostatin-14,

in free form or in pharmaceutically acceptable salt form.

8. A kit or package according to claim 7, wherein the compound of the somatostatin class is octreotide, lanreotide or vapreotide.

9. A kit or package according to claim 6 for simultaneous, separate or sequential use in synergistically effective amounts.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 97/03036

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 239 178 A (SANDOZ LTD) 26 June 1991 see the whole document ---	1-9
A	WO 93 11130 A (SMITHKLINE BEECHAM PLC) 10 June 1993 cited in the application see the whole document ---	1-9
A	SHI E.A.: "Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro" CANCER RESEARCH, vol. 55, 1 May 1995, pages 1982-1988, XP002040888 see the whole document -----	1-9

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 1997	01. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

arnational application No.
PCT/EP 97/03036

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 2 - 5
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte nal Application No
PCT/EP 97/03036

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2239178 A	26-06-91	AT 177788 A	15-02-97
		AU 618270 B	19-12-91
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		CY 1631 A	10-07-92
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WO 9311130 A	10-06-93	AU 2954392 A	28-06-93
		EP 0621865 A	02-11-94
		JP 7501804 T	23-02-95
		US 5491229 A	13-02-96

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/655, A61K 38/31, G01N 33/68 | A1

(11) International Publication Number: **WO 97/05167**

(43) International Publication Date: 13 February 1997 (13.02.97)

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(22) International Filing Date: 17 July 1996 (17.07.96)

(30) Priority Data:
MI95A001670 28 July 1995 (28.07.95) IT

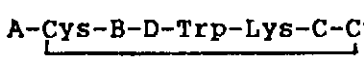
(71)(72) Applicant and Inventor: DEGHENGHI, Romano [IT/CH]; Cheseaux Dessus B1, CH-1264 St. Cergue (CH).

(74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SOMATOSTATIN-ANALOGOUS CYCLIC PEPTIDES WITH INHIBITORY ACTIVITY ON GROWTH HORMONE



(I)

(57) Abstract

Cyclic peptides of general formula (I), wherein the groups are as described in the disclosure, having inhibitory activity on growth hormone. The peptides of formula (I) have also anticancer activity.

FOR THE PURPOSES OF INFORMATION ONLY

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SOMATOSTATIN-ANALOGOUS CYCLIC PEPTIDES WITH INHIBITORY ACTIVITY ON GROWTH HORMONE

The present invention relates to cyclic peptides which are homologues of somatostatin and have inhibitory activity on growth hormone.

BACKGROUND OF THE INVENTION

Somatostatin, or somatotropin-release inhibition factor, is a neuropeptide inhibiting the release of the growth hormone (somatotropin).

A number of somatostatin synthetic analogues are known, which are used in human and animal therapies.

In particular, octreotide has been known for some time, which is a somatostatin synthetic analogue used in therapy for the treatment of syndromes due to gastroenteral-pancreatic endocrine tumours, acromegaly as well as in the post-surgery treatment after pancreas surgery. Octreotide is also indicated as an agent inhibiting gastric secretion. This compound is described in U.S. 4.395.403 (in Sandoz name) and has the formula:

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-NHCH(CH_2OH)CHOHCH$_3$

The octapeptide described above lies within a general formula of monocyclic polypeptides, comprising an hexapeptide residue containing a phenylalanine residue, optionally substituted at the 1- position of the aromatic ring, a cysteine residue at the 2- position, a D-trypthophan residue optionally substituted at the 4- position of the indole ring, a lysine residue at the 5- position, optionally N-alkylated at the position ϵ, an amino acid residue at the 6- position and a cysteine residue at the 7- position, the two sulfur

atoms of the 2 and 7 cysteine residues are linked together and an amino acid residue at the 8- position.

Another somatostatin synthetic analogue, known under the name of Lanreotide, has been recently used in therapy. This compound has formula:

D-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-$ThrNH_2$.

SUMMARY OF THE INVENTION

Now it has been found that compounds of formula (I)

A-Cys-B-D-Trp-Lys-C-Cys-R

wherein

A is D-2AlkTrp, D-βNal, D-Phe;

B is Tyr, Phe;

C is Val, Thr;

R is $ThrNH_2$; $2AlkTrpNH_2$

with the proviso that when A is D-βNal, B is not Tyr, C is not Val and R is not $ThrNH_2$ and the pharmaceutically acceptable salts of these peptides have activity inhibiting the release of the growth hormone and therefore are useful as active principles in human and animal medicine.

The present invention is based on the most surprising finding that the change of some structural characteristics, which had been defined essential in U.S. 4,395,403, not only keeps the somatostatin-like activity, but also shows further advantages in terms of specificity (see for example the expression of receptor sub-types in pituitary adenomas (G.M. Miller et al. J.C.E.M. 80, 1386, 1995) and in bronchial carcinoid tumors (H. Lefabre et al. J.C.E.M. 80, 1423, 1995).

DETAILED DISCLOSURE OF THE INVENTION

The abbreviations for the residues of amino acids

therein used are in agreement with the standard nomenclature for the peptides, therefore, in the formula (I) reported above:

Cys = cysteine;

D-Trp = D-trypthophan;

Lys = L-lysine;

D-AlkTrp = D-2-alkyltryptophan;

D-β-Nal = D-β-naphthylalanine;

D-Phe = D-phenylalanine;

Tyr = L-tyrosine;

Phe = L-phenylalanine;

Val = L-valine;

Thr = L-threonine;

$ThrNH_2$ = L-threonine amide

Alk $TrpNH_2$= L-2-alkyltryptophan amide.

According to the present invention, for alkyl at position 2- of the tryptophan residue it is intended lower alkyl, comprising from 1 to 3 carbon atoms. Examples of lower alkyl are methyl, ethyl, propyl, isopropyl. Among them, the methyl group is most preferred, and the abbreviation MeTrp is used to indicate 2-methyltryptophan.

All the three letter-abbreviations of the amino acids preceded by a "D" indicate the D-configuration of the amino acidic residue.

Preferred compounds according to the present invention are:

D-MeTrp-Cys-Tyr-D-Trp-Lys-Val-Cys-$ThrNH_2$ (Sequence Id no. 1);

D-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-Me$TrpNH_2$ (Sequence Id no. 2);

D-MeTrp-Cys-Phe-D-Trp-Lys-Thr-Cys-MeTrpNH$_2$ (Sequence Id no. 3);

D-Phe-Cys-Tyr-D-Trp-Lys-Cys-MeTrpNH$_2$ (Sequence Id no. 4)

wherein MeTrp is 2-methyltryptophan.

The compound:

D-MeTrp-Cys-Phe-D-Trp-Lys-Thr-Cys-MeTrpNH$_2$ (Sequence Id no. 3),

wherein MeTrp is 2-methyltryptophan,

is most preferred.

The polypeptide compounds according to the present invention can be synthesized according to the usual methods of peptide chemistry, both solid-phase and solution, or by means of the classical methods known in the art. The solid-phase synthesis starts from C-terminal end of peptide. A suitable starting material can be prepared, for example attaching the required protected alpha-amino acid to a chloromethylated resin, a hydroxymethylated resin, a benzhydrylamine resin (BHA), or to a para-methylbenzhydrylamine resin (p-Me-BHA). As example, a chloromethylated resin is sold with the Trade Mark BIOBEADS (R) SX 1 by BioRad Laboratories, Richmond, California. The preparation of the hydroxymethyl resin is described by Bodansky et al., Chem. Ind. (London) 38, 15997, (1966). The BHA resin is described by Pietta and Marshall, Chem. Comm., 650 (1970) and is commercially available by Peninsula Laboratories Inc., Belmont, California.

After the starting attachment, the protecting group of the alpha-amino acid can be removed by means of different acid reagents, comprising trifluoroacetic acid (TFA) or hydrochloric acid (HCl) dissolved in organic

solvents at room temperature. After the removal of the protecting group of the alpha-amino acid, the remaining protected amino acids can be coupled step by step in the desired order. Each protected amino acid can generally be reacted in excess of about three times using a suitable carboxyl activating group, such as dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) dissolved, for example, in methylene chloride (CH_2Cl_2) or dimethylformamide (DMF) and their mixtures. After the desired amino acidic sequence has been completed, the desired peptide can be cleaved from the supporting resin by treatment with a reagent such as hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also the more common protecting groups of the lateral chains. When a chloromethylated resin or a hydroxymethylated resin is used, the treatment with HF leads to the formation of the acid peptide in free form. When a BHA or p-Me-BHA resin is used, the treatment with HF directly leads to the formation of the amide peptide in free form.

The above discussed solid-phase procedure is known in the art and was described by Atherton and Sheppard, Solid Phase Peptide Synthesis (IRL Press, Oxford, 1989).

Some methods in solution, which can be used to synthesize the peptide moieties of the present invention are detailed in Bodansky et al., Peptide Synthesis, 2nd edition, John Wiley & Sons, New York, N.Y. 1976 and in Jones, The Chemical Synthesis of Peptides, (Clarendon Press, Oxford, 1994).

These compounds can be administered to animals and humans at an effective dose which can be easily

determined by the expert in the field and which can vary according to the specie, age, sex and weight of the treated subject. For example, in humans, when intravenously administered, the preferred dose falls in the range from about 0.1 µg to 10 µg of total peptide per kg of body weight. When orally administered, typically higher amounts are necessary. For example, in humans for the oral administration, the dosage level is typically from about 30 µg to about 1000 µg of polypeptide per kg of body weight. The exact level can be easily determined empirically based on the above disclosure.

Compositions comprising as active ingredient the organic and inorganic addition salts of the above described polypeptides and their combinations, optionally, in admixture with a vehicle, diluent, matrix or delayed release coating, are also comprised in the scope of the present invention. The delayed release pharmaceutical forms, comprising bioerodible matrixes suitable for subcutaneous implant, are particularly interesting. Examples of these matrices are described in WO9222600 and WO9512629.

The biological activity of the peptides according to the present invention has been evaluated in vitro and in vivo.

The study of the binding of the peptides on somatostatin receptors has been carried out according to the displacement method which consists in replacing from the receptors the radioligand (11-Tyr radioiodinated somatostatin 14-) before electrophoretic analysis on a denaturant polyacrylaminde gel (Prévost et al. European

J. Cancer, 11, 1589-1592,1993).

The biological activity was tested on rat and human cell lines, namely a GH 3 rat cell line established starting from a pituitary tumor with two 70 and 57 kDa receptors; MCF7 human cell line established starting from a pleural effusion of a breast carcinoma with two main 57 and 42kDa receptors and human cell line established starting from a small cell carcinoma of the lung with a 57 kDa receptor.

The test was effected on the following compounds of the present invention listed below:

D-MeTrp-Cys-Tyr-D-Trp-Lys-Val-Cys-ThrNH_2; (b)

D-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-MeTrpNH; (c)

D-MeTrp-Cys-Phe-D-Trp-Lys-Thr-Cys-MeTrpNH_2; (d)

D-Phe-Cys-Tyr-D-Trp-Lys-Cys-MeTrpNH_2; (e)

wherein MeTrp is 2-methyltryptophan, respectively indicated with the letters b, c, d, e.

As a comparison, the known peptides Lanreotide (W.A. Murphy et al. Life Sci. 40, 2515, 1987), Antarelix (R. Deghenghi et al., Biomed. & Pharmacother. 47, 107, 1993) and somatostatin-14 were used.

The pharmacological study of the displacement of the bond between radiolabelled somatostatin and the receptor by the tested peptides showed that the 70 kDa complex corresponds to the 1 or 4 sub-type receptors, the 57 kDa complex corresponds to the sub-type 2 and the 42 kDa complex corresponds to the sub-type 3 or 5.

The tested peptides were suspended in 0,1% acetic acid at the final concentration of 10mM and stored at 4°C.

Tests were carried out at a 10^{-6} M concentration.

Auto-radiographies in electrophoresis have shown that the 70 kDa complex is suppressed only by somatostatin-14, whereas the 57 kDa complex is displaced by the peptides according to the invention, by Lanreotide, but not by Antarelix. The 42 kDa complex is suppressed by the peptides c, d, e of the invention, which thus prove an action specificity.

Tests with decreasing concentrations, 10^{-6}, 10^{-7}, 10^{-8} M, have shown that the compounds according to the present invention are particularly active compared with the known compounds and somatostatin.

The compounds according to the present invention have inhibitory activity on the release of growth hormone, therefore they are useful as active principles for the preparation of a medicament for the treatment of the diseases characterized by an unbalance of the growth hormone. In particular they are useful for the treatment of endocrine tumors, acromegaly and in the conditions in which the known somatostatin analogues are used.

According to another aspect, the present invention provides cyclic peptides of formula (I) shown above as a support for a radioactive marker, for example ^{125}Iodine or ^{111}Indium or ^{99m}Technetium useful as diagnostic agents for tumors characterized by the presence of somatostatin receptors.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: DEGHENGHI Romano

(B) STREET: Cheseaux Dessus B1

(C) CITY: St. Cergue

(E) COUNTRY: Switzerland

(F) POSTAL CODE (ZIP): 1264

(ii) TITLE OF INVENTION: SOMATOSTATIN-ANALOGOUS CYCLIC PEPTIDES WITH
INHIBITORY ACTIVITY ON GROWTH HORMONE

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: IT MI95A001670

(B) FILING DATE: 28-JUL-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Disulfide-bond

(B) LOCATION:2..7

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION:1

(D) OTHER INFORMATION:/product= "OTHER"

/note= "Trp is D-2-Methyl-Trp"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION:4

(D) OTHER INFORMATION:/product= "OTHER"

/note= "Trp is D-Trp"

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION:8

(D) OTHER INFORMATION:/product= "OTHER"

/note= "Thr is Thr-NH2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Trp Cys Tyr Trp Lys Val Cys Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION:2..7

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Ala is D-betaNal"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Trp is D-Trp"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:8
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Trp is 2-Methyl-Trp-NH2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

12

Ala Cys Tyr Trp Lys Val Cys Trp
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION:2..7

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Trp is D-2-Methyl-Trp"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Trp is D-Trp"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:8

(D) OTHER INFORMATION:/product= "OTHER"
/note= "Trp is 2-Methyl-Trp-NH2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Trp Cys Phe Trp Lys Thr Cys Trp
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION:2..6

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:1
(D) OTHER INFORMATION:/product= "OTHER"
/note= "Phe is D-Phe"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "OTHER"

/note= "Trp is D-Trp"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION:7

(D) OTHER INFORMATION:/product= "OTHER"

/note= "Trp is 2-Methyl-Trp-NH2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Phe Cys Tyr Trp Lys Cys Trp
1 5

CLAIMS

1. Compounds of formula (I)

A-Cys-B-D-Trp-Lys-C-Cys-R

wherein

A is D-AlkTrp, D-βNal,D-Phe;

B is Tyr, Phe;

C is Val, Thr;

R is $ThrNH_2$; $AlkTrpNH_2$

with the proviso that when A is D-βNal, B is not Tyr, C is not Val and R is not Thr NH_2 and the pharmaceutically acceptable salts of said compounds.

2. Compound according to claim 1, selected from the group consisting of:

D-MeTrp-Cys-Tyr-D-Trp-Lys-Val-Cys-$ThrNH_2$ (Sequence Id no. 1);

D-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-$MeTrpNH_2$ (Sequence Id no. 2);

D-MeTrp-Cys-Phe-D-Trp-Lys-Thr-Cys-$MeTrpNH_2$ (Sequence Id no. 3);

D-Phe-Cys-Tyr-D-Trp-Lys-Cys-$MeTrpNH_2$ (Sequence Id no. 4);

wherein MeTrp is 2-methyltryptophane.

3. The use of the compounds of claims 1-2 for the preparation of a medicament with inhibitory activity on the growth hormone.

4. The use of the compounds of claims 1-2 for the preparation of a medicament with antitumor activity.

5. Pharmaceutical compositions containing a therapeutically effective amount of a compound of claims 1-2 as active ingredient in admixture with carriers

and/or pharmaceutically acceptable excipients.

6. Compounds of claims 1-2 as ligands for a radioactive marker.

7. The use of the compounds of claim 6 for the preparation of a diagnostic agent for the detection of tumors.

INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/EP 96/03149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/655 A61K38/31 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 203 031 (UNIV TULANE) 26 November 1986	1,3-7
Y	see the whole document	1-7

X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, April 1987, WASHINGTON US, pages 2502-2506, XP002020260 R.Z.CAI E.A.: "Superactive octapeptide somatostatin analogs containing tryptophan at position 1"	1,3-7
Y	see the whole document	1-7

X	EP,A,0 214 872 (UNIV TULANE) 18 March 1987	1,3-7
Y	see the whole document	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 December 1996	17.12.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Groenendijk, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 18016 (DEGHENGHI ROMANO) 28 November 1991 See especially page 3,lines 13-28;page 5,lines 29-33; SEQ ID Nr.12; claims 1-13 ---	1-7
Y	EP,A,0 505 680 (BIOSIGNAL KUTATO FEJLESZTO KFT) 30 September 1992 See especially example 3 ---	1-7
A	WO,A,92 21383 (MALLINCKRODT MEDICAL INC) 10 December 1992 see the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/03149

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0203031	26-11-86	US-A-	4650787	17-03-87
		US-A-	4725577	16-02-88
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		CA-A-	2081450	12-11-91
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		DE-T-	69105270	13-04-95
		EP-A-	0531461	17-03-93
		ES-T-	2067256	16-03-95
EP-A-0505680	30-09-92	AT-T-	121753	15-05-95
		DE-D-	69202182	01-06-95
		DE-T-	69202182	31-08-95
		ES-T-	2074294	01-09-95
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		US-A-	5480870	02-01-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/03149

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9221383	10-12-92	AU-B-	657770	23-03-95
		AU-A-	2017492	08-01-93
		CA-A-	2102633	04-12-92
		EP-A-	0588905	30-03-94
		JP-T-	6508357	22-09-94

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
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PCT

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WO 02/080975 A1

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(22) International Filing Date: 5 April 2002 (05.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

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60/282,388	6 April 2001 (06.04.2001)	US

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(54) Title: ANTINEOPLASTIC COMBINATIONS SUCH AS RAPAMYCIN TOGETHER WITH GEMCITABINE OR FLUOROURACIL

(57) Abstract: The invention provides the use of a combination of an mTOR inhibitor such as a rapamycin and an antimetabolite antineoplastic agent such as gemsitabine or fluorouracil in the treatment of neoplasms.

ANTINEOPLASTIC COMBINATIONS SUCH AS RAPAMYCIN TOGETHER WITH GEMCITABINE OR FLUOROURACIL

This invention relates to antineoplastic combinations, more particularly to the use of combinations of an mTOR inhibitor (e.g. rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779)) and an antimetabolite antineoplastic agent in the treatment of neoplasms.

BACKGROUND OF THE INVENTION

Rapamycin is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus, which was found to have antifungal activity, particularly against Candida albicans, both in vitro and in vivo [C. Vezina et al., J. Antibiot. 28, 721 (1975); S.N. Sehgal et al., J. Antibiot. 28, 727 (1975); H. A. Baker et al., J. Antibiot. 31, 539 (1978); U.S. Patent 3,929,992; and U.S. Patent 3,993,749]. Additionally, rapamycin alone (U.S. Patent 4,885,171) or in combination with picibanil (U.S. Patent 4,401,653) has been shown to have antitumor activity.

The immunosuppressive effects of rapamycin have been disclosed in FASEB 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); R. Y. Calne et al., Lancet 1183 (1978); and U.S. Patent 5,100,899]. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

Rapamycin is also useful in preventing or treating systemic lupus erythematosus [U.S. Patent 5,078,999], pulmonary inflammation [U.S. Patent 5,080,899], insulin dependent diabetes mellitus [U.S. Patent 5,321,009], skin disorders, such as psoriasis [U.S. Patent 5,286,730], bowel disorders [U.S. Patent 5,286,731], smooth muscle cell proliferation and intimal thickening following vascular injury [U.S. Patents 5,288,711 and 5,516,781], adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1], ocular inflammation [U.S. Patent 5,387,589], malignant carcinomas [U.S. Patent 5,206,018], cardiac inflammatory disease [U.S. Patent 5,496,832], and anemia [U.S. Patent 5,561,138].

Rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) is ester of rapamycin which has demonstrated significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of hydroxyesters of rapamycin, including CCI-779, are disclosed in U.S. Patent 5,362,718.

CCI-779 exhibits cytostatic, as opposed to cytotoxic properties, and may delay the time to progression of tumors or time to tumor recurrence. CCI-779 is considered to have a mechanism of action that is similar to that of sirolimus. CCI-779 binds to and forms a complex with the cytoplasmic protein FKBP, which inhibits an enzyme, mTOR (mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]). Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G_1 to S. The mechanism of action of CCI-779 that results in the G1 S phase block is novel for an anticancer drug.

In vitro, CCI-779 has been shown to inhibit the growth of a number of histologically diverse tumor cells. Central nervous system (CNS) cancer, leukemia (T-cell), breast cancer, prostate cancer, and melanoma lines were among the most sensitive to CCI-779. The compound arrested cells in the G1 phase of the cell cycle.

In vivo studies in nude mice have demonstrated that CCI-779 has activity against human tumor xenografts of diverse histological types. Gliomas were particularly sensitive to CCI-779 and the compound was active in an orthotopic glioma model in nude mice. Growth factor (platelet-derived)-induced stimulation of a human glioblastoma cell line in vitro was markedly suppressed by CCI-779. The growth of several human pancreatic tumors in nude mice as well as one of two breast cancer lines studied in vivo also was inhibited by CCI-779.

DESCRIPTION OF THE INVENTION

This invention provides the use of combinations of an mTOR inhibitor and an antimetabolite antineoplastic agent as antineoplastic combination chemotherapy. In particular, these combinations are useful in the treatment of renal cancer, soft tissue cancer, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small lung cell cancer, prostate cancer,

pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. This invention also provides combinations of an mTOR inhibitor and an antimetabolite antineoplastic agent for use as antineoplastic combination chemotherapy, in which the dosage of either the mTOR inhibitor or the antimetabolite antineoplastic agent or both are used in subtherapeutically effective dosages.

As used in accordance with this invention, the term "treatment" means treating a mammal having a neoplastic disease by providing said mammal an effective amount of a combination of an mTOR inhibitor and an antimetabolite antineoplastic agent with the purpose of inhibiting growth of the neoplasm in such mammal, eradication of the neoplasm, or palliation of the mammal.

As used in accordance with this invention, the term "providing," with respect to providing the combination, means either directly administering the combination, or administering a prodrug, derivative, or analog of one or both of the components of the combination which will form an effective amount of the combination within the body.

mTOR is the mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]. Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S.

mTOR regulates the activity of at least two proteins involved in the translation of specific cell cycle regulatory proteins (Burnett, P.E., PNAS 95: 1432 (1998) and Isotani, S., J. Biol. Chem. 274: 33493 (1999)). One of these proteins p70s6 kinase is phosphorylated by mTOR on serine 389 as well as threonine 412. This phosphorylation can be observed in growth factor treated cells by Western blotting of whole cell extracts of these cells with antibody specific for the phosphoserine 389 residue.

As used in accordance with this invention, an "mTOR inhibitor" means a compound or ligand which inhibits cell replication by blocking progression of the cell

cycle from G1 to S by inhibiting the phosphorylation of serine 389 of p70s6 kinase by mTOR.

The following standard pharmacological test procedure can be used to determine whether a compound is an mTOR inhibitor, as defined herein. Treatment of growth factor stimulated cells with an mTOR inhibitor like rapamycin completely blocks phosphorylation of serine 389 as evidenced by Western blot and as such constitutes a good assay for mTOR inhibition. Thus whole cell lysates from cells stimulated by a growth factor (eg. IGF1) in culture in the presence of an mTOR inhibitor should fail to show a band on an acrylamide gel capable of being labeled with an antibody specific for serine 389 of p70s6K.

Materials:

NuPAGE LDS Sample Buffer	(Novex Cat # NP0007)
NuPAGE Sample Reducing Agent	(Novex Cat # NP0004)
NuPAGE 4-12% Bis-Tris Gel	(Novex Cat # NP0321)
NuPAGE MOPS SDS Running Buffer	(Novex Cat # NP0001)
Nitrocellulose	(Novex Cat # LC2001)
NuPAGE Transfer Buffer	(Novex Cat # NP0006)
Hyperfilm ECL	(Amersham Cat # RPN3114H)
ECL Western Blotting Detection Reagent	(Amersham Cat # RPN2134)

Primary antibody: Phospho-p70 S6 Kinase (Thr389) (Cell Signaling Cat # 9205)
Secondary antibody: Goat anti-rabbit IgG-HRP conjugate (Santa Cruz Cat # sc-2004)

Methods:

A. Preparation of Cell Lysates

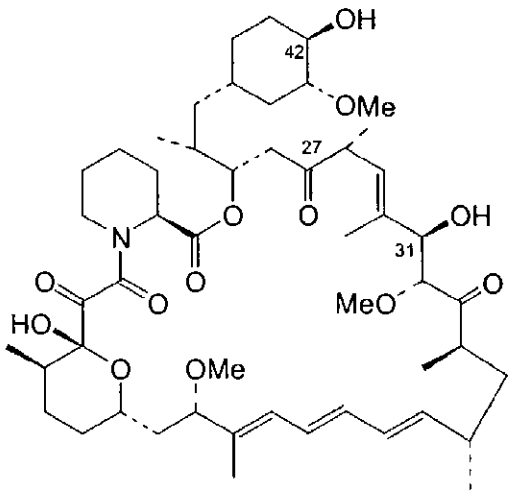
Cell lines were grown in optimal basal medium supplemented with 10% fetal bovine serum and penicillin/treptomycin. For phosphorylation studies, cells were subcultured in 6-well plates. After the cells have completely attached, they were either serum-starved. Treatment with mTOR inhibitors ranged from 2 to 16 hours. After drug treatment, the cells were rinsed once with PBS (phosphate buffered saline without Mg++ and Ca++) and then lysed in 150-200 µl NuPAGE LDS sample buffer per well. The lysates were briefly sonicated and then centrifuged for 15 minutes at 14000 rpm. Lysates were stored at minus -80^{0}C until use.

The test procedure can also be run by incubating the cells in growth medium overnigh, after they have completely attached. The results under both sets of conditions should be the same for an mTOR inhibitor.

B. Western Blot Analysis

1) Prepare total protein samples by placing 22.5 µl of lysate per tube and then add 2.5 µl NuPAGE sample reducing agent. Heat samples at 70^0C for 10 minutes. Electrophoresed using NuPAGE gels and NuPAGE SDS buffers.
2) Transfer the gel to a nitrocellulose membrane with NuPAGE transfer buffer. The membrane are blocked for 1 hour with blocking buffer (Tris buffered saline with 0.1%-Tween and 5% nonfat-milk). Rinse membranes 2x with washing buffer (Tris buffered saline with 0.1%-Tween).
3) Blots/membrane are incubated with the P-p70 S6K (T389) primary antibody (1:1000) in blocking buffer overnight at 4°C in a rotating platform.
4) Blots are rinsed 3x for 10 minutes each with washing buffer, and incubated with secondary antibody (1:2000) in blocking buffer for 1 hour at room temperature.
5) After the secondary antibody binding, blots are washed 3x for 10 minutes each with washing buffer, and 2x for 1 minute each with Tris-buffered saline, followed by chemiluminescent (ECL) detection and then exposed to chemiluminescence films.

As used in accordance with this invention, the term "a rapamycin" defines a class of immunosuppressive compounds which contain the basic rapamycin nucleus (shown below). The rapamycins of this invention include compounds which may be chemically or biologically modified as derivatives of the rapamycin nucleus, while still retaining immunosuppressive properties. Accordingly, the term "a rapamycin" includes esters, ethers, oximes, hydrazones, and hydroxylamines of rapamycin, as well as rapamycins in which functional groups on the rapamycin nucleus have been modified, for example through reduction or oxidation. The term "a rapamycin" also includes pharmaceutically acceptable salts of rapamycins, which are capable of forming such salts by virtue of containing either an acidic or basic moiety.



RAPAMYCIN

It is preferred that the esters and ethers of rapamycin are of the hydroxyl groups at the 42- and/or 31-positions of the rapamycin nucleus, esters and ethers of a hydroxyl group at the 27-position (following chemical reduction of the 27-ketone), and that the oximes, hydrazones, and hydroxylamines are of a ketone at the 42-position (following oxidation of the 42-hydroxyl group) and of 27-ketone of the rapamycin nucleus.

Preferred 42- and/or 31-esters and ethers of rapamycin are disclosed in the following patents, which are all hereby incorporated by reference: alkyl esters (U.S. Patent 4,316,885); aminoalkyl esters (U.S. Patent 4,650,803); fluorinated esters (U.S. Patent 5,100,883); amide esters (U.S. Patent 5,118,677); carbamate esters (U.S. Patent 5,118,678); silyl ethers (U.S. Patent 5,120,842); aminoesters (U.S. Patent 5,130,307); acetals (U.S. Patent 5,51,413); aminodiesters (U.S. Patent 5,162,333); sulfonate and sulfate esters (U.S. Patent 5,177,203); esters (U.S. Patent 5,221,670); alkoxyesters (U.S. Patent 5,233,036); O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Patent 5,258,389); carbonate esters (U.S. Patent 5,260,300); arylcarbonyl and alkoxycarbonyl carbamates (U.S. Patent 5,262,423); carbamates (U.S. Patent 5,302,584); hydroxyesters (U.S. Patent 5,362,718); hindered esters (U.S. Patent 5,385,908); heterocyclic esters (U.S. Patent 5,385,909); gem-disubstituted esters (U.S. Patent 5,385,910); amino alkanoic esters (U.S. Patent 5,389,639); phosphorylcarbamate esters (U.S. Patent 5,391,730); carbamate esters (U.S. Patent

5,411,967); carbamate esters (U.S. Patent 5,434,260); amidino carbamate esters (U.S. Patent 5,463,048); carbamate esters (U.S. Patent 5,480,988); carbamate esters (U.S. Patent 5,480,989); carbamate esters (U.S. Patent 5,489,680); hindered N-oxide esters (U.S. Patent 5,491,231); biotin esters (U.S. Patent 5,504,091); O-alkyl ethers (U.S. Patent 5,665,772); and PEG esters of rapamycin (U.S. Patent 5,780,462). The preparation of these esters and ethers are disclosed in the patents listed above.

Preferred 27-esters and ethers of rapamycin are disclosed in U.S. Patent 5,256,790, which is hereby incorporated by reference. The preparation of these esters and ethers are disclosed in the patents listed above.

Preferred oximes, hydrazones, and hydroxylamines of rapamycin are disclosed in U.S. Patents 5,373,014, 5,378,836, 5,023,264, and 5,563,145, which are hereby incorporated by reference. The preparation of these oximes, hydrazones, and hydroxylamines are disclosed in the above listed patents. The preparation of 42-oxorapamycin is disclosed in 5,023,263, which is hereby incorporated by reference.

Particularly preferred rapamycins include rapamycin [U.S. Patent 3,929,992], CCI-779 [rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid; U.S. Patent 5,362,718], and 42-O-(2-hydroxy)ethyl rapamycin [U.S. Patent 5,665,772].

When applicable, pharmaceutically acceptable salts of the rapamycin can be formed from organic and inorganic acids, for example, acetic, propionic, lactic, citric, tartaric, succinic, fumaric, maleic, malonic, mandelic, malic, phthalic, hydrochloric, hydrobromic, phosphoric, nitric, sulfuric, methanesulfonic, napthalenesulfonic, benzenesulfonic, toluenesulfonic, camphorsulfonic, and similarly known acceptable aids when the rapamycin contains a suitable basic moiety. Salts may also be formed from organic and inorganic bases, such as alkali metal salts (for example, sodium, lithium, or potassium) alkaline earth metal salts, ammonium salts, alkylammonium salts containing 1-6 carbon atoms or dialkylammonium salts containing 1-6 carbon atoms in each alkyl group, and trialkylammonium salts containing 1-6 carbon atoms in each alkyl group, when the rapamycin contains a suitable acidic moiety.

It is preferred that the mTOR inhibitor used in the antineoplastic combinations of this invention is a rapamycin, and more preferred that the mTOR inhibitor is rapamycin, CCI-779, or 42-O-(2-hydroxy)ethyl rapamycin.

As described herein, CCI-779 was evaluated as a representative mTOR inhibitor in the mTOR inhibitor plus antimetabolite combinations of this invention.

The preparation of CCI-779 is described in U.S. Patent 5,362,718, which is hereby incorporated by reference. When CCI-779 is used as an antineoplastic agent, it is projected that initial i.v. infusion dosages will be between about 0.1 and 100 mg/m^2 when administered on a daily dosage regimen (daily for 5 days, every 2-3 weeks), and between about 0.1 and 1000 mg/m^2 when administered on a once weekly dosage regimen. Oral or intravenous infusion are the preferred routes of administration, with intravenous being more preferred.

As used in accordance with this invention, the term "antimetabolite" means a substance which is structurally similar to a critical natural intermediate (metabolite) in a biochemical pathway leading to DNA or RNA synthesis which is used by the host in that pathway, but acts to inhibit the completion of that pathway (i.e., synthesis of DNA or RNA). More specifically, antimetabolites typically function by (1) competing with metabolites for the catalytic or regulatory site of a key enzyme in DNA or RNA synthesis, or (2) substitute for a metabolite that is normally incorporated into DNA or RNA, and thereby producing a DNA or RNA that cannot support replication. Major categories of antimetabolites include (1) folic acid analogs, which are inhibitors of dihydrofolate reductase (DHFR); (2) purine analogs, which mimic the natural purines (adenine or guanine) but are structurally different so they competitively or irreversibly inhibit nuclear processing of DNA or RNA; and (3) pyrimidine analogs. which mimic the natural pyrimidines (cytosine, thymidine, and uracil) but are structurally different so they competitively or irreversibly inhibit nuclear processing of DNA or RNA.

The following are representative examples of antimetabolites of this invention.

5-Fluorouracil (5-FU; 5-fluoro-2,4(1*H*,3*H*)-pyrimidinedione) is commercially available in a topical cream (FLUOROPLEX or EFUDEX) a topical solution (FLUOROPLEX or EFUDEX), and as an injectable containing 50 mg/mL 5-fluorouracil (ADRUCIL or flurouracil).

Floxuradine (2'-deoxy-5-fluorouridine) is commercially available as an injectable containing 500 mg/vial of floxuradine (FUDR or floxuradine).

Thioguanine (2-amino-1,7-dihydro-6-*H*-purine-6-thione) is commercially available in 40 mg oral tablets (thioguanine).

Cytarabine (4-amino-1-(beta)-D-arabinofuranosyl-2(1H)-pyrimidinone) is commercially available as a liposomal injectable containing 10 mg/mL cytarabine (DEPOCYT) or as a liquid injectable containing between 1mg - 1g/vial or 20 mg/mL (cytarabine or CYTOSAR-U).

Fludarabine (9-H-Purin-6-amine,2-fluoro-9-(5-O-phosphono-(beta)-D-arabino-furanosyl) is commercially available as a liquid injectable containing 50 mg/vial (FLUDARA).

6-Mercaptopurine (1,7-dihydro-6*H*-purine-6-thione) is commercially available in 50 mg oral tablets (PURINETHOL).

Methotrexate (MTX; *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]-benzoyl]-L-glutamic acid) is commercially available as a liquid injectable containing between 2.5 - 25 mg/mL and 20 mg - 1 g/vial (methotrexate sodium or FOLEX) and in 2.5 mg oral tablets (methotrexate sodium).

Gemcitabine (2'-deoxy-2',2'-difluorocytidine monohydrochloride ((beta)-isomer)), is commercially available as a liquid injectable containing between 200 mg - 1g/vial (GEMZAR).

Capecitabine (5'-deoxy-5-fluoro-N-[(pentyloxy)carbonyl]-cytidine) is commercially available as a 150 or 500 mg oral tablet (XELODA).

Pentostatin ((R)-3-(2-deoxy-(beta)-D-*erythro*-pentofuranosyl)-3,6,7,8-tetra-hydroimidazo[4,5-*d*][1,3]diazepin-8-ol) is commercially available as a liquid injectable containing 10 mg/vial (NIPENT).

Trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]-quinazoline mono-D-glucuronate) is commercially available as a liquid injectable containing between 25 - 200 mg/vial (NEUTREXIN).

Cladribine (2-chloro-6-amino-9-(2-deoxy-(beta)-D-erythropento-furanosyl)-purine) is commercially available as a liquid injectable containing 1 mg/mL (LEUSTATIN).

The following table briefly summarizes some of the recommended dosages for the antimetabolites listed above.

Drug	Dosage	Regimen
5-Fluorouracil	12 mg/kg oral	daily for 4 days
	6 mg/kg oral	days 6, 8, 10, 12
		no drug on days 5, 7, 9, and 11; doses cut in half if toxicity observed
	370 - 600 mg/m^2 i.v.	daily for 5 days, every 3-4 weeks
Floxuradine (FUDR)	0.1-0.6 mg/kg	daily by arterial infusion
Cytarabine (DEPOCYT)	50 mg	every 14 days for 5 doses during induction period; followed by every 28 days for maintenance
Cytarabine (injectable)	100 mg/m^2	daily for 7 days
	2-3 g/m^2	twice daily for 2-6 days
Fludarabine (FLUDARA)	25 mg/m^2	30 min infusion for 5 consecutive days; every 28 days
6-Mercaptopurine	2.5-5 mg/kg	daily for induction
(PURINETHOL)	1.5-2.5 mg/kg	daily for maintenance
Methotrexate	15-30 mg oral	daily for 5 day course; repeated 3-5 times
Gemcitabine (GEMZAR)	1000 mg/m^2/30 min	single agent: once weekly for 7 weeks, followed by 1 week rest, then once weekly for 3 out of every 4 weeks
	1000 -1250 mg/m^2/ 30 min	combination therapy: days 1, 8, 15 per 28 day cycle, or days 1 and 8 per 21 day cycle
Capecitabine (XELODA)	2500 mg/m^2	daily for 2 weeks followed by 1 week rest period
Pentostatin (NIPENT)	4 mg/m^2	as bolus injection or diluted as i.v. infusion; every other week
Trimetrexate (NEUTREXIN)	45 mg/m^2	i.v. infusion once daily for 21 days
Cladribine (LEUSTATIN)	0.09 mg/kg/day	continuous infusion for 7 consecutive days

This invention also covers the use of an mTOR inhibitor plus an antimetabolite in which a biochemical modifying agent is part of the chemotherapeutic regimen. The term "biochemical modifying agent" is well known and understood to those skilled in the art as an agent given as an adjunct to antimetabolite therapy, which serves to potentiate its antineoplastic activity, as well as counteract the side effects of the antimetabolite. Leucovorin and levofolinate are typically used as biochemical modifying agents for methotrexate and 5-FU therapy.

Leucovorin (5-formyl-5,6,7,8-tetrahydrofolic acid) is commercially available as an injectable liquid containing between 5 - 10 mg/mL or 50 - 350 mg/vial (leucovorin calcium or WELLCOVORIN) and as 5 - 25 mg oral tablets (leucovorin calcium).

Levofolinate (pharmacologically active isomer of 5-formyltetrahydrofolic acid) is commercially available as an injectable containing 25 - 75 mg levofolinate (ISOVORIN) or as 2.5 - 7.5 mg oral tablets (ISOVORIN).

Preferred mTOR inhibitor plus antimetabolite combinations of this invention include CCI-779 plus gemcitabine; CCI-779 plus 5-fluorouracil; and CCI-779 plus 5-fluorouracil plus leucovorin. It is preferred that the CCI-779 plus gemcitabine combination be used in treating pancreatic cancer and that the CCI-779 plus 5-fluorouracil combination (with or without leucovorin) be used in treating colorectal cancer.

The antineoplastic activity of the CCI-779 plus antimetabolite combination was confirmed in *in vitro* and *in vivo* standard pharmacological test procedures using combinations of CCI-779 plus gemcitabine; and CCI-779 plus 5-fluorouracil as representative combinations of this invention. The following briefly describes the procedures used and the results obtained.

Human rhabdomyosarcoma lines Rh30 and Rh1 and the human glioblastoma line SJ-GBM2 were used for *in vitro* combination studies with CCI-779 and antimetabolite agents. *In vivo* studies used a human neuroblastoma (NB1643) and human colon line GC3.

Dose response curves were determined for each of the drugs of interest. The cell lines Rh30, Rh1 and SJ-G2 were plated in six-well cluster plates at $6x10^3$, $5x10^3$ and $2.5x10^4$ cells/well respectively. After a 24 hour incubation period, drugs were added in either 10%FBS+RPMI 1640 for Rh30 and Rh1 or 15%FBS+DME for SJ-G2. After seven days exposure to drug containing media, the nuclei were released by treating the cells with a hypotonic solution followed by a detergent. The nuclei were then counted with a Coulter Counter. The results of the experiments were graphed and the IC_{50} (drug concentration producing 50% inhibition of growth) for each drug was determined by extrapolation. Because the IC50s varied slightly from experiment to experiment, two values that bracketed the IC50 of each drug were used in the interaction studies. The point of maximum interaction between two drugs occurs

when they are present in a 1:1 ratio if the isobole is of standard shape. Therefore, each of the three approximate IC_{50} concentrations of CCI-779 was mixed in a 1:1 ratio with each of three approximated IC_{50}s of gemcitabine or 5-FU. This resulted in nine 1:1 combinations of drugs in each experiment plus three IC_{50} concentrations for CCI-779 and the other drug. This protocol usually resulted in at least one combination for each drug containing an IC_{50} value. The 1:1 combination of IC_{50} concentrations for CCI-779 and each chemotherapy drug was then used to calculate additivity, synergism, or antagonism using Berenbaum's formula: $x/X_{50}+y/Y_{50},=1,<1,>1$. If the three concentrations of CCI-779 tested alone didn't produce an IC that matched any of the three ICs of the other compound tested alone, all the 1:1 combinations were checked to see if their ICs fell between the appropriate ICs of drugs tested singly. If they did, the effect was considered additive.

The results obtained in the *in vitro* standard pharmacological test procedure showed that in no case did the combinations yield less than a 50% inhibition of growth indicating that the combinations were at least additive and produced no evidence of antagonism.

Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME), 4 weeks of age, were immune-deprived by thymectomy, followed 3 weeks later by whole-body irradiation (1200 cGy) using a ^{137}Cs source. Mice received 3×10^6 nucleated bone marrow cells within 6-8 h of irradiation. Tumor pieces of approximately 3 mm^3 were implanted in the space of the dorsal lateral flanks of the mice to initiate tumor growth. Tumor-bearing mice were randomized into groups of seven prior to initiating therapy. Mice bearing tumors each received drug when tumors were approximately 0.20-1 cm in diameter. Tumor size was determined at 7-day intervals using digital Vernier calipers interfaced with a computer. Tumor volumes were calculated assuming tumors to be spherical using the formula $[(\pi/6) \times d^3]$, where d is the mean diameter. CCI-779 was given on a schedule of 5 consecutive days for 2 weeks with this cycle repeated every 21 days for 3 cycles. This resulted in CCI-779 being given on days 1-5, 8-12 (cycle 1); 21-25, 28-32 (cycle 2); and 42-46, 49-53 (cycle 3). The schedule of the other chemotherapy drug for each study was as follows:

Gemcitabine on days 1, 4, 8 in cycle 1 only

The combination of CCI-779 and gemcitabine was evaluated in a human colon (GC3) mouse xenograft test procedure. In this test procedure, CCI-779 was given daily x 5 for 2 consecutive weeks every 21 days for 3 cycles and gemcitabine given on days 1, 4, and 8 in the first cycle only. The presence of CCI-779 did not enhance tumor regression seen in the first cycle with gemcitabine treatment. However, groups treated with CCI-779 were delayed in the time required to reach 2-3x the original pretreatment tumor volume (versus gemcitabine alone), indicating that there was at least an additive benefit derived from the combination treatment.

Based on the results of these standard pharmacological test procedures, combinations of an mTOR inhibitor plus an antimetabolite chemotherapeutic agent are useful as antineoplastic therapy. More particularly, these combinations useful in treating treatment of renal carcinoma, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. As these combinations contain at least two active antineoplastic agents, the use of such combinations also provides for the use of combinations of each of the agents in which one or both of the agents is used at subtherapeutically effective dosages, thereby lessening toxicity associated with the individual chemotherapeutic agent.

In providing chemotherapy, multiple agents having different modalities of action are typically used as part of a chemotherapy "cocktail." It is anticipated that the combinations of this invention will be used as part of a chemotherapy cocktail that may contain one or more additional antineoplastic agents depending on the nature of the neoplasia to be treated. For example, this invention also covers the use of the mTOR inhibitor/antimetabolite combination used in conjunction with other chemotherapeutic agents, such as alkylating agents (i.e., cisplatin, carboplatin, streptazoin, melphalan, chlorambucil, carmustine, methclorethamine, lomustine, bisulfan, thiotepa, ifofamide, or cyclophosphamide); hormonal agents (i.e., estramustine, tamoxifen, toremifene, anastrozole, or letrozole); antibiotics (i.e., plicamycin, bleomycin, mitoxantrone, idarubicin, dactinomycin, mitomycin, doxorubicin, or daunorubicin); immunomodulators (i.e., interferons, IL-2, or BCG);

antimitotic agents (i.e., vinblastine, vincristine, teniposide, or vinorelbine); topoisomerase inhibitors (i.e., topotecan, irinotecan, or etoposide); and other agents (i.e., hydroxyurea, trastuzumab, altretamine, retuximab, paclitaxel, docetaxel, L-asparaginase, or gemtuzumab ozogamicin).

As used in this invention, the combination regimen can be given simultaneously or can be given in a staggered regimen, with the mTOR inhibitor being given at a different time during the course of chemotherapy than the antimetabolite. This time differential may range from several minutes, hours, days, weeks, or longer between administration of the two agents. Therefore, the term combination does not necessarily mean administered at the same time or as a unitary dose, but that each of the components are administered during a desired treatment period. The agents may also be administered by different routes. For example, in the combination of an mTOR inhibitor plus an antimetabolite, it is anticipated that the mTOR inhibitor will be administered orally or parenterally, with parenterally being preferred, while the antimetabolite may be administered parenterally, orally, or by other acceptable means. For the CCI-779 combination with gemcitabine, it is preferred that the gemcitabine be administered parenterally. For the CCI-779 combination with 5-FU and leucovorin, it is preferred that the 5-FU and leucovorin are administered parenterally. These combination can be administered daily, weekly, or even once monthly. As typical for chemotherapeutic regimens, a course of chemotherapy may be repeated several weeks later, and may follow the same timeframe for administration of the two agents, or may be modified based on patient response.

Accordingly this invention also provides a product comprising an mTOR inhibitor and an antimetabolite antineoplastic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm in a mammal.

As typical with chemotherapy, dosage regimens are closely monitored by the treating physician, based on numerous factors including the severity of the disease, response to the disease, any treatment related toxicities, age, health of the patient, and other concomitant disorders or treatments.

Based on the results obtained with the representative CCI-779 plus antimetabolite combinations, it is projected that the initial i.v. infusion dosage of the mTOR inhibitor will be between about 0.1 and 100 mg/m^2, with between about 2.5

and 70 mg/m^2 being preferred. It is also preferred that the mTOR inhibitor be administered by i.v., typically over a 30 minute period, and administered about once per week. The initial dosages of the antimetabolite component will depend on the component used, and will be based initially on physician experience with the agents chosen.

Based on the results obtained with the CCI-779 plus antimetabolite combinations, it is projected that for the mTOR inhibitor plus gemcitabine combination, the initial i.v. infusion dosage of the mTOR inhibitor will be between about 0.1 and 100 mg/m^2, with between about 2.5 and 70 mg/m^2 being preferred, and the initial i.v. infusion dosage of gemcitabine will be between about 400 and 1500 mg/m^2, with between about 800 and 1000 mg/m^2 being preferred. It is initially projected that patients will receive a 30 minute i.v. infusion of the mTOR inhibitor, followed immediately or preceded by a 30 minute i.v. infusion of gemcitabine on days 1 and 8 of a 21 day treatment cycle. After one or more treatment cycles, the dosages can be adjusted upwards or downwards depending on the results obtained and the side effects observed.

Based on the results obtained, when CCI-779 is used in combination with 5-FU and leucovorin, it is projected that in an mTOR inhibitor plus 5-FU plus leucovorin regimen, the initial i.v. infusion dosage of the mTOR inhibitor will be between about 0.1 and 100 mg/m^2, with between about 2.5 and 70 mg/m^2 being preferred; the initial i.v. infusion dosage of leucovorin will be between about 50 and 500 mg/m^2, with about 200 mg/m^2 being preferred; and the initial i.v. infusion dosage of 5-FU will be between about 500 and 7500 mg/m^2, with between about 1000 and 5000 mg/m^2 being preferred. It is initially projected that the combination will be administered according to the following regimen: patients will receive a 1 hour i.v. infusion of leucovorin once weekly during each 6 week treatment cycle; immediately following each dose of leucovorin, 5-FU is administered as a 24-hour continuous i.v. infusion. The mTOR inhibitor will be administered beginning on day 8, of cycle 1, and will be given once weekly as a 30 minute i.v. infusion. Each 6 week treatment cycle is followed by a 1 week rest before beginning the next 6 week treatment cycle. After one or more treatment cycles, the dosages can be adjusted upwards or downwards depending on the results obtained and the side effects observed.

For commercially available antimetabolites, the existing dosage form can be used, with the dosages divided as need be. Alternatively, such agents or antimetabolites that are not commercially available can be formulated according to standard pharmaceutical practice. Oral formulations containing the active compounds of this invention may comprise any conventionally used oral forms, including tablets, capsules, buccal forms, troches, lozenges and oral liquids, suspensions or solutions. Capsules may contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (e.g. corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium lauryl sulfate, microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. Preferred surface modifying agents include nonionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidol silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

In some cases it may be desirable to administer the compounds directly to the airways in the form of an aerosol.

The compounds may also be administered parenterally or intraperitoneally. Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary

conditions of storage and use, these preparation contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

For the purposes of this disclosure, transdermal administrations are understood to include all administrations across the surface of the body and the inner linings of bodily passages including epithelial and mucosal tissues. Such administrations may be carried out using the present compounds, or pharmaceutically acceptable salts thereof, in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semi-permeable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

CLAIMS

What is claimed is:

1. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising an mTOR inhibitor and an antimetabolite antineoplastic agent.

2. A method of treating a neoplasm according to claim 1 wherein either the mTOR inhibitor, the antimetabolite, or both are provided in subtherapeutically effective amounts.

3. A method according to claim 2 in which the mTOR inhibitor is provided in a subtherapeutically effective amount.

4. A method according to claim 2 or claim 3 in which the antimetabolite is provided in a subtherapeutically effective amount.

5. A method according to any one of claims 1 to 4, wherein the neoplasm is one of the following:
renal cancer; soft tissue sarcoma; breast cancer; a neuroendocrine tumor of the lung; cervical cancer; uterine cancer;. a head and neck cancer; glioma; non-small cell lung cancer; prostate cancer; pancreatic cancer; lymphoma; melanoma; small cell lung cancer; ovarian cancer; colon cancer; esophageal cancer; gastric cancer; leukemia; colorectal cancer; or unknown primary cancer.

6. A method according to any one of claims 1 to 5, wherein the combination further comprises a biochemical modifying agent.

7. A method according to claim 6, wherein the biochemical modifying agent is leucovorin or levofolinate.

8. A method according to any one of claims 1 to 5 in which the antimetabolite is gemcitabine.

9. A method according to claim 8, wherein the neoplasm is pancreatic cancer.

10. A method according to any one of claims 1 to 5 in which the antimetabolite is 5-fluorouracil.

11. A method according to claim 10, in which the combination further comprises a biochemical modifying agent.

12. A method according to any one of claims 1 to 5 which comprises providing to said mammal an effective amount of a combination of an mTOR inhibitor, 5-fluorouracil, and leucovorin.

13. A method according to any one of claims 10 to 12, wherein the neoplasm is colorectal cancer.

14. A method according to any one of claims 1 to 13, wherein the mTOR inhibitor is a rapamycin.

15. A method according to claim 14, wherein the rapamycin is rapamycin.

16. A method according to claim 14, wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

17. A method according to claim 14, wherein the rapamycin is rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid.

18. An antineoplastic combination which comprises an effective amount of an mTOR inhibitor and an antimetabolite antineoplastic agent.

19. The combination of claim 18, which further comprises a biochemical modifying agent.

20. The combination according to claim 19, wherein the mTOR inhibitor is a rapamycin.

21. The combination according to claim 19, wherein the rapamycin is rapamycin.

22. The combination according to claim 19, wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

23. The combination according to claim 19, wherein the rapamycin is rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid.

24. A product comprising an mTOR inhibitor and an antimetabolite antineoplastic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm in a mammal.

25. A product as claimed in claim 24 further comprising a biochemical modifying agent.

26. A product as claimed in claim 24 or claim 25 wherein the neoplasm is one of the following:
renal cancer; soft tissue sarcoma; breast cancer; a neuroendocrine tumor of the lung; cervical cancer; uterine cancer;. a head and neck cancer; glioma; non-small cell lung cancer; prostate cancer; pancreatic cancer; lymphoma; melanoma; small cell lung cancer; ovarian cancer; colon cancer; esophageal cancer; gastric cancer; leukemia; colorectal cancer; or unknown primary cancer.

27. A product as claimed in any one of claims 24 to 26. wherein the mTOR inhibitor is a rapamycin.

28. A product according to claim 27, wherein the rapamycin is rapamycin.

29. A product according to claim 27, wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

30. A product according to claim 27, wherein the rapamycin is rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid.

31. A product according to any one of claims 24 to 30 in which the antimetabolite is gemcitabine or 5-fluorouracil.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 02/10912

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K45/06 A61K31/436 A61K31/7068 A61K31/513 A61K31/519 A61P35/00 //(A61K31/7068,31:436),(A61K31/7068,31:519,31:436), (A61K31/513,31:436),(A61K31/519,31:513,31:436)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, BIOSIS, CHEM ABS Data, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 52514 A (ELI LILLY GES MBH ;KONWALINKA GUENTHER (AT); MARGREITER RAIMUND (A) 21 October 1999 (1999-10-21)	1-9,14, 18-21, 24-28,31
Y	claims 6-15 ---	1-31
X	US 5 066 493 A (SEHGAL SURENDRA N ET AL) 19 November 1991 (1991-11-19)	1-6, 10-15, 18-21, 24-28,31
Y	claims ---	1-31
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2002	26/08/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Herrera, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ENG C P ET AL: "Activity of rapamycin (AY-22,989) against transplanted tumors." THE JOURNAL OF ANTIBIOTICS. JAPAN OCT 1984, vol. 37, no. 10, October 1984 (1984-10), pages 1231-1237, XP001098253 ISSN: 0021-8820	1-6, 10-15, 18-21, 24-28,31
Y	abstract	1-31
Y	ALEXANDRE J ET AL: "LA RAPAMYCINE ET LE CCI-779//RAPAMYCIN AND CCI-779" CANCER BULLETIN, MEDICAL ARTS PUB, HOUSTON, US, vol. 10, no. 86, October 1999 (1999-10), pages 808-811, XP001078856 ISSN: 0008-5448 abstract	1-31
Y	ALEXANDRE J ET AL: "PHASE I STUDY OF CCI-779,A NOVEL RAPAMYCIN ANALOG: PRELIMINARY RESULTS" PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW YORK, NY, US, vol. 41, no. 41, March 2000 (2000-03), page 613 XP001071217 ISSN: 0197-016X abstract	1-31
P,X	PUNT C J A ET AL: "A phase I study of escalating doses of CCI-779 in combination with 5-fluorouracil and leucovorin in patients with advanced solid tumors." EUROPEAN JOURNAL OF CANCER, vol. 37, no. Supplement 6, October 2001 (2001-10), page S17 XP001093978 11th European Cancer Conference;Lisbon, Portugal; October 21-25, 2001, October, 2001 ISSN: 0959-8049 abstract	1-7, 10-31

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BONI J ET AL: "Pharmacokinetics of escalating doses of CCI-779 in combination with 5-fluorouracil and leucovorin in patients with advanced solid tumors." EUROPEAN JOURNAL OF CANCER, vol. 37, no. Supplement 6, October 2001 (2001-10), page S68 XP001093977 11th European Cancer Conference;Lisbon, Portugal; October 21-25, 2001, October, 2001 ISSN: 0959-8049 abstract	1-7, 10-31

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Continuation of Box I.2

The subject-matter of present claims is defined by means of the functional features: "mTOR inhibitor" and "antimetabolite antineoplastic agent"

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 02/10912

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: –
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 02/10912

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9952514	A	21-10-1999	WO	9952514 A2	21-10-1999
			AU	3126799 A	01-11-1999
			BR	9909617 A	12-12-2000
			CA	2327431 A1	21-10-1999
			CN	1312719 T	12-09-2001
			EP	1071433 A2	31-01-2001
			HU	0102671 A2	28-12-2001
			JP	2002511405 T	16-04-2002
			NO	20005179 A	13-10-2000
			PL	343769 A1	10-09-2001
			TR	200002969 T2	22-01-2001
			TR	200101633 T2	21-06-2002
			TW	466112 B	01-12-2001
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			JP	55073616 A	03-06-1980
			ZA	7905449 A	26-11-1980

Form PCT/ISA/210 (patent family annex) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(10) International Publication Number
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(51) International Patent Classification[7]: A61K 31/395, A61P 35/00, A61K 31/4706 // (A61K 31/395, 31:4706)

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(30) Priority Data:
60/310,646 7 August 2001 (07.08.2001) US

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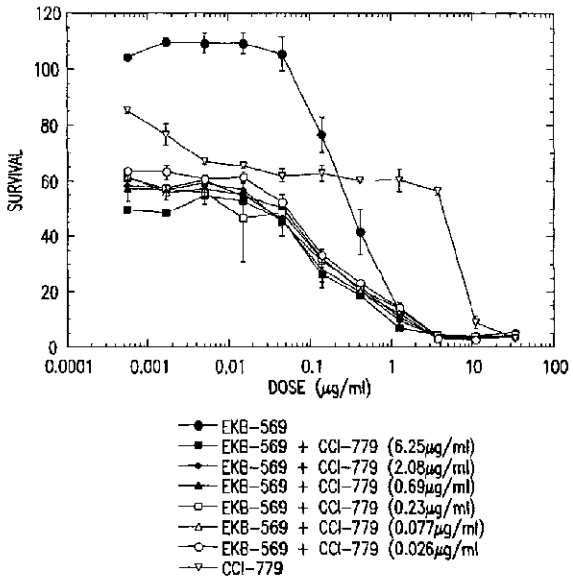
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTINEOPLASTIC COMBINATIONS



(57) Abstract: This invention provides the use of a combination of CCI-779 and EKB-569 in the treatment of neoplasms.

WO 03/020266 A1

ANTINEOPLASTIC COMBINATIONS

BACKGROUND OF THE INVENTION

This invention relates to the use of combinations of rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) and 4-dimethylamino-but-2-enoic acid [4-(3-chloro-4-fluoro-phenylamino)-3-cyano-7-ethoxy-quinolin-6-yl]-amide (EKB-569).

Rapamycin is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus, which was found to have antifungal activity, particularly against Candida albicans, both in vitro and in vivo [C. Vezina et al., J. Antibiot. 28, 721 (1975); S.N. Sehgal et al., J. Antibiot. 28, 727 (1975); H. A. Baker et al., J. Antibiot. 31, 539 (1978); U.S. Patent 3,929,992; and U.S. Patent 3,993,749]. Additionally, rapamycin alone (U.S. Patent 4,885,171) or in combination with picibanil (U.S. Patent 4,401,653) has been shown to have antitumor activity.

The immunosuppressive effects of rapamycin have been disclosed in FASEB 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); R. Y. Calne et al., Lancet 1183 (1978); and U.S. Patent 5,100,899]. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

Rapamycin is also useful in preventing or treating systemic lupus erythematosus [U.S. Patent 5,078,999], pulmonary inflammation [U.S. Patent 5,080,899], insulin dependent diabetes mellitus [U.S. Patent 5,321,009], skin disorders, such as psoriasis [U.S. Patent 5,286,730], bowel disorders [U.S. Patent 5,286,731], smooth muscle cell proliferation and intimal thickening following vascular injury [U.S. Patents 5,288,711 and 5,516,781], adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1], ocular inflammation [U.S. Patent 5,387,589], malignant carcinomas [U.S. Patent 5,206,018], cardiac inflammatory disease [U.S. Patent 5,496,832], and anemia [U.S. Patent 5,561,138].

Rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) is ester of rapamycin which has demonstrated significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of hydroxyesters of rapamycin, including CCI-779, are disclosed in U.S. Patent 5,362,718.

CCI-779 exhibits cytostatic, as opposed to cytotoxic properties, and may delay the time to progression of tumors or time to tumor recurrence. CCI-779 is considered to have a mechanism of action that is similar to that of sirolimus. CCI-779 binds to and forms a complex with the cytoplasmic protein FKBP, which inhibits an enzyme, mTOR (mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]). Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G_1 to S. The mechanism of action of CCI-779 that results in the G1 S phase block is novel for an anticancer drug.

In vitro, CCI-779 has been shown to inhibit the growth of a number of histologically diverse tumor cells. Central nervous system (CNS) cancer, leukemia (T-cell), breast cancer, prostate cancer, and melanoma lines were among the most sensitive to CCI-779. The compound arrested cells in the G1 phase of the cell cycle.

In vivo studies in nude mice have demonstrated that CCI-779 has activity against human tumor xenografts of diverse histological types. Gliomas were particularly sensitive to CCI-779 and the compound was active in an orthotopic glioma model in nude mice. Growth factor (platelet-derived)-induced stimulation of a human glioblastoma cell line in vitro was markedly suppressed by CCI-779. The growth of several human pancreatic tumors in nude mice as well as one of two breast cancer lines studied in vivo also was inhibited by CCI-779.

Protein tyrosine kinases are a class of enzymes that catalyze the transfer of a phosphate group from ATP or GTP to tyrosine residue located on protein substrates. Protein tyrosine kinases clearly play a role in normal cell growth. Many of the growth factor receptor proteins function as tyrosine kinases and it is by this process that they effect signaling. The interaction of growth factors with these receptors is a necessary event in normal regulation of cell growth. However, under certain conditions, as a

result of either mutation or overexpression, these receptors can become deregulated; the result of which is uncontrolled cell proliferation which can lead to tumor growth and ultimately to the disease known as cancer [Wilks A.F., *Adv. Cancer Res.*, *60*, 43 (1993) and Parsons, J.T.; Parsons, S.J., *Important Advances in Oncology*, DeVita V.T. Ed., J.B. Lippincott Co., Phila., 3 (1993)]. Among the growth factor receptor kinases and their proto-oncogenes that have been identified and which are targets of the compounds of this invention are the epidermal growth factor receptor kinase (EGF-R kinase, the protein product of the erbB oncogene), and the product produced by the erbB-2 (also referred to as the neu or HER2) oncogene. Since the phosphorylation event is a necessary signal for cell division to occur and since overexpressed or mutated kinases have been associated with cancer, an inhibitor of this event, a protein tyrosine kinase inhibitor, will have therapeutic value for the treatment of cancer and other diseases characterized by uncontrolled or abnormal cell growth. For example, overexpression of the receptor kinase product of the erbB-2 oncogene has been associated with human breast and ovarian cancers [Slamon, D. J., et. al., *Science, 244*, 707 (1989) and *Science*, *235* , 1146 (1987)]. Deregulation of EGF-R kinase has been associated with epidermoid tumors [Reiss, M., et. al., *Cancer Res.*, *51*, 6254 (1991)], breast tumors [Macias, A., et. al., *Anticancer Res.*, *7*, 459 (1987)], and tumors involving other major organs [Gullick, W.J., *Brit. Med. Bull.*, *47*, 87 (1991)]. Because of the importance of the role played by deregulated receptor kinases in the pathogenesis of cancer, many recent studies have dealt with the development of specific PTK inhibitors as potential anti-cancer therapeutic agents [some recent reviews: Burke. T.R., *Drugs Future, 17*, 119 (1992) and Chang, C.J.; Geahlen, R.L., *J. Nat. Prod.*, *55*, 1529 (1992)].

4-Dimethylamino-but-2-enoic acid [4-(3-chloro-4-fluoro-phenylamino)-3-cyano-7-ethoxy-quinolin-6-yl]-amide (EKB-569) is an EGFR kinase inhibitor which has significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of EGFR kinase inhibitors, such as EKB-569, are disclosed in U.S. Patent 6,002,008.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows cytotoxicity curves of EKB-569, CCI-779, and combinations of EKB-569 + CCI-779 in HCT116 cells.

FIG. 2 shows isobolograms (at the 50% effect level) of a EKB-569 + CCI-779 combination.

FIG. 3 shows isobolograms for EKB-569 + CCI-779 combinations derived from different endpoints ranging from 50-65%.

FIG. 4 shows a 3-dimensional analysis of the synergistic interaction of a EKB-569 + CCI-779 combination.

FIG. 5 shows a contour plot of the 3-dimensional synergy plot of a EKB-569 + CCI-779 combination.

DESCRIPTION OF THE INVENTION

This invention provides the use of combinations of CCI-779 and EKB-569 as antineoplastic combination chemotherapy. In particular, these combinations are useful in the treatment of renal cancer, soft tissue cancer, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small lung cell cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. This invention also provides combinations of CCI-779 and EKB-569 for use as antineoplastic combination chemotherapy, in which the dosage of either CCI-779 or EKB-569 or both are used in subtherapeutically effective dosages.

As used in accordance with this invention, the term "treatment" means treating a mammal having a neoplastic disease by providing said mammal an effective amount of a combination of CCI-779 and EKB-569 with the purpose of inhibiting growth of the neoplasm in such mammal, eradication of the neoplasm, or palliation of the mammal.

As used in accordance with this invention, the term "providing," with respect to providing the combination, means either directly administering the combination, or administering a prodrug, derivative, or analog of one or both of the components of the combination which will form an effective amount of the combination within the body.

The preparation of CCI-779 is described in U.S. Patent 5,362,718, which is hereby incorporated by reference. An improved preparation of CCI-779 is disclosed in US patent application SN 09/670,358, which is hereby incorporated by reference. When CCI-779 is used as an antineoplastic agent, it is projected that initial i.v. infusion dosages will be between about 0.1 and 100 mg/m^2 when administered on a daily dosage regimen (daily for 5 days, every 2-3 weeks), and between about 0.1 and 1000 mg/m^2 when administered on a once weekly dosage regimen. Oral or intravenous infusion are the preferred routes of administration, with intravenous being more preferred.

EKB-569 can be prepared according to the procedures described in US Patent 6,002,008, which is incorporated by reference. Preferred procedures for the preparation of EKB-569 are provided herein. When EKB-569 is used as an antineoplastic agent it is projected that the initial oral dosage will be between 1 and 100 mg per day. Depending on patient tolerance, EKB-569 can be administered daily for a treatment period, such as 14 days, followed by a rest period (no drug administered), or can be administered on a continuous basis for a longer treatment period (for example, 6 months or longer).

The antineoplastic activity of the CCI-779 plus EKB-569 combination was confirmed in *in vitro* standard pharmacological test procedure; the following briefly describes the procedure used and the results obtained.

Cell Proliferation Procedure - HCT 116 colon adenocarcinoma cells were maintained in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 50 μg/ml gentamicin (Life Technologies) under 7% CO_2 at 37°C. Cells were plated in 96-well microtiter dishes (6000 cells/well) in 200 μl RPMI 1640 medium containing 5% FBS and 50 μg/ml gentamicin and incubated overnight at 37°C. Compound dilutions were prepared in the same medium, at 5X final concentration, and 50 μl of the drug dilution was added to the cell-containing wells. For studies involving combinations of two drugs, serial dilutions of one compound were prepared in the presence of a fixed dose of a second compound. Alternatively, a checkerboard dilution series was employed. Cells were cultured for three days in the presence of the drugs. Untreated cells were

included as controls. The percentage of surviving cells was determined using sulforhodamine B (SRB, Sigma-Aldrich, St Louis, MO), a protein binding dye. Cellular protein was precipitated in each well by the addition of 50 μl of 50% cold trichloroacetic acid. After 1 hour, the plates were washed extensively in water and dried. SRB dye reagent (0.4% SRB in 1% acetic acid, 80 μl per well) was added and plates were kept at room temperature for ten minutes. Plates were then washed thoroughly in 1% acetic acid and dried. Cell-associated dye was dissolved in 10 mM Tris (150 μl) and the absorbance was read at 540 nm in a microtiter plate reader. The concentration of compound that caused a fixed percentage inhibition of growth was determined by plotting cell survival (relative to untreated cells) against the compound dose.

Synergy Evaluation - Isobolograms were used to study the interaction of two pharmacological agents. Here, the concentration of each drug alone which produces a certain endpoint (e.g 50% inhibition of cell growth, IC_{50}), is plotted on the two graphical axes. The straight line connecting the two points represents equally effective concentrations of all combinations of the two drugs if the interaction is purely additive. A shift of the isobologram to the left of the predicted cytotoxicity (curve with concave side up) represents a synergistic interaction. Conversely, a shift to the right (isobologram with the convex side up) represents an antagonistic interaction. When isobolograms for different endpoints were plotted on the same graph, the concentration of each drug was expressed as the fraction of the concentration of each drug alone that produced the same effect. This produces a symmetrical isobologram with unit-less measures on each axis, and allows a direct comparison of different endpoints.

A second model for studying drug interactions was proposed by Prichard and Shipman [Antiviral Research. *14:* 181-206 (1990)]. This is a 3-dimensional model: one for each drug and the third for the biological effect. Theoretical additive interactions are calculated from the individual dose-response curves, based on a dissimilar sites model of additivity (Bliss independence). The calculated additive surface, representing predicted cytotoxicity is subtracted from the experimental surface to reveal areas of enhanced toxicity (synergy) or reduced toxicity (antagonism). The resulting surface appears as a horizontal plane at 0% inhibition

above the calculated additive surface, if the interaction is additive. Peaks and valleys deviating from this plane are indicative of synergy and antagonism, respectively. MacSynergyII, a Microsoft Excel-based software was used to perform all calculations automatically. This spreadsheet calculates the theoretical additive interactions, and locates and quantifies synergistic or antagonistic interactions that are significant at the 95% confidence levels. The results were plotted as a 3-dimensional plot, or as a contour plot.

Results - HCT 116 cells were chosen as they express low, but detectable levels of EGFR, and are sensitive to inhibition by EGFR inhibitors. The cells are somewhat resistant to CCI-779, but are inhibited by high doses (5-10 μg/ml) of this drug. HCT-116 cells were cultured in the presence of EKB-569 alone, CCI-779 alone, or a dilution series of EKB-569 with fixed doses of CCI-779. Following growth for 3 days, cell survival was determined using the SRB test procedure. Cytotoxicity curves are shown in Fig. 1. EKB-569 produced an IC_{50} value of 0.31 μg/ml in HCT116 cells. When this compound was combined with 2.08 μg/ml CCI-779 (which caused 41% inhibition of growth when administered alone), the IC_{50} value is reduced to 0.03 μg/ml, a 10-fold decrease. When combined with 0.026 μg/ml CCI-779 (which alone inhibits cell proliferation by 36 %), the IC_{50} value dropped to 0.051 μg/ml, a 6-fold decrease. Similar results were observed when dose-response curves were produced with CCI-779 in the presence of fixed doses of EKB-569. To identify the nature of this drug interaction, isobolograms (at 50% effect level) of the combination of EKB-569 and CCI-779 were generated (Fig. 2). The isobologram was deeply indented with the concave side up, indicating a substantial synergistic interaction between the two drugs. At the most synergistic point, 0.03 μg/ml of EKB-569 combined with 0.077 μg/ml CCI-779 was iso-effective with 0.31 μg/ml of EKB-569 alone or 4.3 μg/ml CCI-779 alone (IC_{50} for each drug alone). Thus, a 10-fold reduction in the dose of EKB-569 and a 50-fold reduction in the dose of CCI-779 was required to inhibit cell proliferation by 50% when the drugs were combined, compared to either drug alone. Isobolograms derived from different endpoints, ranging from 50 to 65% were also examined. As shown in Fig 3., the isobolograms produced were almost superimposable, indicating synergy at all effect levels tested.

The interaction between EKB-569 and CCI-779 was also evaluated using a 3-dimensional analysis. Here, pharmacological interactions are presented in a 3-dimensional plot with the plane at 0% representing additive interaction, and peaks and valleys representing areas of synergy or antagonism, respectively, between the two drugs. In Fig. 4, the combination of EKB-569 and CCI-779 resulted in a broad area of synergistic interaction, consistent with the results shown in the isobologram studies. A contour plot of the 3-dimensional synergy plot facilitates the identification of the concentrations of drugs at which greatest synergistic toxicity occurs (Fig. 5). A broad area of synergy was observed at 0.0005 to 3 μg/ml CCI-779 and 0.16 to 0.4 μg/ml EKB-569. Within this area, two peaks of maximum synergy occurred at 0.0005 to 0.003 μg/ml and 0.05 to 0.3 μg/ml of CCI-779 and 0.25 to 0.37 μg/ml EKB-569.

Based on the results of these standard pharmacological test procedures, combinations of CCI-779 plus EKB-569 acted synergistically together, and are useful as antineoplastic therapy. More particularly, these combinations are useful in the treatment of renal carcinoma, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. As these combinations contain at least two active antineoplastic agents, the use of such combinations also provides for the use of combinations of each of the agents in which one or both of the agents is used at subtherapeutically effective dosages, thereby lessening toxicity associated with the individual chemotherapeutic agent.

In providing chemotherapy, multiple agents having different modalities of action are typically used as part of a chemotherapy "cocktail." It is anticipated that the combinations of this invention will be used as part of a chemotherapy cocktail that may contain one or more additional antineoplastic agents depending on the nature of the neoplasia to be treated. For example, this invention also covers the use of the CCI-779/EKB-923 combination used in conjunction with other chemotherapeutic agents, such as antimetabolites (i.e., 5-fluorouracil, floxuradine, thioguanine,

cytarabine, fludarabine, 6-mercaptopurine, methotrexate, gemcitabine, capecitabine, pentostatin, trimetrexate, or cladribine); DNA crosslinking and alkylating agents (i.e., cisplatin, carboplatin, streptazoin, melphalan, chlorambucil, carmustine, methclorethamine, lomustine, bisulfan, thiotepa, ifofamide, or cyclophosphamide); hormonal agents (i.e., , tamoxifen, roloxifen, toremifene, anastrozole, or letrozole); antibiotics (i.e., plicamycin, bleomycin, mitoxantrone, idarubicin, dactinomycin, mitomycin, doxorubicin or daunorubicin); immunomodulators (i.e., interferons, IL-2, or BCG); antimitotic agents (i.e., estramustine, paclitaxel, docetaxel, vinblastine, vincristine, or vinorelbine); topoisomerase inhibitors (i.e., topotecan, irinotecan, etoposide, or teniposide.); and other agents (i.e., hydroxyurea, trastuzumab, altretamine, retuximab, L-asparaginase, or gemtuzumab ozogamicin).

As used in this invention, the combination regimen can be given simultaneously or can be given in a staggered regimen, with CCI-779 being given at a different time during the course of chemotherapy than EKB-923. This time differential may range from several minutes, hours, days, weeks, or longer between administration of the two agents. Therefore, the term combination does not necessarily mean administered at the same time or as a unitary dose, but that each of the components are administered during a desired treatment period. The agents may also be administered by different routes. For example, in the combination of CCI-779 plus EKB-569, it is anticipated that the CCI-779 will be administered orally or parenterally, with parenterally being preferred, while the EKB-569 may be administered parenterally, orally, or by other acceptable means. These combination can be administered daily, weekly, or even once monthly. As typical for chemotherapeutic regimens, a course of chemotherapy may be repeated several weeks later, and may follow the same timeframe for administration of the two agents, or may be modified based on patient response.

As typical with chemotherapy, dosage regimens are closely monitored by the treating physician, based on numerous factors including the severity of the disease, response to the disease, any treatment related toxicities, age, health of the patient, and other concomitant disorders or treatments.

Based on the results obtained with the CCI-779 plus EKB-569 combinations, it is projected that the initial i.v. infusion dosage of CCI-779 will be between about 0.1

and 100 mg/m^2, with between about 2.5 and 70 mg/m^2 being preferred. It is also preferred that the CCI-779 be administered by i.v., typically over a 30 minute period, and administered about once per week. The initial daily dosages of EKB-569 will be between about 1 and 100 mg, with between 5 and 75 mg being preferred. After one or more treatment cycles, the dosages can be adjusted upwards or downwards depending on the results obtained and the side effects observed.

Oral formulations containing the active compounds of this invention may comprise any conventionally used oral forms, including tablets, capsules, buccal forms, troches, lozenges and oral liquids, suspensions or solutions. Capsules may contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (e.g. corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium lauryl sulfate, microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. Preferred surface modifying agents include nonionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

In some cases it may be desirable to administer the compounds directly to the airways in the form of an aerosol.

The compounds may also be administered parenterally or intraperitoneally. Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparation contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

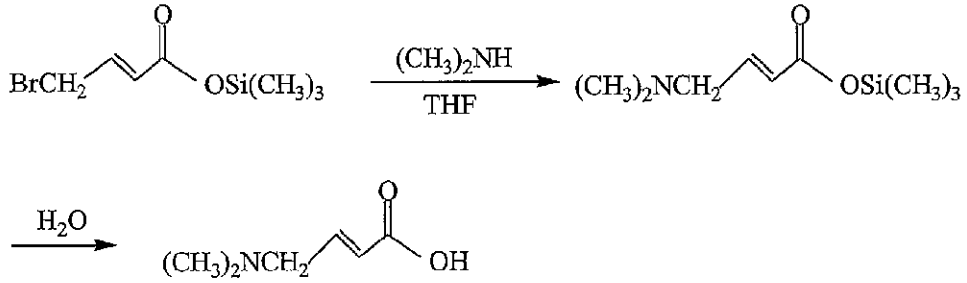
For the purposes of this disclosure, transdermal administrations are understood to include all administrations across the surface of the body and the inner linings of bodily passages including epithelial and mucosal tissues. Such administrations may be carried out using the present compounds, or pharmaceutically acceptable salts thereof, in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semi-permeable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

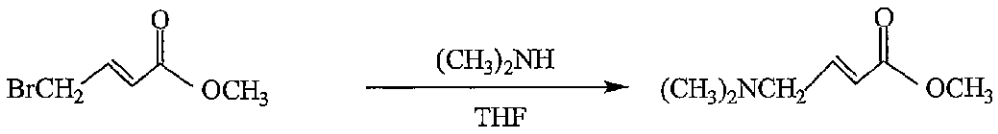
The following provides the preparation of EKB-569 from commercially available starting materials or starting materials that can be made according to available literature procedures.

Preparation of 4-dimethylaminocrotonic acid from TMS-4-bromocrotonate



211 ml dimethylamine (2M in THF, 0.422 moles) was added drop-wise to a solution of 50 g TMS-4-bromocrotonate (0.211 moles, 75.9% by GC-MS) in 250 ml of THF at 0-50°C under N_2. The reaction mixture was stirred at room temperature for 30 minutes. A white solid by-product was filtered off. 2 ml water was added to the filtrate followed by seeding. The crystals formed were filtered and washed with ether to give 18.3 g (from two crops) off -white solid product. Yield was 67.2% (98% purity by GC-MS, NMR was consistent with the structure).

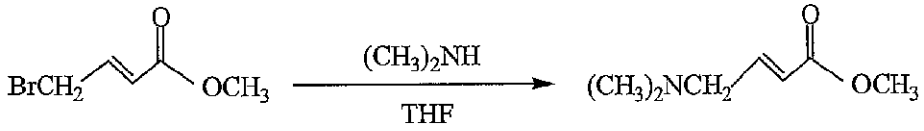
Preparation of methyl 4-dimethylaminocrotonate from methyl-4-bromocrotonate



120 ml dimethylamine (2M in THF, 0.24 moles) was added drop-wise to a solution of 20 g methyl 4-bromocrotonate (85% purity, 0.095 moles) in 150 ml of THF at 0-50°C under N_2. The reaction mixture was stirred for 15 minutes at room temperature. TLC

(9:1 CH_2Cl_2: MeOH with few drops of Et_3N) showed residual methyl 4-bromo-crotonate. The reaction mixture was heated to 40-450C for 15 minutes. A white solid by-product was filtered off. The filtrate was evaporated to give a yellow oil (14 g). The yellow oil was dissolved in 100 ml CH_2Cl_2 and washed with H_2O twice. The aqueous layer was back extracted with 100 ml CH_2Cl_2. The CH_2Cl_2 layers were combined, dried over $MgSO_4$ and filtered. The filtrate was evaporated to give an oil (12 g). Yield was 88%. NMR indicated desired product with trace methyl 4- bromocrotonate.

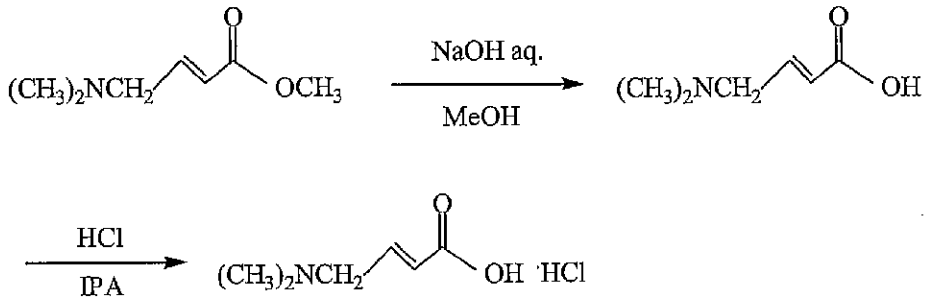
Preparation of Methyl 4-N,N-dimethylaminocrotonate hydrochloride on large scale:



A 3L flask was charged with tetrahydrofuran (0.71 kg, 0.80 L). Methyl 4-bromocrotonate (0.20 kg, 0.13 L, d = 1.522 g/mL) was added and rinsed with tetrahydrofuran (0.18 kg, 0.20 L). The solution was stirred and cooled to 0-10°C. An additional funnel was charged with a solution of dimethylamine in tetrahydrofuran and added over (1 h 15 min) keeping the temperature at 0-10°C. The mixture was stirred for a minimum of 30 mins and checked for reaction completion by TLC. The reaction was complete when there is ≤ 2% detectable starting material (methyl 4-bromo-crotonate) present. The mixture was filtered cold on a Buchner funnel into a 3 L multi-neck flask, rinsed with pre-chilled (0-10°C) tetrahydrofuran (2 x 0.18 kg, 2 x 0.20 L), and suction maintained until dripping stops. The flask was equipped with an agitator, thermometer, and a setup for vacuum distillation. The solution was concentrated by distillation under a reduced pressure of (125-200 mm Hg) and at a maximum pot temperature of (40°C) to a pot volume of (200 mL). Isopropanol (0.22 kg, 0.28 L) was added and the mixture cooled to 0-10°C. The distillation stillhead was replaced with an addition funnel charged with a solution of HCl in isopropanol, which was added over 45 min until pH of 2.0-3.0 was reached, while maintaining a temperature 0-10°C. The mixture was held for a minimum 30 min, and fileted cold on a Buchner funnel, rinsed with isopropanol (2 x 0.12 kg, 2 x 0.15 L). The filter cake was dammed and

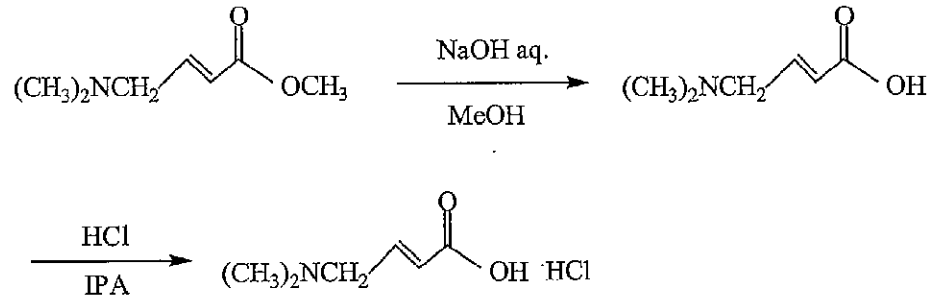
suction maintained until dripping stopped. The product was dried in a vacuum oven at 50°C and 10 mm Hg for 18-20 h.

Preparation of 4-dimethylaminocrotonic acid hydrochloride from methyl 4-dimethylaminocrotonate



A NaOH solution (3.35 g in 25 ml H_2O, 0.084 moles) was added drop-wise to a solution of 12 g methyl 4-dimethylaminocrotonate (0.084 moles) in 100 ml MeOH at room temperature. The reaction mixture was heated to 40-45°C for 1 hour then cooled to room temperature. The pH was adjusted to 1~2 with 5 N HCl. The mixture was concentrated to a thick oil which was triturated with dehydrated alcohol to form a solid. The solid by-product was filtered off. The filtrate was evaporated to an oil which was triturated with IPA. Seven (7.0) g of white solid product was obtained. Yield was 50% with the purity 86.3 % by GC-MS.

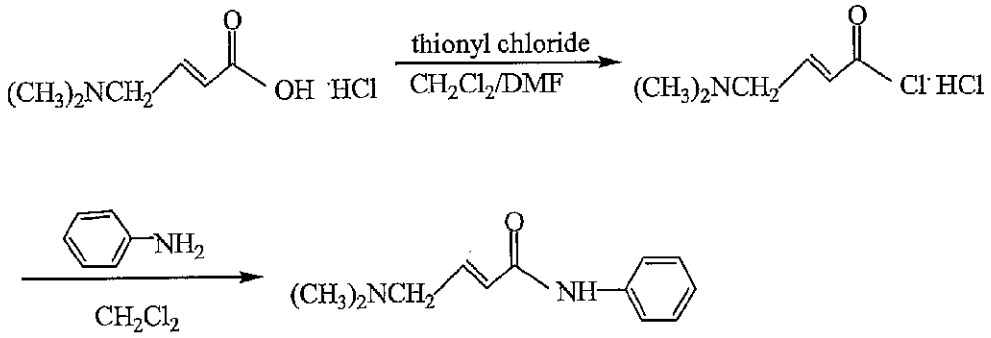
Preparation of 4-N,N-dimethylaminocrotonic acid hydrochloride on large scale



A 2 L multi-neck flask was equipped with agitator, thermometer, addition funnel, and nitrogen protection. The flask was charged with ethanol (0.39 kg, 0.50 L). Methyl 4-N,N-dimethylamino crotonate hydrochloride (0.125 kg) was added and rinsed with

ethanol (0.10 kg, 0.125 L). The suspension was stirred and cooled to 0-10°C. The addition funnel was charged with sodium hydroxide (50%) (0.11 kg, 0.072 L, d=1.53 g/mL) and addd over 20 min keeping the temperature at 0-10°C. A slight exotherm was observed and the mixture turned yellow. The mixture was stirred for a minimum of 15 min, and then warmed to 18-22°C, and held for a minimum of 4 h. The reaction was checked for completion by TLC . The reaction is complete when there is ≤ 2% detectable starting material (methyl 4-N,N-dimethylaminocrotonate hydrochloride) present. The mixture was cooled to 0-10°C. An addition funnel was charged with a solution of HCl in isopropanol and added over 40 min until pH 2.0-3.0 was attained, while maintaining the pot temperature of 0-10°C. The mixture was sturred for a minimum of 30 min, and filtered cold on a Buchner funnel into a 2 L multi-neck flask, rinsed with cold ethanol (0-10°C) (2 x 0.05 kg, 2 x 0.063 L) with suction maintained until dripping stops. The flask was equpped with an agitator, thermometer, and setup for vacuum distillation. Solvent was removed under a reduced pressure of 50-100 mm Hg and at a maximum pot temperature of (40°C) to a pot volume of 160-180 mL. Isopropanol (0.049 kg, 0.063 L) was added, and the mixture warmed to 35-40°C over 10 min. Acetone (0.10 kg, 0.13 L) was added over 20 min while maintaining the pot temperature at 35-40°C. The mixture was seeded and cooled to ambient temperature 20-25°C, and held there for a minimum of 12-18 h. The mixture was cooled to 0-10°C, held there for a minimum of 1 h. A mixture of isopropanol (0.049 kg, 0.063 L) and acetone (0.10 kg, 0.13 L) was prepared, stirred to homogenize, and cooled to 0-10°C. The mixture was filtered cold on a Buchner funnel, rinsed with isopropanol/ acetone (2 x 0.074 kg, 2 x 0.096 L), and the filter caked dammed while maintaining suction until dripping stopped. The product was dried in a vacuum oven at 50°C and 10 mm Hg for 18-20 h.

Preparation of 4-dimethylaminocrotonyl anilide from 4-dimethylaminocrotonic acid hydrochloride



Thionyl chloride (0.36 ml, 0.005 moles) was added drop-wise to a solution of 0.33 g 4-dimethylaminocrotonic acid hydrochloride (0.002 moles) in 15 ml CH_2Cl_2 containing 2 drops of DMF at 0°C under N2. The reaction mixture was refluxed for 30 min. Then 0.72 ml aniline (0.008 moles) was added drop-wise to the reaction mixture at 0°C and stirred for 1 hour at room temperature. A solid by-product was filtered. The filtrate was evaporated to give an oil (0.6 g). GC-MS data shows that the oil is 11.7% 4-dimethylaminocrotonic acid hydrochloride and 85% of desired product.

Preparation and isolation of 4-N,N-dimethylaminocrotonoylchloride hydrochloride

A well stirred suspension 4-dimethylaminocrotonic acid hydrochloride (5.0 g, 30 mmol) in cold (0°C) THF (40 mL) and DMF (2 pipet drops) was treated with oxalyl chloride (3.15 mL, 36 mmol). The mixture was stirred at 20-25 °C for 3 h then cooled to 0°C and held for 30 min. The solids were collected on Buchner funnel (under a blanket of nitrogen) and washed with cold (0°C) THF (3 x 5 mL). The product was dried under vacuum (~ 1 torr) at 40-50°C for 3 h to give 4.0 g of 4-dimethylaminocrotonoyl chloride hydrochloride. This material is characterized as its methyl ester by treatment of the solid with methanol.

Alternatively, the title compound can be prepared in CH_3CN and used directly for the coupling step:

Preparation of EKB-569

A 3 L multi-neck flask was equipped with an agitator, thermometer, dip tube, and nitrogen protection. The flask was charged with N-methyl pyrrolidinone (0.77 kg, 0.75 L, d=1.033 g/mL). At ambient temperature, 4-[3-chloro-4-fluorophenyl]amino-6-amino-3-cyano-7-ethoxy quinoline (0.0748 kg) [see, US Patent 6,002,008] was added and the mixture stirred while heating to 40-45 °C and hold for 15 min. The flask was cooled to 0-10°C. The mixture containing 4-N,N-dimethylaminocrotonoyl chloride hydrochloride was transferred via dip tube and positive nitrogen pressure to the 3 L flask over 30-45 min, while maintaining 0-10°C. The mixture was kept at 0-10°C for a minimum of 2 h. The reaction was checked for completion by HPLC. The reaction is complete when there is $\leq 2\%$ of the starting material (4-[3-chloro-4-fluorophenyl]-amino-6-amino-3-cyano-7-ethoxy quinoline) present. A 12 L multi-neck flask equipped with agitator, thermometer, dip tube, and nitrogen protection was charged with water (2.61 kg, 2.61 L). Sodium bicarbonate (0.209 kg) was added and stirred until a solution was obtained. The solution was cooled to 20-24°C. The NMP-CH_3CN mixture was transferred, via dip tube and positive nitrogen pressure, to the 12 L flask over 45-60 min, while maintaining 20-24°C. The mixture was maintained at 20-24°C for a minimum of 1 h, and filtered on a Buchner funnel, and rinsed with water (3 x 0.40 kg, 3 x 0.40 L) with suction being maintained until dripping stops. The product was dried in a vacuum oven at 50°C and 10 mm Hg for 28-30 h to give 78.5 g (86% yield) of product.

CLAIMS

1. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising CCI-779 and EKB-569.

2. The method according to claim 1, wherein the neoplasm is renal cancer.

3. The method according to claim 1, wherein the neoplasm is soft tissue sarcoma.

4. The method according to claim 1, wherein the neoplasm is breast cancer.

5. The method according to claim 1, wherein the neoplasm is a neuroendocrine tumor of the lung.

6. The method according to claim 1, wherein the neoplasm is cervical cancer.

7. The method according to claim 1, wherein the neoplasm is uterine cancer.

8. The method according to claim 1, wherein the neoplasm is a head and neck cancer.

9. The method according to claim 1, wherein the neoplasm is glioma.

10. The method according to claim 1, wherein the neoplasm is non-small cell lung cancer.

11. The method according to claim 1, wherein the neoplasm is prostate cancer.

12. The method according to claim 1, wherein the neoplasm is pancreatic cancer.

13. The method according to claim 1, wherein the neoplasm is lymphoma.

14. The method according to claim 1, wherein the neoplasm is melanoma.

15. The method according to claim 1, wherein the neoplasm is small cell lung cancer.

16. The method according to claim 1, wherein the neoplasm is ovarian cancer.

17. The method according to claim 1, wherein the neoplasm is colon cancer.

18. The method according to claim 1, wherein the neoplasm is esophageal cancer.

19. The method according to claim 1, wherein the neoplasm is gastric cancer.

20. The method according to claim 1, wherein the neoplasm is leukemia.

21. The method according to claim 1, wherein the neoplasm is colorectal cancer.

22. The method according to claim 1, wherein the neoplasm is unknown primary cancer.

23. A method according to any one of Claims 1 to 22 which comprises providing to said mammal an effective amount of a combination comprising CCI-779

and EKB-569, wherein either CCI-779, EKB-569, or both are provided in subtherapeutically effective amounts.

24. A method according to claim 23 in which CCI-779 is provided in a subtherapeutically effective amount.

25. A method according to claim 23 in which EKB-569 is provided in a subtherapeutically effective amount.

26. A method according to claim 23 in which both CCI-779 and EKB-569 are provided in subtherapeutically effective amounts.

27. An antineoplastic combination which comprises an antineoplastic effective amount of a combination of CCI-779 and EKB-569.

28. A composition according to claim 27 in which CCI-779 is provided in a subtherapeutically effective amount.

29. A composition according to claim 27 in which EKB-569 is provided in a subtherapeutically effective amount.

30. A composition according to claim 27 in which both CCI-779 and EKB-569 are provided in subtherapeutically effective amounts.

31. A product comprising CCI-779 and EKB-569 as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm in a mammal

32. A product according to claim 31 in which the neoplasm is one of the following: renal cancer, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, a head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer or unknown primary cancer.

33. Use of CCI-779 and EKB-569 in the preparation of a medicament for the treatment of neoplasm in a mammal.

34. A use according to claim 33 in which the neoplasm is one of the following: renal cancer, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, a head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer or unknown primary cancer.

35. A use according to claim 33 or 34 in which CCI-779 is provided in a subtherapeutically effective amount.

36. A use according to claim 33 or 34 in which EKB-569 is provided in a subtherapeutically effective amount.

37. A use according to claim 33 or 34 in which both CCI-779 and EKB-569 are provided in subtherapeutically effective amounts.

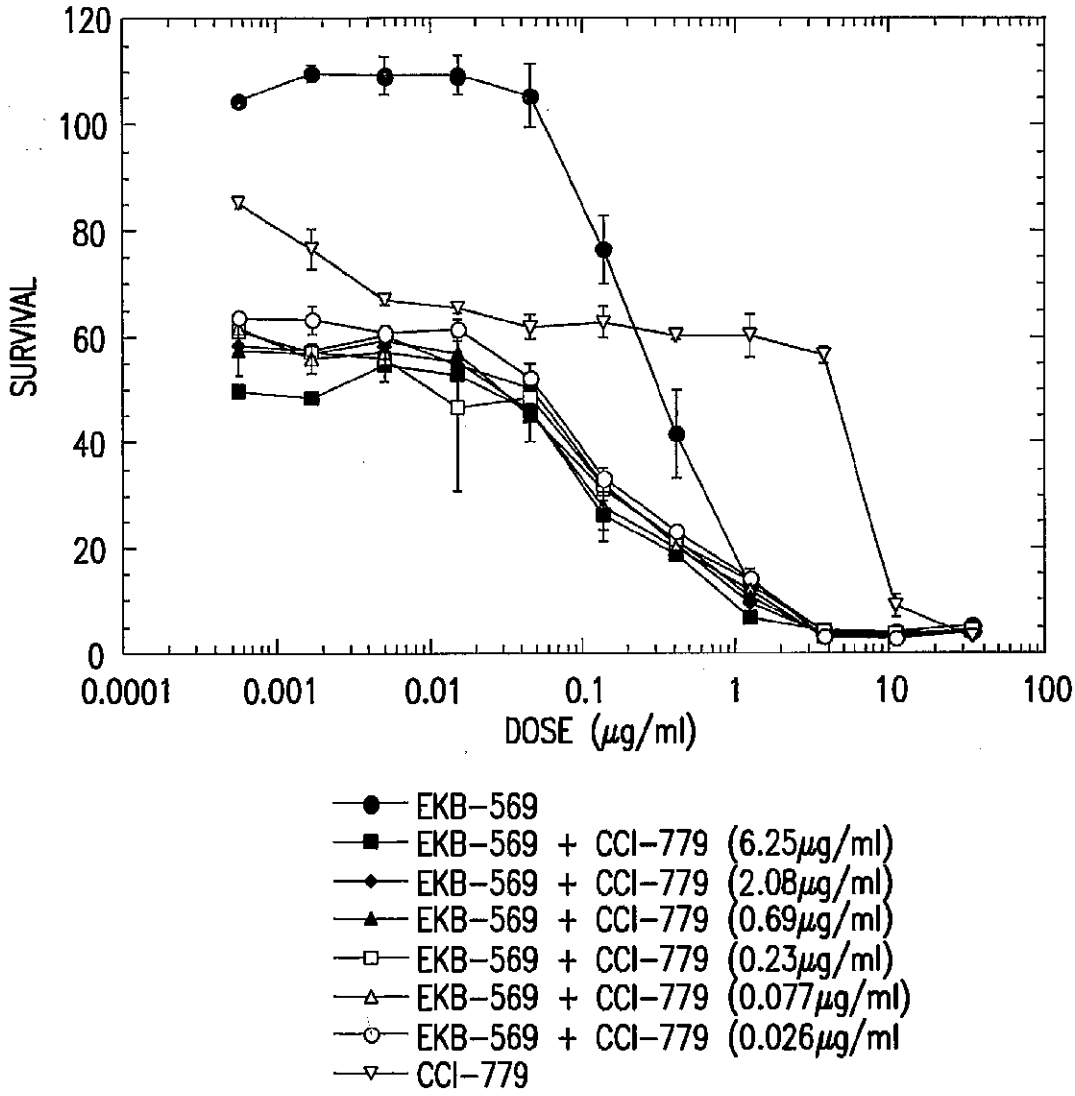

120
100
80
60
40
20
0
SURVIVAL
0.0001
0.001
0.01
0.1
1
10
100
DOSE (μg/ml)
EKB-569
EKB-569 + CCI-779 (6.25μg/ml)
EKB-569 + CCI-779 (2.08μg/ml)
EKB-569 + CCI-779 (0.69μg/ml)
EKB-569 + CCI-779 (0.23μg/ml)
EKB-569 + CCI-779 (0.077μg/ml)
EKB-569 + CCI-779 (0.026μg/ml
CCI-779

FIG.1

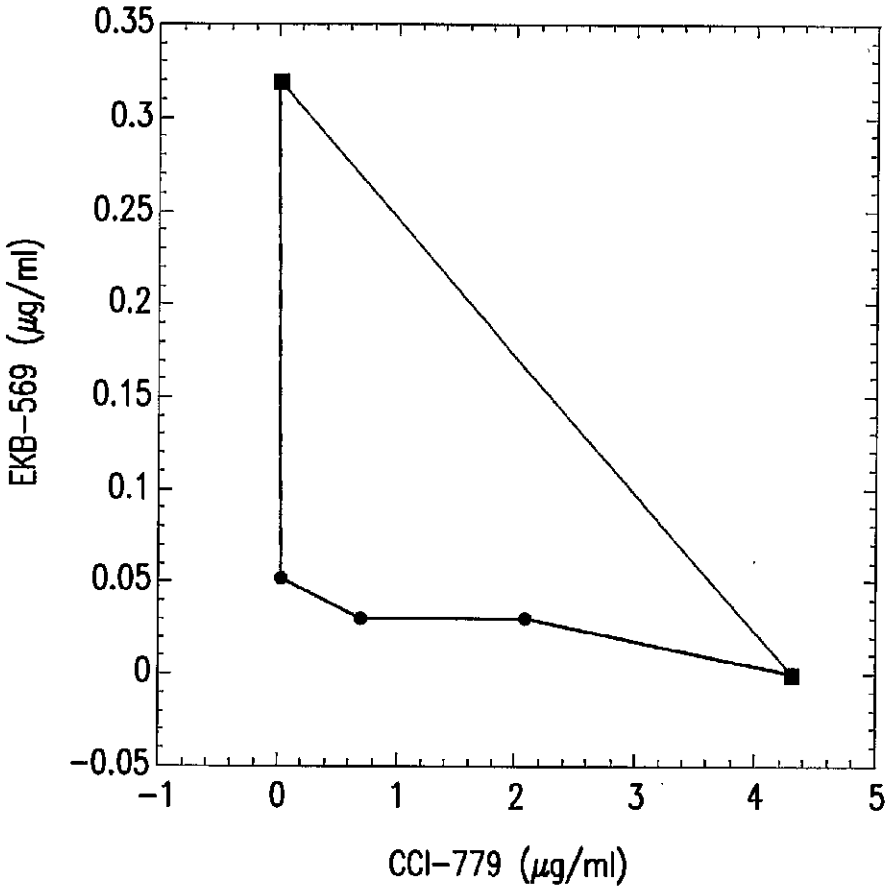


FIG.2

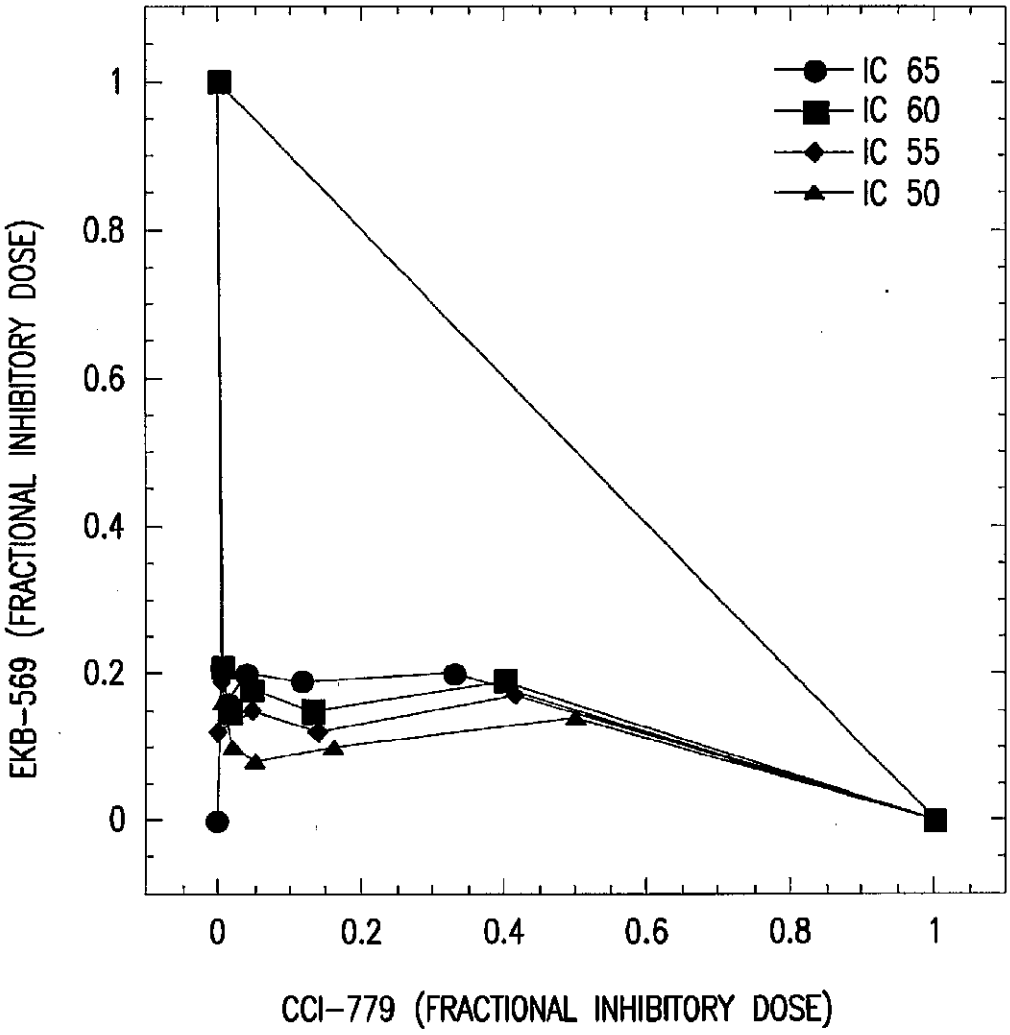
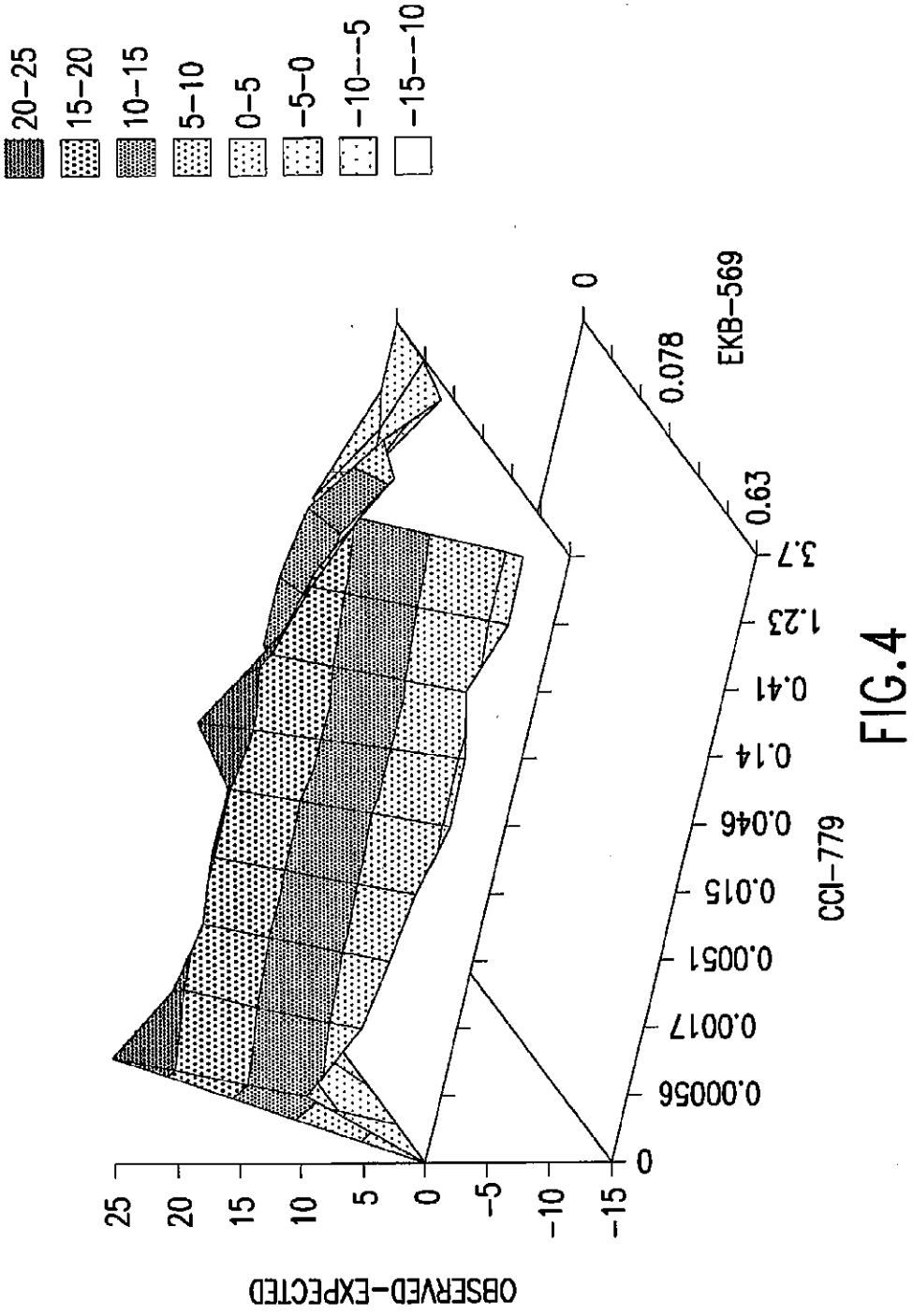

IC 65
IC 60
IC 55
IC 50
EKB-569 (FRACTIONAL INHIBITORY DOSE)
CCI-779 (FRACTIONAL INHIBITORY DOSE)
0
0.2
0.4
0.6
0.8
1

FIG.3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5–10
0–5
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-15–-10
OBSERVED–EXPECTED
25
20
15
10
5
0
-5
-10
-15
EKB–569
0
0.078
0.63
3.7
CCI–779
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0.046
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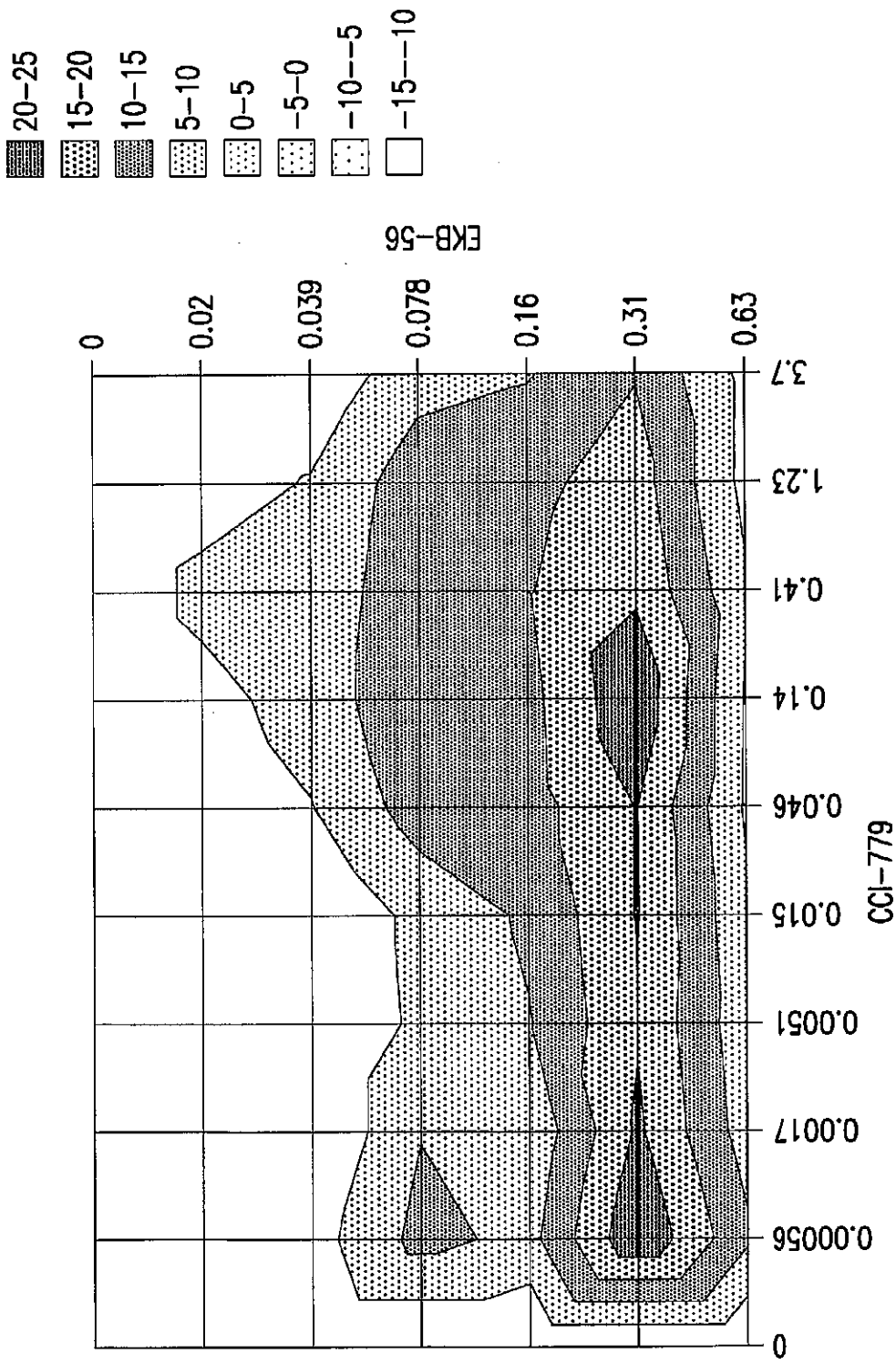
0.41
1.23
3.7

FIG.4


20–25
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10–15
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–5–0
–10–5
–15–10
EKB–56
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0.078
0.16
0.31
0.63
3.7
1.23
0.41
0.14
0.046
0.015
0.0051
0.0017
0.00056
0
CCI–779

FIG.5

INTERNATIONAL SEARCH REPORT

PCT/US 02/24841

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/395 A61P35/00 A61K31/4706 //(A61K31/395,31:4706)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EMBASE, EPO-Internal, WPI Data, PAJ, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUPTA R A ET AL: "Combinations for cancer prevention." NATURE MEDICINE, (2000 SEP) 6 (9) 974-5., XP002223840 the whole document, especially from page 974, right-hand column, line 17 to page 975, left-hand column, line 18 ---	1-37
A	TORRANCE C J ET AL: "Combinatorial chemoprevention of intestinal neoplasia." NATURE MEDICINE, (2000 SEP) 6 (9) 1024-8, XP002223841 the whole document --- -/--	1-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 2002	10/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Venturini, F

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

PCT/US 02/24841

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GREENBERGER L M (REPRINT) ET AL: "EKB - 569: a new irreversible inhibitor of epidermal growth factor receptor tyrosine kinase for the treatment of cancer." CLINICAL CANCER RESEARCH, (NOV 2000) VOL. 6, SUPP. 'S!, PP. 388-388. PUBLISHER: AMER ASSOC CANCER RESEARCH, PO BOX 11806, BIRMINGHAM, AL 35202. ISSN: 1078-0432., XP001120789 WYETH AYERST RES, PEARL RIVER, NY 10965 abstract	1-37
A	GEOERGER B ET AL: "ANTITUMOR ACTIVITY OF THE RAPAMYCIN ANALOG CCI-779 IN HUMAN PRIMITIVE NEUROECTODERMAL TUMOR/MEDULLOBLASTOMA MODELS AS SINGLE AGENT AND IN COMBINATION CHEMOTHERAPY" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 4, no. 61, 15 February 2001 (2001-02-15), pages 1527-1532, XP001079526 ISSN: 0008-5472 the whole document	1-37
A	US 5 066 493 A (SEHGAL SURENDRA N ET AL) 19 November 1991 (1991-11-19) the whole document	1-37
P,A	WO 02 13802 A (AMERICAN HOME PROD) 21 February 2002 (2002-02-21) the whole document	1-37

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

PCT/US 02/24841

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/US 02/24841

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5066493 A	19-11-1991	US 5206018 A	27-04-1993
		US 4885171 A	05-12-1989
		BE 877700 A1	14-01-1980
		JP 55073616 A	03-06-1980
		ZA 7905449 A	26-11-1980
WO 0213802 A	21-02-2002	AU 8313901 A	25-02-2002
		WO 0213802 A2	21-02-2002
		US 2002045638 A1	18-04-2002

Form PCT/ISA/210 (patent family annex) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 September 2005 (09.09.2005)

PCT

(10) International Publication Number
WO 2005/082411 A1

(51) International Patent Classification[7]: A61K 39/395, 31/40, 31/44

(21) International Application Number: PCT/US2005/005547

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(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/546,850 23 February 2004 (23.02.2004) US

(71) Applicants *(for all designated States except US)*: **SUGEN, INC.** [US/US]; 230 East Grand Avenue, South San Francisco, CA-94080 (US). **DANA-FARBER CANCER INSTITUTE INC** [US/US]; 44 Binney Street, Boston, MA 02115 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHRISTENSEN, James, G.** [US/US]; 4276 Kerwood Court, San Diego, CA 92130 (US). **SALGIA, Ravi** [US/US]; 1426 N. Vernon Avenue, Park ridge, IL 60068 (US).

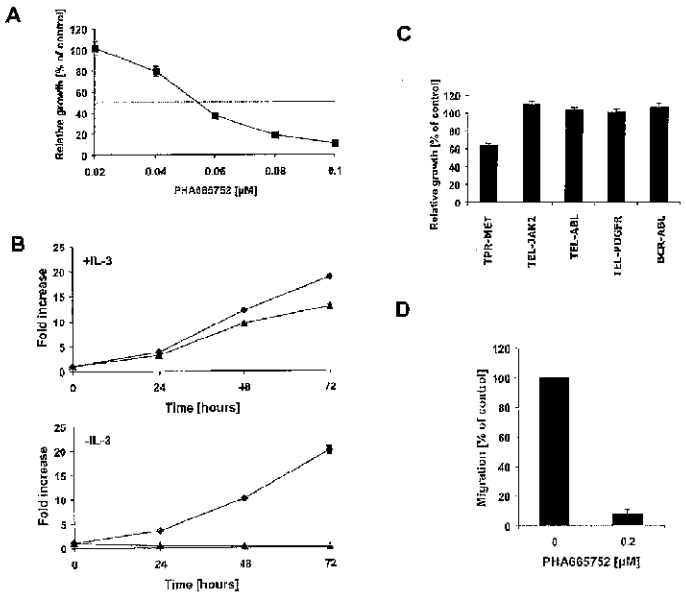
(74) Agent: **GROLZ, Edward, W.**; Scully, Scott, Murphy & Presser, 400 Old Country Road, Ste 300, Garden City, NY 11530 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: METHOD OF TREATING ABNORMAL CELL GROWTH USING C-MET AND-TOR INHIBITORS



(57) Abstract: The invention provides a method of treataing abnormal cell growth in a mammal, such as a human, by administering to the mammal a therapeutically effective amount of a c-MET inhibitor and a mammalian target of rapamycin (mTOR) inhibitor. Figure 1 shows PHA665752 inhibition of cell growth of TPR-MET transformed BaF3 cells.

WO 2005/082411 A1

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

Published:

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METHOD OF TREATING ABNORMAL CELL GROWTH USING C-MET AND M-TOR INHIBITORS

This application claims the benefit of U.S. Provisional Application Serial No. 60/546,850, filed February 23, 2004, the disclosure of which is incorporated herein by reference in its entirety.

Field of the Invention

This invention relates to methods of treatment of abnormal cell growth, such as cancer, in mammals. In particular, the invention provides methods of treatment of abnormal cell growth using a c-MET inhibitor and an mTOR inhibitor.

Background

c-MET receptor tyrosine kinase (RTK) has been shown in many human cancers to be involved in oncogenesis, tumor progression with enhanced cell motility and invasion, as well as metastasis (see, e.g., Ma, P.C., Maulik, G., Christensen, J. & Salgia, R. (2003b). *Cancer Metastasis Rev*, 22, 309-25; Maulik, G., Shrikhande, A., Kijima, T., Ma, P.C., Morrison, P.T. & Salgia, R. (2002b). *Cytokine Growth Factor Rev*, 13, 41-59). c-MET can be activated through overexpression or mutations in various human cancers including small cell lung cancer (SCLC) (Ma, P.C., Kijima, T., Maulik, G., Fox, E.A., Sattler, M., Griffin, J.D., Johnson, B.E. & Salgia, R. (2003a). *Cancer Res*, 63, 6272-6281). Several c-MET inhibitors are known, including small molecule, ligand and antibody inhibitors (see references herein).

It would be desirable to have novel methods of treating abnormal cell growth, such as cancers, using such c-MET inhibitors in combination with other agents that enhance the efficacy of the c-MET inhibitors.

Summary of the Invention

In one embodiment, the invention provides a method of treating abnormal cell growth in a mammal, such as a human, by administering to the mammal a therapeutically effective amount of a c-MET inhibitor and an mTOR inhibitor.

mTOR is an important signaling intermediate molecule downstream of the PI3K/AKT pathway that inhibits apoptosis, and is important in nutritional status checkpoint (see, e.g., Grunwald, V., DeGraffenried, L., Russel, D., Friedrichs, W.E., Ray, R.B. & Hidalgo, M. (2002). *Cancer Res*, 62, 6141-5; Nave, B.T., Ouwens, M., Withers, D.J., Alessi, D.R. & Shepherd, P.R. (1999). *Biochem J*, 344 Pt 2, 427-31; Scott, P.H., Brunn, G.J., Kohn, A.D., Roth, R.A. & Lawrence, J.C., Jr. (1998). *Proc Natl Acad Sci U S A*, 95, 7772-7; Stolovich, M., Tang, H., Hornstein, E., Levy, G., Cohen, R., Bae, S.S., Birnbaum, M.J. & Meyuhas, O. (2002). *Mol Cell Biol*, 22, 8101-13). mTOR is a large (M_r ~289,000) multidomain serine/threonine kinase, and is a member of the PI3K family of protein kinases based on homology within its catalytic domain.

Mammalian target of rapamycin ("mTOR") regulates the activity of at least two proteins involved in the translation of specific cell cycle regulatory proteins. One of these proteins, p70s6 kinase, is phosphorylated by mTOR on serine 389 as well as threonine 412. This phosphorylation can be observed in growth factor treated cells by Western blotting of whole cell extracts of these cells with antibody specific for the phosphoserine 389 residue. As used herein, the term "mTOR

inhibitor" means a compound or ligand which inhibits cell replication by blocking progression of the cell cycle from G1 to S by inhibiting the phosphorylation of serine 389 of p70s6 kinase by mTOR. One skilled in the art can readily determine if a compound, such as a rapamycin derivative, is an mTOR inhibitor. A specific method of making such determination is disclosed in U.S. Patent Application Publication No. 2003/0008923, the disclosure of which is incorporated herein by reference in its entirety.

A preferred mTOR inhibitor, rapamycin, is described in U.S. Patent No. 3,929,992, the disclosure of which is incorporated herein by reference in its entirety. Rapamycin is also know by its USAN generic name, sirolimus.

As used herein, the term "rapamycin derivatives" includes compounds having the rapamycin core structure as defined in U.S. Patent Application Publication No. 2003/0008923, which may be chemically or biologically modified while still retaining mTOR inhibiting properties. Such derivatives include esters, ethers, oximes, hydrazones, and hydroxylamines of rapamycin, as well as compounds in which functional groups on the rapamycin core structure have been modified, for example, by reduction or oxidation. Pharmaceutically acceptable salts of such compounds are also considered to be rapamycin derivatives.

Specific examples of esters and ethers of rapamycin are esters and ethers of the hydroxyl groups at the 42- and/or 31-positions of the rapamycin nucleus, and esters and ethers of a hydroxyl group at the 27-position (following chemical reduction of the 27-ketone). Specific examples of oximes, hydrazones, and hydroxylamines are of a ketone at the 42-position (following oxidation of the 42-hydroxyl group) and of 27-ketone of the rapamycin nucleus.

Examples of 42- and/or 31-esters and ethers of rapamycin are disclosed in the following patents, which are hereby incorporated by reference in their entireties: alkyl esters (U.S. Pat. No. 4,316,885); aminoalkyl esters (U.S. Pat. No. 4,650,803); fluorinated esters (U.S. Pat. No. 5,100,883); amide esters (U.S. Pat. No. 5,118,677); carbamate esters (U.S. Pat. No. 5,118, 678); silyl ethers (U.S. Pat. No. 5,120,842); aminoesters (U.S. Pat. No. 5,130,307); acetals (U.S. Pat. No. 5,51,413); aminodiesters (U.S. Pat. No. 5,162,333); sulfonate and sulfate esters (U.S. Pat. No. 5,177,203); esters (U.S. Pat. No. 5,221,670); alkoxyesters (U.S. Pat. No. 5,233,036); O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Pat. No. 5,258,389); carbonate esters (U.S. Pat. No. 5,260,300); arylcarbonyl and alkoxycarbonyl carbamates (U.S. Pat. No. 5,262,423); carbamates (U.S. Pat. No. 5,302,584); hydroxyesters (U.S. Pat. No. 5,362,718); hindered esters (U.S. Pat. No. 5,385,908); heterocyclic esters (U.S. Pat. No. 5,385,909); gem-disubstituted esters (U.S. Pat. No. 5,385,910); amino alkanoic esters (U.S. Pat. No. 5,389,639); phosphorylcarbamate esters (U.S. Pat. No. 5, 391,730); carbamate esters (U.S. Pat. No. 5,411,967); carbamate esters (U. S. Pat. No. 5,434,260); amidino carbamate esters (U.S. Pat. No. 5,463,048) ; carbamate esters (U.S. Pat. No. 5,480,988); carbamate esters (U.S. Pat. No. 5,480,989); carbamate esters (U.S. Pat. No. 5,489,680); hindered N- oxide esters (U.S. Pat. No. 5,491,231); biotin esters (U.S. Pat. No. 5, 504,091); O-alkyl ethers (U.S. Pat. No. 5,665,772); and PEG esters of rapamycin (U.S. Pat. No. 5,780,462).

Examples of 27-esters and ethers of rapamycin are disclosed in U. S. Pat. No. 5,256,790, which is hereby incorporated by reference in its entirety.

Examples of oximes, hydrazones, and hydroxylamines of rapamycin are disclosed in U.S. Pat. Nos. 5,373,014, 5,378,836, 5,023,264, and 5, 563,145, which are hereby incorporated by reference. The preparation of these oximes, hydrazones, and hydroxylamines is disclosed in the above listed patents. The preparation of 42-oxorapamycin is disclosed in U.S. Pat. No. 5,023,263, which is hereby incorporated by reference.

Other compounds within the scope of "rapamycin derivatives" include those compounds and classes of compounds referred to as "rapalogs" in, for example, WO 98/02441 and references cited therein, and "epirapalogs" in, for example, WO 01/14387 and references cited therein, the disclosures of which are incorporated herein by reference in their entireties.

Another compound within the scope of "rapamycin derivatives" is everolimus, a 4-O-(2-hydroxyethyl)-rapamycin derived from a macrolide antibiotic produced by Streptomyces hygroscopicus (Novartis). Everolimus is also known as Certican, RAD-001 and SDZ-RAD.

Another preferred mTOR inhibitor is tacrolimus, a macrolide lactone immunosuppressant isolated from the soil fungus Streptomyces tsukubaensis. Tacrolimus is also known as FK 506, FR 900506, Fujimycin, L 679934, Tsukubaenolide, Protopic and Prograf.

Another preferred mTOR inhibitor is ABT-578 an antiproliferative agent (Abbott Laboratories). ABT-578 is believed to inhibit smooth muscle cell proliferation with a cytostatic effect resulting from the inhibition of mTOR.

Other preferred mTOR inhibitors include AP-23675, AP-23573, and AP-23841 (Ariad).

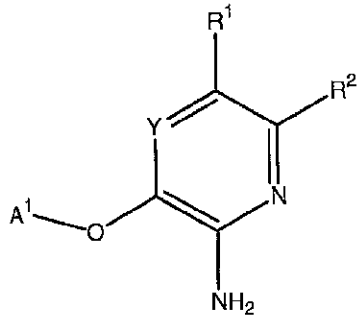
Preferred rapamycin derivatives include everolimus, CCI-779 [rapamycin 42-ester with 3-hydroxy-2- (hydroxymethyl)-2-methylpropionic acid; U.S. Pat. No. 5,362,718]; 7-epi-rapamycin; 7-thiomethyl-rapamycin; 7-epi-trimethoxyphenyl-rapamycin; 7-epi-thiomethyl-rapamycin; 7-demethoxy-rapamycin; 32- demethoxy-rapamycin; 2-desmethyl-rapamycin; and 42-O-(2-hydroxy)ethyl rapamycin [U.S. Pat. No. 5,665,772].

In one embodiment, the c-MET inhibitor is a small molecule c-MET inhibitor. Examples of c-MET inhibitors include the 5-aralkylsulfonyl-3-(pyrrole-2ylmethylidene)-2-indolinone compounds disclosed in U.S. Patent No. 6,599,902, and the compounds disclosed in WO 2001/60814, the disclosures of which are incorporated herein in their entireties. One skilled in the art can readily identify those compounds suitable as c-MET inhibitors by carrying out the assays as described, for example, in U.S. Patent No. 6,599,902.

Preferred c-MET inhibitors include those having c-MET inhibitory activity as defined by any one or more of IC_{50}, Ki, or percent inhibition. One skilled in the art can readily determine if a compound has such activity by carrying out the appropriate assay. In one embodiment, particularly preferred compounds have a c-MET IC_{50} of less than 5 μM, or less than 2 μM, or less than 1 μM, or less than 500 nM, or less than 400 nM, or less than 300 nM, or less than 200 nM, or less than 100 nM, or less than 50 nM. In another embodiment, particularly preferred compounds have a c-MET Ki of less than 5 μM or less than 2 μM, or less than 1 μM, or less than 500 nM, or less than

400 nM, or less than 300 nM, or less than 200 nM, or less than 100 nM, or less than 50 nM. In another embodiment, particularly preferred compounds have a c-MET inhibition at 1 μM of at least 10% or at least 20% or at least 30% or at least 40% or at least 50% or at least 60% or at least 70% or at least 80% or at least 90%. Methods of determining these c-MET activity values are described in U.S. Provisional Patent Application No. 60/449,588, filed February 26, 2003, and U.S. Provisional Application No. 60/540,229, filed January 29, 2004, published as WO 04/076412, the disclosures of which are incorporated herein by reference in their entireties.

In one embodiment, the c-MET inhibitor is a compound of formula **1**



1

wherein:

Y is N or CR^{12};

R^1 is selected from C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups;

R^2 is hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, and each hydrogen in R^2 is optionally substituted by one or more R^8 groups;

R^3 is halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, each hydrogen in R^3 is optionally substituted by one or more R^8 groups, and R^3 groups on adjacent atoms may combine to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

each R^4, R^5, R^6 and R^7 is independently hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl; or any two of R^4, R^5, R^6 and R^7 bound to the same nitrogen atom may, together with the nitrogen to which they are bound, be combined to form a 3 to 12 membered heteroalicyclic or 5-12 membered heteroaryl group optionally containing 1 to 3 additional heteroatoms selected from N, O, and S; or

any two of R^4, R^5, R^6 and R^7 bound to the same carbon atom may be combined to form a C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic or 5-12 membered heteroaryl group; and each hydrogen in R^4, R^5, R^6 and R^7 is optionally substituted by one or more R^8 groups;

each R^8 is independently halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -CN, -O-C_{1-12} alkyl, -O-$(CH_2)_nC_{3-12}$ cycloalkyl, -O-$(CH_2)_nC_{6-12}$ aryl, -O-$(CH_2)_n$(3-12 membered heteroalicyclic) or -O-$(CH_2)_n$(5-12 membered heteroaryl); and each hydrogen in R^8 is optionally substituted by one or more R^{11} groups;

A^1 is $-(CR^9R^{10})_n$-A^2 except that:

(i) when Y is N and R^1 is substituted or unsubstituted aryl or substituted or unsubstituted heteroaryl, A^1 is $-(CR^9R^{10})_n$-A^2 and n is not zero; and

(ii) when Y is N and R^2 is H and A^1 is m-chlorobenzyl, R^1 is not unsubstituted piperazine;

each R^9 and R^{10} is independently hydrogen, halogen, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -$S(O)_mR^4$, -$SO_2NR^4R^5$, -$S(O)_2OR^4$, -NO_2, $-NR^4R^5$, -$(CR^6R^7)_nOR^4$, -CN, -$C(O)R^4$, -$OC(O)R^4$, -$NR^4C(O)R^5$, -$(CR^6R^7)_nC(O)OR^4$, -$(CR^6R^7)_nNCR^4R^5$, -$NR^4C(O)NR^5R^6$, -$NR^4S(O)_pR^5$ or -$C(O)NR^4R^5$; R^9 and R^{10} may combine to form a C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, C_{6-12} aryl or 5-12 membered heteroaryl ring; and each hydrogen in R^9 and R^{10} is optionally substituted by one or more R^3 groups;

A^2 is C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic, and A^2 is optionally substituted by one or more R^3 groups;

each R^{11} is independently halogen, C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -O-C_{1-12} alkyl, -O-$(CH_2)_nC_{3-12}$ cycloalkyl, -O-$(CH_2)_nC_{6-12}$ aryl, -O-$(CH_2)_n$(3-12 membered heteroalicyclic), -O-$(CH_2)_n$(5-12 membered heteroaryl) or -CN, and each hydrogen in R^{11} is optionally substituted by one or more groups selected from halogen, -OH, -CN, -C_{1-12} alkyl which may be partially or fully halogenated, -O-C_{1-12} alkyl which may be partially or fully halogenated, -CO, -SO and -SO_2;

R^{12} is hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -$S(O)_mR^4$, -$SO_2NR^4R^5$, -$S(O)_2OR^4$, -NO_2, $-NR^4R^5$, -$(CR^6R^7)_nOR^4$, -CN, -$C(O)R^4$, -$OC(O)R^4$, -$O(CR^6R^7)_nR^4$, -$NR^4C(O)R^5$, -$(CR^6R^7)_nC(O)OR^4$, -$(CR^6R^7)_nNCR^4R^5$, -$C(=NR^6)NR^4R^5$, -$NR^4C(O)NR^5R^6$, -$NR^4S(O)_pR^5$ or -$C(O)NR^4R^5$, and each hydrogen in R^{12} is optionally substituted by one or more R^3 groups;

R^1 and R^2 or R^1 and R^{12} may be combined together to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

m is 0, 1 or 2;

n is 0, 1, 2, 3 or 4; and

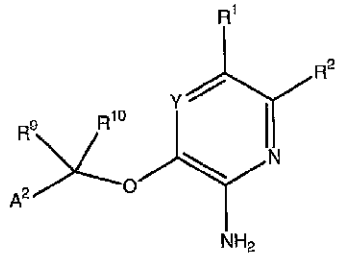
p is 1 or 2;

or a pharmaceutically acceptable salt, solvate or hydrate thereof.

In a particular aspect of this embodiment, Y is N. In a preferred aspect, R^1 is not piperazine. In another preferred aspect, R^1 is not heteroalicyclic.

In another particular aspect of this embodiment, Y is CR^{12}.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, the compound has formula **1a**



1a

wherein A^2 is C_{6-12} aryl or 5-12 membered heteroaryl optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^1 is selected from C_{6-12} aryl and 5-12 membered heteroaryl, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is selected from C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, A^2 is substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^2 is hydrogen, R^9 and R^{10} are independently C_{1-4} alkyl, and A^2 is phenyl substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a furan, thiopene, pyrrole, pyrroline, pyrrolidine, dioxolane, oxazole, thiazole, imidazole, imidazoline, imidazolidine, pyrazole, pyrazoline, pyrazolidine, isoxazole, isothiazole, oxadiazole, triazole, thiadiazole, pyran, pyridine, piperidine, dioxane, morpholine, dithiane, thiomorpholine, pyridazine, pyrimidine, pyrazine, piperazine, triazine, trithiane or phenyl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a furan, thiopene, pyrrole, pyrroline, pyrrolidine, dioxolane, oxazole, thiazole, imidazole, imidazoline, imidazolidine, pyrazole, pyrazoline, pyrazolidine, isoxazole, isothiazole, oxadiazole, triazole, thiadiazole, pyran, pyridine, piperidine, dioxane, morpholine, dithiane, thiomorpholine, pyridazine,

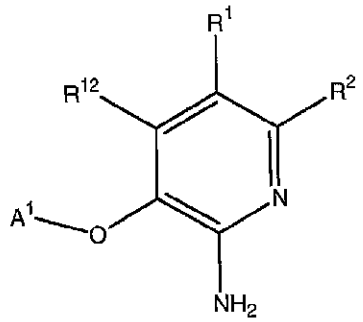
pyrimidine, pyrazine, triazine, trithiane or phenyl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups. In a more particular aspect, R^1 is not heteroalicyclic.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a fused ring heteroaryl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a $-SO_2NR^4R^5$ group.

Specific compounds of this embodiment, and methods of synthesizing compounds of this embodiment, are described in U.S. Provisional Patent Application No. 60/449,588, filed February 26, 2003, and U.S. Provisional Application No. 60/540,229, filed January 29, 2004, published as WO 04/076412, the disclosures of which are incorporated herein by reference in their entireties.

In another embodiment, the c-MET inhibitor is a compound of formula **2**



2

wherein:

R^1 is selected from C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups;

R^2 is hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, and each hydrogen in R^2 is optionally substituted by one or more R^8 groups;

R^3 is halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, each hydrogen in R^3 is optionally substituted by one or more R^8 groups, and R^3 groups on adjacent atoms may combine to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

each R^4, R^5, R^6 and R^7 is independently hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl; or any two of R^4, R^5, R^6 and R^7 bound to the same nitrogen atom may, together with the nitrogen to which they are bound, be combined to form a 3 to 12 membered heteroalicyclic or 5-12 membered heteroaryl group optionally containing 1 to 3 additional heteroatoms selected from N, O, and S; or any two of R^4, R^5, R^6 and R^7 bound to the same carbon atom may be combined to form a C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic or 5-12 membered heteroaryl group; and each hydrogen in R^4, R^5, R^6 and R^7 is optionally substituted by one or more R^8 groups;

each R^8 is independently halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -CN, -O-C_{1-12} alkyl, -O-$(CH_2)_nC_{3-12}$ cycloalkyl, -O-$(CH_2)_nC_{6-12}$ aryl, -O-$(CH_2)_n$(3-12 membered heteroalicyclic) or -O-$(CH_2)_n$(5-12 membered heteroaryl); and each hydrogen in R^6 is optionally substituted by one or more R^{11} groups;

A^1 is $-(CR^9R^{10})_n-A^2$;

each R^9 and R^{10} is independently hydrogen, halogen, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$; R^9 and R^{10} may combine to form a C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, C_{6-12} aryl or 5-12 membered heteroaryl ring; and each hydrogen in R^9 and R^{10} is optionally substituted by one or more R^3 groups;

A^2 is C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic, and A^2 is optionally substituted by one or more R^3 groups;

each R^{11} is independently halogen, C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -O-C_{1-12} alkyl, -O-$(CH_2)_nC_{3-12}$ cycloalkyl, -O-$(CH_2)_nC_{6-12}$ aryl, -O-$(CH_2)_n$(3-12 membered heteroalicyclic), -O-$(CH_2)_n$(5-12 membered heteroaryl) or -CN, and each hydrogen in R^{11} is optionally substituted by one or more groups selected from halogen, -OH, -CN, -C_{1-12} alkyl which may be partially or fully halogenated, -O-C_{1-12} alkyl which may be partially or fully halogenated, -CO, -SO and $-SO_2$;

R^{12} is hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, and each hydrogen in R^{12} is optionally substituted by one or more R^3 groups;

R^1 and R^2 or R^1 and R^{12} may be combined together to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

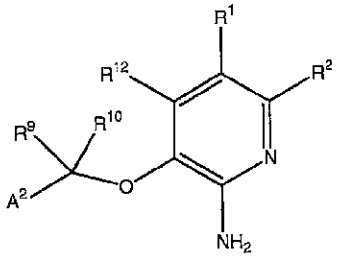
m is 0, 1 or 2;

n is 0, 1, 2, 3 or 4; and

p is 1 or 2;

or a pharmaceutically acceptable salt, solvate or hydrate thereof.

In a particular aspect of this embodiment, the compound has formula **2a**



2a

wherein A^2 is C_{6-12} aryl or 5-12 membered heteroaryl optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^1 is selected from C_{6-12} aryl and 5-12 membered heteroaryl, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is selected from C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, A^2 is substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^2 is hydrogen, R^9 and R^{10} are independently C_{1-4} alkyl, and A^2 is phenyl substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a furan, thiopene, pyrrole, pyrroline, pyrrolidine, dioxolane, oxazole, thiazole, imidazole, imidazoline, imidazolidine, pyrazole, pyrazoline, pyrazolidine, isoxazole, isothiazole, oxadiazole, triazole, thiadiazole, pyran, pyridine, piperidine, dioxane, morpholine, dithiane, thiomorpholine, pyridazine, pyrimidine, pyrazine, piperazine, triazine, trithiane or phenyl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

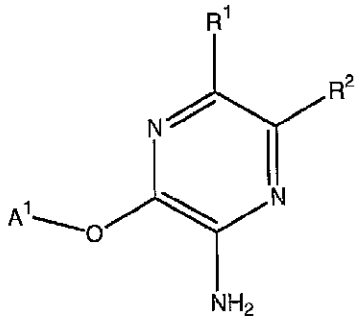
In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a fused ring heteroaryl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a $-SO_2NR^4R^5$ group.

Specific compounds of this embodiment, and methods of synthesizing compounds of this embodiment, are described in U.S. Provisional Patent Application No. 60/449,588, filed February

26, 2003, and U.S. Provisional Application No. 60/540,229, filed January 29, 2004, published as WO 04/076412, the disclosures of which are incorporated herein by reference in their entireties.

In another embodiment, the c-MET inhibitor is a compound of formula **3**



3

wherein:

R^1 is selected from C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups;

R^2 is hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, and each hydrogen in R^2 is optionally substituted by one or more R^8 groups;

R^3 is halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-O(CR^6R^7)_nR^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-C(=NR^6)NR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$, each hydrogen in R^3 is optionally substituted by one or more R^8 groups, and R^3 groups on adjacent atoms may combine to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

each R^4, R^5, R^6 and R^7 is independently hydrogen, halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl; or any two of R^4, R^5, R^6 and R^7 bound to the same nitrogen atom may, together with the nitrogen to which they are bound, be combined to form a 3 to 12 membered heteroalicyclic or 5-12 membered heteroaryl group optionally containing 1 to 3 additional heteroatoms selected from N, O, and S; or any two of R^4, R^5, R^6 and R^7 bound to the same carbon atom may be combined to form a C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic or 5-12 membered heteroaryl group; and each hydrogen in R^4, R^5, R^6 and R^7 is optionally substituted by one or more R^8 groups;

each R^8 is independently halogen, C_{1-12} alkyl, C_{2-12} alkenyl, C_{2-12} alkynyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -CN, $-O-C_{1-12}$ alkyl, $-O-(CH_2)_nC_{3-12}$ cycloalkyl, $-O-(CH_2)_nC_{6-12}$ aryl, $-O-(CH_2)_n$(3-12 membered heteroalicyclic) or

-O-$(CH_2)_n$(5-12 membered heteroaryl); and each hydrogen in R^8 is optionally substituted by one or more R^{11} groups;

A^1 is $-(CR^9R^{10})_n-A^2$ except that:

(i) when R^1 is substituted or unsubstituted aryl or substituted or unsubstituted heteroaryl, A^1 is $-(CR^9R^{10})_n-A^2$ and n is not zero; and

(ii) when R^2 is H and A^1 is m-chlorobenzyl, R^1 is not unsubstituted piperazine;

each R^9 and R^{10} is independently hydrogen, halogen, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-S(O)_2OR^4$, $-NO_2$, $-NR^4R^5$, $-(CR^6R^7)_nOR^4$, -CN, $-C(O)R^4$, $-OC(O)R^4$, $-NR^4C(O)R^5$, $-(CR^6R^7)_nC(O)OR^4$, $-(CR^6R^7)_nNCR^4R^5$, $-NR^4C(O)NR^5R^6$, $-NR^4S(O)_pR^5$ or $-C(O)NR^4R^5$; R^9 and R^{10} may combine to form a C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, C_{6-12} aryl or 5-12 membered heteroaryl ring; and each hydrogen in R^9 and R^{10} is optionally substituted by one or more R^3 groups;

A^2 is C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic, and A^2 is optionally substituted by one or more R^3 groups;

each R^{11} is independently halogen, C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{6-12} aryl, 3-12 membered heteroalicyclic, 5-12 membered heteroaryl, -O-C_{1-12} alkyl, -O-$(CH_2)_nC_{3-12}$ cycloalkyl, -O-$(CH_2)_nC_{6-12}$ aryl, -O-$(CH_2)_n$(3-12 membered heteroalicyclic), -O-$(CH_2)_n$(5-12 membered heteroaryl) or -CN, and each hydrogen in R^{11} is optionally substituted by one or more groups selected from halogen, -OH, -CN, -C_{1-12} alkyl which may be partially or fully halogenated, -O-C_{1-12} alkyl which may be partially or fully halogenated, -CO, -SO and $-SO_2$;

R^1 and R^2 may be combined together to form a C_{6-12} aryl, 5-12 membered heteroaryl, C_{3-12} cycloalkyl or 3-12 membered heteroalicyclic group;

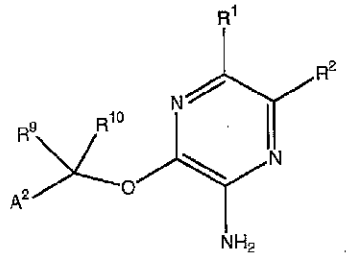
m is 0, 1 or 2;

n is 0, 1, 2, 3 or 4; and

p is 1 or 2;

or a pharmaceutically acceptable salt, solvate or hydrate thereof.

In a particular aspect of this embodiment, the compound has formula **3a**



3a

wherein A^2 is C_{6-12} aryl or 5-12 membered heteroaryl optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^1 is selected from C_{6-12} aryl and 5-12 membered heteroaryl, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is selected from C_{3-12} cycloalkyl, 3-12 membered heteroalicyclic, $-O(CR^6R^7)_nR^4$, $-C(O)R^4$, $-C(O)OR^4$, -CN, $-NO_2$, $-S(O)_mR^4$, $-SO_2NR^4R^5$, $-C(O)NR^4R^5$, $-NR^4C(O)R^5$, $-C(=NR^6)NR^4R^5$, C_{1-8} alkyl, C_{2-8} alkenyl, and C_{2-8} alkynyl; and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, A^2 is substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment, R^2 is hydrogen, R^9 and R^{10} are independently C_{1-4} alkyl, and A^2 is phenyl substituted by at least one halogen atom.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a furan, thiopene, pyrrole, pyrroline, pyrrolidine, dioxolane, oxazole, thiazole, imidazole, imidazoline, imidazolidine, pyrazole, pyrazoline, pyrazolidine, isoxazole, isothiazole, oxadiazole, triazole, thiadiazole, pyran, pyridine, piperidine, dioxane, morpholine, dithiane, thiomorpholine, pyridazine, pyrimidine, pyrazine, piperazine, triazine, trithiane or phenyl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a furan, thiopene, pyrrole, pyrroline, pyrrolidine, dioxolane, oxazole, thiazole, imidazole, imidazoline, imidazolidine, pyrazole, pyrazoline, pyrazolidine, isoxazole, isothiazole, oxadiazole, triazole, thiadiazole, pyran, pyridine, piperidine, dioxane, morpholine, dithiane, thiomorpholine, pyridazine, pyrimidine, pyrazine, triazine, trithiane or phenyl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups. In still more particular aspects, R^1 is not heteroalicyclic.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a fused ring heteroaryl group, and each hydrogen in R^1 is optionally substituted by one or more R^3 groups.

In particular aspects of this embodiment, and in combination with any other particular aspects of this embodiment not inconsistent with the following definition of R^1, R^1 is a $-SO_2NR^4R^5$ group.

Specific compounds of this embodiment, and methods of synthesizing compounds of this embodiment, are described in U.S. Provisional Patent Application No. 60/449,588, filed February 26, 2003, and U.S. Provisional Application No. 60/540,229, filed January 29, 2004, published as WO 04/076412, the disclosures of which are incorporated herein by reference in their entireties.

In another embodiment, the c-MET inhibitor is a compound of formula **4**

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(54) **Pharmaceutical resorption-improved somatostatin compositions, their preparation and use.**

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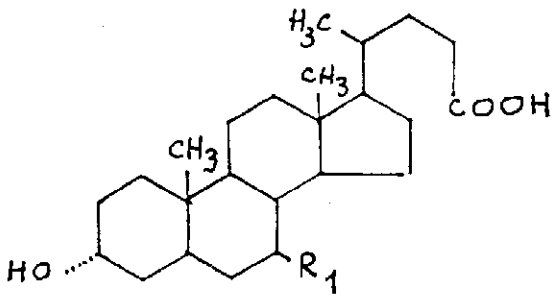
Description

The invention relates to pharmaceutical compositions containing a somatostatin and a resorption promoter and having an improved somatostatin bioavailability, and to concomitant administration of a somatostatin and a resorption promoter.

The invention provides a pharmaceutical composition which contains a somatostatin and at least one cholanic acid of the formula I



I

in which R_1 = α- or β-OH as a resorption promoter with a gallstone eliminating/preventing or size reducing activity, adapted for administration via a transmucosal route.

The naturally occuring somatostatin is one of the contemplated compounds and is a tetradecapeptide having the structure:-

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp
* *
Cys-Ser-Thr-Phe-Thr-Lys

This hormone is produced by the hypothalmus gland as well as other organs, e.g. the GI tract, and mediates, together with GRP, q.v. the neuroregulation of pituitary growth hormone release. In addition to inhibition of GH release by the pituitary, somatostatin is a potent inhibitor of a number of systems, including central and peripheral neural, gastrointestinal and vascular smooth muscle. It also inhibits the release of insulin and glucagon.

The term "somatostatin" includes its analogues or derivatives thereof. By derivatives and analogues is understood straight-chain, bridged or cyclic polypeptides wherein one or more amino acid units have been omitted and/or replaced by one or more other amino radical(s) of and/or wherein one or more functional groups have been replaced by one or more other functional groups and/or one or more groups have been replaced by one or several other isosteric groups. In general, the term covers all modified derivatives of a biologically active peptide which exhibit a qualitatively similar effect to that of the unmodified somatostatin peptide.

Agonist analogues of somatostatin are thus useful in replacing natural somatostatin in its effect on regulation of physiological functions.

Some of these offer improvements, e.g. in bioavailability and duration of action. However, little clinical evidence has yet been published on non-parenteral e.g. orally effective therapeutic somatostatin compositions.

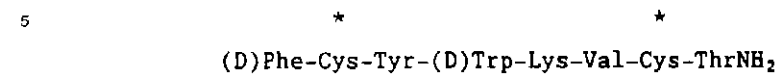
Preferred known somatostatins are:-

a)

* *
(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr-ol

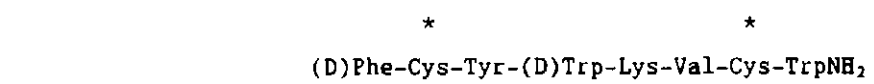
(Generic name Octreotide)

b)



(described in EP 203,031 A2; Generic name Vapreotide)

c)



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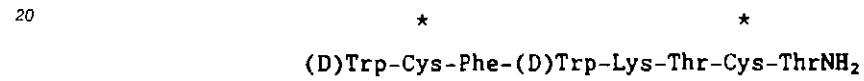

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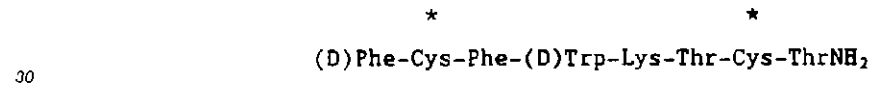

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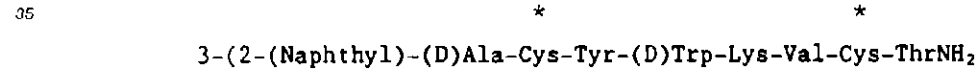
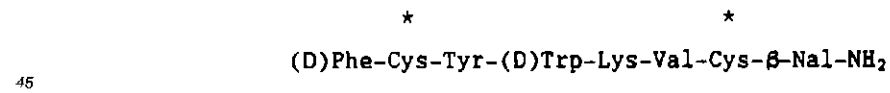
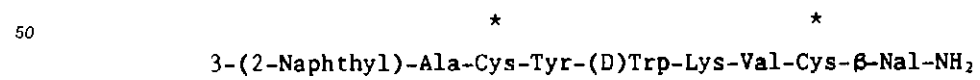


(described in EP 214.872 A2 and EP 215.171 A2; Generic name Angiopeptin)

g)



h)



i)

(D)Phe-Cys-β-Nal-(D)Trp-Lys-Val-Cys-Thr-NH_2

wherein in each of compounds a) to i) there is a bridge between the amino acids marked with a *.

Other contemplated somatostatins include:-

H-Cys-Phe-Phe-(D)Trp-Lys-Thr-Phe-Cys-OH

(See Vale et al., Metabolism, 27, Suppl. 1, 139 (1978)).

Asn-Phe-Phe-(D)Trp-Lys-Thr-Phe-Gaba

(See European Pat. Publication No. 1295)

MeAla-Tyr-(D)Trp-Lys-Val-Phe

(See Verber et al., Life Sciences, 34, 1371-1378 (1984), EP 0351.354 A2 and EP 70 021)) also known as cyclo (N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe).

NMePhe-His-(D)Trp-Lys-Val-Ala

(See R.F.Nutt et al., Klin.Wochenschr. (1986) 64 (Suppl. VII)

H-Cys-His-His-Phe-Phe-(D)Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

(See EP-A-200,188)

wherein in the above mentioned amino acids there is a bridge between the amino acids marked with a *.

The contents of all the above publications including the specific compounds are specifically referred to. The term derivative includes also the corresponding derivatives bearing a sugar residue.

When somatostatins bear a sugar residue, this is preferably coupled to a N-terminal amino group and/or to at least one amino group present in a peptide side chain, more preferably to a N-terminal amino group. Such compounds and their preparation are disclosed, e.g. in WO 88/02756.

The term octreotide derivatives includes those including the moiety

-D-Phe-Cys-Phe-DTrp-Lys-Thr-Cys-

having a bridge between the Cys residues.

Particularly preferred derivatives are N$^{\alpha}$-[α-glucosyl-(1-4-deoxyfructosyl]-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr-ol and N$^{\alpha}$-[β-deoxyfructosyl-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr-ol, each having a bridge between the -Cys- moieties, preferably in acetate salt form and described in Examples 2 and 1 respectively of the above mentioned application.

The somatostatins may exist e.g. in free form, salt form or in the form of complexes thereof. Acid addition salts may be formed with e.g. organic acids, polymeric acids and inorganic acids. Acid addition salts include e.g. the hydrochloride and acetates. Complexes are e.g. formed from somatostatins on addition of inorganic substances, e.g inorganic salts or hydroxides such as Ca- and Zn-salts and/or an addition of polymeric organic substance.

The acetate salt is a preferred salt for such formulations. The pamoate salt is also useful.

The pamoate may be obtained in conventional manner, e.g. by reacting embonic acid (pamoic acid) with octreotide, e.g. in free base form. The octreotide pamoate is described in the UK Patent application GB 2,234,896 A, Example 10.

The reaction may be effected in a polar solvent, e.g. at room temperature.

The somatostatins are indicated for use in the treatment of disorders wherein long term application of the drug is envisaged, e.g. disorders with an aetiology comprising or associated with excess GH-secretion, e.g. in the treatment of acromegaly, for use in the treatment of gastrointestinal disorders, for example, in the treatment of peptic ulcers, enterocutaneous and pancreaticocutaneous fistula, irritable bowel syndrome, dumping syndrome, watery diarrhea syndrome, acute pancreatitis and gastroenterophathic endocrine tumors (e.g. vipomas, GRFomas, glucagonomas, insulinomas, gastrinomas and carcinoid tumors) as well as gastro-intestinal bleeding, breast cancer and complications concerned with diabetes. Further the somatostatins are indicated for the treatment of arthritis conditions, e.g. rheumatoid arthritis.

As a side effect of therapy in which somatostatins are administered sometimes, at least for octreotide, the formation of symptomatic gallstones has been observed. However, little has been published on the mechanism of formation of these gallstones or their compositions.

The cholanic acids of the formula I described before, as well as their pharmaceutically acceptable salts, are generally known per se. The compound having the 7α-hydroxyl group has been described in the Merck Index, Ninth Edition (1976), under monograph number 2010, in which it is denoted as chenodeoxycholic acid. The compound having the 7β-hydroxyl group is denoted as ursodeoxycholic acid and has been described under monograph number 9551.

Both cholanic acids are of natural origin and are, whether alone or, preferably, in combination, medically indicated in cholesterin gallstone dissolution treatment as described in Digestive Diseases and Sciences, Vol. 31, No. 10,1032-1040 (1986), Die Medizinische Welt, No. 45, 1489-1495 (1987), Gut, Vol. 28, No. 10, October 1987, A.1360. The cholanic acids are orally administered.

Based on data in "Drug Evaluations", 6th edition, American Medical Association, Chicago, Illinois the daily dosis for gallstone treatment is about at least 1000 mg/75 kg of body weight per day for chenodeoxycholic acid and 1200 - 1500 mg/75 kg of body weight per day for urso deoxycholic acid.

According to "The Pharmacological Basis of Therapeutics", Eighth edition, Goodman and Gilman, Pergamon Press, the dissolution of gallstones can also be performed by administering a combination of chenodeoxycholic acid and ursodeoxycholic acid at lower dosages, e.g. 5 mg/kg body weight/day = 375 mg/75 kg of body weight/day of each compound (page 931).

As generally known for peptides, the development of appropriate and effective somatostatin administration forms has caused many problems.

Being peptides, the somatostatins are easily decomposed by the indigestion juices after oral administration. Moreover, transport across the mucous membranes in the body, whether the stomach or intestines or in the mouth, the nose or the rectum is inefficient. For this reason the somatostatins are parenterally administered to humans. No other commercially available route of administration is available, despite the well known disadvantages of parenteral administration.

According to Gastroenterology Vol. 96, No. 5, Part 2, page A 580 (1989), Abstract of Papers, gallstones formed under the influence of a long term therapy with the specific somatostatin Sandostatin® (= Octreotide) were treated during further octreotide administration with ursodeoxy cholic acid resulting in the dissolution of the stones in just one patient. The somatostatin was given parenterally at a known dose of about 450 microgram, the ursodeoxycholic acid orally and thus no pharmaceutical composition was given in which somatostatins were combined with urso- and/or chenodeoxycholic acid.

We have now surprisingly discovered that both cholanic acids mentioned before, i.e. one of them or both in combination, can be used, e.g. at low dosages, for an improved resorption of a somatostatin through the mucous membrane e.g. of the gastrointestinal tract, as determined in the following experiment:

Wistar rats (about 300 g body weight) were fasted for 12 hours and anaesthetised with urethane (2 doses of 0.7 mg/kg i.p.). The abdomen was opened to gain access to the gastrointestinal tract in particular the jejunum.

A solution of the pharmaceutical composition according to the invention or of the somatostatin as a control or of a somatostatin in combination with sodium taurocholate, a resorption enhancer, known to be used in combination with e.g. salmon calcitonin or insulin for an improved resorption through the mucous membrane of the gastrointestinal tract, was injected into the gastrointestinal tract, e.g. the jejunum. Blood samples (e.g. 1 ml) are obtained 20 minutes, and 1 and 2 hours after administration from the vena cava. The blood is centrifuged and the somatostatin concentration in the plasma determined in conventional manner using a radio-immunoassay method.

The relative bioavailability (AUC_o^2 = Area under the curve over 2 hours) is determined and expressed in the following table, in which the AUC is expressed as ng/ml.hour and C_{max} as ng/ml:

TABLE A

Administered 0.5 ml of NaCl (0.9 % W/V) solution	AUC_o^2		C_{max}		Bioavail.	
containing further:	median	mean	sem	mean	sem	rel. %
100 microg. of octreotide (reference)	2.06	2.26	0.46	2.23	0.55	100 %
100 microg. of octreotide + 5 mg of chenodeoxycholic acid	80.78	84.26	21.29	63.76	18.48	3921.36
100 microg. of octreotide + 5 mg of ursodeoxycholic acid	8.35	10.33	2.94	10.76	2.98	405.39
100 microg. of octreotide + 5 mg of sodium taurocholate	2.91	5.40	2.22	4.02	1.47	141.26
sem = standard error of the mean.						

The invention thus provides a pharmaceutical composition containing a somatostatin, its analgoues and derivatives, and at least one cholanic acid of the formula I, mentioned before, and having, when administered to the jejunum of a rat a relative somatostatin bioavailability of at least 400%, compared with the same composition without the resorption promoter.

Whereas the sodium taurocholate containing solution exhibits only a small octreotide bioavailability improvement in relation to the reference (improvement factor = 1.4), the solutions with ursodeoxycholic acid and especially chenodeoxycholic acid show considerably better improvement factors of about 4 and 39 respectively.

The invention thus provides the administration of the cholanic acids of formula I via the same transmucosal, preferably oral, route as the administration of a somatostatin.

The invention preferably provides pharmaceutical compositions containing a somatostatin, especially octreotide or its derivatives, chenodeoxycholic acid and/or ursodeoxycholic acid and/or their pharmaceutically acceptable salts.

In a further aspect the invention provides a process for the production of a pharmaceutical composition of the invention, which comprises working up the somatostatin and the resorption promoter.

The cholanic acid may be worked up in any pharmaceutically acceptable condition, e.g. as a precursor, or as a salt, e.g. an alkaline metal salt.

The pharmaceutical composition may be produced in conventional manner, using, if desired, appropriate excipients for the route of administration particularly contemplated, e.g. nasal, rectal or, preferably, oral.

Preferably the composition contains no water and is in the solid state. The invention relates particularly to pharmaceutical compositions for nasal, rectal and oral administration.

The compositions may be prepared by conventional techniques to be in conventional forms for transmucosal administration, for example, capsules, tablets, suppositories, dispersible powders. Preferably the cholanic acid of formula I is the sole bile salt present.

The exact dose of somatostatin to be used may be ascertained in conventional animal or clinical studies. For example, the dose may be ascertained by comparative bioavailability trials with other formulations with a known therapeutic effect, the dose being chosen to produce in the steady state comparable drug levels to therapeutically effective levels, e.g. on parenteral administration.

In the compositions of the invention the dosage of the cholanic acids can be chosen within wide ranges: at lower dosages the cholanic acids show the resorption promoting effect on somatostatins, at higher

dosages, the cholanic acids - additionally - have a gallstone dissolving effect. In dosages between these extreme limits the cholanic acids surprisingly inhibit gallstone formation and thus have a gallstone prophylactic effect.

The compositions of the invention in which the amount of cholanic acid is chosen such that it has a resorption promoting and additionally a gallstone prophylactic effect are the most preferred.

The compositions of the invention open up the possibility of a more convenient method of administration of sandostatin compositions.

The compositions are preferably so formulated that the therapeutic daily dose of somatostatin and the daily dose of cholanic acid for a resorption promoting and preferably additionally a gallstone prophylactic effect are incorporated in the same dosage units. However, the invention also relates to preparations, in which the daily dosages of somatostatin and of cholanic acids are separate, but are administrable both for the same transmucosal route, whether rectal, nasal or oral.

Hence, the invention also provides a package containing unit dosages of a cholanic acid of formula I and of a somatostatin, as a combined preparation for simultaneous, separate or sequential use via the same transmucosal route, preferably the oral route, in a therapy for which the somatostatin is indicated.

Such unit dosages may be made in analogous manner to the pharmaceutical compositions of the invention.

Conveniently, the compositions are in unit dosage form or may be divided into or administered as unit dosages each preferably containing e.g. up to 35 mg of a somatostatin, the exact amount depending on e.g. the active ingredient, the type of illness to be treated and the mode of administration. When the used somatostatin is octreotide or vapreotide preferably 1 to 10 mg is used per unit dosage form.

The unit dosage form contains preferably 5 to 500, especially up to 250 mg of one of the cholanic acids or of a mixture of both for a resorption promoting effect on the somatostatin used. For an additional effective gallstone prophylaxis the unit dosage may contain such an amount of cholanic acid, that its daily dosis is up to 1500 mg, e.g. up to 1000 mg. If a combination of urso- and chenodeoxycholic acids is used, the dosage form may contain such amounts that their daily doses are e.g. up to 1000 mg/day, e.g. up to 400 mg/day of each component.

The invention is illustrated by the following pharmaceutical compositions:

EXAMPLE 1:

Capsules for oral application:	
Octreotide	2,3 mg* (aequivalent to 2 mg somatostatin)
Chenodeoxycholic acid	150 mg
Microcrystalline cellulose	100 mg
Lactose	50 mg

* the acetate salt

EXAMPLE 2:

Suppositoria for rectal application:	
Octreotide	5,8 mg* (aequivalent to 5 mg somatostatin)
anhydrous citric acid	0.78
Trisodium citrate-hydrate	0.50
Mannitol	48.651
Chenodeoxycholic acid	150.0
Suppositorium base A**	1300.0
	1500.0 mg

* the acetate salt

** Witepsol ^{R} H.12, linear (C_{10-18}) fatty acid glyceride, Melting range 32-33.5 °, solidification range 29-33 °C Obtainable from Dynamit Nobel, Germany.

EXAMPLE 3:

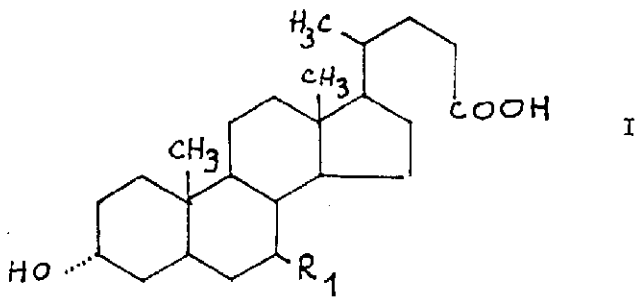
Solutions for nasal application:	
Octreotide	2,3 mg
Sodium citrate - dihydrate	10,00 mg
Citric acid - monohydrate	10,00 mg
Chenodeoxycholic acid	25,00 mg
Ethylenediaminetetracetic acid disodium salt	1,0 mg
Benzalkonium chloride	0,2 mg
	48,5 mg

The powder is sprayed in a quantity of about 6 mg per nostril. When administered 4 times daily, the dose of octreotide is sufficient for therapeutic purposes.

Instead of octreotide other somatostatins can be formulated. Instead of chenodeoxycholic acid the same amount of ursodeoxycholic acid can be used.

Claims

1. A pharmaceutical composition, containing a somatostatin and at least one cholanic acid of formula I



I

wherein R_1 = α- or β-OH. as a resorption promoter with a gallstone eliminating/preventing or size reducing activity, adapted for administration via a transmucosal route.

2. A composition according to claim 1, having, when administered to the jejunum of a rat, a relative somatostatin bioavailability of at least 400 %, compared with the same compositions without the resorption promoter.

3. A composition according to claim 2, in which the cholanic acid of formula I is ursodeoxycholic acid.

4. A composition according to claim 2, in which the cholanic acid of formula I is chenodeoxycholic acid.

5. A composition according to any one of claims 1 to 4, in which the somatostatin is octreotide.

6. A composition according to any one of claims 1 to 5 for nasal, rectal or oral administration.

7. A composition according to any one of claims 1 to 6, containing up to 35 mg of a somatostatin per dosage unit.

8. A composition according to any one of claims 1 to 7 containing from 1 to 10 mg of octreotide per dosage unit.

9. A composition according to any one of claims 1 to 8 containing from 5 to 500 mg of the cholanic acid compound per dosage unit.

10. A composition according to any one of claims 1 to 9 in solid state.

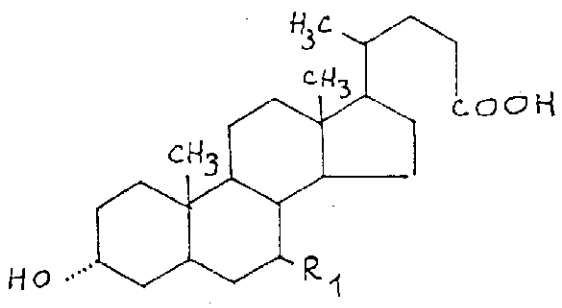
11. A process for the production of a pharmaceutical composition of any one of claims 1 to 10 which comprises working up the somatostatin and the resorption promoter.

12. A package containing unit dosages of a cholanic acid of formula I given in claim 1 and of a somatostatin, as a combined preparation for simultaneous, separate or sequential use via the same transmucosal route in a therapy for which the somatostatin is indicated.

13. A package according to claims 12, for oral application of the unit dosages.

Patentansprüche

1. Pharmazeutische Komposition die ein Somatostatin und als Resorptionsförderer mit gallensteineliminierender, - verhindernder oder-verkleinernder Wirkung mindestens eine Cholansäure der Formel I



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enthält
worin R_1 = α - oder β - OH, zur Applikation über den transmukosalen Weg ausgestaltet.

2. Komposition gemäss Anspruch 1 welche eine relative Somatostatinbioverfügbarkeit von mindestens 400% gegenüber der gleichen Komposition ohne Resorptionsförderer aufweist, wenn sie ins Jejunum einer Ratte gebracht wird.

3. Komposition gemäss Anspruch 2, mit Ursodeoxycholsäure als Cholansäure der Formel 1.

4. Komposition gemäss Anspruch 2, mit Chenodeoxycholsäure als Cholansäure der Formel 1.

5. Komposition gemäss einem der Ansprüche 1 bis 4 mit Octreotid als Somatostatin.

6. Komposition gemäss einem der Ansprüche 1 bis 5 zur nasalen, rektalen oder oralen Applikation.

7. Komposition gemäss einem der Ansprüche 1 bis 6 mit bis 35 mg eines Somatostatins per Dosierungseinheit.

8. Komposition gemäss einem der Ansprüche 1 bis 7 mit 1 bis 10 mg Octreotid pro Dosierungseinheit.

9. Komposition gemäss einem der Ansprüche 1 bis 8 mit 5 bis 500 mg der Cholansäureverbindung pro Dosierungseinheit.

10. Komposition gemäss einem der Ansprüche 1 bis 9 in festem Zustand.

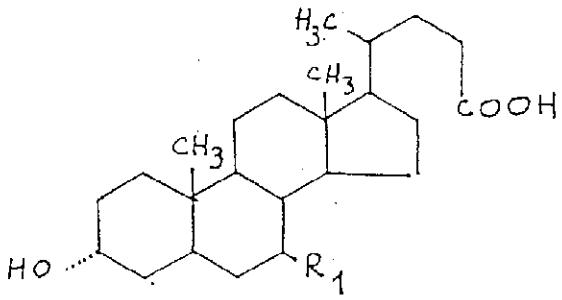
11. Verfahren zur Herstellung einer pharmazeutischen Komposition gemäss einem der Ansprüche 1 bis 10, dadurch gekennzeichnet, dass man darin das Somatostatin und den Resorptionsförderer verarbeitet.

12. Verpackung mit Dosierungseinheiten einer Cholansäure der Formel 1 und eines Somatostatins als Kombinationspräparat für den gleichzeitigen separaten oder nachfolgenden Einsatz über den gleichen transmukosalen Applikationsweg in einer Therapie für welche das Somatostatin vorgesehen ist.

13. Verpackung gemäss Anspruch 12, zur oralen Applikation der Dosierungseinheiten.

Revendications

1. Une composition pharmaceutique, contenant une somatostatine et au moins un acide cholanique de formule I



I

dans laquelle R_1 = α- or β-OH, comme promoteur de résorption ayant une activité permettant d'éliminer les calculs biliaires, d'empêcher la formation des calculs biliaires ou de réduire la dimension des calculs biliaires, et adaptée pour l'administration à travers les muqueuses.

2. Une composition selon la revendication 1 ayant, après administration au jejunum d'un rat, une biodisponibilité relative de somatostatine d'au moins 400%, par rapport à la même composition exempte de promoteur de résorption.

3. Une composition selon la revendication 2, dans laquelle l'acide cholanique de formule I est l' acide ursodésoxycholique.

4. Une composition selon la revendication 2, dans laquelle l' acide cholanique de formule I est l'acide chénodésoxycholique.

5. Une composition selon l'une quelconque des revendications 1 à 4, dans laquelle la somatostatine est l'octréotide.

6. Une composition selon l'une quelconque des revendications 1 à 5, pour l'administration nasale, rectale ou orale.

7. Une composition selon l'une quelconque des revendications 1 à 6, contenant jusqu'à 35 mg de somatostatine par dose unitaire.

8. Une composition selon l'une quelconque des revendications 1 à 7, contenant de 1 à 10 mg d'octréotide par dose unitaire.

9. Une composition selon l'une quelconque des revendications 1 à 8, contenant de 5 à 500 mg d'acide cholanique par dose unitaire.

10. Une composition selon l'une quelconque des revendications 1 à 9, sous forme solide.

11. Un procédé de préparation d'une composition pharmaceutique selon l'une quelconque des revendications 1 à 10, qui comprend le traitement de la somatostatine et du promoteur de résorption.

12. Un emballage contenant des doses unitaires d'un acide cholanique de formule I spécifié à la revendication 1 et d'une somatostatine, comme préparation combinée pour l'administration simultanée, séparée ou séquentielle à travers les muqueuses dans une thérapie pour laquelle la somatostatine est indiquée.

13. Un emballage selon la revendication 12, pour l'administration par voie orale de doses unitaires.

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(54) Title: ANTINEOPLASTIC COMBINATIONS

(57) Abstract: This invention provides the use of a combination of an mTOR inhibitor and an antineoplastic alkylating agent in the treatment of neoplasms.

ANTINEOPLASTIC COMBINATIONS

This invention relates to the use of combinations of an mTOR inhibitor (e.g rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779)) and an antineoplastic alkylating agent in the treatment of neoplasms, to the use of an mTOR inhibitor and an antineoplastic alkylating agent in the preparation of a medicament for the treatment of a neoplasm, to a product comprising an mTOR inhibitor and an antineoplastic alkylating agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm, and to pharmaceutical compositions comprising an mTOR inhibitor, an antineoplastic alkylating agent and a pharmaceutically acceptable carrier.

BACKGROUND OF THE INVENTION

Rapamycin is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus, which was found to have antifungal activity, particularly against Candida albicans, both in vitro and in vivo [C. Vezina et al., J. Antibiot. 28, 721 (1975); S.N. Sehgal et al., J. Antibiot. 28, 727 (1975); H. A. Baker et al., J. Antibiot. 31, 539 (1978); U.S. Patent 3,929,992; and U.S. Patent 3,993,749]. Additionally, rapamycin alone (U.S. Patent 4,885,171) or in combination with picibanil (U.S. Patent 4,401,653) has been shown to have antitumor activity.

The immunosuppressive effects of rapamycin have been disclosed in FASEB 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); R. Y. Calne et al., Lancet 1183 (1978); and U.S. Patent 5,100,899]. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

Rapamycin is also useful in preventing or treating systemic lupus erythematosus [U.S. Patent 5,078,999], pulmonary inflammation [U.S. Patent 5,080,899], insulin dependent diabetes mellitus [U.S. Patent 5,321,009], skin disorders, such as psoriasis [U.S. Patent 5,286,730], bowel disorders [U.S. Patent 5,286,731], smooth muscle cell proliferation and intimal thickening following vascular

injury [U.S. Patents 5,288,711 and 5,516,781], adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1], ocular inflammation [U.S. Patent 5,387,589], malignant carcinomas [U.S. Patent 5,206,018], cardiac inflammatory disease [U.S. Patent 5,496,832], and anemia [U.S. Patent 5,561,138].

Rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) is ester of rapamycin which has demonstrated significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of hydroxyesters of rapamycin, including CCI-779, are disclosed in U.S. Patent 5,362,718.

CCI-779 exhibits cytostatic, as opposed to cytotoxic properties, and may delay the time to progression of tumors or time to tumor recurrence. CCI-779 is considered to have a mechanism of action that is similar to that of sirolimus. CCI-779 binds to and forms a complex with the cytoplasmic protein FKBP, which inhibits an enzyme, mTOR (mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]). Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S. The mechanism of action of CCI-779 that results in the G1 S phase block is novel for an anticancer drug.

In vitro, CCI-779 has been shown to inhibit the growth of a number of histologically diverse tumor cells. Central nervous system (CNS) cancer, leukemia (T-cell), breast cancer, prostate cancer, and melanoma lines were among the most sensitive to CCI-779. The compound arrested cells in the G1 phase of the cell cycle.

In vivo studies in nude mice have demonstrated that CCI-779 has activity against human tumor xenografts of diverse histological types. Gliomas were particularly sensitive to CCI-779 and the compound was active in an orthotopic glioma model in nude mice. Growth factor (platelet-derived)-induced stimulation of a human glioblastoma cell line in vitro was markedly suppressed by CCI-779. The growth of several human pancreatic tumors in nude mice as well as one of two breast cancer lines studied in vivo also was inhibited by CCI-779.

DESCRIPTION OF THE INVENTION

This invention provides the use of combinations of an mTOR inhibitor and an antineoplastic alkylating agent as antineoplastic combination chemotherapy. In particular, these combinations are useful in the treatment of renal cancer, soft tissue cancer, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small lung cell cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. This invention also provides combinations of an mTOR inhibitor and an antineoplastic alkylating agent for use as antineoplastic combination chemotherapy, in which the dosage of either the mTOR inhibitor or the antineoplastic alkylating agent or both are used in subtherapeutically effective dosages.

In another aspect, the invention provides the use of combinations of an mTOR inhibitor and an antineoplastic alkylating agent in the preparation of a medicament for the treatment of a neoplasm. In a further aspect, the invention provides a product comprising an mTOR inhibitor and an antineoplastic alkylating agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm in a mammal. In a still further aspect, the invention provides a pharmaceutical composition comprising an mTOR inhibitor, an antineoplastic alkylating agent and a pharmaceutically acceptable carrier.

As used in accordance with this invention, the term "treatment" means treating a mammal having a neoplastic disease by providing said mammal an effective amount of a combination of an mTOR inhibitor and an antineoplastic alkylating agent with the purpose of inhibiting growth of the neoplasm in such mammal, eradication of the neoplasm, or palliation of the mammal.

As used in accordance with this invention, the term "providing," with respect to providing the combination, means either directly administering the combination, or administering a prodrug, derivative, or analog of one or both of the components of the combination which will form an effective amount of the combination within the body.

mTOR is the mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]. Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation,

translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S.

mTOR regulates the activity of at least two proteins involved in the translation of specific cell cycle regulatory proteins (Burnett, P.E., PNAS 95: 1432 (1998) and Isotani, S., J. Biol. Chem. 274: 33493 (1999)). One of these proteins p70s6 kinase is phosphorylated by mTOR on serine 389 as well as threonine 412. This phosphorylation can be observed in growth factor treated cells by Western blotting of whole cell extracts of these cells with antibody specific for the phosphoserine 389 residue.

As used in accordance with this invention, an "mTOR inhibitor" means a compound or ligand which inhibits cell replication by blocking progression of the cell cycle from G1 to S by inhibiting the phosphorylation of serine 389 of p70s6 kinase by mTOR.

The following standard pharmacological test procedure can be used to determine whether a compound is an mTOR inhibitor, as defined herein. Treatment of growth factor stimulated cells with an mTOR inhibitor like rapamycin completely blocks phosphorylation of serine 389 as evidenced by Western blot and as such constitutes a good assay for mTOR inhibition. Thus whole cell lysates from cells stimulated by a growth factor (eg. IGF1) in culture in the presence of an mTOR inhibitor should fail to show a band on an acrylamide gel capable of being labeled with an antibody specific for serine 389 of p70s6K.

Materials:

NuPAGE LDS Sample Buffer	(Novex Cat # NP0007)
NuPAGE Sample Reducing Agent	(Novex Cat # NP0004)
NuPAGE 4-12% Bis-Tris Gel	(Novex Cat # NP0321)
NuPAGE MOPS SDS Running Buffer	(Novex Cat # NP0001)
Nitrocellulose	(Novex Cat # LC2001)
NuPAGE Transfer Buffer	(Novex Cat # NP0006)
Hyperfilm ECL	(Amersham Cat # RPN3114H)
ECL Western Blotting Detection Reagent	(Amersham Cat # RPN2134)

Primary antibody: Phospho-p70 S6 Kinase (Thr389) (Cell Signaling Cat #9205)
Secondary antibody: Goat anti-rabbit IgG-HRP conjugate (Santa Cruz Cat #sc-2004)

Methods:

A. Preparation of Cell Lysates

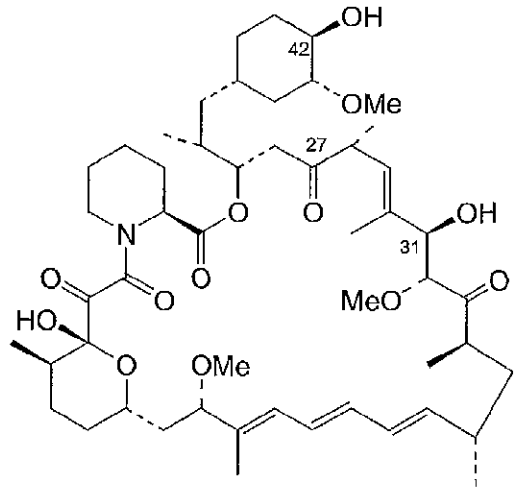
Cell lines were grown in optimal basal medium supplemented with 10% fetal bovine serum and penicillin/treptomycin. For phosphorylation studies, cells were subcultured in 6-well plates. After the cells have completely attached, they were either serum-starved. Treatment with mTOR inhibitors ranged from 2 to 16 hours. After drug treatment, the cells were rinsed once with PBS (phosphate buffered saline without Mg++ and Ca++) and then lysed in 150-200 µl NuPAGE LDS sample buffer per well. The lysates were briefly sonicated and then centrifuged for 15 minutes at 14000 rpm. Lysates were stored at minus -80^{0}C until use.

The test procedure can also be run by incubating the cells in growth medium overnight after they have completely attached. The results under both sets of conditions should be the same for an mTOR inhibitor.

B. Western Blot Analysis

1) Prepare total protein samples by placing 22.5 µl of lysate per tube and then add 2.5 µl NuPAGE sample reducing agent. Heat samples at 70^{0}C for 10 minutes. Electrophoresed using NuPAGE gels and NuPAGE SDS buffers.
2) Transfer the gel to a nitrocellulose membrane with NuPAGE transfer buffer. The membrane are blocked for 1 hour with blocking buffer (Tris buffered saline with 0.1%-Tween and 5% nonfat-milk). Rinse membranes 2x with washing buffer (Tris buffered saline with 0.1%-Tween).
3) Blots/membrane are incubated with the P-p70 S6K (T389) primary antibody (1:1000) in blocking buffer overnight at 4°C in a rotating platform.
4) Blots are rinsed 3x for 10 minutes each with washing buffer, and incubated with secondary antibody (1:2000) in blocking buffer for 1 hour at room temperature.
5) After the secondary antibody binding, blots are washed 3x for 10 minutes each with washing buffer, and 2x for 1 minute each with Tris-buffered saline, followed by chemiluminescent (ECL) detection and then exposed to chemiluminescence films.

As used in accordance with this invention, the term "a rapamycin" defines a class of immunosuppressive compounds which contain the basic rapamycin nucleus (shown below). The rapamycins of this invention include compounds which may be chemically or biologically modified as derivatives of the rapamycin nucleus, while still retaining immunosuppressive properties. Accordingly, the term "a rapamycin" includes esters, ethers, oximes, hydrazones, and hydroxylamines of rapamycin, as well as rapamycins in which functional groups on the rapamycin nucleus have been modified, for example through reduction or oxidation. The term "a rapamycin" also includes pharmaceutically acceptable salts of rapamycins, which are capable of forming such salts, either by virtue of containing an acidic or basic moiety.



RAPAMYCIN

It is preferred that the esters and ethers of rapamycin are of the hydroxyl groups at the 42- and/or 31-positions of the rapamycin nucleus, esters and ethers of a hydroxyl group at the 27-position (following chemical reduction of the 27-ketone), and that the oximes, hydrazones, and hydroxylamines are of a ketone at the 42-position (following oxidation of the 42-hydroxyl group) and of 27-ketone of the rapamycin nucleus.

Preferred 42- and/or 31-esters and ethers of rapamycin are disclosed in the following patents, which are all hereby incorporated by reference: alkyl esters (U.S.

Patent 4,316,885); aminoalkyl esters (U.S. Patent 4,650,803); fluorinated esters (U.S. Patent 5,100,883); amide esters (U.S. Patent 5,118,677); carbamate esters (U.S. Patent 5,118,678); silyl ethers (U.S. Patent 5,120,842); aminoesters (U.S. Patent 5,130,307); acetals (U.S. Patent 5,51,413); aminodiesters (U.S. Patent 5,162,333); sulfonate and sulfate esters (U.S. Patent 5,177,203); esters (U.S. Patent 5,221,670); alkoxyesters (U.S. Patent 5,233,036); O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Patent 5,258,389); carbonate esters (U.S. Patent 5,260,300); arylcarbonyl and alkoxycarbonyl carbamates (U.S. Patent 5,262,423); carbamates (U.S. Patent 5,302,584); hydroxyesters (U.S. Patent 5,362,718); hindered esters (U.S. Patent 5,385,908); heterocyclic esters (U.S. Patent 5,385,909); gem-disubstituted esters (U.S. Patent 5,385,910); amino alkanoic esters (U.S. Patent 5,389,639); phosphorylcarbamate esters (U.S. Patent 5,391,730); carbamate esters (U.S. Patent 5,411,967); carbamate esters (U.S. Patent 5,434,260); amidino carbamate esters (U.S. Patent 5,463,048); carbamate esters (U.S. Patent 5,480,988); carbamate esters (U.S. Patent 5,480,989); carbamate esters (U.S. Patent 5,489,680); hindered N-oxide esters (U.S. Patent 5,491,231); biotin esters (U.S. Patent 5,504,091); O-alkyl ethers (U.S. Patent 5,665,772); and PEG esters of rapamycin (U.S. Patent 5,780,462). The preparation of these esters and ethers are disclosed in the patents listed above.

Preferred 27-esters and ethers of rapamycin are disclosed in U.S. Patent 5,256,790, which is hereby incorporated by reference. The preparation of these esters and ethers are disclosed in the patents listed above.

Preferred oximes, hydrazones, and hydroxylamines of rapamycin are disclosed in U.S. Patents 5,373,014, 5,378,836, 5,023,264, and 5,563,145, which are hereby incorporated by reference. The preparation of these oximes, hydrazones, and hydroxylamines are disclosed in the above listed patents. The preparation of 42-oxorapamycin is disclosed in 5,023,263, which is hereby incorporated by reference.

Particularly preferred rapamycins include rapamycin [U.S. Patent 3,929,992], CCI-779 [rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid; U.S. Patent 5,362,718], and 42-O-(2-hydroxy)ethyl rapamycin [U.S. Patent 5,665,772].

When applicable, pharmaceutically acceptable salts of the rapamycin can be formed from organic and inorganic acids, for example, acetic, propionic, lactic, citric, tartaric, succinic, fumaric, maleic, malonic, mandelic, malic, phthalic, hydrochloric, hydrobromic, phosphoric, nitric, sulfuric, methanesulfonic, napthalenesulfonic, benzenesulfonic, toluenesulfonic, camphorsulfonic, and similarly known acceptable aids when the rapamycin contains a suitable basic moiety. Salts may also be formed from organic and inorganic bases, such as alkali metal salts (for example, sodium, lithium, or potassium) alkaline earth metal salts, ammonium salts, alkylammonium salts containing 1-6 carbon atoms or dialkylammonium salts containing 1-6 carbon atoms in each alkyl group, and trialkylammonium salts containing 1-6 carbon atoms in each alkyl group, when the rapamycin contains a suitable acidic moiety.

It is preferred that the mTOR inhibitor used in the antineoplastic combinations of this invention is a rapamycin, and more preferred that the mTOR inhibitor is rapamycin, CCI-779, or 42-O-(2-hydroxy)ethyl rapamycin.

As described herein, CCI-779 was evaluated as a representative mTOR inhibitor in the mTOR inhibitor plus antimetabolite combinations of this invention.

The preparation of CCI-779 is described in U.S. Patent 5,362,718, which is hereby incorporated by reference. When CCI-779 is used as an antineoplastic agent, it is projected that initial i.v. infusion dosages will be between about 0.1 and 100 mg/m^2 when administered on a daily dosage regimen (daily for 5 days, every 2-3 weeks), and between about 0.1 and 1000 mg/m^2 when administered on a once weekly dosage regimen. Oral or intravenous infusion are the preferred routes of administration, with intravenous being more preferred.

As used in accordance with this invention, the term "antineoplastic alkylating agent" means a substance which reacts with (or "alkylates") many electron-rich atoms in cells to form covalent bonds. The most important reactions with regard to their antitumor activities are reactions with DNA bases. Some alkylating agents are monofunctional and react with only one strand of DNA. Others are bifunctional and react with an atom on each of the two strands of DNA to produce a "cross-link" that covalently links the two strands of the DNA double helix. Unless repaired, this lesion

will prevent the cell from replicating effectively. The lethality of the monofunctional alkylating agents results from the recognition of the DNA lesion by the cell and the response of the cell to that lesion. (Colvin OM. Antitumor Alkylating Agents. In Cancer Principles & Practice of Oncology 6th Edition. ed. DeVita VT, Hellman S, Rosenberg SA. Lippincott Williams & Wilkins. Philadelphia 2001. p. 363.)

Antineoplastic alkylating agents are roughly classified, according to their structure or reactive moiety, into several categories which include nitrogen mustards, such as mustargen, cyclophosphamide, ifosfamide, melphalan, and chlorambucil; azidines and epoxides, such as thiotepa, mitomycin C, dianhydrogalactitol, and dibromodulcitol; alkyl sulfonates, such as busulfan; nitrosoureas, such as bischloroethylnitrosourea (BCNU), cyclohexyl-chloroethylnitrosourea (CCNU), and methylcyclohexylchloroethylnitrosourea (MeCCNU); hydrazine and triazine derivatives, such as procarbazine, dacarbazine, and temozolomide; and platinum compounds. Platinum compounds are platinum containing agents that react preferentially at the N7 position of guanine and adenine residues to form a variety of monofunctional and bifunctional adducts. (Johnson SW, Stevenson JP, O'Dwyer PJ. Cisplatin and Its Analogues. In Cancer Principles & Practice of Oncology 6th Edition. ed. DeVita VT, Hellman S, Rosenberg SA. Lippincott Williams & Wilkins. Philadelphia 2001. p. 378.) These compounds include cisplatin, carboplatin, platinum IV compounds, and multinuclear platinum complexes.

The following are representative examples of antineoplastic alkylating agents of this invention.

Meclorethamine is commercially available as an injectable (MUSTARGEN).

Cyclophosphamide is commercially available as an injectable (cyclophosphamide, lyophilized CYTOXAN, or NEOSAR) and in oral tablets (cyclophosphamide or CYTOXAN).

Ifosfamide is commercially available as an injectable (IFEX).

Melphalan is commercially available as an injectable (ALKERAN) and in oral tablets (ALKERAN).

Chlorambucil is commercially available in oral tablets (LEUKERAN).

Thiotepa is commercially available as an injectable (thiotepa or THIOPLEX).

Mitomycin is commercially available as an injectable (mitomycin or MUTAMYCIN).

Busulfan is commercially available as an injectable (BUSULFEX) and in oral tablets (MYLERAN).

Lomustine (CCNU) is commercially available in oral capsules (CEENU).

Carmustine (BCNU) is commercially available as an intracranial implant (GLIADEL) and as an injectable (BICNU).

Procarbazine is commercially available in oral capsules (MATULANE).

Temozolomide is commercially available in oral capsules (TEMODAR).

Cisplatin is commercially available as an injectable (cisplatin, PLATINOL, or PLATINOL-AQ).

Carboplatin is commercially available as an injectable (PARAPLATIN).

The following table briefly summarizes some of the recommended dosages for the antineoplastic alkylating agents listed above.

Table 1. Recommended Dosages of Antineoplastic Alkylating Agents

Drug	Dosage	Regimen
Mustargen	0.4 mg/kg	each course given as a singe dose or in divided doses of 0.1 to 0.2 mg/kg/day.
Cyclophosphamide	40-50 mg/kg i.v. 10-15 mg/kg i.v. 3-5 mg/kg i.v. 1-5 mg/kg oral	in divided doses over a period of 2-5 days every 7-10 days twice weekly daily
Ifosfamide	1.2 g/m^2 i.v.	daily for 5 consecutive days; repeated every 3 weeks or after recovery from hematologic toxicity.
Melphalan	6 mg orally 10 mg orally 0.15 mg/kg orally 16 mg/m^2 i.v.	daily for 2-3 weeks followed by 4 weeks rest, then 2 mg daily maintenance dosage daily for 7-10 days followed by 2 mg daily maintenance after white blood cell count has recovered. daily for 7 days, followed by a rest period of at least 14 days, then 0.005 mg/kg daily maintenance. single infusion over 15-20 minutes every 2 weeks for 4 doses, followed by a rest period, then administered at 4 week intervals for maintenance.
Chlorambucil	0.1-0.2 mg/kg orally	daily for 3-6 weeks
Thiotepa	0.3-0.4 mg/kg i.v.	every 1-4 weeks
Mitomycin	20 mg/m^2 i.v.	every 6-8 weeks
Busulfan	1.8 mg/m^2 orally	daily
Lomustine	130 mg/m^2 orally	every 6 weeks

Drug	Dosage	Regimen
Carmustine	150-200 mg/m^2 i.v.	every 6 weeks
Procarbazine	2-4 mg/kg orally 1-2 mg/kg orally	daily for first week, then 4-6 mg/kg until maximum response is achieved maintentance
Temozolomide	150 mg/m^2 orally	once daily for 5 days per 28-day treatment cycle
Cisplatin	20 mg/m^2 i.v. 75-100 mg/m^2 i.v.	daily for 5 days per cycle once every 4 week cycle
Carboplatin	360 mg/m^2 i.v.	once every 4 week cycle

Preferred mTOR inhibitor plus antineoplastic alkylating agent combinations of this invention include CCI-779 plus cisplatin; CCI-779 plus cyclophosphamide; CCI-779 plus carboplatin; and CCI-779 plus BCNU.

The antineoplastic activity of the mTOR inhibitor plus antineoplastic alkylating agent combinations were confirmed using CCI-779 as a representative mTOR inhibitor in *in vitro* and *in vivo* standard pharmacological test procedures using combinations of CCI-779 plus cisplatin; CCI-779 plus cyclophosphamide; and CCI-779 plus BCNU as representative combinations of this invention. The following briefly describes the procedures used and the results obtained.

Human rhabdomyosarcoma lines Rh30 and Rh1 and the human glioblastoma line SJ-GBM2 were used for *in vitro* combination studies with CCI-779 and alkylating agents. *In vivo* studies used a human neuroblastoma (NB1643) and human colon line GC3.

Dose response curves were determined for each of the drugs of interest. The cell lines Rh30, Rh1 and SJ-G2 were plated in six-well cluster plates at $6x10^3$, $5x10^3$ and $2.5x10^4$ cells/well respectively. After a 24 hour incubation period, drugs were added in either 10%FBS+RPMI 1640 for Rh30 and Rh1 or 15%FBS+DME for SJ-G2. After seven days exposure to drug containing media, the nuclei were released by treating the cells with a hypotonic solution followed by a detergent. The nuclei were then counted with a Coulter Counter. The results of the experiments were graphed and the IC_{50} (drug concentration producing 50% inhibition of growth) for each drug was determined by extrapolation. Because the IC50s varied slightly from experiment

to experiment, two values that bracketed the IC50 of each drug were used in the interaction studies. The point of maximum interaction between two drugs occurs when they are present in a 1:1 ratio if the isobole is of standard shape. Therefore, each of the three approximate IC_{50} concentrations of CCI-779 was mixed in a 1:1 ratio with each of three approximated IC_{50}s of cisplatin, BCNU, and melphanan. This resulted in nine 1:1 combinations of drugs in each experiment plus three IC_{50} concentrations for CCI-779 and the other drug. This protocol usually resulted in at least one combination for each drug containing an IC_{50} value. The 1:1 combination of IC_{50} concentrations for CCI-779 and each chemotherapy drug was then used to calculate additivity, synergism, or antagonism using Berenbaum's formula: $x/X_{50}+y/Y_{50},=1,<1,>1$. If the three concentrations of CCI-779 tested alone didn't produce an IC that matched any of the three ICs of the other compound tested alone, all the 1:1 combinations were checked to see if their ICs fell between the appropriate ICs of drugs tested singly. If they did, the effect was considered additive.

The results obtained in the *in vitro* standard pharmacological test procedure showed when tested against Rh30 tumor line, the combination of CCI-779 plus cisplatin was synergistic; the combination was greater than additive but did not reach levels of being mathematically synergystic against the Rh1 tumor cell line, and was additive against the SJ-G2 tumor cell line. A combination of CCI-779 plus BCNU was synergistic against the SJ-G2 tumor cell line and greater than additive but did not reach levels of being mathematically synergystic against the Rh30 cell line, and additive against the Rh1 cell line. The combination of CCI-779 plus melphanan was additive against each of the cell lines.

Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME), 4 weeks of age, were immune-deprived by thymectomy, followed 3 weeks later by whole-body irradiation (1200 cGy) using a ^{137}Cs source. Mice received 3×10^6 nucleated bone marrow cells within 6-8 h of irradiation. Tumor pieces of approximately 3 mm^3 were implanted in the space of the dorsal lateral flanks of the mice to initiate tumor growth. Tumor-bearing mice were randomized into groups of seven prior to initiating therapy. Mice bearing tumors each received drug when tumors were approximately 0.20-1 cm in diameter. Tumor size was determined at 7-day intervals using digital Vernier calipers interfaced with a computer. Tumor volumes were calculated assuming tumors

to be spherical using the formula [(π/6) x d^3], where d is the mean diameter. CCI-779 was given on a schedule of 5 consecutive days for 2 weeks with this cycle repeated every 21 days for 3 cycles. This resulted in CCI-779 being given on days 1-5, 8-12 (cycle 1); 21-25, 28-32 (cycle 2); and 42-46, 49-53 (cycle 3). The schedule of the other chemotherapy drug for each study was as follows:

Cyclophosphamide on days 1 and 8 every 21 days for 3 cycles

The combination of CCI-779 and cyclophosphamide was evaluated using a human rhabdosarcoma (Rh18) using the mouse xenograft test procedure described above. In this test procedure, the effect of CCI-779 with cyclophosphamide (44 mg/kg) was additive. When combined as suboptimum dosages, CCI-779 plus cyclophosphamide was equivalent to cyclophosphamide given at an optimum dosage.

Based on the results of these standard pharmacological test procedures, combinations of an mTOR inhibitor plus an antineoplastic alkylating agent are useful as antineoplastic therapy. More particularly, these combinations are useful in the treatment of renal carcinoma, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. As these combinations contain at least two active antineoplastic agents, the use of such combinations also provides for the use of combinations of each of the agents in which one or both of the agents is used at subtherapeutically effective dosages, thereby lessening toxicity associated with the individual chemotherapeutic agent.

In providing chemotherapy, multiple agents having different modalities of action are typically used as part of a chemotherapy "cocktail." It is anticipated that the combinations of this invention will be used as part of a chemotherapy cocktail that may contain one or more additional antineoplastic agents depending on the nature of the neoplasia to be treated. For example, this invention also covers the use of the mTOR inhibitor/alkylating agent combination used in conjunction with other chemotherapeutic agents, such as antimetabolites (i.e., 5-fluorouracil, floxuradine,

thioguanine, cytarabine, fludarabine, 6-mercaptopurine, methotrexate, gemcitabine, capecitabine, pentostatin, trimetrexate, or cladribine); hormonal agents (i.e., estramustine, tamoxifen, toremifene, anastrozole, or letrozole); antibiotics (i.e., plicamycin, bleomycin, mitoxantrone, idarubicin, dactinomycin, mitomycin, or daunorubicin); immunomodulators (i.e., interferons, IL-2, or BCG); antimitotic agents (i.e., vinblastine, vincristine, teniposide, or vinorelbine); topoisomerase inhibitors (i.e., topotecan, irinotecan, etoposide, or doxorubicin); and other agents (i.e., hydroxyurea, trastuzumab, altretamine, retuximab, paclitaxel, docetaxel, L-asparaginase, or gemtuzumab ozogamicin).

As used in this invention, the combination regimen can be given simultaneously or can be given in a staggered regimen, with the mTOR inhibitor being given at a different time during the course of chemotherapy than the alkylating agent. This time differential may range from several minutes, hours, days, weeks, or longer between administration of the two agents. Therefore, the term combination does not necessarily mean administered at the same time or as a unitary dose, but that each of the components are administered during a desired treatment period. The agents may also be administered by different routes. For example, in the combination of an mTOR inhibitor plus an alkylating agent, it is anticipated that the mTOR inhibitor will be administered orally or parenterally, with parenterally being preferred, while the alkylating agent may be administered parenterally, orally, or by other acceptable means. These combination can be administered daily, weekly, or even once monthly. As typical for chemotherapeutic regimens, a course of chemotherapy may be repeated several weeks later, and may follow the same timeframe for administration of the two agents, or may be modified based on patient response.

As typical with chemotherapy, dosage regimens are closely monitored by the treating physician, based on numerous factors including the severity of the disease, response to the disease, any treatment related toxicities, age, health of the patient, and other concomitant disorders or treatments.

Based on the results obtained with the CCI-779 plus alkylating agent combinations, it is projected that the initial i.v. infusion dosage of the mTOR inhibitor will be between about 0.1 and 100 mg/m^2, with between about 2.5 and 70 mg/m^2 being preferred. It is also preferred that the mTOR inhbitor be administered by i.v.,

typically over a 30 minute period, and administered about once per week. The initial dosages of the alkylating agent component will depend on the component used, and will be based initially on physician experience with the agents chosen. After one or more treatment cycles, the dosages can be adjusted upwards or downwards depending on the results obtained and the side effects observed.

For commercially available alkylating agents, the existing dosage form can be used, with the dosages divided as need be. Alternatively, such agents or alkylating agents that are not commercially available can be formulated according to standard pharmaceutical practice. Oral formulations containing the active compounds of this invention may comprise any conventionally used oral forms, including tablets, capsules, buccal forms, troches, lozenges and oral liquids, suspensions or solutions. Capsules may contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (e.g. corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium lauryl sulfate, microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. Preferred surface modifying agents include nonionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

In some cases it may be desirable to administer the compounds directly to the airways in the form of an aerosol.

The compounds may also be administered parenterally or intraperitoneally. Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparation contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

For the purposes of this disclosure, transdermal administrations are understood to include all administrations across the surface of the body and the inner linings of bodily passages including epithelial and mucosal tissues. Such administrations may be carried out using the present compounds, or pharmaceutically acceptable salts thereof, in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semi-permeable membrane covering a reservoir

containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

CLAIMS

What is claimed is:

1. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising an mTOR inhibitor and an antineoplastic alkylating agent.

2. The method according to Claim 1, wherein the neoplasm is renal cancer.

3. The method according to Claim 1, wherein the neoplasm is soft tissue sarcoma.

4. The method according to Claim 1, wherein the neoplasm is breast cancer.

5. The method according to Claim 1, wherein the neoplasm is a neuroendocrine tumor of the lung.

6. The method according to Claim 1, wherein the neoplasm is cervical cancer.

7. The method according to Claim 1, wherein the neoplasm is uterine cancer.

8. The method according to Claim 1, wherein the neoplasm is a head and neck cancer.

9. The method according to Claim 1, wherein the neoplasm is glioma.

10. The method according to Claim 1, wherein the neoplasm is non-small cell lung cancer.

11. The method according to Claim 1, wherein the neoplasm is prostate cancer.

12. The method according to Claim 1, wherein the neoplasm is pancreatic cancer.

13. The method according to Claim 1, wherein the neoplasm is lymphoma.

14. The method according to Claim 1, wherein the neoplasm is melanoma.

15. The method according to Claim 1, wherein the neoplasm is small cell lung cancer.

16. The method according to Claim 1, wherein the neoplasm is ovarian cancer.

17. The method according to Claim 1, wherein the neoplasm is colon cancer.

18. The method according to Claim 1, wherein the neoplasm is esophageal cancer.

19. The method according to Claim 1, wherein the neoplasm is gastric cancer.

20. The method according to Claim 1, wherein the neoplasm is leukemia.

21. The method according to Claim 1, wherein the neoplasm is colorectal cancer.

22. The method according to Claim 1, wherein the neoplasm is unknown primary cancer.

23. The method according to any one of Claims 1 to 22, wherein the antineoplastic alkylating agent is selected from the group consisting of mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, thiotepa,

mitomycin, busulfan, lomustine, carmustine, procarbazine, temozolomide, cisplatin, and carboplatin.

24. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising an mTOR inhibitor and an antineoplastic alkylating agent, wherein either the mTOR inhibitor, the alkylating agent, or both are provided in subtherapeutically effective amounts.

25. The method according to Claim 24 in which the mTOR inhibitor is provided in a subtherapeutically effective amount.

26. The method according to Claim 24 in which the alkylating agent is provided in a subtherapeutically effective amount.

27. The method according to Claim 24 in which both the mTOR inhibitor and the alkylating agent are provided in subtherapeutically effective amounts.

28. The method according to any one of Claims 1 to 27, wherein the mTOR inhibitor is a rapamycin.

29. The method according to Claim 28, wherein the rapamycin is rapamycin.

30. The method according to Claim 28, wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

31. The method according to Claim 28 wherein the rapamycin is CCI-779.

32. An antineoplastic combination which comprises an effective amount of an mTOR inhibitor and an antineoplastic alkylating agent.

33. The combination according to Claim 32, wherein the mTOR inhibitor is a rapamycin.

34. The combination according to Claim 33, wherein the rapamycin is rapamycin.

35. The combination according to Claim 33, wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

36. The combination according to Claim 33 wherein the rapamycin is CCI-779.

37. The use of an mTOR inhibitor and an antineoplastic alkylating agent in the preparation of a medicament for the treatment of a neoplasm.

38. Use according to Claim 37 wherein the neoplasm is renal cancer, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, a head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer or unknown primary cancer.

39. Use according to Claim 37 or 38, wherein the antineoplastic alkylating agent is selected from the group consisting of meclorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, thiotepa, mitomycin, busulfan, lomustine, carmustine, procarbazine, temozolomide, cisplatin, and carboplatin.

40. Use according to any one of Claims 37 to 39 wherein either the mTOR inhibitor, the alkylating agent, or both are provided in subtherapeutically effective amounts.

41. Use according to any one of Claims 37 to 40 wherein the mTOR inhibitor is a rapamycin.

42. Use according to Claim 41 wherein the rapamycin is rapamycin.

43. Use according to Claim 41 wherein the rapamycin is 42-O-(2-hydroxy)ethyl rapamycin.

44. Use according to Claim 41 wherein the rapamycin is CCI-779.

45. A product comprising an mTOR inhibitor and an antineoplastic alkylating agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a neoplasm in a mammal.

46. A product according to Claim 45 wherein the mTOR inhibitor is CCI-779.

47. A pharmaceutical composition comprising an mTOR inhibitor, an antineoplastic alkylating agent and a pharmaceutically acceptable carrier.

48. A pharmaceutical composition according to Claim 47 wherein the mTOR inhibitor is CCI-779.

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(71) Applicant: WYETH [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).

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(74) Agents: MILOWSKY, Arnold, S.; Wyeth, Patent Law Department, Five Giralda Farms, Madison, NJ 07940-0874 et al. (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/098416 A3

(54) Title: ANTINEOPLASTIC COMBINATIONS

(57) Abstract: This invention provides the use of a combination of an mTOR inhibitor and an antineoplastic alkylating agent in the treatment of neoplasms.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/16737

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/395 A61K31/675 A61P35/00 //(A61K31/395,31:675)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, CHEM ABS Data, EMBASE, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHI Y ET AL: "Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro." CANCER RESEARCH, (1995 MAY 1) 55 (9) 1982-8. , vol. 55, no. 9, - 1995 pages 1982-8, XP002040888 page 1982, right-hand column, line 31-40 discussion page 1985, left-hand column, line 30 -page 1987, left-hand column, line 19; figure 7 --- -/--	1-23, 28-39, 41-48

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 2002	20/12/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Venturini, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GEOERGER B ET AL: "ANTITUMOR ACTIVITY OF THE RAPAMYCIN ANALOG CCI-779 IN HUMAN PRIMITIVE NEUROECTODERMAL TUMOR/MEDULLOBLASTOMA MODELS AS SINGLE AGENT AND IN COMBINATION CHEMOTHERAPY" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 4, no. 61, 15 February 2001 (2001-02-15), pages 1527-1532, XP001079526 ISSN: 0008-5472 page 1528, right-hand column, line 30-45 discussion page 1529, right-hand column, line 15-22; figures 1,6; table 1 ---	1-23, 28-39, 41-48
X	US 5 066 493 A (SEHGAL SURENDRA N ET AL) 19 November 1991 (1991-11-19) column 1, line 61 -column 2, line 19 column 4, line 50 -column 5, line 8; claims ---	1-23, 28-39, 41-48
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E	WO 02 066019 A (NOVARTIS ERFIND VERWALT GMBH ;NOVARTIS AG (CH); LANE HEIDI (CH); 0) 29 August 2002 (2002-08-29) page 4, line 27 -page 5, line 33 page 8, line 6-10 page 14, line 24-31 page 17, line 11-25; claims ---	1-23, 28-39, 41-48
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A	BONI J ET AL: "PHARMACOKINETICS OF ESCALATING DOSES OF CCI-779 IN COMBINATION WITH 5-FLUOROURACIL AND LEUCOVORIN IN PATIENTS WITH ADVANCED SOLID TUMORS" , EUROPEAN JOURNAL OF CANCER, PERGAMON PRESS, OXFORD, GB, VOL. 37, NR. SUPPL 6, PAGE(S) S68 XP001084492 ISSN: 0959-8049 abstract ---	1-48
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INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/US 02/16737

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Electronic Acknowledgement Receipt

EFS ID:	3320341
Application Number:	12094173
International Application Number:	PCT/EP06/68656
Confirmation Number:	9572
Title of Invention:	NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	01095
Filer:	Gregory Houghton./Linda adams
Filer Authorized By:	Gregory Houghton.
Attorney Docket Number:	34768-US-PCT
Receipt Date:	19-MAY-2008
Filing Date:	
Time Stamp:	10:51:49
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

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Payment Type	Deposit Account
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RAM confirmation Number	5921
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Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. 1.492 (National application filing, search, and examination fees)	

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1		cvtltr_dec.pdf	120019 ea557bad174be14dcf9182100c1cb5d0a3205715	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal of New Application	1	2
	Oath or Declaration filed	3	5

Warnings:

Information:

2		IDS.pdf	50962 d3e473c23135611102f8b69f0f5dfbb9716b1e206	yes	4

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Information Disclosure Statement Letter	1	2
	Information Disclosure Statement (IDS) Filed	3	4

Warnings:

Information:

3		prelim_amend.pdf	27968 ceb6b8eb2f74a35ad340e13da9be5c19ce68b752	yes	6

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	2
	Abstract	3	3
	Claims	4	5
	Applicant Arguments/Remarks Made in an Amendment	6	6

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4	Application Data Sheet	ADS.pdf	97400 5338346812811 7ac40ca370ca3e1addc392a2c5f	no	4
Warnings:					
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5	Foreign Reference	WO1997005167_A.pdf	609726 8d078a128cbc13727de292affb94c7f7210c2071	no	22
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6	Foreign Reference	WO1997047317_A1.pdf	937866 d4c89ad17a679f3b1dc91fb940e769cdaed843d6	no	32
Warnings:					
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7	Foreign Reference	WO2002080975_A1.pdf	1204549 96afb764bf280857711a3b7a466e3b4bb8fa36fe3	no	28
Warnings:					
Information:					
8	Foreign Reference	WO2002098416_A2_A3.pdf	1246677 696fa4230dd5bdafc64f3a13b1a7e8c5d63a61ad	no	27
Warnings:					
Information:					
9	Foreign Reference	WO2003020266_A1.pdf	1336745 356b4df138dc2197d7fb41e3ea3bf00b5d9a48c9	no	31
Warnings:					
Information:					
10	Foreign Reference	WO2005082411_A1.pdf	844407 c101acb035a5047d7d9d244e1fdf9eacd3c95419	no	14
Warnings:					
Information:					
11	Foreign Reference	EP462071_B1.pdf	563328 3a4de5fe45bdf4969d11bac3584af3a69f567ded	no	11
Warnings:					
Information:					
12	Fee Worksheet (PTO-06)	fee-info.pdf	8556 e33f2db5027a03dc53dc57fd59c2c55e2f0e118	no	2

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory Houghton./Linda adams
Attorney Docket Number:	34768-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
National Stage Fee	1631	1	310	310
Natl Stage Search Fee - Report provided	1642	1	410	410
National Stage Exam - all other cases	1633	1	210	210
Pages:				
Claims:				
Independent claims in excess of 3	1614	4	210	840
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1770**

Amendments to the Specification:

A copy of the abstract is herein provided on the following separate sheet.

REMARKS/ARGUMENTS

The foregoing amendments to the specification is to place the Abstract on a separate sheet. The amendments to the claims are to place the claims in better form and remove multiple dependencies. No new matter has been added. Should the Examiner have any questions, please contact the undersigned attorney.

Respectfully submitted,

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
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(862) 778-2614

Gregory D. Houghton
Attorney for Applicants
Reg. No. 47,666

Date:

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claim 1. (Original): A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 2. (Original) A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

Claim 3. (Original) A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 4. (Original) A method for inducing endocrine tumor regression, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 5. (Original) A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 6. (Original) A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 7. (Original) A method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 8. (Currently Amended) A method according to ~~any one of claims 1 to 7~~claim 1, comprising administering in addition a therapeutically effective amount of at least one second drug substance.

Claim 9. (Original) A method according to claim 8, wherein a second drug substance is somastatin or a somastatin analogue.

Claim 10. (Currently Amended) The use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to ~~any one of claims 1 to 9~~claim 1.

Claim 11. (Currently Amended) A method according to ~~any one of claims 1 to 9~~claim 1, ~~or the use according to claim 10~~, wherein an mTOR inhibitor is selected from rapamycin or a rapamycin derivative.

Claim 12. (Original) A method according to claim 10, wherein an mTOR inihibitor is 40-O-(2-hydroxyethyl)-rapamycin.

Abstract

A method for treating endocrine tumors by administration of an mTOR inhibitor, optionally in combination with another drug.

CASE 34678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF

MARKS ET AL.

INTERNATIONAL APPLICATION NO:

FILED:

U.S. APPLICATION NO: Not Yet Known

35 USC §371 DATE: Herewith

FOR: NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Sir:

Prior to the examination of the above-referenced patent application, please enter the following preliminary amendments.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of the claims which begins on page 4 of this paper.

Remarks/Arguments begin on page 6 of this paper.

Doc code :IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-08)
Approved for use through 05/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	
	Filing Date	
	First Named Inventor	Peter Wayne Marks
	Art Unit	
	Examiner Name	
	Attorney Docket Number	34768-US-PCT

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5538739		1996-07-23	BODMER DAVID, ET AL	

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20040176339		2004-09-09	SHERMAN MATTHEW L.	
	2	20020183240		2002-12-05	GIBBONS JAMES J.	

If you wish to add additional U.S. Published Application citation information please click the Add button.

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	02/080975	WO		2002-10-17	WYETH CORP		☐
	2	2005/082411	WO		2005-09-09	CHRISTENSEN JAMES G.		☐

EFS Web 2.1.1

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Peter Wayne Marks
	Art Unit	
	Examiner Name	
	Attorney Docket Number	34768-US-PCT

	3	02/098416	WO		2002-12-12	WYETH CORP		☐
	4	97/05167	WO		1997-02-13	DEGHENGHI ROMANO		☐
	5	0 462 071	EP		1991-12-18	SANDOZ LTD		☐
	6	03/020266	WO		2003-03-13	WYETH CORP		☐
	7	97/47317	WO		1997-12-18	CIBA GEIGY AG		☐

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1		☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	34678-US-PCT
	Application Number	
Title of Invention	NEUROENDOCRINE TUMOR TREATMENT	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Applicant Information:

Applicant 1

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
	Peter	Wayne	Marks	

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Citizenship under 37 CFR 1.41(b) i: US

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Postal Code	06525	Country i	US

Applicant 2

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
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Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

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Citizenship under 37 CFR 1.41(b) i: US

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Address 2			
City	Madison	State/Province	NJ
Postal Code	07940	Country i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	34678-US-PCT
	Application Number	
Title of Invention	NEUROENDOCRINE TUMOR TREATMENT	

Customer Number	01095		
Email Address		Add Email	Remove Email

Application Information:

Title of the Invention	NEUROENDOCRINE TUMOR TREATMENT		
Attorney Docket Number	34678-US-PCT	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Suggested Class (if any)		Sub Class (if any)	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)		Suggested Figure for Publication (if any)	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S. C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Enter either Customer Number or complete the Representative Name section below. If both sections are completed the Customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	01095		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	a 371 of international	PCT/EP2006/068656	2006-11-20

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

PTO/SB/14 (07-07)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	34678-US-PCT
	Application Number	
Title of Invention	NEUROENDOCRINE TUMOR TREATMENT	

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

Application Number	Country [i]	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
0523658.3	GB	2005-11-21	(•) Yes ( ) No
0601082.1	GB	2006-01-19	(•) Yes ( ) No
0602747.8	GB	2006-02-10	(•) Yes ( ) No
0607942.0	GB	2006-04-21	(•) Yes ( ) No
0609272.0	GB	2006-05-10	(•) Yes ( ) No
0609912.1	GB	2006-05-18	(•) Yes ( ) No
06120660.3	EP	2006-09-14	(•) Yes ( ) No

Remove

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Assignee Information:

Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office.

Assignee 1

If the Assignee is an Organization check here. [X]

Organization Name	NOVARTIS AG		
Mailing Address Information:			
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Address 2			
City	Basel	**State/Province**	
Country [i]	CH	Postal Code	4056
Phone Number		Fax Number	
Email Address			

PTO/SB/14 (07-07)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	34678-US-PCT
	Application Number	
Title of Invention	NEUROENDOCRINE TUMOR TREATMENT	

Additional Assignee Data may be generated within this form by selecting the **Add** button.

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.

Signature	Gregory Houghton			Date (YYYY-MM-DD)	5/14/08
First Name	Gregory G.	Last Name	Houghton	Registration Number	47666

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Case 34678-US-PCT

Assistant Commissioner for Patents
Washington, D.C. 20231

Attn: Licensing and Review

PETITION FOR RETROACTIVE LICENSE (37 CFR 5.25)

Sir:

It is respectfully requested that this petition for a retroactive license for foreign filing attached hereto be granted under the provisions of 37 CFR 5.25.

Attached is a copy of United Kingdom Patent Application Nos. 0523658.3, 0601082.1, 0602747.8, 0607942.0, 0609272.0 and 0609912.1 and European Patent Application No. 06120660.3, the material which was filed abroad without a license for foreign filing. The title of the invention is Organic Compounds.

With respect to the material for which a retroactive license is requested, it was filed in the United Kingdom on November 21, 2005, January 19, 2006, February 10, 2006, April 21, 2006, May 10, 2006, May 18, 2006 and with the European Patent Office on September 14, 2006.

Also attached is a Declaration of Hans Schaller which confirms that, in accordance with 37 CFR 5.25 (a)(3)(i)-(iii),

a) the subject matter in questions was not under a secrecy order at the time it was filed abroad, and that it is not currently under a secrecy order;
b) the license is being sought after discovery of the proscribed foreign filing; and
c) an explanation of why the material was filed abroad through error and without deceptive intent without the required license under 37 CFR 5.11 first having been obtained.

Please charge the $200 fee for this Petition for Retroactive License to Deposit Account No. 19-0134 in the name of Novartis Corporation.

Respectfully submitted,

Gregory C. Houghton
Attorney for Applicant
Reg. No. 47,666
Phone No. (862) 778-2614

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080

Date: May 19, 2008

DECLARATION UNDER §5.25(3)

I, Hans Schaller, hereby state:

i) That I am a European Patent Attorney of the Patent and Trademark Department of Novartis*) and was in charge of the above identified application and the European Attorney who filed the above-identified patent application's priority applications at the United Kingdom's Patent Office and the European Patent Office. I am also the European Attorney who filed the above-identified PCT Application at the World Intellectual Property Organization.

ii) The subject matter of the following priority patent applications, UK Patent Application Nos. 0523658.3, 0601082.1, 0602747.8, 0607942.0, 0609272.0, and 0609912.1 and European Patent Application No. 06120660.3, in questions, were filed to acquire a priority filing date.

iii) A PCT Application No. PCT/EP2006/068656 claiming priority to the applications outlined in section ii) above was filed at the end of the priority year.

iv) The applications were filed without knowledge of the inventors, of whom, subsequently include United States citizens. At the time that said patent applications were filed, it was not recognized that it contained subject matter of United States origin. The patent applications were filed abroad through error and without deceptive intent without the require license under §5.11 first having been obtained.

v) The subject matter of the patent applications outlined in section ii) and iii) were not under a secrecy order in the United States at the time they were filed abroad, and they are not currently under a secrecy order in the United States.

vi) It was first determined on May 8, 2008 that UK priority applications in section ii) above were inadvertently filed abroad in the United Kingdom on November 21, 2005, January 19, 2006, February 10, 2006, April 21, 2006, May 10, 2006, May 18, 2006 and European Patent Application in section ii) above was a priority application inadvertently filed abroad in European Patent Office on September 14, 2006 without a foreign filing license under 35 U.S.C. 184. Also, a PCT Application No. PCT/EP2006/068656 was inadvertently filed with World Intellectual Property Organization on November 20, 2006 without a foreign filing license under 35 U.S.C. 184. Upon this discovery of the need for a retroactive foreign filing license under 34 U.S.C. 184, this license has been diligently and promptly sought after.

Signed Hans Schaller

Hans Schaller

Date: 13-May-2008

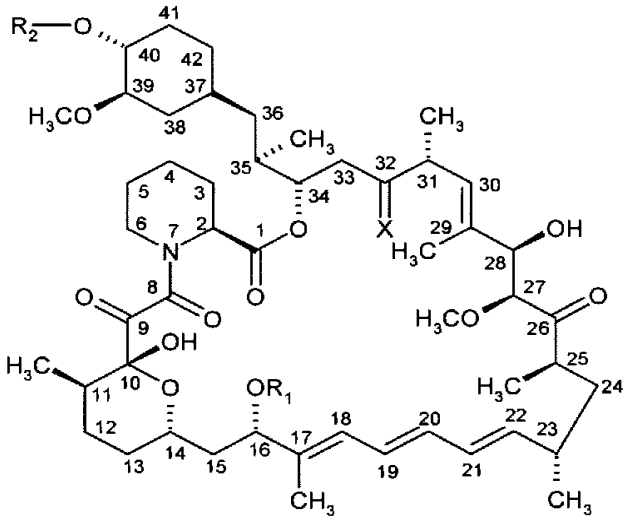
*) Since 1-Jan-2008 I am no longer employed with Novartis but with Kopecky & Schwarz, Patentanwaltskanzlei, Wipplingerstrasse 30, A-1010 WIEN AUSTRIA

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.

Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.

The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small

intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcionoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). The most frequent symptoms of carcinoid syndrome are flushing and diarrhea. Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation, with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms and pellagra-like skin lesions with hyperkeratosis are also seen in a number of patients. A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as pancreatic neuroendocrine tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract. Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming

resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®,
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the

podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as

active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial
27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapammycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/20-Nov-05

Abstract

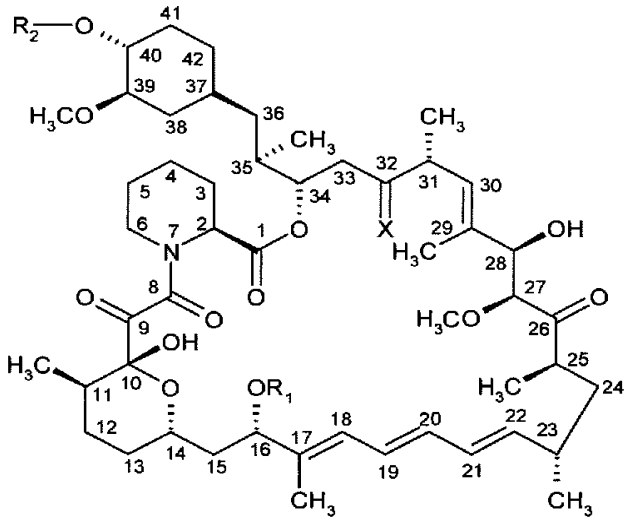
A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.

Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.

The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small

intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcionoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). The most frequent symptoms of carcinoid syndrome are flushing and diarrhea. Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation, with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms and pellagra-like skin lesions with hyperkeratosis are also seen in a number of patients. A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as pancreatic neuroendocrine tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically

acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the

podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- calcineurin inhibitors, e.g. cyclosporin A or FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- bcr-abl tyrosine kinase inhibitors;
- c-kit receptor tyrosine kinase inhibitors;
- PDGF receptor tyrosine kinase inhibitors, e.g. Gleevec (imatinib);
- p38 MAP kinase inhibitors,
- VEGF receptor tyrosine kinase inhibitors,
- PKC inhibitors, e.g. as disclosed in WO 0238561 or WO 0382859, e.g. the compound of Example 56 or 70;
- JAK3 kinase inhibitors, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO04052359 or WO05066156;
- S1P receptor agonists or modulators, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;

- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- adhesion molecule inhibitors, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- CCR9 antagonists,
- MIF inhibitors,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- antibodies which bind to TNF-alpha, such as infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. PDE4B-inhibitors,
- caspase ihibitors,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, pleuromutilins, fluoroquinolones, e.g. metronidazole, ciprofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-

Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.

Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/20-Nov-05

Abstract

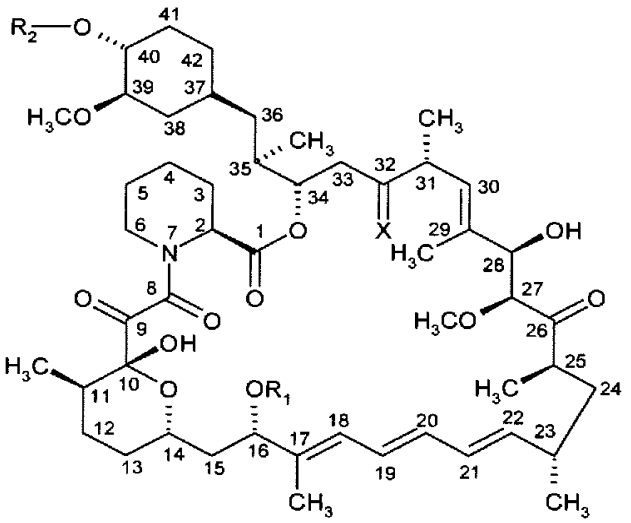
A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.

Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.

The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under

1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin,

idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,
- alkylating agents, such as dacarbazine,
- streptozotocin.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- calcineurin inhibitors, e.g. cyclosporin A or FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- bcr-abl tyrosine kinase inhibitors;
- c-kit receptor tyrosine kinase inhibitors;
- PDGF receptor tyrosine kinase inhibitors, e.g. Gleevec (imatinib);
- p38 MAP kinase inhibitors,
- VEGF receptor tyrosine kinase inhibitors,
- PKC inhibitors, e.g. as disclosed in WO 0238561 or WO 0382859, e.g. the compound of Example 56 or 70;
- JAK3 kinase inhibitors, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO04052359 or WO05066156;
- S1P receptor agonists or modulators, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol

optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;

- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- adhesion molecule inhibitors, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- CCR9 antagonists,
- MIF inhibitors,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- antibodies which bind to TNF-alpha, such as infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. PDE4B-inhibitors,
- caspase ihibitors,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, pleuromutilins, fluoroquinolones, e.g. metronidazole, ciprofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance

with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/10-Feb-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

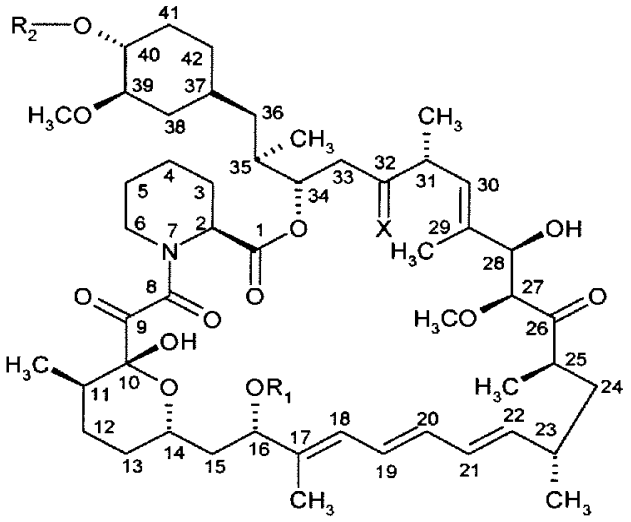
5

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a

therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;

such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising
a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the

podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;

- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as

active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial
27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/19-Apr-06

Abstract

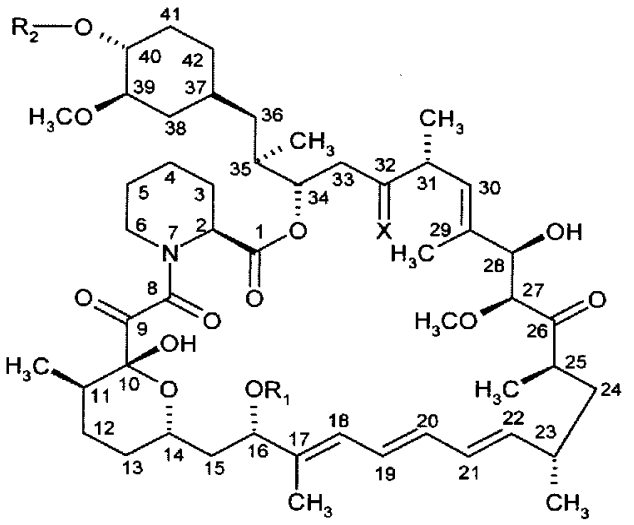
A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a

therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;

such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising
a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the

podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;

- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as

active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial
27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.
A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).
A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with

Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/10-May-06

Abstract

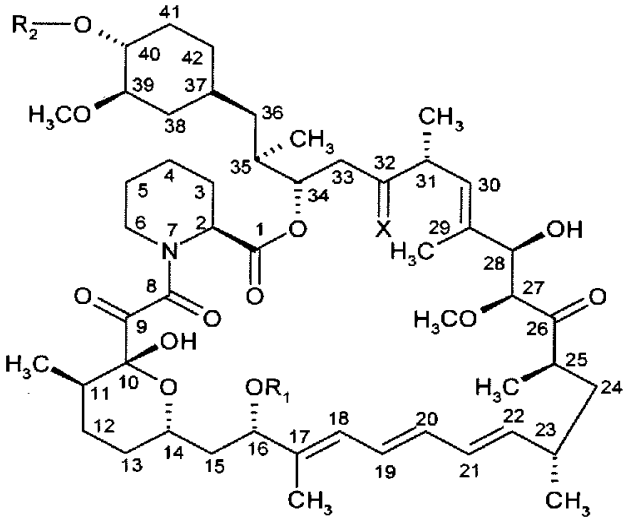
A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.
A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.
Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors, e.g. it was found that suppression of the ASK1/JNK pathway is responsible for resistancy of cells against endocrine agent treatment and that mTOR inhibitors, e.g. Compound A, are able to restore that pathway.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or

inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method for reducing or avoiding resistance of endocrine cancer cells in the treatment with endocrine agents, comprising treating resistant cells with an effective amount of a combination of an mTOR inhibitor and an endocrine agent.

An "endocrine agent" e.g. includes an aromatase inhibitor, such as letrozole, or an estrogen inhibitor, e.g. tamoxifen.

Resistant cancer cells inlcude such wherein the ASK/JNK pathway is blocked at least partially, or totally.

1.10 A method as indicated under 1.1 to 1.9, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.11 A method as indicated under 1.1 to 1.10, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.11 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.11 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.11 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTOR inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.11 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter or hereinbefore.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter or hereinbefore.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,

- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,
-alkylating agents, such as dacarbazine,
- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490),

prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,

- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting

antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. In vitro findings

Compound A is able to restore activity of endocrine agents, like estrogen inhibitors and/or aromatase inhibitors in cells which are otherwise resistant to endocrine agent treatment. Several studies have implicated aberrant acitivty of the Akt kinase as a significant mechanism by which breast cancer tumors are unresponsive to endocrine therapy.

For evaluating that, response in MCF-7 breast cancer cells expressing either wild-type (control) or constitutively-active Akt (myrAkt) and a dominant-negative ASK1 (DNASK1) was investigated. It was found that DNASK1 cells expressed are much more resistant to the inhibitory growth effects of endocrine agent treatment, such as endocrine agents like estrogen receptor inhibitors, e.g. tamoxifen, or aromatase inhibitors, e.g. letrozole. At the molecular level, treatment with endocrine agents results in phosphorylation (activation) of cJUN in the control cells, but not in either the myrAkt1 or DANSK1 cells. Co-treatment of resistant myrAkt1 MCF-7 cells with Compound A, however, restores activation of the ASK/JNK pathway and increases endocrine therapy sensivity.

D. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.
A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).
A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/18-May-06

Abstract

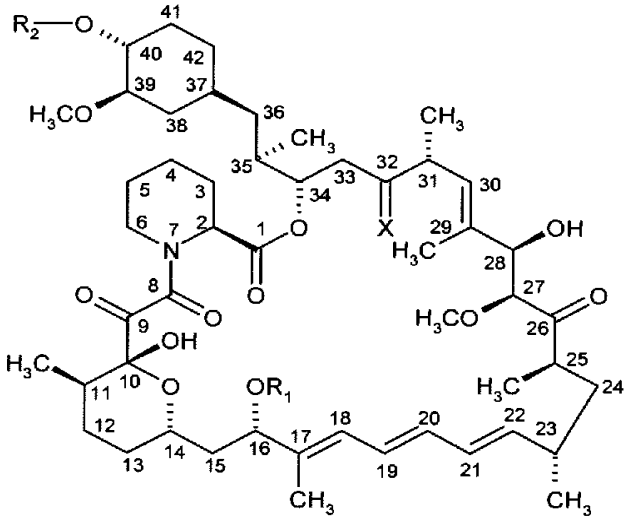
A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors, e.g. it was found that suppression of the ASK1/JNK pathway is responsible for resistancy of cells against endocrine agent treatment and that mTOR inhibitors, e.g. Compound A, are able to restore that pathway.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or

inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method for reducing or avoiding resistance of endocrine cancer cells in the treatment with endocrine agents, comprising treating resistant cells with an effective amount of a combination of an mTOR inhibitor and an endocrine agent.

An "endocrine agent" e.g. includes an aromatase inhibitor, such as letrozole, or an estrogen inhibitor, e.g. tamoxifen.

Resistant cancer cells inlcude such wherein the ASK/JNK pathway is blocked at least partially, or totally.

1.10 A method as indicated under 1.1 to 1.9, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.11 A method as indicated under 1.1 to 1.10, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.11 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.11 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.11 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTOR inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.11 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter or hereinbefore.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter or hereinbefore.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.

LHRH peptidomimetics, e.g. such as disclosed in US6627609, teverelix, D-63153; perifosine, erucyl phosphocholine, AN-152, AN-238, AN-215, lobaplatin, disorazol E, ZEN-014, ZEN-017, RC-3095, AE-941 (Neovastat), cetorelix.
ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold

under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;

- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,

- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;
- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),

- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more

pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye)

determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. In vitro findings

Compound A is able to restore activity of endocrine agents, like estrogen inhibitors and/or aromatase inhibitors in cells which are otherwise resistant to endocrine agent treatment. Several studies have implicated aberrant acitivty of the Akt kinase as a significant mechanism by which breast cancer tumors are unresponsive to endocrine therapy.

For evaluating that, response in MCF-7 breast cancer cells expressing either wild-type (control) or constitutively-active Akt (myrAkt) and a dominant-negative ASK1 (DNASK1) was investigated. It was found that DNASK1 cells expressed are much more resistant to the inhibitory growth effects of endocrine agent treatment, such as endocrine agents like estrogen receptor inhibitors, e.g. tamoxifen, or aromatase inhibitors, e.g. letrozole. At the molecular level, treatment with endocrine agents results in phosphorylation (activation) of cJUN in the control cells, but not in either the myrAkt1 or DANSK1 cells. Co-treatment of resistant myrAkt1 MCF-7 cells with Compound A, however, restores activation of the ASK/JNK pathway and increases endocrine therapy sensivity.

D. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.
A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).
A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/14-Sep-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	
Title of Invention:	NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory Houghton./Linda Adams
Attorney Docket Number:	34678-US-PCR

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Petition fee- 37 CFR 1.17(g) (Group II)	1463	1	200	200
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**200**

Electronic Acknowledgement Receipt

EFS ID:	3321822
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	NEUROENDOCRINE TUMOR TREATMENT USING MTOR INHIBITORS
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Gregory Houghton./Linda Adams
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)

1	Miscellaneous Incoming Letter	RetroactiveLicensePetition1.pdf	870617 73b3ac57a20741f34375f0f5f36c8e062088e337	no	138
Warnings:					
Information:					
2	Fee Worksheet (PTO-06)	fee-info.pdf	8194 4f35728d7360dcb528bc27a5f61b54a228fab5dc	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	878811	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

4. A method for inducing endocrine tumor regression, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method according to any one of claims 1 to 7, comprising administering in addition a therapeutically effective amount of at least one second drug substance.

9. A method according to claim 8, wherein a second drug substance is somastatin or a somastatin analogue.

10. The use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to any one of claims 1 to 9.

11. A method according to any one of claims 1 to 9, or the use according to claim 10, wherein an mTOR inhibitor is selected from rapamycin or a rapamycin derivative.

12. A method according to claim 10, wherein an mTOR inihibitor is 40-O-(2-hydroxyethyl)-rapamycin.

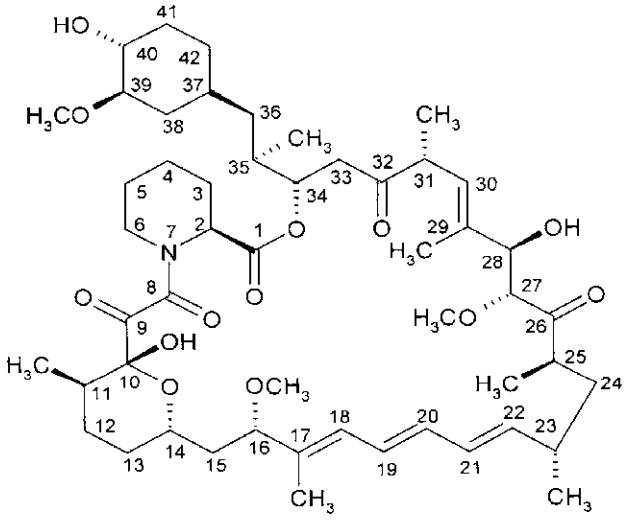
SC/17-Nov-06

Neuroendocrine tumor treatment

The present invention relates to organic compounds, more specifically to the use of mTOR inhibitors in neuroendocrine tumor treatment.

An mTOR inhibitor as used herein is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit mTOR activity via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin is a known macrolide antibiotic produced by Streptomyces hygroscopicus of formula



Other mTOR inhibitors include rapamycin derivatives, for example including rapamycin substituted in position 40 and/or 16 and/or 32.

Examples of other mTOR inhibitors include 40-O-alkyl-rapamycin derivatives, e.g. 40-O-hydroxyalkyl-rapamycin derivatives, for example 40-O-(2-hydroxy)-ethyl-rapamycin (everolimus),

rapamycin derivatives which are substituted in 40 position by heterocyclyl, e.g. 40-epi-(tetrazolyl)-rapamycin (also known as ABT578),

32-deoxo-rapamycin derivatives and 32-hydroxy-rapamycin derivatives, such as 32-deoxorapamycin,
16-O-substituted rapamycin derivatives such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32(S or R) -dihydro-rapamycin, or 16-pent-2-ynyloxy-32(S or R)-dihydro-40-O-(2-hydroxyethyl)-rapamycin,
rapamycin derivatives which are acylated at the oxygen in position 40, e.g. 40-[3-hydroxy-2-(hydroxy-methyl)-2-methylpropanoate]-rapamycin (also known as CCI779 or temsirolimus),
rapamycin derivatives (also sometimes designated as rapalogs) as disclosed in WO9802441 or WO0114387, e.g. including AP23573, such as 40-O-dimethylphosphinyl-rapamycin,
compounds disclosed under the name biolimus (biolimus A9), including 40-O-(2-ethoxy)ethyl-rapamycin, and compounds disclosed under the name TAFA-93, AP23464, AP23675 or AP23841; or
mTOR inhibitors as e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385 and WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, AP23573, AP23464, AP23675 or AP23841, e.g. AP23573, and/or
compounds disclosed under the name TAFA-93, and/or
compounds disclosed under the name biolimus.

More preferably an mTOR inhibitor is selected from the group consisting of
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or

16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)-rapamycin (also known as ABT578), and/or
AP23573,
such as 40-O-(2-hydroxyethyl)-rapamycin.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors (NETs), are found in the endocrine system. Carcinoid tumors, are a special type of tumor, generally classified as endocrine tumors. Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen. Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors. Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas). The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial, pulmonary or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed

as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are

unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic

nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are

predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (that is why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was found that mTOR inhibitors may be used for the treatment of such special type of tumors

In accordance with the particular findings the present invention provides in several aspects:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.4 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.5 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.6 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 The use of an mTOR inhibitor for the manufacture of a medicament for use in any method of 1.1 to 1.7 above.

1.9 A pharmaceutical composition comprising an mTOR inhibitor in association with at least one pharmaceutically acceptable excipient, e.g. appropriate carrier and/or diluent, e.g. including fillers, binders, disintegrants, flow conditioners, lubricants, sugars or sweeteners, fragrances, preservatives, stabilizers, wetting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers; for use in any method or use of 1.1 to 1.7 above.

Endocrine tumors as indicated herein e.g. include neuroendocrine tumors, e.g. including carcinoid tumors, pancreatic neuroendocrine tumors and tumors in parathyroid, pancreas, and pituitary glands.

Carcinoid tumors as indicated herein e.g. include typical and atypical carcinoids, ranging from low-grade malignant typical to intermediate atypical to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma; e.g. including carcinoids arising from the

- foregut e.g., bronchial, pulmonary or gastric carcinoids, e.g. including primary foregut tumors confined to the thymus, lung, stomach, and duodenum; e.g. carcinoid tumors of the

GI tract, e.g. located in the colon, stomach or small intestine, e.g. small-intestinal carcinoids, e.g. including

- midgut, e.g., small intestine or appendiceal carcinoids, e.g. located in the distal ileum, cecum, and proximal colon, or
- hindgut, e.g., rectal carcinoids.

Carcinoid lung tumors as indicated herein e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC) (Kulchitsky cells, argentaffin cells), bronchial carcinoid tumors, bronchial adenomas, e.g. including bronchial adenomas such as a small cell carcinoma and large cell neuroendocrine tumors, typical carcinoids or atypical carcinoids associated with large bronchopulmonary carcinoid tumors or small-cell carcinomas, pulmonary carcinoids, neuroendocrine lung tumors, large-cell neuroendocrine carcinoma of the lung, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Pancreatic neuroendocrine tumors as indicated herein e.g. include islet cell tumors, APUDomas, insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs associated with hypercalcemia, gastrinomas, VIPomas, somatostatinomas, GRFomas.

Endocrine or neuroendocrine tumor symptoms as indicated herein e.g. include hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, shortness of breath, flushing and diarrhea, endocrine or neuroendocrine syndromes carcinoid syndrome, e.g. including manifestations of either typical or atypical pulmonary carcinoid tumors, Cushing's syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia, ectopic growth hormone-releasing hormone secretion, ectopic secretion of corticotropin, cardiac manifestations secondary to fibrosis of the endocardium (endocardial fibrosis), valvular regurgitation (valvular heart disease), tricuspid insufficiency, secondary right-sided heart failure, wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension, recurrent pneumonia, cough, chest pain.

Tumors in parathyroid, pancreas and pituitary glands as indicated herein, e.g. include pituitary tumors, nervous system tumor, such as adenomas, multiple endocrine neoplasia type 1 (MEN 1).

Pituitary tumor symptoms as indicated herein include symptoms that are associated with excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones, loss of vision, clinically nonfunctioning tumor, e.g. associated with hypopituitarism underactive pituitary gland, e.g. associated with failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue, prolactinoma, a benign tumor that produces the prolactin hormone, acromegaly, e.g. associated with excessive somatic growth and multiple systemic, medical consequences, Cushing's disease, nerve damage, growth disturbances, changes in hormonal balance, double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

Disorders associated with endocrine tumors include endocrine or neuroendocrine tumor symptoms and pituitary tumor symptoms, such as indicated above.
Disorders include diseases.

An mTOR inhibitor may be used, e.g. in any method of 1.1 to 1.8 as described herein alone or in combination with one or more, at least one, second drug substance.

In other aspects the present invention provides

2.1 A combination of an mTOR inhibitor with at least one second drug substance, e.g. for any use as indicated under 1.1 to 1.8 above.

2.2 A pharmaceutical combination comprising an mTOR inhibitor in combination with at least one second drug substance, e.g. for any use as indicated under 1.1 to 1.8 above.

2.3 A pharmaceutical composition comprising an mTOR inhibitor in combination with at least one second drug substance and one or more pharmaceutically acceptable excipient(s), e.g. for any use as indicated under 1.1 to 1.8 above.

2.4 The use of an mTOR inhibitor for the manufacture of a medicament for use in combination with a second drug substance, e.g. for any use as indicated under 1.1 to 1.8 above.

2.5 Any method of 1.1 to 1.8 above comprising co-administering, concomitantly or in sequence, a therapeutically effective amount of an mTOR inhibitor and at least one second drug substance, e.g. in the form of a pharmaceutical combination or composition.

2.6 An mTOR inhibitor in combination with at least one second drug substance for use in the preparation of a medicament, e.g. for use in any method of 1.1 to 1.8 above.

2.7 Any method as indicated under 2.1 to 2.6 above, wherein the mTOR inhibitor is administered intermittently.

Combinations include fixed combinations, in which an mTOR inhibitor and at least one second drug substance are in the same formulation; kits, in which an mTOR inhibitor and at least one second drug substance in separate formulations are provided in the same package, e.g. with instruction for co-administration; and free combinations in which an mTOR inhibitor and at least one second drug substance are packaged separately, but instruction for concomitant or sequential administration are given.

In another aspect the present invention provides

2.8 A pharmaceutical package comprising a first drug substance which is an mTOR inhibitor and at least one second drug substance, beside instructions for combined administration;

2.9 A pharmaceutical package comprising an mTOR inhibitor beside instructions for combined administration with at least one second drug substance;

2.10 A pharmaceutical package comprising at least one second drug substance beside instructions for combined administration with an mTOR inhibitor;

e.g. for use in any method of 1.1 to 1.8 above.

Treatment with combinations according to the present invention may provide improvements compared with single treatment.

In another aspect the present invention provides

2.11 A pharmaceutical combination comprising an amount of an mTOR inhibitor and an amount of a second drug substance, wherein the amounts are appropriate to produce a synergistic therapeutic effect.

2.12- A method for improving the therapeutic utility of a an mTOR inhibitor comprising co-administering, e.g. concomitantly or in sequence, a therapeutically effective amount of an mTOR inhibitor and a second drug substance.

2.13 A method for improving the therapeutic utility of a second drug substance comprising co-administering, e.g. concomitantly or in sequence, a therapeutically effective amount of an mTOR inhibitor and a second drug substance.

e.g. for use in any method of 1.1 to 1.8 above.

In a method of 2.11 to 2.13 above the activity of an mTOR inhibitor or a second drug substance may be enhanced compared with single treatment, e.g. combined treatment may result in synergistic effects or may overcome resistance against an mTOR inhibitor or a chemotherapeutic agent, e.g. when used in any method according to 1.1 to 1.8 as described above.

A (pharmaceutical) combination, e.g. composition as indicated under 2.1 to 2.13 comprises

a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter or hereinbefore.

Treatment of disorders (diseases) according to the present invention includes prophylaxis (prevention).

For such treatment, the appropriate dosage will, of course, vary depending upon, for example, the chemical nature and the pharmacokinetic data of a compound used, the individual host, the mode of administration and the nature and severity of the conditions being treated. However, in general, for satisfactory results in larger mammals, for example

humans, an indicated daily dosage includes a range

- from about 0.0001 g to about 1.5 g, such as 0.001 g to 1.5 g;
- from about 0.001 mg/kg body weight to about 20 mg/kg body weight, such as 0.01 mg/kg body weight to 20 mg/kg body weight,

for example administered in divided doses up to four times a day.

In a method, for use or in a combination, pharmaceutical combination or pharmaceutical composition provided by the present invention an mTOR inhibitor, such as rapamycin or rapamycin derivative, may be administered as appropriate, e.g. in dosages which are known for mTOR inhibitors, by any administration route, e.g. enterally, e.g. orally, or parenterally. E.g. everolimus may be administered, e.g. orally, in dosages from 0.1 mg up to 15 mg, such as 0.1 mg to 10 mg. e.g. 0.1 mg, 0.25 mg, 0.5 mg, 0.75 mg, 1 mg, 2.5 mg, 5 mg, or 10 mg, more preferably from 0.5 mg to 10 mg, e.g. in the form of (dispersible) tablets; e.g. comprising everolimus in the form of a solid dispersion; e.g. a weekly dosage may include up to 70 mg, e.g. 10 to 70, such as 30 to 50 mg, depending on the disease being treated. Rapamycin or e.g. temsirolimus may be administered parenterally in similar dosage ranges.

A second drug substance may be administered in combination therapy as appropriate, e.g. according to a method as conventional, e.g. analogously to administration indications given for a specified drug for single treatment.

A second drug substance according to the present invention may be administered by any conventional route, for example enterally, e.g. including nasal, buccal, rectal, oral, administration; parenterally, e.g. including intravenous, intraarterial, intramuscular, intracardiac, subcutanous, intraosseous infusion, transdermal (diffusion through the intact skin), transmucosal (diffusion through a mucous membrane), inhalational administration; topically; e.g. including epicutaneous, intranasal, intratracheal administration; intraperitoneal (infusion or injection into the peritoneal cavity); epidural (peridural) (injection or infusion into the epidural space); intrathecal (injection or infusion into the cerebrospinal fluid); intravitreal (administration via the eye); or via medical devices, e.g. for local delivery, e.g. stents; e.g. in form of coated or uncoated tablets, capsules, (injectable) solutions, infusion solutions, solid solutions, suspensions, dispersions, solid dispersions; e.g. in the form of ampoules, vials, in the form of creams, gels, pastes, inhaler powder, foams, tinctures, lip sticks, drops, sprays, or in the form of suppositories.

A second drug substance according to the present invention may be administered in the form of a pharmaceutically acceptable salt, or in free form; optionally in the form of a solvate. Pharmaceutical compositions according to the present invention may be manufactured according, e.g. analogously, to a method as conventional, e.g. by mixing, granulating, coating, dissolving or lyophilizing processes. Unit dosage forms may contain, for example, from about 0.1 mg to about 1500 mg, such as 1 mg to about 1000 mg.
Pharmaceutical compositions comprising a combination of the present invention and pharmaceutical compositions comprising a second drug substance as described herein, may be provided as appropriate, e.g. according, e.g. analogously, to a method as conventional, or as described herein for a pharmaceutical composition of the present invention.

By the term "second drug substance" as used herein is meant either an mTOR inhibitor other than the first drug substance or a chemotherapeutic agent other than an mTOR inhibitor, preferably any chemotherapeutic agent other an mTOR inhibitor.
For example, a second drug substance as used herein includes e.g.
-an anticancer drug, preferably an anti-endocrine tumor agent,
- an anti-inflammatory and/or immunomodulatory and/or antiallergic drug,
- a combination of an anticancer drug with an anti-inflammatory and/or immunomodulatory drug and/or antiallergic drug.
A second drug substance also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

In another aspect the present invention provides

3. Any method, combination, pharmaceutical combination, pharmaceutical composition or use as indicated under 1.1 to 1.9 and 2.1 to 2.13 above wherein an mTOR inhibitor is selected from rapamycin or a rapamycin derivative, such as
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin (also known as everolimus), and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or

16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, AP23573, AP23464, AP23675 or AP23841, e.g. AP23573, and/or
compounds disclosed under the name TAFA-93, and/or
compounds disclosed under the name biolimus;
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

In a preferred aspect the present invention provides any method, combination, pharmaceutical combination, pharmaceutical composition, or use as indicated under 1.1 to 1.9 and 2.1 to 2.13 above for treating neuroendocrine tumors.

In another preferred aspect the present invention provides any method, combination, pharmaceutical combination, pharmaceutical composition, or use as indicated under 1.1 to 1.9 and 2.1 to 2.13 above for treating carcinoid tumors.

In another preferred aspect the present invention any method, combination, pharmaceutical combination, pharmaceutical composition, or use as indicated under 1.1 to 1.9 and 2.1 to 2.13 above for treating pituitary tumors.

Anticancer drugs which are prone to be useful as a combination partner with an mTOR inhibitor, e.g. prone to be useful according to the present invention, e.g. include

i. a steroid; e.g. prednisone.
ii. an adenosine-kinase-inhibitor; which targets, decreases or inhibits nucleobase, nucleoside, nucleotide and nucleic acid metabolisms, such as 5-Iodotubercidin, which is also known as 7H-pyrrolo[2,3-d]pyrimidin-4-amine, 5-iodo-7-β-D-ribofuranosyl-(9CI).
iii. an adjuvant; which enhances the 5-FU-TS bond as well as a compound which targets, decreases or inhibits, alkaline phosphatase, such as leucovorin, levamisole.
iv. an adrenal cortex antagonist; which targets, decreases or inhibits the activity of the adrenal cortex and changes the peripheral metabolism of corticosteroids, resulting in a decrease in 17-hydroxycorticosteroids, such as mitotane.

v. an AKT pathway inhibitor; such as a compound which targets, decreases or inhibits Akt, also known as protein kinase B (PKB), such as deguelin, which is also known as 3H-bis[1]benzopyrano[3,4-b:6',5'-e]pyran-7(7aH)-one, 13,13a-dihydro-9,10-dimethoxy-3,3-dimethyl-, (7aS, 13aS)-(9CI); and triciribine, which is also known as 1,4,5,6,8-pentaazaacenaphthylen-3-amine, 1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-(9CI).

vi. an alkylating agent; which causes alkylation of DNA and results in breaks in the DNA molecules as well as cross-linking of the twin strands, thus interfering with DNA replication and transcription of RNA, such as chlorambucil, cyclophosphamide, dacarbazine, lomustine, procarbazine, e.g. in the form of a hydrochloride, thiotepa, melphalan, temozolomide (TEMODAR®), carmustine, ifosfamide, mitomycin, altretamine, busulfan, machlorethamine hydrochloride, nitrosourea (BCNU or Gliadel), streptozocin, estramustine. Cyclophosphamide can be administered, e.g., in the form as it is marketed, e.g., under the trademark CYCLOSTIN®; and ifosfamide as HOLOXAN®.

vii. an angiogenesis inhibitor; which targets, decreases or inhibits the production of new blood vessels, e.g. which targets methionine aminopeptidase-2 (MetAP-2), macrophage inflammatory protein-1 (MIP-1alpha), CCL5, TGF-beta, lipoxygenase, cyclooxygenase, and topoisomerase, or which indirectly targets p21, p53, CDK2 and collagen synthesis, e.g. including fumagillin, which is known as 2,4,6,8-decatetraenedioic acid, mono[(3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methyl-2-butenyl)oxiranyl]-1-oxaspiro[2.5]oct-6-yl] ester, (2E,4E,6E,8E)- (9CI); shikonin, which is also known as 1,4-naphthalenedione, 5,8-dihydroxy-2-[(1R)-1-hydroxy-4-methyl-3-pentenyl]- (9CI); tranilast, which is also known as benzoic acid, 2-[[3-(3,4-dimethoxyphenyl)-1-oxo-2-propenyl]amino]-(9CI); ursolic acid; suramin; bengamide or a derivative thereof, thalidomide, TNP-470.

viii. an anti-androgen; which blocks the action of androgens of adrenal and testicular origin which stimulate the growth of normal and malignant prostatic tissue, such as nilutamide; bicalutamide (CASODEX®), which can be formulated, e.g., as disclosed in US4636505.

ix. an anti-estrogen; which antagonizes the effect of estrogens at the estrogen receptor level, e.g. including an aromatase inhibitor, which inhibits the estrogen production, i. e. the conversion of the substrates androstenedione and testosterone to estrone and estradiol, respectively,

e.g. including atamestane, exemestane, formestane, aminoglutethimide, roglethimide, pyridoglutethimide, trilostane, testolactone, ketokonazole, vorozole, fadrozole, anastrozole, letrozole, toremifene; bicalutamide; flutamide; tamoxifen, tamoxifen citrate; tamoxifen; fulvestrant; raloxifene, raloxifene hydrochloride. Tamoxifen may be e.g. administered in the form as it is marketed, e.g., NOLVADEX®; and raloxifene hydrochloride is marketed as EVISTA®. Fulvestrant may be formulated as disclosed in US4659516 and is marketed as FASLODEX®.

x. an anti-hypercalcemia agent; which is used to treat hypercalcemia, such as gallium (III) nitrate hydrate; and pamidronate disodium.

xi. an antimetabolite; which inhibits or disrupts the synthesis of DNA resulting in cell death, such as 6-mercaptopurine; cytarabine; fludarabine; flexuridine; fluorouracil; 5-fluorouracil(5-FU), floxuridine (5-FUdR), capecitabine; raltitrexed; methotrexate; cladribine; gemcitabine; gemcitabine hydrochloride; thioguanine; 6-thioguanine, hydroxyurea; DNA de-methylating agents, such as 5-azacytidine and decitabine; edatrexate; folic acid antagonists such as pemetrexed. Capecitabine and gemcitabine can be administered e.g. in the marketed form, such as XELODA® and GEMZAR®.

xii. an apoptosis inducer; which induces the normal series of events in a cell that leads to its death, e.g. selectively inducing the X-linked mammalian inhibitor of apoptosis protein XIAP, or e.g. downregulating BCL-xL; such as ethanol, 2-[[3-(2,3-dichlorophenoxy)propyl]amino]-(9CI); gambogic acid; embelin, which is also known as 2,5-cyclohexadiene-1,4-dione, 2,5-dihydroxy-3-undecyl- (9CI); arsenic trioxide.

xiii. an aurora kinase inhibitor; which targets, decreases or inhibits later stages of the cell cycle from the G2/M check point all the way through to the mitotic checkpoint and late mitosis; such as binucleine 2, which is also known as methanimidamide, N'-[1-(3-chloro-4-fluorophenyl)-4-cyano-1H-pyrazol-5-yl]-N,N-dimethyl- (9CI).

xiv. a Bruton's Tyrosine Kinase (BTK) inhibitor; which targets, decreases or inhibits human and murine B cell development; such as terreic acid.

xv. a calcineurin inhibitor; which targets, decreases or inhibits the T cell activation pathway, such as cypermethrin, which is also known as cyclopropanecarboxylic acid, 3-(2,2-dichloroethenyl)-2,2-dimethyl-,cyano(3-phenoxyphenyl)methyl ester (9CI); deltamethrin, which is also known as cyclopropanecarboxylic aci, 3-(2,2-dibromoethenyl)-2,2-dimethyl-(S)-cyano(3-phenoxyphenyl)methyl ester, (1R,3R)-(9CI); fenvalerate, which is also known as benzeneacetic acid, 4-chloro-α-(1-methylethyl)-

,cyano(3-phenoxyphenyl)methyl ester (9CI); and Tyrphostin 8; but excluding cyclosporin or FK506.

xvi. a CaM kinase II inhibitor; which targets, decreases or inhibits CaM kinases; constituting a family of structurally related enzymes that include phosphorylase kinase, myosin light chain kinase, and CaM kinases I-IV; such as 5-isoquinolinesulfonic acid, 4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester (9CI); benzenesulfonamide, N-[2-[[[3-(4-chlorophenyl)-2-propenyl]methyl]amino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy-(9CI).

xvii. a CD45 tyrosine phosphatase inhibitor; which targets, decreases or inhibits dephosphorylating regulatory pTyr residues on Src-family protein-tyrosine kinases, which aids in the treatment of a variety of inflammatory and immune disorders; such as phosphonic acid, [[2-(4-bromophenoxy)-5-nitrophenyl]hydroxymethyl]-(9CI).

xviii. a CDC25 phosphatase inhibitor; which targets, decreases or inhibits overexpressed dephosphorylate cyclin-dependent kinases in tumors; such as 1,4-naphthalenedione, 2,3-bis[(2-hydroyethyl)thio]-(9CI).

xix. a CHK kinase inhibitor; which targets, decreases or inhibits overexpression of the antiapoptotic protein Bcl-2; such as debromohymenialdisine. Targets of a CHK kinase inhibitor are CHK1 and/or CHK2.

xx. a controlling agent for regulating genistein, olomucine and/or tyrphostins; such as daidzein, which is also known as 4H-1-benzopyran-4-one, 7-hydroxy-3-(4-hydroxyphenyl)-(9CI); Iso-Olomoucine, and Tyrphostin 1.

xxi. a cyclooxygenase inhibitor; e.g. including Cox-2 inhibitors; which targets, decreases or inhibits the enzyme cox-2 (cyclooxygenase-2); such as 1H-indole-3-acetamide, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-N-(2-phenylethyl)-(9CI); 5-alkyl substituted 2-arylaminophenylacetic acid and derivatives, e.g. celecoxib (CELEBREX®), rofecoxib (VIOXX®), etoricoxib, valdecoxib; or a 5-alkyl-2-arylaminophenylacetic acid, e.g., 5-methyl-2-(2'-chloro-6'-fluoroanilino)phenyl acetic acid, lumiracoxib; and celecoxib.

xxii. a cRAF kinase inhibitor; which targets, decreases or inhibits the up-regulation of E-selectin and vascular adhesion molecule-1 induced by TNF; such as 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodo-1,3-dihydroindol-2-one; and benzamide, 3-(dimethylamino)-N-[3-[(4-hydroxybenzoyl)amino]-4-methylphenyl]-(9CI). Raf kinases play an important role as extracellular signal-regulating kinases in cell differentiation, proliferation, and apoptosis. A target of a cRAF kinase inhibitor includes, but is not limited, to RAF1.

xxiii. a cyclin dependent kinase inhibitor; which targets, decreases or inhibits cyclin dependent kinase playing a role in the regulation of the mammalian cell cycle; such as N9-isopropyl-olomoucine; olomoucine; purvalanol B, which is also known as Benzoic acid, 2-chloro-4-[[2-[[(1R)-1-(hydroxymethyl)-2-methylpropyl]amino]-9-(1-methylethyl)-9H-purin-6-yl]amino]- (9CI); roascovitine; indirubin, which is also known as 2H-indol-2-one, 3-(1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,3-dihydro- (9CI); kenpaullone, which is also known as indolo[3,2-d][1]benzazepin-6(5H)-one, 9-bromo-7,12-dihydro- (9CI); purvalanol A, which is also known as 1-Butanol, 2-[[6-[(3-chlorophenyl)amino]-9-(1-methylethyl)-9H-purin-2-yl]amino]-3-methyl-, (2R)- (9CI); indirubin-3'-monooxime. Cell cycle progression is regulated by a series of sequential events that include the activation and subsequent inactivation of cyclin dependent kinases (Cdks) and cyclins. Cdks are a group of serine/threonine kinases that form active heterodimeric complexes by binding to their regulatory subunits, cyclins. Examples of targets of a cyclin dependent kinase inhibitor include, but are not limited to, CDK, AHR, CDK1, CDK2, CDK5, CDK4/6, GSK3beta, and ERK.

xxiv. a cysteine protease inhibitor; which targets, decreases or inhibits cystein protease which plays a vital role in mammalian cellular turnover and apotosis; such as 4-morpholinecarboxamide,N-[(1S)-3-fluoro-2-oxo-1-(2-phenylethyl)propyl]amino]-2-oxo-1-(phenylmethyl)ethyl]-(9CI).

xxv. a DNA intercalator; which binds to DNA and inhibits DNA, RNA, and protein synthesis; such as plicamycin, dactinomycin.

xxvi. a DNA strand breaker; which causes DNA strand scission and results in inhibition of DNA synthesis, ininhibition of RNA and protein synthesis; such as bleomycin.

xxvii. an E3 Ligase inhibitor; which targets, decreases or inhibits the E3 ligase which inhibits the transfer of ubiquitin chains to proteins, marking them for degradation in the proteasome; such as N-((3,3,3-trifluoro-2-trifluoromethyl)propionyl)sulfanilamide.

xxviii. an endocrine hormone; which by acting mainly on the pituitary gland causes the suppression of hormones in males, the net effect being a reduction of testosterone to castration levels; in females, both ovarian estrogen and androgen synthesis being inhibited; such as leuprolide; megestrol, megestrol acetate.

xxix. compounds targeting, decreasing or inhibiting the activity of the epidermal growth factor family of receptor tyrosine kinases (EGFR, ErbB2, ErbB3, ErbB4 as homo- or heterodimers), such as compounds, proteins or antibodies which inhibit members of the EGF receptor tyrosine kinase family, e.g. EGF receptor, ErbB2, ErbB3 and ErbB4

or bind to EGF or EGF-related ligands, and are in particular those compounds, proteins or monoclonal antibodies generically and specifically disclosed in WO 9702266, e.g. the compound of ex. 39, EP0564409, WO9903854, EP0520722, EP0566226, EP0787722, EP0837063, US5747498, WO9810767, WO9730034, WO9749688, WO9738983 and, especially, WO9630347, e.g. a compound known as CP 358774, WO9633980, e.g. a compound known as ZD 1839; and WO 9503283, e.g. a compound known as ZM105180, e.g including trastuzumab (HERCEPTIN®), cetuximab, iressa, OSI-774, CI-1033, EKB-569, GW-2016, E1.1, E2.4, E2.5, E6.2, E6.4, E2.11, E6.3 or E7.6.3, 7H-pyrrolo-[2,3-d]pyrimidine derivatives which are e.g. disclosed in WO03013541, erlotinib, gefitinib. Erlotinib can be administered in the form as it is marketed, e.g. TARCEVA®, and gefitinib as IRESSA®, human monoclonal antibodies against the epidermal growth factor receptor including ABX-EGFR.

xxx. an EGFR, PDGFR tyrosine kinase inhibitor; such as EGFR kinase inhibitors including tyrphostin 23, tyrphostin 25, tyrphostin 47, tyrphostin 51 and tyrphostin AG 825; 2-propenamide, 2-cyano-3-(3,4-dihydroxyphenyl)-N-phenyl-(2E)-(9CI); tyrphostin Ag 1478; lavendustin A; 3-pyridineacetonitrile, α-[(3,5-dichlorophenyl)methylene]-, (αZ)-(9CI); an example of an EGFR, PDGFR tyrosine kinase inhibitor e.g. includes tyrphostin 46. PDGFR tyrosine kinase inhibitor including tyrphostin 46. Targets of an EGFR kinase inhibitor include guanylyl cyclase (GC-C) HER2, EGFR, PTK and tubulin.

xxxi. a farnesyltransferase inhibitor; which targets, decreases or inhibits the Ras protein;such as a-hydroxyfarnesylphosphonic acid; butanoic acid, 2-[[(2S)-2-[[(2S,3S)-2-[[(2R)-2-amino-3-mercaptopropyl]amino]-3-methylpentyl]oxy]-1-oxo-3-phenylpropyl]amino]-4-(methylsulfonyl)-,1-methylethyl ester, (2S)-(9cl); manumycin A; L-744,832 or DK8G557, tipifarnib (R115777), SCH66336 (lonafarnib), BMS-214662,

xxxii. a Flk-1 kinase inhibitor; which targets, decreases or inhibits Flk-1 tyrosine kinase activity; such as 2-propenamide, 2-cyano-3-[4-hydroxy-3,5-bis(1-methylethyl)phenyl]-N-(3-phenylpropyl)-(2E)-(9CI). A target of a Flk-1 kinase inhibitor includes, but is not limited to, KDR.

xxxiii. a Glycogen synthase kinase-3 (GSK3) inhibitor; which targets, decreases or inhibits glycogen synthase kinase-3 (GSK3); such as indirubin-3'-monooxime. Glycogen Synthase Kinase-3 (GSK-3; tau protein kinase I), a highly conserved, ubiquitously expressed serine/threonine protein kinase, is involved in the signal transduction cascades of multiple cellular processes. which is a protein kinase that has been shown to be involved in the regulation of a diverse array of cellular functions, including protein

synthesis, cell proliferation, cell differentiation, microtubule assembly/disassembly, and apoptosis.

xxxiv. a histone deacetylase (HDAC) inhibitor; which inhibits the histone deacetylase and which possess anti-proliferative activity; such as compounds disclosed in WO0222577, especially N-hydroxy-3-[4-[[(2-hydroxyethyl)[2-(1H-indol-3-yl)ethyl]-amino]methyl]phenyl]-2E-2-propenamide, and N-hydroxy-3-[4-[[[2-(2-methyl-1H-indol-3-yl)-ethyl]-amino]methyl]phenyl]-2E-2-propenamide and pharmaceutically acceptable salts thereof; suberoylanilide hydroxamic acid (SAHA); [4-(2-amino-phenylcarbamoyl)-benzyl]-carbamic acid pyridine-3-ylmethyl ester and derivatives thereof; butyric acid, pyroxamide, trichostatin A, oxamflatin, apicidin, depsipeptide; depudecin; trapoxin, HC toxin, which is also known as cyclo[L-alanyl-D-alanyl-(I IS,2S)-I I-amino-I -oxooxiraneoctanoyl-D-prolyl] (9CI); sodium phenylbutyrate, suberoyl bis-hydroxamic acid; Trichostatin A, BMS-27275, pyroxamide, FR-901228, valproic acid.

xxxv. a HSP90 inhibitor; which targets, decreases or inhibits the intrinsic ATPase activity of HSP90; degrades, targets, decreases or inhibits the HSP90 client proteins via the ubiquitin proteosome pathway. Compounds targeting, decreasing or inhibiting the intrinsic ATPase activity of HSP90 are especially compounds, proteins or antibodies which inhibit the ATPase activity of HSP90, e.g., 17-allylamino,17-demethoxygeldanamycin (17AAG), a geldanamycin derivative; other geldanamycin-related compounds; radicicol and HDAC inhibitors. Other examples of an HSP90 inhibitor include geldanamycin,17-demethoxy-17-(2-propenylamino)-(9CI). Potential indirect targets of an HSP90 inhibitor include FLT3, BCR-ABL, CHK1, CYP3A5*3 and/or NQ01*2.

xxxvi. a I-kappa B-alpha kinase inhibitor (IKK); which targets, decreases or inhibits NF-kappaB, such as 2-propenenitrile, 3-[(4-methylphenyl)sulfonyl]-(2E)-(9CI).

xxxvii. an insulin receptor tyrosine kinase inhibitor; which modulates the activities of phosphatidylinositol 3-kinase, microtubule-associated protein, and S6 kinases; such as hydroxyl-2-naphthalenylmethylphosphonic acid, LY294002.

xxxviii. a c-Jun N-terminal kinase (JNK) kinase inhibitor; which targets, decreases or inhibits Jun N-terminal kinase; such as pyrazoleanthrone and/or epigallocatechin gallate. Jun N-terminal kinase (JNK), a serine-directed protein kinase, is involved in the phosphorylation and activation of c-Jun and ATF2 and plays a significant role in metabolism, growth, cell differentiation, and apoptosis. A target for a JNK kinase inhibitor includes, but is not limited to, DNMT.

xxxix a microtubule binding agent; which acts by disrupting the microtubular network that is essential for mitotic and interphase cellular function; such as vinblastine, vinblastine sulfate; vinca alkaloids, such as vincristine, vincristine sulfate; vindesine; vinorelbine; taxanes, such as docetaxel; paclitaxel; discodermolides; cochicine, epothilones and derivatives thereof, e.g. epothilone B or a derivative thereof. Paclitaxel is marketed as TAXOL®; docetaxel as TAXOTERE®; vinblastine sulfate as VINBLASTIN R.P®; and vincristine sulfate as FARMISTIN®. Also included are the generic forms of paclitaxel as well as various dosage forms of paclitaxel. Generic forms of paclitaxel include, but are not limited to, betaxolol hydrochloride. Various dosage forms of paclitaxel include, but are not limited to albumin nanoparticle paclitaxel marketed as ABRAXANE®; ONXOL®, CYTOTAX®. Discodermolide can be obtained, e.g., as disclosed in US5010099. Also included are Epotholine derivatives which are disclosed in US6194181, WO98/0121, WO9825929, WO9808849, WO9943653, WO9822461 and WO0031247. Especially preferred are Epotholine A and/or B.

xl. a mitogen-activated protein (MAP) kinase-inhibitor; which targets, decreases or inhibits Mitogen-activated protein, such as benzenesulfonamide, N-[2-[[[3-(4-chlorophenyl)-2-propenyl]methyl]amino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy-(9Cl). The mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. They regulate several physiological and pathological cellular phenomena, including inflammation, apoptotic cell death, oncogenic transformation, tumor cell invasion, and metastasis.

xli. a MDM2 inhibitor; which targets, decreases or inhibits the interaction of MDM2 and the p53 tumor suppressor; such as trans-4-iodo, 4'-boranyl-chalcone.

xlii. a MEK inhibitor; which targets, decreases or inhibits the kinase activity of MAP kinase MEK; such as Nexavar® (sorafenib tosylate), butanedinitrile, bis[amino[2-aminophenyl)thio]methylene]-(9Cl). A target of a MEK inhibitor includes, but is not limited to ERK. An indirect target of a MEK inhibitor includes, but is not limited to, cyclin D1.

xliii: a matrix metalloproteinase inhibitor (MMP) inhibitor; which targets, decreases or inhibits a class of protease enzyme that selectively catalyze the hydrolysis of polypeptide bonds including the enzymes MMP-2 and MMP-9 that are involved in promoting the loss of tissue structure around tumors and facilitating tumor growth, angiogenesis, and metastasissuch as actinonin, which is also known as

butanediamide, N-4-hydroxy-N1-[(1S)-1-[[(2S)-2-(hydroxymethyl)-1-pyrrolidinyl]carbonyl]-2-methylpropyl]-2-pentyl-, (2R)-(9CI); epigallocatechin gallate; collagen peptidomimetic and non-peptidomimetic inhibitors; tetracycline derivatives, e.g., hydroxamate peptidomimetic inhibitor batimastat; and its orally-bioavailable analogue marimastat, prinomastat,, metastat, neovastat, tanomastat, TAA211, BMS-279251, BAY 12-9566, MMI270B or AAJ996. A target of a MMP inhibitor includes, but is not limited to, polypeptide deformylase.

xliv. a NGFR tyrosine-kinase-inhibitor; which targets, decreases or inhibits nerve growth factor dependent $p140^{c\text{-}trk}$ tyrosine phosphorylation; such as tyrphostin AG 879. Targets of a NGFR tyrosine-kinase-inhibitor include, but are not limited to, HER2, FLK1, FAK, TrkA, and/or TrkC. An indirect target inhibits expression of RAF1.

xlv. a p38 MAP kinase inhibitor, including a SAPK2/p38 kinase inhibitor; which targets, decreases or inhibits p38-MAPK, which is a MAPK family member, such as phenol, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-(9CI). An example of a a SAPK2/p38 kinase inhibitor includes, but is not limited to, benzamide, 3-(dimethylamino)-N-[3-[(4-hydroxybenzoyl)amino]-4-methylphenyl]-(9CI). A MAPK family member is a serine/threonine kinase activated by phosphorylation of tyrosine and threonine residues. This kinase is phosphorylated and activated by many cellular stresses and inflammatory stimuli, thought to be involved in the regulation of important cellular responses such as apoptosis and inflammatory reactions.

xlvi. a p56 tyrosine kinase inhibitor; which targets, decreases or inhibits p56 tyrosine kinase, which is an enzyme that is a lymphoid-specific src family tyrosine kinase critical for T-cell development and activation; such as damnacanthal, which is also known as 2-anthracenecarboxaldehyde,9,10-dihydro-3-hydroxy-1methoxy-9,10-dioxo-(9CI), Tyrphostin 46. A target of a p56 tyrosine kinase inhibitor includes, but is not limited to, Lck. Lck is associated with the cytoplasmic domains of CD4, CD8 and the beta-chain of the IL-2 receptor, and is thought to be involved in the earliest steps of TCR-mediated T-cell activation.

xlvii. a PDGFR tyrosine kinase inhibitor; targeting, decreasing or inhibiting the activity of the C-kit receptor tyrosine kinases (part of the PDGFR family), such as targeting, decreasing or inhibiting the activity of the c-Kit receptor tyrosine kinase family, especially inhibiting the c-Kit receptor. Examples of targets of a PDGFR tyrosine kinase inhibitor includes, but are not limited to PDGFR, FLT3 and/or c-KIT; such as tyrphostin AG 1296; tyrphostin 9; 1,3-butadiene-1,1,3-tricarbonitrile,2-amino-4-(1H-

indol-5-yl)-(9Cl); N-phenyl-2-pyrimidine-amine derivative, e. g. imatinib, IRESSA®. PDGF plays a central role in regulating cell proliferation, chemotaxis, and survival in normal cells as well as in various disease states such as cancer, atherosclerosis, and fibrotic disease. The PDGF family is composed of dimeric isoforms (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD), which exert their cellular effects by differentially binding to two receptor tyrosine kinases. PDGFR-α and PDGFR-ß have molecular masses of ~170 and 180 kDa, respectively.

xlviii. a phosphatidylinositol 3-kinase inhibitor; which targets, decreases or inhibits PI 3-kinase; such as wortmannin, which is also known as 3H-Furo[4,3,2-de]indeno[4,5-h]-2-benzopyran-3,6,9-trione, 11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-, (1S,6bR,9aS,11R,11bR)- (9Cl); 8-phenyl-2-(morpholin-4-yl)-chromen-4-one; quercetin, quercetin dihydrate. PI 3-kinase activity has been shown to increase in response to a number of hormonal and growth factor stimuli, including insulin, platelet-derived growth factor, insulin-like growth factor, epidermal growth factor, colony-stimulating factor, and hepatocyte growth factor, and has been implicated in processes related to cellular growth and transformation. An example of a target of a phosphatidylinositol 3-kinase inhibitor includes, but is not limited to, Pi3K.

xlix. a phosphatase inhibitor; which targets, decreases or inhibits phosphatase; such as cantharidic acid; cantharidin; and L-leucinamide, N-[4-(2-carboxyethenyl)benzoyl]glycyl-L-α-glutamyl-(E)-(9Cl). Phosphatases remove the phosphoryl group and restore the protein to its original dephosphorylated state. Hence, the phosphorylation- dephosphorylation cycle can be regarded as a molecular "on-off" switch.

l. platinum agent; which contains platinum and inhibit DNA synthesis by forming interstrand and intrastrand cross-linking of DNA molecules; such as carboplatin; cisplatin; oxaliplatin; cisplatinum; satraplatin and platinum agents such as ZD0473. Carboplatin can be administered, e.g., in the form as it is marketed, e.g. CARBOPLAT®; and oxaliplatin as ELOXATIN®.

li. a protein phosphatase inhibitor, including a PP1 and PP2 inhibitor and a tyrosine phosphatase inhibitor; which targets, decreases or inhibits protein phosphatase. Examples of a PP1 and PP2A inhibitor include cantharidic acid and/or cantharidin. Examples of a tyrosine phosphatase inhibitor include, but are not limited to, L-P-

bromotetramisole oxalate; 2(5H)-furanone,4-hydroxy-5-(hydroxymethyl)-3-(1-oxohexadecyl)-, (5R)-(9CI); and benzylphosphonic acid.

The term "a PP1 or PP2 inhibitor", as used herein, relates to a compound which targets, decreases or inhibits Ser/Thr protein phosphatases. Type I phosphatases, which include PP1, can be inhibited by two heat-stable proteins known as Inhibitor-1 (I-1) and Inhibitor-2 (I-2). They preferentially dephosphorylate a subunit of phosphorylase kinase. Type II phosphatases are subdivided into spontaneously active (PP2A), CA^{2+}-dependent (PP2B), and Mg^{2+}-dependent (PP2C) classes of phosphatases.

The term "tyrosine phosphatase inhibitor", as used here, relates to a compounds which targets, decreases or inhibits tyrosine phosphatase. Protein tyrosine phosphatases (PTPs) are relatively recent additions to the phosphatase family. They remove phosphate groups from phosphorylated tyrosine residues of proteins. PTPs display diverse structural features and play important roles in the regulation of cell proliferation, differentiation, cell adhesion and motility, and cytoskeletal function. Examples of targets of a tyrosine phosphatase inhibitor include, but are not limited to, alkaline phosphatase (ALP), heparanase, PTPase, and/or prostatic acid phosphatase.

iii. a PKC inhibitor and a PKC delta kinase inhibitor: The term "a PKC inhibitor", as used herein, relates to a compound which targets, decreases or inhibits protein kinase C as well as its isozymes. Protein kinase C (PKC), a ubiquitous, phospholipid-dependent enzyme, is involved in signal transduction associated with cell proliferation, differentiation, and apoptosis. Examples of a target of a PKC inhibitor include, but are not limited to, MAPK and/or NF-kappaB. Examples of a PKC inhibitor include, but are not limited to, 1-H-pyrrolo-2,5-dione,3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-(9CI); bisindolylmaleimide IX; sphingosine, which is known as 4-octadecene-1,3-diol, 2-amino-, (2S,3R,4E)- (9CI); staurosporine, which is known as 9,13-Epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one, staurosporine derivatives such as disclosed in EP0296110, e. g. midostaurin; 2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11-(methylamino)-, (9S,10R,11R,13R)- (9CI); tyrphostin 51; and hypericin, which is also known as phenanthro[1,10,9,8-opqra]perylene-7,14-dione, 1,3,4,6,8,13-hexahydroxy-10,11-dimethyl-, stereoisomer (6CI,7CI,8CI,9CI), UCN-01,safingol, BAY 43-9006, bryostatin 1, perifosine;ilmofosine ; RO 318220 and RO 320432; GO 6976 ; Isis 3521; LY333531/LY379196. The term "a PKC delta kinase inhibitor", as used herein, relates to a compound which targets, decreases or inhibits the delta isozymes of PKC. The

delta isozyme is a conventional PKC isozymes and is Ca^{2+}-dependent. An example of a PKC delta kinase inhibitor includes, but is not limited to, Rottlerin, which is also known as 2-Propen-1-one, 1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-, (2E)- (9CI).

liii. a polyamine synthesis inhibitor; which targets, decreases or inhibits polyamines spermidine; such as DMFO, which is also known as (-)-2-difluoromethylornithin; N1, N12-diethylspermine 4HCl. The polyamines spermidine and spermine are of vital importance for cell proliferation, although their precise mechanism of action is unclear. Tumor cells have an altered polyamine homeostasis reflected by increased activity of biosynthetic enzymes and elevated polyamine pools.

liv. a proteosome inhibitor; which targets, decreases or inhibits proteasome, such as aclacinomycin A; gliotoxin; PS-341; MLN 341; bortezomib; velcade. Examples of targets of a proteosome inhibitor include, but are not limited to, O(2)(-)-generating NADPH oxidase, NF-kappaB, and/or farnesyltransferase, geranyltransferase I.

lv. a PTP1B inhibitor; which targets, decreases or inhibits PTP1B, a protein tyrosine kinase inhibitor; such as L-leucinamide, N-[4-(2-carboxyethenyl)benzoyl]glycyl-L-α-glutamyl-,(E)-(9CI).

lvi. a protein tyrosine kinase inhibitor including a SRC family tyrosine kinase inhibitor; a Syk tyrosine kinase inhibitor; and a JAK-2 and/or JAK-3 tyrosine kinase inhibitor;

The term "a protein tyrosine kinase inhibitor", as used herein, relates to a compound which which targets, decreases or inhibits protein tyrosine kinases. Protein tyrosine kinases (PTKs) play a key role in the regulation of cell proliferation, differentiation, metabolism, migration, and survival. They are classified as receptor PTKs and non-receptor PTKs. Receptor PTKs contain a single polypeptide chain with a transmembrane segment. The extracellular end of this segment contains a high affinity ligand-binding domain, while the cytoplasmic end comprises the catalytic core and the regulatory sequences. Examples of targets of a tyrosine kinase inhibitor include, but are not limited to, ERK1, ERK2, Bruton's tyrosine kinase (Btk), JAK2, ERK ½, PDGFR, and/or FLT3. Examples of indirect targets include, but are not limited to, TNFalpha, NO, PGE2, IRAK, iNOS, ICAM-1, and/or E-selectin. Examples of a tyrosine kinase inhibitor include, but are not limited to, tyrphostin AG 126; tyrphostin Ag 1288; tyrphostin Ag 1295; geldanamycin; and genistein.

Non-receptor tyrosine kinases include members of the Src, Tec, JAK, Fes, Abl, FAK, Csk, and Syk families. They are located in the cytoplasm as well as in the nucleus.

They exhibit distinct kinase regulation, substrate phosphorylation, and function. Deregulation of these kinases has also been linked to several human diseases.

The term "a SRC family tyrosine kinase inhibitor", as used herein, relates to a compound which which targets, decreases or inhibits SRC. Examples of a SRC family tyrosine kinase inhibitor include, but are not limited to, PP1, which is also known as 1H-pyrazolo[3,4-d]pyrimidin-4-amine, 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)- (9CI); and PP2, which is also known as 1H-Pyrazolo[3,4-d]pyrimidin-4-amine, 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)- (9CI).

The term "a Syk tyrosine kinase inhibitor", as used herein, relates to a compound which targets, decreases or inhibits Syk. Examples of targets for a Syk tyrosine kinase inhibitor include, but are not limited to, Syk, STAT3, and/or STAT5. An example of a Syk tyrosine kinase inhibitor includes, but is not limited to, piceatannol, which is also known as 1,2-benzenediol, 4-[(1E)-2-(3,5-dihydroxyphenyl)ethenyl]- (9CI).

The term "a Janus (JAK-2 and/or JAK-3) tyrosine kinase inhibitor", as used herein, relates to a compound which targets, decreases or inhibits janus tyrosine kinase. Janus tyrosine kinase inhibitor are shown anti-leukemic agents with anti-thrombotic, anti-allergic and immunosuppressive properties. Targets of a JAK-2 and/or JAK-3 tyrosine kinase inhibitor include, but are not limited to, JAK2, JAK3, STAT3. An indirect target of an JAK-2 and/or JAK-3 tyrosine kinase inhibitor includes, but is not limited to CDK2. Examples of a JAK-2 and/or JAK-3 tyrosine kinase inhibitor include, but are not limited to, Tyrphostin AG 490; and 2-naphthyl vinyl ketone.

Compounds which target, decrease or inhibit the activity of c-Abl family members and their gene fusion products, e. g. include PD180970 ; AG957; or NSC 680410.

lvii. a retinoid; which target, decrease or inhibit retinoid dependent receptors; such as isotretinoin, tretinoin.

lviii. a RNA polymerase II elongation inhibitor; which targets, decreases or inhibits insulin-stimulated nuclear and cytosolic p70S6 kinase in CHO cells; targets, decreases or inhibits RNA polymerase II transcription, which may be dependent on casein kinase II; and targets, decreases or inhibits germinal vesicle breakdown in bovine oocytes; such as 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole.

lvix. a serine/threonine kinase inhibitor; which inhibits serine/threonine kinases; such as 2-aminopurine, also known as 1H-purin-2-amine(9CI). . An example of a target of a serine/threonine kinase inhibitor includes, but is not limited to, dsRNA-dependent protein kinase (PKR). Examples of indirect targets of a serine/threonine kinase

inhibitor include, but are not limited to, MCP-1, NF-kappaB, eIF2alpha, COX2, RANTES, IL8,CYP2A5, IGF-1, CYP2B1, CYP2B2, CYP2H1, ALAS-1, HIF-1, erythropoietin, and/or CYP1A1.

lx. a sterol biosynthesis inhibitor; which inhibits the biosynthesis of sterols such as cholesterol; such as terbinadine. Examples of targets for a sterol biosynthesis inhibitor include, but are not limited to, squalene epoxidase, and CYP2D6.

lxi. a topoisomerase inhibitor; including a topoisomerase I inhibitor and a topoisomerase II inhibitor. Examples of a topoisomerase I inhibitor include, but are not limited to, topotecan, gimatecan, irinotecan, camptothecan and its analogues, 9-nitrocamptothecin and the macromolecular camptothecin conjugate PNU-166148 (compound A1 in WO9917804); 10-hydroxycamptothecin acetate salt; etoposide; idarubicin hydrochloride; irinotecan hydrochloride; teniposide; topotecan, topotecan hydrochloride; doxorubicin; epirubicin, epirubicin hydrochloride; mitoxantrone, mitoxantrone hydrochloride; daunorubicin, daunorubicin hydrochloride, dasatinib (BMS-354825). Irinotecan can be administered, e.g., in the form as it is marketed, e.g., under the trademark CAMPTOSAR®. Topotecan can be administered, e.g., in the form as it is marketed, e.g., under the trademark HYCAMTIN®. The term "topoisomerase II inhibitor", as used herein, includes, but is not limited to, the anthracyclines, such as doxorubicin, including liposomal formulation, e.g., CAELYX®, daunorubicin, including liposomal formulation, e.g., DAUNOSOME®, epirubicin, idarubicin and nemorubicin; the anthraquinones mitoxantrone and losoxantrone; and the podophillotoxines etoposide and teniposide. Etoposide is marketed as ETOPOPHOS®; teniposide as VM 26-BRISTOL®; doxorubicin as ADRIBLASTIN® or ADRIAMYCIN®; epirubicin as FARMORUBICIN® idarubicin as ZAVEDOS®; and mitoxantrone as NOVANTRON®.

lxii. VEGFR tyrosine kinase inhibitor; which targets, decreases and/or inhibits the known angiogenic growth factors and cytokines implicated in the modulation of normal and pathological angiogenesis. The VEGF family (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and their corresponding receptor tyrosine kinases [VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR), and VEGFR-3 (Flt-4)] play a paramount and indispensable role in regulating the multiple facets of the angiogenic and lymphangiogenic processes. An example of a VEGFR tyrosine kinase inhibitor includes 3-(4-dimethylaminobenzylidenyl)-2-indolinone. Compounds which target, decrease or inhibit the activity of VEGFR are especially compounds, proteins or antibodies which inhibit the VEGF receptor tyrosine kinase, inhibit a VEGF receptor or bind to VEGF, and are in particular those

compounds, proteins or monoclonal antibodies generically and specifically disclosed in WO9835958, e. g.1- (4- chloroanilino)-4- (4-pyridylmethyl) phthalazine or a pharmaceutical acceptable salt thereof, e. g. the succinate, or in WO0009495, WO0027820, WO0059509, WO9811223, WO0027819 and EP0769947; e.g. those as described by M. Prewett et al in Cancer Research 59 (1999) 5209-5218, by F. Yuan et al in Proc. Natl. Acad. Sci. USA, vol. 93, pp. 14765-14770, Dec. 1996, by Z. Zhu et al in Cancer Res. 58,1998,3209-3214, and by J. Mordenti et al in Toxicologic Pathology, Vol. 27, no. 1, pp 14-21,1999; in WO0037502 and WO9410202; Angiostatin, described by M. S. O'Reilly et al, Cell 79,1994,315-328; Endostatin described by M. S. O'Reilly et al, Cell 88,1997,277-285;anthranilic acid amides; ZD4190; ZD6474; SU5416; SU6668; or anti-VEGF antibodies or anti-VEGF receptor antibodies, e. g. RhuMab (bevacizumab). By antibody is meant intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies formed from at least 2 intact antibodies, and antibodies fragments so long as they exhibit the desired biological activity. an example of an VEGF-R2 inhibitor e.g. includes axitinib,

lxiii. a gonadorelin agonist, such as abarelix, goserelin, goserelin acetate,

lxiv. a compound which induce cell differentiation processes, such as retinoic acid, alpha-, gamma- or 8- tocopherol or alpha-, gamma- or 8-tocotrienol.

lxv. a bisphosphonate, e.g. including etridonic, clodronic, tiludronic, pamidronic, alendronic, ibandronic, risedronic and zoledronic acid.

lxvi. a heparanase inhibitor which prevents heparan sulphate degradation, e. g. PI-88,

lxvii. a biological response modifier, preferably alymphokine or interferons, e. g. interferon alpha,

lxviii. a telomerase inhibitor, e. g. telomestatin,

lxix. mediators, such as inhibitors of catechol-O-methyltransferase, e.g. entacapone,

lxx: ispinesib, permetrexed (Alimta®), sunitinib (SU11248), diethylstilbestrol (DES), BMS224818 (LEA29Y),

lxxi somatostatin or a somatostatin analogue, such as octreotide (Sandostatin® or Sandostatin LAR®).

lxxii. Growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

Cancer treatment, such as endocrine tumor treatment with an mTOR inhibitor, optionally in combination with an anticancer drug, such as indicated herein, may be associated with

radiotherapy. Edocrine tumor treatment with an mTOR inhibitor, optionally in combination with an anticancer drug, may be a second line treatment, e.g. following treatment with another anticancer drug.

A preferred anticancer drug as a second drug substance in endocrine tumor treatment e.g. includes 5-fluorouracil, dacarbazine, streptozotocin, a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, interferon alpha or somatostatin or a somatostatin analogue, such as octreotide.

Preferably a second drug substance is somatostatin or a somatostatin analogue, such as octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

Anti-inflammatory and/or immunomodulatory drugs which are prone to be useful in combination with an mTOR inhibitor e.g. prone to be useful according to the present invention, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; e.g. sodium, mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97,

KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®), thalidomide, lenalidomide,
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, such as inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- mediators, e.g. agonists, of the G protein coupled receptor GPBAR1,
- mediators, e.g. inhibitors, of ceramide kinase activity,

- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine.

Anti-inflammatory drugs which are prone to be useful in combination with an mTOR inhibitor, e.g. prone to be useful according to the present invention, include e.g. non-steroidal antiinflammatory agents (NSAIDs) such as propionic acid derivatives (alminoprofen, benoxaprofen, bucloxic acid, carprofen, fenbufen, fenoprofen, fluprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen, miroprofen, naproxen, oxaprozin, pirprofen, pranoprofen, suprofen, tiaprofenic acid, and tioxaprofen), acetic acid derivatives (indomethacin, acemetacin, alclofenac, clidanac, diclofenac, fenclofenac, fenclozic acid, fentiazac, furofenac, ibufenac, isoxepac, oxpinac, sulindac, tiopinac, tolmetin, zidometacin, and zomepirac), fenamic acid derivatives (flufenamic acid, meclofenamic acid, mefenamic acid, niflumic acid and tolfenamic acid), biphenylcarboxylic acid derivatives (diflunisal and flufenisal), oxicams (isoxicam, piroxicam, sudoxicam and tenoxican), salicylates (acetyl salicylic acid, sulfasalazine) and the pyrazolones (apazone, bezpiperylon, feprazone, mofebutazone, oxyphenbutazone, phenylbutazone); cyclooxygenase-2 (COX- 2) inhibitors such as celecoxib; inhibitors of phosphodiesterase type IV (PDE-IV); antagonists of the chemokine receptors, especially CCR-1, CCR-2, and CCR-3; cholesterol lowering agents such as HMG-CoA reductase inhibitors (lovastatin, simvastatin and pravastatin, fluvastatin, atorvastatin, and other statins), sequestrants (cholestyramine and colestipol), nicotinic acid, fenofibric acid derivatives (gemfibrozil, clofibrat, fenofibrate and benzafibrate), and probucol; anticholinergic agents such as muscarinic antagonists (ipratropium bromide); other compounds such as theophylline, sulfasalazine and aminosalicylates, e.g. 5-aminosalicylic acid and prodrugs thereof, antirheumatics.

Antiallergic drugs which are prone to be useful in combination with an mTOR inhibitor, e.g. prone to be useful according to the present invention, e.g. include antihistamines (H1-histamine antagonists), e.g. bromopheniramine, chlorpheniramine, dexchlorpheniramine, triprolidine, clemastine, diphenhydramine, diphenylpyraline, tripelennamine, hydroxyzine, methdilazine, promethazine, trimeprazine, azatadine, cyproheptadine, antazoline, pheniramine pyrilamine, astemizole, terfenadine, loratadine, cetirizine, fexofenadine, descarboethoxyloratadine, and non-steroidal anti- asthmatics such as β2-agonists (terbutaline, metaproterenol, fenoterol, isoetharine, albuterol, bitolterol, salmeterol and pirbuterol), theophylline, cromolyn sodium, atropine, ipratropium bromide, leukotriene antagonists (zafirlukast, montelukast, pranlukast, iralukast, pobilukast, SKB-106,203), leukotriene biosynthesis inhibitors (zileuton, BAY-1005); bronchodilators, antiasthmatics (mast cell stabilizers).

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further, both the first agent and the co-agent are not the identical ingredient.
The structure of the drug substances identified by code numbers, generic or trade names may be taken from the Internet, actual edition of the standard compendium "The Merck Index" or from databases, e.g., Patents International, e.g., IMS World Publications, or the publications mentioned above and below. The corresponding content thereof is hereby incorporated by reference.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy may be determined by measurement of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. In vitro studies

Compound A is able to restore activity of endocrine agents, like estrogen inhibitors and/or aromatase inhibitors in cells which are otherwise resistant to endocrine agent treatment. Several studies have implicated aberrant acitivty of the Akt kinase as a significant mechanism by which breast cancer tumors are unresponsive to endocrine therapy.

D. Clinical Trials

In clinical trial studies involving patients having carcinoid or islet cell cancer inhibition of S6K1 activity and a reduction of chromogranin A may be observed when administering either Compound A alone, or a combination of Sandostatin LAR® (30 mg daily) and compound A (5 mg daily). Response evaluation may be performed every 12 weeks. Study duration: 6 months).

Also synergistic effects of such combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.

A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).

A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

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(57) Abstract: A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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(57) Abstract: A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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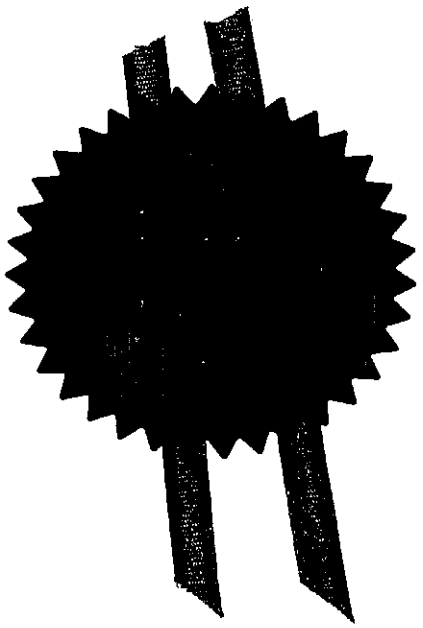

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0609912.1

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1. Your reference: *(optional)* 18 MAY 2006 — TX/4-34678P6/NFI 8102

2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:

Novartis AG
Lichtstrasse 35
CH - 4056 Basel
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Patents ADP number *(if you know it)*: 7125487008

If the applicant is a corporate body, give the country/state of its incorporation: Switzerland

3. Title of the invention: Organic Compounds

4. Name of your agent *(if you have one)*:

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Novartis Pharmaceuticals UK Limited
Patents and Trademarks
Wimblehurst Road
Horsham, West Sussex
RH12 5AB

Patents ADP number *(if you know it)*: 07181522002

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Signature(s): Date: 18/05/2006

12. Name, e-mail address, telephone, Fax and/or mobile number, if any, of a contact point for the applicant:

Mrs S Schnerr

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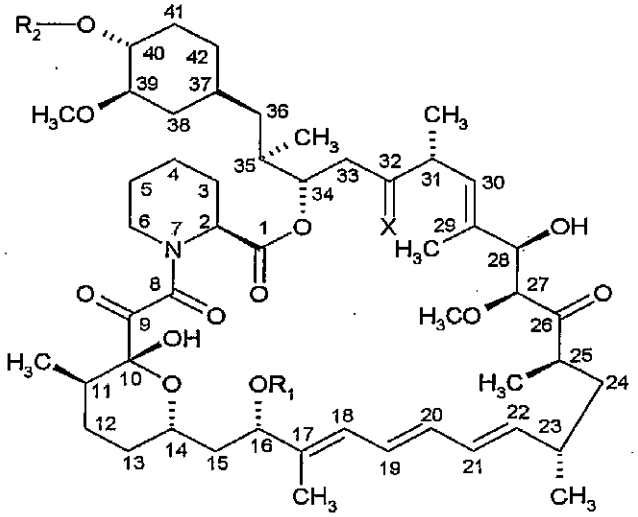
DUPLICATE

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9813273.

Preferred mTOR inhibitors include

rapamycin, and/or

40-O-(2-hydroxyethyl)-rapamycin, and/or

32-deoxorapamycin, and/or

16-pent-2-ynyloxy-32-deoxorapamycin, and/or

16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or

16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or

40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or

40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or

the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or

compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.
Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum. Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrebronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to

excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including decarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2006;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands.

Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions.

The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence.

Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an

endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.
Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors, e.g. it was found that suppression of the ASK1/JNK pathway is responsible for resistancy of cells against endocrine agent treatment and that mTOR inhibitors, e.g. Compound A, are able to restore that pathway.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method for reducing or avoiding resistance of endocrine cancer cells in the treatment with endocrine agents, comprising treating resistant cells with an effective amount of a combination of an mTOR inhibitor and an endocrine agent.

An "endocrine agent" e.g. includes an aromatase inhibitor, such as letrozole, or an estrogen inhibitor, e.g. tamoxifen.
Resistant cancer cells inlcude such wherein the ASK/JNK pathway is blocked at least partially, or totally.

1.10 A method as indicated under 1.1 to 1.9, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.11 A method as indicated under 1.1 to 1.10, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.11 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.11 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.11 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTOR inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.11 comprising
a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter or hereinbefore.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter or hereinbefore.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
lspinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), dpifamib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066018, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAå® or Somatuline Autogelå®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;

- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;
- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-[4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl]-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,

- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g.

cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x

the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. In vitro findings

Compound A is able to restore activity of endocrine agents, like estrogen inhibitors and/or aromatase inhibitors in cells which are otherwise resistant to endocrine agent treatment. Several studies have implicated aberrant acitivty of the Akt kinase as a significant mechanism by which breast cancer tumors are unresponsive to endocrine therapy.

For evaluating that, response in MCF-7 breast cancer cells expressing either wild-type (control) or constitutively-active Akt (myrAkt) and a dominant-negative ASK1 (DNASK1) was investigated. It was found that DNASK1 cells expressed are much more resistant to the inhibitory growth effects of endocrine agent treatment, such as endocrine agents like estrogen receptor inhibitors, e.g. tamoxifen, or aromatase inhibitors, e.g. letrozole. At the

molecular level, treatment with endocrine agents results in phosphorylation (activation) of cJUN in the control cells, but not in either the myrAkt1 or DANSK1 cells. Co-treatment of resistant myrAkt1 MCF-7 cells with Compound A, however, restores activation of the ASK/JNK pathway and increases endocrine therapy sensivity.

D. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.
A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).
A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendocrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

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Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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1. Your reference: *(optional)* TX/4-34678P2/NFI 8102

2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:

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Patents ADP number *(if you know it)*:

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If the applicant is a corporate body, give the country/state of its incorporation: Switzerland

3. Title of the invention: Organic Compounds

4. Name of your agent *(if you have one)*:

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)*: 07181522002 ✓

	Country	Application number *(if you know it)*	Date of filing *(day / month / year)*
5. Priority declaration: Are you claiming priority from one or more earlier-filed patent applications? If so, please give details of the application(s):			

	Number of earlier UK application	Date of filing *(day / month / year)*
6. Divisionals etc: Is this application a divisional application, or being made following resolution of an entitlement dispute about an earlier application? If so, please give the application number and filing date of the earlier application:		

7. Inventorship: (Inventors must be individuals not companies) (Please tick the appropriate boxes)

Are all the applicants named above also inventors?	YES ☐	NO ☒
If yes, are there any other inventors?	YES ☐	NO ☐

8. Are you paying the application fee with this form? YES ☒ NO ☐

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9. Accompanying documents: not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form:	
Description:	12 BH
Claim(s):	3
Abstract:	1
Drawing(s):	

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Country	Application number	Date of filing (day / month / year)

10. If you are also filing any of the following, state how many against each item.

Priority documents:	
Statement of inventorship and right to grant of a patent (Patents Form 7/77):	
Request for search (Patents Form 9A/77):	1
Request for substantive examination (Patents Form 10/77):	
Any other documents: (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature(s): P. Wardle-Smith Date: 19/01/2006

12. Name, e-mail address, telephone, Fax and/or mobile number, if any, of a contact point for the applicant:

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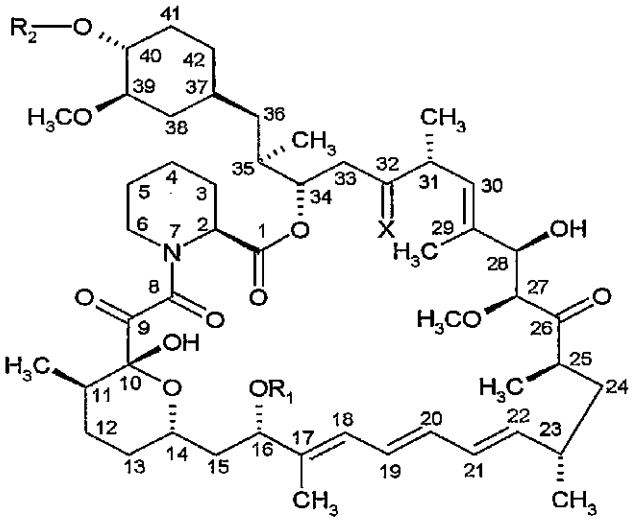
Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.
A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.
Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors. Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas. The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcionoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). The most frequent symptoms of carcinoid syndrome are flushing and diarrhea. Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:283-288) which may result in valvular regurgitation, with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms and pellagra-like skin lesions with hyperkeratosis are also seen in a number of patients. A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-659). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as pancreatic neuroendocrine tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of

an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof s therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoats]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),

- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,
- alkylating agents, such as dacarbazine,
- streptozotocin.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- calcineurin inhibitors, e.g. cyclosporin A or FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- bcr-abl tyrosine kinase inhibitors;
- c-kit receptor tyrosine kinase inhibitors;
- PDGF receptor tyrosine kinase inhibitors, e.g. Gleevec (imatinib);
- p38 MAP kinase inhibitors,
- VEGF receptor tyrosine kinase inhibitors,
- PKC inhibitors, e.g. as disclosed in WO 0238561 or WO 0382859, e.g. the compound of Example 56 or 70;
- JAK3 kinase inhibitors, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-

methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO04052359 or WO05066156;

- S1P receptor agonists or modulators, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- adhesion molecule inhibitors, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- CCR9 antagonists,
- MIF inhibitors,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- antibodies which bind to TNF-alpha, such as infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. PDE4B-inhibitors,
- caspase ihibitors,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, pleuromutilins, fluoroquinolones, e.g. metronidazole, ciprofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;

- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention

could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.

Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/20-Nov-05

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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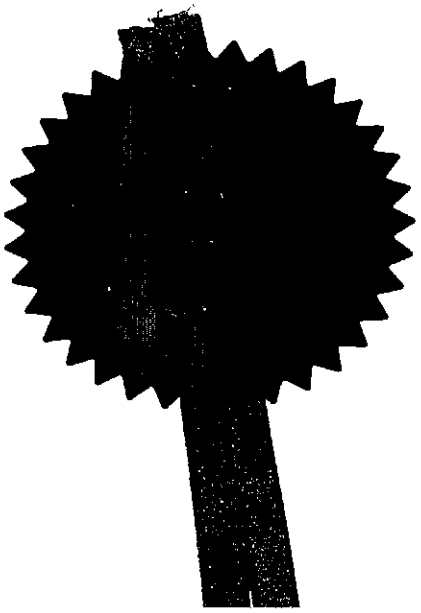

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Novartis AG
Lichtstrasse 35
CH - 4056 Basel
Switzerland

Patents ADP number *(if you know it)*: 7123487008

If the applicant is a corporate body, give the country/state of its incorporation: Switzerland

3. Title of the invention: Organic Compounds

4. Name of your agent *(if you have one)*:

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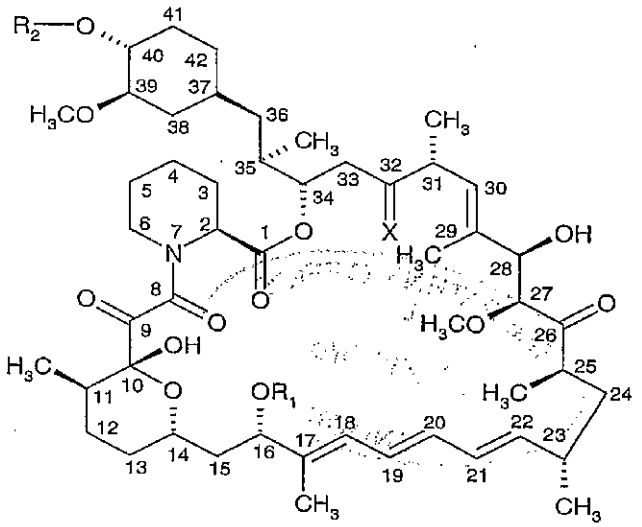
ORGANIC COMPOUNDS

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors. Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas. The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to

metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcionoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). The most frequent symptoms of carcinoid syndrome are flushing and diarrhea. Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation, with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms and pellagra-like skin lesions with hyperkeratosis are also seen in a number of patients. A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction,

comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as pancreatic neuroendocrine tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract. Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-

pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy-,methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;

such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibitor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising
a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®,
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone--Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,
-alkylating agents, such as dacarbazine,
- streptozotocin.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carcinlod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

CLAIMS

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-

methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug

substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapammycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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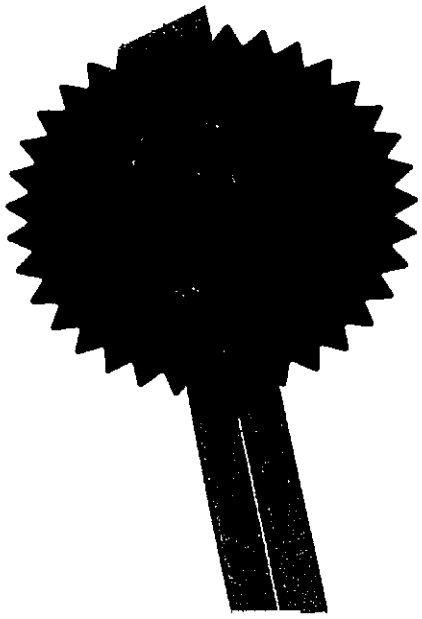

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2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:

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Lichtstrasse 35
CH - 4056 Basel
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Patents ADP number *(if you know it)*: 7125487008

If the applicant is a corporate body, give the country/state of its incorporation: Switzerland

3. Title of the invention: Organic Compounds

4. Name of your agent *(if you have one)*:

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Patents ADP number *(if you know it)*: 07181522002

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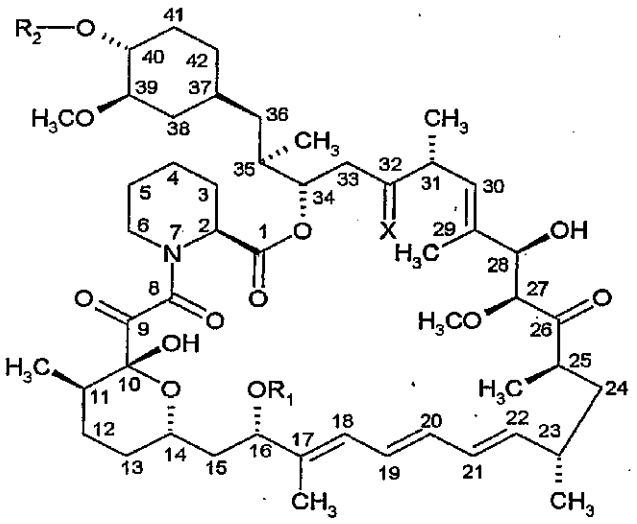
DUPLICATE

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9408010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO8641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include

rapamycin, and/or

40-O-(2-hydroxyethyl)-rapamycin, and/or

32-deoxorapamycin, and/or

16-pent-2-ynyloxy-32-deoxorapamycin, and/or

16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or

16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or

40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or

40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or

the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or

compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally, potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.
Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to

excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):648-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands.

Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an

endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.
Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such

tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin,

16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]- rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;

such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
lapinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherapeutic agents" in WO0206619, e.g. on pages 5 and 6 under i) to x), in more detail on pages 8 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, lanreotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sanvar® or Dorisad®, lanreotide, e.g. sold under the trade name Somatuline LA® or Somatuline Autogel®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),

- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,
- alkylating agents, such as dacarbazine,
- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin

AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 activity,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,

- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting

antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasivsness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeuticaily effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/19-Apr-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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10 MAY 2006

Application number GB

0609272.0

1. Your reference: *(optional)*

TX/4-34678P5/NFI 8102

2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:

Novartis AG
Lichtstrasse 35
CH -.4056 Basel
Switzerland

Patents ADP number *(if you know it)*:

71254870005

If the applicant is a corporate body, give the country/state of its incorporation:

Switzerland

3. Title of the invention:

Organic Compounds

4. Name of your agent *(if you have one)*:

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Novartis Pharmaceuticals UK Limited
Patents and Trademarks
Wimblehurst Road
Horsham, West Sussex
RH12 5AB

Patents ADP number *(if you know it)*:

07181522002

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Request for search (Patents Form 9A/77):	1
Request for substantive examination (Patents Form 10/77):	
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11. I/We request the grant of a patent on the basis of this application.

Signature(s): Novartis Pharmaceuticals UK Ltd Date: 10/05/2006

12. Name, e-mail address, telephone, Fax and/or mobile number, if any, of a contact point for the applicant: Mrs S Schnarr phone: 01403 323 069 fax : 01403 323 629

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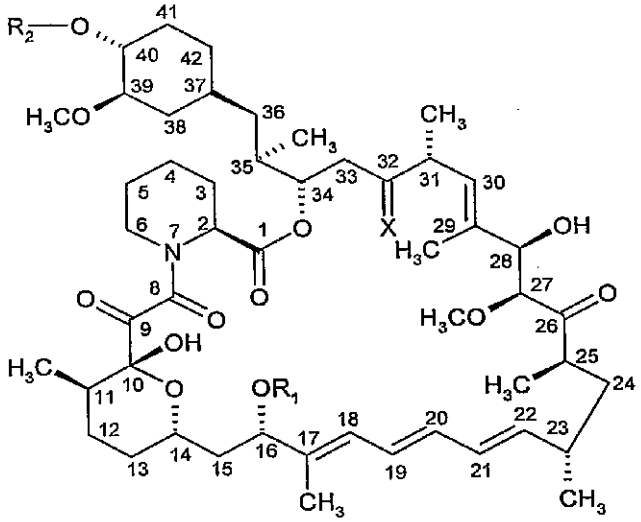
DUPLICATE

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-5}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-6})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a

therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides

1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrins, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inhibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;

such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising
a) a first agent which is an mTOR inhibitor and
b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.
ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, lanreotids (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sanser® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LA® or Somatuline Autogel®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the

podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®

- 5-Fluorouracil,
- alkylating agents, such as dacarbazine,
- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,
- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;

- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phosphopholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;

- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as

active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somactatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.

A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).

A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with

Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/10-May-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

Box No. VIII (ii) DECLARATION: ENTITLEMENT TO APPLY FOR AND BE GRANTED A PATENT

The declaration must conform to the standardized wording provided for in Section 212; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (ii). If this Box is not used, this sheet should not be included in the request.

Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent (Rules 4.17(ii) and 51*bis*.1(a)(ii)), in a case where the declaration under Rule 4.17(iv) is not appropriate:

in relation to this international application,

Novartis AG and Novartis Pharma GmbH, are entitled to apply for and be granted a patent by virtue of the following:

an assignment from:

Peter Wayne MARKS, citizen of United States Of America, 145 Rimmon Road, Woodbridge, CT 06525-1913, US, dated 13.12.2006

David LEBWOHL, citizen of United States Of America, 55 Pomeroy Road, Madison, New Jersey 07940, US, dated 20.12.2006

to Novartis AG in respect of all designated States with the exception of AT (Austria) and Novartis Pharma GmbH in respect of Austria

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (ii)".

Document made available under the Patent Cooperation Treaty (PCT)

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World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

Europäisches Patentamt GD1	European Patent Office DG1	Office européen des brevets DG1

Bescheinigung	Certificate	Attestation
Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.	The attached documents are exact copies of the European patent application described on the following page, as originally filed.	Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.	Patent application No.	Demande de brevet n°

06120660.3 / EP06120660

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP06120660

08 DEC 2006

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
p.o.

R.C. van Dijk

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Anmeldung Nr:
Application no.:
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Anmelder/Applicant(s)/Demandeur(s):

Novartis AG
Lichtstrasse 35
4002 Basel/CH

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Organic Compounds

In anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

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A61K31/00

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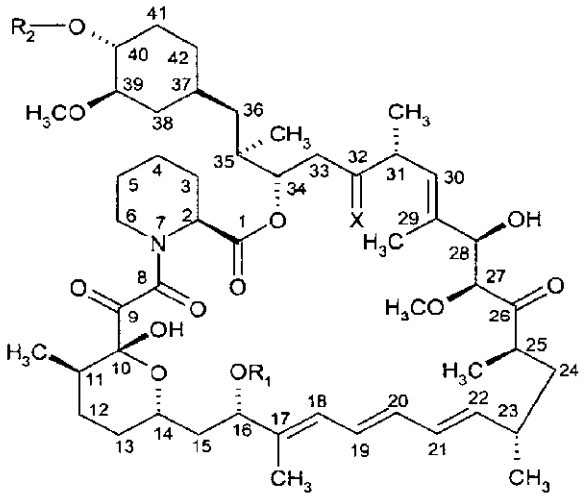
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR

Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.

A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.

Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Endocrine, e.g. neuroendocrine tumors, are found in the endocrine system
Carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.
Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.
Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.
The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).
All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).
In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.
Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and

diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:263-268) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

As part of the endocrine system that regulates hormones, the pituitary gland controls many of the other glands through secretion. Our "master gland," the pituitary makes some hormones, but also acts as an intermediary between the brain and other endocrine glands. Our hormones and the pituitary gland accomplish many homeostatic and specialized functions, like bone growth and uterine contractions.

Neurons carry messages regarding the production of hormones between the pituitary gland and the hypothalamus. Both are located at the base of the brain, nestled in a rounded part of bone, carefully protected. They are connected by a bunch of neurons called the

infundibulum. Together, they work to regulate all the hormones that circulate in the bloodstream, controlling things like growth and hair pigmentation. Hormones are the long-distance messangers that can inform cells when to become active or stay dormant. The pituitary gland controls the thyroid, adrenal glands, ovaries and testes, even though it's only the size of a pea.

There are different parts of the pituitary gland that have selective functions. The posterior lobe, called the neurohypophysis, releases the hormones vasopressin and oxytocin, but doesn't produce them. Vasopressin is an anti-diuretic that controls how the kidneys absorb water. Oxytocin is a special hormone only present during childbirth to speed contractions. The anterior lobe of the pituitary gland is called the adenohypophysis. It produces a variety of hormones, such as prolactin that stimulates lactation in women. Melanocyte spurs the body to produce melanin for skin and hair pigmentation. Follicle-stimulating hormone indicates where and when hair should grow during development. The very important growth hormone controls bone growth to determine height, especially active during adolescence. Hormones control glands as well. The thyroid reacts to thyrotropin, the adrenal glands are stimulated by adrenocorticotropin, and the sex glands are affected by luteinizing hormone. The pituitary gland is responsible for many stages and aspects of our maturation.

Pituitary tumors are in general noncancerous (benign), comprising only 10 percent of brain tumors. However, because of the location of the pituitary gland, at the base of the skull, a pituitary tumor grows upward. And, eventually, many pituitary tumors press against the optic nerves, causing vision problems. Symptoms vary depending upon what type of tumor is growing and what area of the pituitary gland is affected. Pituitary tumors can cause symptoms that are caused by excess production of pituitary hormones and symptoms caused by reduced production of pituitary hormones. Other symptoms may be due to the proximity of these tumors to local brain structures, such as the optic nerves leading to loss of vision. Each individual also experiences symptoms differently, and the symptoms many resemble other conditions or medical problems. Always consult your physician for a diagnosis.

The most common type of pituitary tumor is called a clinically nonfunctioning tumor, because patients do not have the classic pituitary syndromes from excess hormones, such as in acromegaly. These types of tumors may be detected during an evaluation of an incidental problem. A clinically nonfunctioning tumor may cause hypopituitarism, or an underactive pituitary gland, which may lead to failure of sexual function, reduced sperm production, and cessation of a woman's menstrual period, along with fatigue.

Another common pituitary tumor is called a prolactinoma, a benign tumor that produces the prolactin hormone. Prolactin stimulates breast milk production after childbirth. Women with a prolactinoma may have reduced or absent menstrual cycles along with breast milk production.

An uncommon pituitary tumor causes excess growth hormone production (a hormone necessary for normal childhood growth) resulting in acromegaly. In adults, such tumors lead to excessive somatic growth and multiple systemic, medical consequences. Another uncommon pituitary tumor results in Cushing's disease, a disorder of excess steroid production.

Multiple endocrine neoplasia type 1 (MEN 1) is a relatively uncommon inherited disease. Individuals who inherit the gene for MEN 1 have an increased chance of developing overactivity and enlargement of certain endocrine glands. The endocrine glands most commonly affected by MEN 1 are the parathyroid, pancreas, and pituitary glands. Almost everyone who inherits MEN 1 develops overactivity of the parathyroid glands (hyperparathyroidism) at some stage in their life. The other endocrine glands become overactive less frequently, however, people who inherit MEN 1 will usually develop overactivity in more than one endocrine gland. Overactivity in different endocrine glands may occur simultaneously or at separate times during a persons life. MEN 1 can lead to overactivity and enlargement of the three endocrine glands listed above (the endocrine glands which start with the letter "P"). People who inherit the gene for MEN 1 are predisposed to developing an overactivity in hormone production from the parathyroid glands, pituitary gland and pancreas (thetas why physicians will measure hormones in the blood to check for overproduction of each specific hormone). Increased hormone production is usually associated with enlargement of these glands. Endocrine gland enlargement and hormone overproduction does not usually occur in all areas of an endocrine gland at the same point in time. Some parts of overactive endocrine glands grow more rapidly than others, and produce more hormone than other parts of the same gland. The parts of an endocrine gland which grow most rapidly become "lumpy". These lumps are usually benign. Benign lumps in endocrine glands are known as adenomas.

Adenomas are benign (not cancerous), and do not spread to other parts of the body. Pituitary adenomas (pituitary tumors, nervous system tumor) can lead to nerve damage, growth disturbances, and changes in hormonal balance. Symptoms of pituitary adenomas can vary considerably, largely depending on whether or not the tumor is secreting one or

more of a variety of hormones. Even if the tumor is not producing any hormones, its location at the base of the brain can cause significant symptoms. Symptoms may e.g. include double or blurred vision, loss of peripheral vision, sudden blindness, headache, dizziness, loss of consciousness, nausea, weakness, unexplained weight changes, amenorrhea, erectile dysfunction in men, decreased sexual desire, especially in men, growth of skull, hands, and feet , deepening of voice, changes in facial appearance (due to changes in facial bones), wider spacing of teeth, joint pain, increased sweating, purple stretch marks on the abdomen, increased hair growth, fat deposits where the neck meets the spine, moodiness or depression, easy bruising, palpitations (rapid or irregular heartbeat), tremor, increased appetite, feeling warm or hot, difficulty falling asleep, anxiousness, frequent bowel movements, lump in the front of the neck (enlarged thyroid).

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors, e.g. it was found that suppression of the ASK1/JNK pathway is responsible for resistancy of cells against endocrine agent treatment and that mTOR inhibitors, e.g. Compound A, are able to restore that pathway.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or

inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom. Tumors of the endocrine system also include pituitary tumors.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method for reducing or avoiding resistance of endocrine cancer cells in the treatment with endocrine agents, comprising treating resistant cells with an effective amount of a combination of an mTOR inhibitor and an endocrine agent.

An "endocrine agent" e.g. includes an aromatase inhibitor, such as letrozole, or an estrogen inhibitor, e.g. tamoxifen.

Resistant cancer cells inlcude such wherein the ASK/JNK pathway is blocked at least partially, or totally.

1.10 A method as indicated under 1.1 to 1.9, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.11 A method as indicated under 1.1 to 1.10, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.11 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating carcinoid tumors.

In another preferred aspect the present invention a method of 1.1 to 1.11 for treating pituitary tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.11 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.11 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTOR inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.11 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter or hereinbefore.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter or hereinbefore.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g.

LHRH peptidomimetics, e.g. such as disclosed in US6627609, teverelix, D-63153; perifosine, erucyl phosphocholine, AN-152, AN-238, AN-215, lobaplatin, disorazol E, ZEN-014, ZEN-017, RC-3095, AE-941 (Neovastat), cetorelix.

ispinesib, oxaliplatin, triciribine, permetrexed (Alimta®), sunitinib (SU11248), temozolidine, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil(5-FU),floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin, diethylstilbestrol (DES), tipifarnib, bortezomib and drugs such as disclosed as "chemotherpeutic agents" in WO02066019, e.g. on pages 5 and 6 under i) to x), in more detail on pages 6 to 11, and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold

under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;

- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,
- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase 11 inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,

-alkylating agents, such as dacarbazine,

- streptozotocin.

WO02066019 is introduced herein by reference, specifically regarding the "second drug substance" indication therein.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- mediators, e.g. inhibitors, of calcineurin, e.g. cyclosporin A, FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- mediators, e.g. inhibitors, of bcr-abl tyrosine kinase activity;
- mediators, e.g. inhibitors, of c-kit receptor tyrosine kinase activity;
- mediators, e.g. inhibitors, of PDGF receptor tyrosine kinase activity, e.g. Gleevec (imatinib);
- mediators, e.g. inhibitors, of p38 MAP kinase activity,
- mediators, e.g. inhibitors, of VEGF receptor tyrosine kinase activity,

- mediators, e.g. inhibitors, of PKC activity, e.g. as disclosed in WO0238561 or WO0382859, e.g. the compound of Example 56 or 70;
- mediators, e.g. inhibitors, of JAK3 kinase activity, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO2004052359 or WO2005066156;
- mediators, e.g. agonists or modulators of S1P receptor activity, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- mediators, e.g. inhibitors of adhesion molecule activities, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- mediators, e.g. antagonists of CCR9 acitiviy,
- mediators, e.g. inhibitors, of MIF activity,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- mediators, e.g. inhibitors, of TNF-alpha activity, e.g. including antibodies which bind to TNF-alpha, e.g. infliximab (Remicade®),

- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. mediators, e.g. inhibitors of PDE4B activity,
- mediators, e.g. inhibitors, of caspase activity,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;
- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, such as sulfadiazine, sulfisoxazole; sulfones, such as dapsone; pleuromutilins, fluoroquinolones, e.g. metronidazole, quinolones such as ciprofloxacin; levofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antiviral drugs, such as ribivirin, vidarabine, acyclovir, ganciclovir, zanamivir, oseltamivir phosphate, famciclovir, atazanavir, amantadine, didanosine, efavirenz, foscarnet, indinavir, lamivudine, nelfinavir, ritonavir, saquinavir, stavudine, valacyclovir, valganciclovir, zidovudine;.
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more

pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye)

determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.

Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. In vitro findings

Compound A is able to restore activity of endocrine agents, like estrogen inhibitors and/or aromatase inhibitors in cells which are otherwise resistant to endocrine agent treatment. Several studies have implicated aberrant acitivty of the Akt kinase as a significant mechanism by which breast cancer tumors are unresponsive to endocrine therapy.

For evaluating that, response in MCF-7 breast cancer cells expressing either wild-type (control) or constitutively-active Akt (myrAkt) and a dominant-negative ASK1 (DNASK1) was investigated. It was found that DNASK1 cells expressed are much more resistant to the inhibitory growth effects of endocrine agent treatment, such as endocrine agents like estrogen receptor inhibitors, e.g. tamoxifen, or aromatase inhibitors, e.g. letrozole. At the molecular level, treatment with endocrine agents results in phosphorylation (activation) of cJUN in the control cells, but not in either the myrAkt1 or DANSK1 cells. Co-treatment of resistant myrAkt1 MCF-7 cells with Compound A, however, restores activation of the ASK/JNK pathway and increases endocrine therapy sensivity.

D. Clinical Trial

27 patients (16 carcinoid, 11 islet cells) are tretaed with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.

Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with, e.g. 30 mg, of Sandostatin LAR® daily are investigated, e.g.

A randomized, double-blind, placebo controlled study of compound A in 420 patients who are receiving therapy with Sandostatin LAR® for advanced midgut carcinoid tumors. Patients continue baseline Sandostatin LAR® therapy and are randomized to receive Compound A 10 mg/day or placebo. Primary endpoint is progression free survival (PFS). Secondary endpoints include overall survival, carcinoid-associated symptoms of flushing and diarrhea, pharmakinetics and pharmadynamics. For efficacy assessment progression and response are assessed per RECIST criteria. Due to the nature of neuroendocrine tumors, all patients must have triphasic CT scans or MRI. Scans are repeated every two months. Aim: Compound A in combination with Sandostatin LAR® for treatment of advanced progressing midgut tumor (carcinoid tumor).

A single-arm placebo controlled study of Compound A 10 mg/day in 100 patients with measurable advanced (metastatic or unresentable) pancreatic neuroendcrine tumors (islet cell tumor) after failure of cytotxic chemotherapy as a monotherapy. Primary goal is to determine the response rate. A cohort of 44 patients receiving chronic treatment with Sandostain LAR® for secretory pancreatic tumors are also be treated with Compound A, 10 mg a day, in addtion to Sandostatin LAR®.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0-(2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/14-Sep-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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EP06/68636

The Patent Office
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NP10 8QQ

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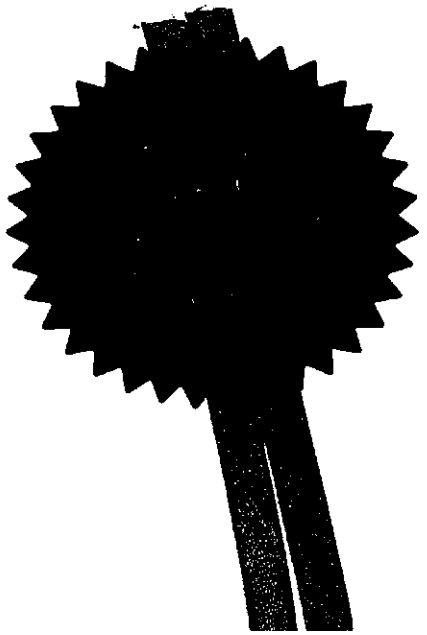

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Dated 23 October 2006

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Patents Form 1/77

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Request for grant of a patent

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Application number GB 0602747.8

1. Your reference: *(optional)* TX/4-34678P3/NFI 8102

2. Full name, address and postcode of the applicant or of each applicant *(underline all surnames)*:
Novartis AG
Lichtstrasse 35
CH - 4056 Basel
Switzerland

Patents ADP number *(if you know it)*: 07125487008

If the applicant is a corporate body, give the country/state of its incorporation: Switzerland

3. Title of the invention: Organic Compounds

4. Name of your agent *(if you have one)*:

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*:
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Patents and Trademarks
Wimblehurst Road
Horsham, West Sussex
RH12 5AB

Patents ADP number *(if you know it)*: 07181522002 ✓

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Country	Application number *(if you know it)*	Date of filing *(day / month / year)*

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7. Inventorship: (Inventors must be individuals not companies) (Please tick the appropriate boxes)

Are all the applicants named above also inventors? YES ☐ NO ☒

If yes, are there any other inventors? YES ☐ NO ☐

8. Are you paying the application fee with this form? YES ☒ NO ☐

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Request for search (Patents Form 9A/77):	1
Request for substantive examination (Patents Form 10/77):	
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12. Name, e-mail address, telephone, Fax and/or mobile number, if any, of a contact point for the applicant:

Mrs S Schnerr

phone: 01483 323 069
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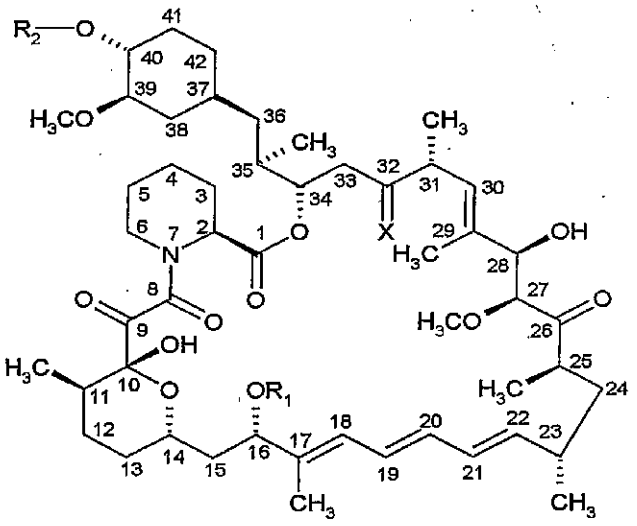
Organic Compounds

The present invention relates to organic compounds, more specifically to a new use of mTOR inhibitors.

An mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target of rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.

Rapamycin, having mTOR-inhibition properties, is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Other mTOR inhibitors include substituted rapamycin, e. g. rapamycin substituted in position 40 and/or 16 and/or 32, for example a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-6}alkynyl,

R_2 is H,-CH_2-CH_2-OH, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X is = O, (H, H) or (H, OH), provided that R_2 is other than H when X is =O and R_1 is CH_3, or a prodrug thereof when R_2 is-CH_2-CH_2-OH, e. g. a physiologically hydrolysable ether thereof, for instance -CH_2-CH_2-O-(C_{1-8})alkyl.

Representative examples of compounds of formula I include e. g. 32-deoxorapamycin, 16-O-substituted rapamycins such as 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin(also known as CCI779) or 40-epi-(tetrazolyl)- rapamycin (also known as ABT578), the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, e. g. AP23573, AP23464, AP23675 or AP23841 and compounds disclosed under the name TAFA-93 and biolimus.
A preferred compound is e. g. 40-0- (2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO9409010 (referred hereinafter as Compound A), or 32-deoxorapamycin or 16-pent-2-ynyloxy-32 (S) -dihydro- rapamycin as disclosed in WO9641807, or a compound as disclosed in WO9516691.
Further examples of other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273.

Preferred mTOR inhibitors include
rapamycin, and/or
40-O-(2-hydroxyethyl)-rapamycin, and/or
32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32-deoxorapamycin, and/or
16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, and/or
16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, and/or
40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779) and/or
40-epi-(tetrazolyl)- rapamycin (also known as ABT578), and/or
the so-called rapalogs, e. g. as disclosed in WO9802441, WO0114387 and WO0364383, such as AP23573, AP23464, AP23675 or AP23841 and/or
compounds disclosed under the name TAFA-93 or biolimus.

mTOR inhibitors, on the basis of observed activity, have been found to be useful e. g. as immunosuppressant, e. g. in the treatment of acute allograft rejection and have additionally potent antiproliferative properties which make them useful for cancer chemotherapy, particularly for the treatment of solid tumors, especially of advanced solid tumors.

Neuroendocrine tumors, e.g. including carcinoid tumors, are a special type of tumor, generally classified as carcinoid tumors or endocrine tumors.

Carcinoid tumors belong to the family of neuroendocrine tumors which derive from the neuroendocrine cell system. In the intestinal tract, these tumors develop deep in the mucosa, growing slowly and extending into the underlying submucosa and mucosal surface. This results in the formation of small firm nodules, which bulge into the intestinal lumen.

Pancreatic neuroendocrine tumors (islet cell tumors), which were formerly classified as APUDomas (tumors of the amine precursor uptake and decarboxylation system), comprise less than half of all neuroendicrine tumors and only 1-2% of all pancreatic tumors.

Pancreatic NETs can arise either in the pancreas (insulinomas, glucagonomas, nonfunctioning pancreatic NETs, pancreatic NETs causing hypercalcemia) or at both pancreatic and extrapancreatic sites (gastrinomas, VIPomas, somatostatinomas, GRFomas.

The hormones secreted by pancreatic NETs depend upon the cell of origin and are physiologically involved in a network of autocrine, paracrine, endocrine and neurotransmitter communication. While hormone secretion is not observed in all cases of pancreatic NET, the apparently "nonfunctioning" (i.e., non-secreting) pancreatic NETs tend to be more aggressive and present with symptoms of tumor bulk (see e.g. Barakat et al, Endocrine-related cancer 2004;11:1-18 and Tomassetti et al, Ann Oncol 2001;12(Suppl 2):S95-S99).

All pancreatic NETs, with the exception of 90% of insulinomas, have long-term metastatic potential. Most are overtly malignant at the time of diagnosis, and 60% or more present with liver metastases. The most common cause of death from pancreatic NET is hepatic failure (Warner RRP, Gastroenterology 2005;128:1668-16842005).

In a recent review, the 5-year survival rate in a series of 83 consecutive patients with pancreatic NETs has been reported to be 55.3% which points to an unmet medical need for continued treatment in patients with pancreatic NETs whose disease has progressed following 1 or more courses of chemotherapy.

Carcinoid tumors have historically been classified, according to their point of origin in embryonic development, as arising from the foregut (e.g., bronchial or gastric carcinoid), midgut (e.g., small intestine or appendiceal carcinoid), or hindgut (e.g., rectal carcinoid), see e.g. Kulke M., Cancer Treatment Reviews 2003;29:363-370.

Primary foregut tumors are confined to the thymus, lung, stomach, and duodenum.

Midgut carcinoids are located in the distal ileum, cecum, and proximal colon. One interesting subset of this group is appendiceal carcinoids, which are often benign and rarely give rise to metastatic disease. The midgut carcinoids dominate the malignant carcinoid tumors, particularly when the carcinoid syndrome is present.

The hindgut tumors are primarily located in the distal colon and rectum.

Data suggest that the incidence of pulmonary and gastric carcinoid has increased in the past two decades.

According to histopathologic criteria, carcinoids can be divided into typical (TC) and atypical (AC) carcinoids. Carcinoids can be placed in a spectrum of neuroendocrine tumors, ranging from low-grade malignant TC to intermediate AC to high-grade large-cell neuroendocrine carcinoma and small-cell lung carcinoma.

Carcinoid lung tumors e.g. include neuroendocrine carcinoma, Kulchitsky cell carcinoma (KCC), bronchial carcinoid tumors, bronchial adenomas, typical carcinoids, atypical carcinoids, carcinoid syndrome, small-cell carcinomas, Kulchitsky cells, argentaffin cells, pulmonary carcinoids, neuroendocrine lung tumors, (primary) pulmonary neoplasms, bronchopulmonary carcinoid tumors, lung neoplasms, lung cancers, pulmonary cancers, intrabronchial mass.

Bronchial carcinoid tumors may originate from the neurosecretory cells of bronchial mucosa and were previously classified as bronchial adenomas. Bronchial carcinoids are now classed as low-grade malignant neoplasms because of their potential to cause local invasion, their tendency for local recurrence, and their occasional metastases to extrathoracic sites.

Bronchial carcinoids belong to a group of neuroendocrine tumors, which cover a range of tumors ranging from bronchial carcinoid at one of the spectrum, with a small cell carcinoma, or possibly large cell neuroendocrine tumors at the other end. They demonstrate a wide range of clinical and biologic behaviors, including the potential to synthesize and secrete peptide hormones and neuroamines, particularly adrenocorticotropic hormone (ACTH), serotonin, somatostatin, and bradykinin.

Bronchial carcinoid tumors may arise from Kulchitsky cells (argentaffin cells) within the bronchial mucosa. The predominant distribution of cells are believed to occur at the bifurcation of the lobar bronchi. These cells are neurosecretory cells, which belong to the amine precursor uptake and decarboxylation (APUD) system. They have the capacity to synthesize serotonin (5-hydroxytryptamine), 5-hydroxytryptophan, ACTH, norepinephrine, bombesin, calcitonin, antidiuretic hormone (ADH), and bradykinin.

Large-cell neuroendocrine carcinoma of the lung is a newly recognized clinicopathologic entity, which is distinct from small-cell carcinoma and has a poor prognosis.

Typical carcinoid tumors of the lung represent the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumor. These tumors characteristically grow slowly and tend to metastasize infrequently. Atypical carcinoid tumors have a more aggressive histologic and clinical picture. They metastasize at a considerably higher rate than do typical carcinoid tumors. Carcinoid syndrome has been reported in association with very large bronchopulmonary carcinoid tumors or in the presence of metastatic disease. It is noted much less frequently in association with carcinoids of pulmonary origin than those originating within the gastrointestinal tract. Endocrine syndromes found in association with small cell carcinoma of the lung are found less commonly with carcinoid tumors of the lung; however, some endocrine abnormalities have been attributed to both typical and atypical pulmonary carcinoid tumors.

Carcinoid tumors of the GI tract may display an aggressive biology similar to that of adenocarcinomas, particularly when they are located in the colon, stomach, and small intestine, see e.g. Modlin IM et al, Gastroenterology 2005;128:1717-1751. For small-intestinal carcinoids, which are the most frequent cause of carcinoid syndrome due to metastatic disease in the liver, the incidence of metastasis increases proportionally with the size of the primary tumor (Tomassetti et al 2001, ibidem).

The incidence and survival data available suggest that clinical trials of new anticancer agents in patients with midgut carcinoid tumors may provide the opportunity to address an unmet medical need in a growing segment of the population of patients with carcinoids.

Carcinoid syndrome is caused by hypersecretion of numerous hormone products by the tumor cells, including kinins, prostaglandins, substance P, gastrin, corticotrophin and chromogranin A (see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644). Various endocrine or neuroendocrine syndromes can be initial clinical manifestations of either typical or atypical pulmonary carcinoid tumors. Carcinoid syndrome, hypercortisolism and Cushing syndrome, inappropriate secretion of ADH, increased pigmentation secondary to

excess MSH, and ectopic insulin production resulting in hypoglycemia are some of the endocrinopathies that can be produced by a pulmonary carcinoid tumor in a patient who is otherwise asymptomatic.

The most common symptoms are hemoptysis, cough, recurrent pulmonary infection, fever, chest discomfort and chest pain, unilateral wheezing, and shortness of breath, flushing and diarrhea. Paraneoplastic syndromes are rare and include carcinoid syndrome, Cushing's syndrome, and ectopic growth hormone-releasing hormone secretion.

Other less frequent symptoms include cardiac manifestations secondary to fibrosis of the endocardium (Jacobsen MB et al, Eur Heart J 1995;16:283-288) which may result in valvular regurgitation (valvular heart disease), with varying degrees of heart failure in patients with cardiac manifestations. Wheezing or asthma-like symptoms, pellagra-like skin lesions with hyperkeratosis, abdominal pain, telangiectasias and paroxysmal hypotension are also seen in a number of patients. Patients with pulmonary carcinoid often show symptoms like recurrent pneumonia, cough, hemoptysis or chest pain. The majority of pulmonary carcinoid tumors are in the perihilar area. Ectopic secretion of corticotropin from pulmonary carcinoid tumors may also account for Cushing's syndrome. Early in the course, symptoms are usually episodic and may be provoked by stress, catecholamines, and ingestion of food or alcohol. During acute paroxysms, systolic blood pressure typically falls 20 to 30 mmHg. Endocardial fibrosis can cause valvular heart disease, usually affecting the proximal side of the tricuspid and pulmonary valves and leading to tricuspid insufficiency and secondary right-sided heart failure.

A recent review of chemotherapeutic treatment of carcinoids reports that the sensitivity of these tumors to various cytotoxic drugs is low, and combination does not increase their effectiveness. Based on their review of various combination therapies, including dacarbazine/fluorouracil or 5-fluorouracil/ epirubicin, the authors conclude that that they are unable to recommend a specific chemotherapeutic regimen for patients with well-differentiated neuroendocrine malignancies of the GI tract (Arnold R, Rinke A et al, Clinical Gastroenterology 2005;19(4):649-656). The apparent refractoriness of such tumors to currently available therapies points to an unmet medical need for treatment in this patient population.

It was now surprisingly found that mTOR inhibitors may be used for the treatment of such special type of tumors.

In accordance with the particular findings the present invention provides:

1.1 A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.2 A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.4 A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.5 A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Endocrine tumors include neuroendocrine tumors, such as described above, e.g. including pancreatic neuroendocrine and pulmonary tumors. Carcinoid tumors are neuroendocrine tumors and include carcinoid tumors such as described above, e.g. including carcinoid tumors arising from the foregut, e.g., bronchial or gastric carcinoid; midgut, e.g., small intestine or appendiceal carcinoid tumors; or hindgut, e.g. rectal carcinoid tumors; such as carcinoid tumors of the GI tract, e.g. including advanced low grade neuroendicrine carcinoma (LGNET). Symptoms of carcinoid cancer include e.g. a carcinoid syndrom.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides 1.6 A method for the treatment of a disease associated with endocrine tumors,

comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.7 A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

1.9 A method as indicated under 1.1 to 1.8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus;
such as 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin,
e.g. 40-O-(2-hydroxyethyl)-rapamycin (herein also designated as "compound A").

1.10 A method as indicated under 1.1 to 1.9, wherein the mTOR inhibitor is administered intermittently.

In a preferred aspect the present invention provides a method of 1.1 to 1.10 for treating neuroendocrine tumors.

In another preferred aspect the present invention a method of 1.1 to 1.10 for treating carcinoid tumors.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 An mTOR inhibitor for use in any method as defined under 1.1 to 1.10 above.

3.1 An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.10 above.

4.1 A pharmaceutical combination, e.g. composition, for use in any method as defined under 1.1 to 1.10 above comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination, e.g. composition, use as indicated under 1.1 to 1.10 comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent, e. g. as defined hereinafter.

6. Any method as defined above comprising co-administration, e. g. concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent, e. g. as indicated hereinafter.

By the term"chemotherapeutic agent" is meant especially any chemotherapeutic agent other than an mTOR inhibitor.

Such chemotherapeutic agents include e.g. those which are listed as chemotherapeutic agents in WO02066019 and include agents which are active in the treatment of carcinoid cancer, such as

- somastatin, e.g. octreotide, and a somatostatin analogue, e.g. including such as disclosed and referred to in WO9747317, preferably octreotide, e.g. sold under the trade name Sandostatin® or Sandostatin LAR®, laureotide (BIM23014), vapreotide (RC-160), e.g. sold under the trade name Sansar® or Dorised®, lanreotide, e.g. sold under the trade name Somatuline LAâ® or Somatuline Autogelâ®, SOM230;
- interferons, e.g. interferon alpha, e.g. sold under the trade name Roferon®, Intron A®,
- filgrastim or pegfilgrastim, e.g. sold under the trade name Neupogen® or Neulasta®,

- growth Hormone–Receptor Antagonists, such as pegvisomant (a pegylated form of mutant growth hormone),
- receptor tyrosine kinase inhibitors, such as SU011248 (receptor tyrosine kinase inhibitor that has a spectrum of activity that includes not only PDGFR and C-kit, but also the VEGF receptor),
- topoisomerase II inhibitors, e.g.including, anthracyclines such as doxorubicin (Adriamycin®, including liposomal formulation, e.g. CAELYX®), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS®
- 5-Fluorouracil,
-alkylating agents, such as dacarbazine,
- streptozotocin.

Other chemotherapeutic agents e.g. include agents which may be combined with mTOR inhibitors, e.g. to result in beneficial effects.

Such other chemotherapeutic which may be combined with mTOR inhibitors, e.g. to result in beneficial effects, e.g. include

- calcineurin inhibitors, e.g. cyclosporin A or FK 506;
- ascomycins having immuno-suppressive properties, e.g. ABT-281, ASM981;
- corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide; mizoribine;
- mycophenolic acid or salt; mycophenolate mofetil;
- 15-deoxyspergualine or an immunosuppressive homologue, analogue or derivative thereof;
- bcr-abl tyrosine kinase inhibitors;
- c-kit receptor tyrosine kinase inhibitors;
- PDGF receptor tyrosine kinase inhibitors, e.g. Gleevec (imatinib);
- p38 MAP kinase inhibitors,
- VEGF receptor tyrosine kinase inhibitors,
- PKC inhibitors, e.g. as disclosed in WO 0238561 or WO 0382859, e.g. the compound of Example 56 or 70;
- JAK3 kinase inhibitors, e.g. N-benzyl-3,4-dihydroxy-benzylidene-cyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (Tyrphostin AG 490), prodigiosin 25-C (PNU156804), [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P131), [4-(3'-

bromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] (WHI-P154), [4-(3',5'-dibromo-4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline] WHI-P97, KRX-211, 3-{(3R,4R)-4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl}-3-oxo-propionitrile, in free form or in a pharmaceutically acceptable salt form, e.g. mono-citrate (also called CP-690,550), or a compound as disclosed in WO04052359 or WO05066156;

- S1P receptor agonists or modulators, e.g. FTY720 optionally phosphorylated or an analog thereof, e.g. 2-amino-2-[4-(3-benzyloxyphenylthio)-2-chlorophenyl]ethyl-1,3-propanediol optionally phosphorylated or 1-{4-[1-(4-cyclohexyl-3-trifluoromethyl-benzyloxyimino)-ethyl]-2-ethyl-benzyl}-azetidine-3-carboxylic acid or its pharmaceutically acceptable salts;
- immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., Blys/BAFF receptor, MHC, CD2, CD3, CD4, CD7, CD8, CD25, CD28, CD40, CD45, CD52, CD58, CD80, CD86, IL-12 receptor, IL-17 receptor, IL-23 receptor or their ligands;
- other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (for ex. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y;
- adhesion molecule inhibitors, e.g. LFA-1 antagonists, ICAM-1 or -3 antagonists, VCAM-4 antagonists or VLA-4 antagonists,
- CCR9 antagonists,
- MIF inhibitors,
- 5-aminosalicylate (5-ASA) agents, such as sulfasalazine, Azulfidine®, Asacol®, Dipentum®, Pentasa®, Rowasa®, Canasa®, Colazal®, e.g. drugs containing mesalamine; e.g mesalazine in combination with heparin;
- antibodies which bind to TNF-alpha, such as infliximab (Remicade®),
- nitric oxide releasing non-steriodal anti-inlammatory drugs (NSAIDs), e.g. including COX-inhibiting NO-donating drugs (CINOD);
- phospordiesterase, e.g. PDE4B-inhibitors,
- caspase ihibitors,
- 'multi-functional anti-inflammatory' drugs (MFAIDs), e.g. cytosolic phoshpholipase A2 (cPLA2) inhibitors, such as membrane-anchored phospholipase A2 inhibitors linked to glycosaminoglycans;

- antibiotics, such as penicillins, cephalosporins, erythromycins, tetracyclines, sulfonamides, pleuromutilins, fluoroquinolones, e.g. metronidazole, ciprofloxacin; probiotics and commensal bacteria e.g. Lactobacillus, Lactobacillus reuteri;
- antidiarrheal agents, e.g. including diphenoxylate, loperamide, codeine.

Preferably a chemotherpeutic agent is octreotide, sold under the trade name Sandostatin® or Sandostatin LAR®.

In another aspect the present invention provides a pharmaceutical combination, e.g. composition, comprising as a first agent an mTOR inhibitor and as a second agent 5-Fluorouracil, decarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

A chemotherapeutic agent also include agents which are useful in the treatment of symptoms associated with carciniod tumors, such as carcinoid associated diarrhea (e.g. cyproheptadine), carcinoid associated wheezing (e.g. bronchodilators), carcinoid associated heart failure (e.g. diuretics, serotonine inhibitors).

Pharmaceutical combinations include fixed combinations, in which two or more pharmaceutically active agents are in the same formulation; kits, in which two or more pharmaceutically active agents in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the pharmaceutically active agents are packaged separately, but instruction for simultaneous or sequential administration are given.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference, e.g. comprised are likewise the pharmaceutical acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e. g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention may be prepared and administered

as described in the cited documents or in the product description, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i. e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the mTOR inhibitors in treating endocrine tumors as hereinabove specified, may be demonstrated in vitro, in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. In Vitro

A. 1 Antiproliferative activity in combination with other agents

A cell line, e. g. the Compound A resistant A549 line(IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 andHCT116 lines (IC_{50} in the, micromolar range), is added to 96-well plates (1,500 cells/well in100 ul medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (an mTOR inhibitor other than Compound A or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells.

The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where:CI ~ 1 = the interaction is nearly additive; 0.85-0.9 = slight synergism; < 0.85 = synergy. In this assay, mTOR inhibitors, e.g. the compound A, show interesting antiproliferative activity in combination with another chemotherapeutic agent, e.g. such as defined above, e.g. in combination with somastatin or a somastatin analogue.

Furthermore, in this assay Compound A may potentiate the loss of A549 cell viability and cell death when it is used in combination with a second drug, such as octreotide.

B. In vitro assay

The phosphorylation status of downstream markers S6 (the inhibition of S6K1 activity) is used as a read out, reflecting the immediate pharmacodynamic effect of the mTOR inhibitor, e.g. in the p70S6 kinase 1 (S6K1) assay, see e.g. WO2005064343.
Carcionoid efficacy is determined by measurment of chromogranin A which is inter alia hypersecreted in carcionoid cells, see e.g. Davis et al, Gynecology & Obstetrics 1973;137:637-644.

C. Clinical Trial
27 patients (16 carcinoid, 11 islet cells) are treted with Sandostatin LAR® 30mg, and compound A, 5 mg, daily . Response evaluation is performed every 12 weeks. Study duration: 6 months.

In that study practically total inhibition of S6K1 activity and a reduction of more of 50% of chromogranin A is observed.
Also synergistic effects of the combination are obtained.

Further clinical studies using Compound A in an amount of 5 mg or 10 mg daily (5 to 70 mg weekly) in monotherapy, and in combination therapy together with 30 mg of Sandostatin LAR® daily are investigated.

Patent claims

1. A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

2. A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

3 A method for inducing endocrine tumor regression, e. g. tumor mass reduction, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

4. A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to ia subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

5. A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

6. A method for the treatment of a disease associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

7. A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

8. A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent against endocrine, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

9. A method of any one of claims 1 to 8, wherein an mTRO inihibor is rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin, 16-pent-2- ynyloxy-32 (S orR)-

dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40- [3-hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin, 40-epi-(tetrazolyl)- rapamycin, a rapalog, or a compound disclosed under the name TAFA-93 or biolimus.

10. A method of claim 9 wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R) -dihydro-rapamycin or 16-pent-2- ynyloxy-32 (S orR)-dihydro-40-0- (2-hydroxyethyl)-rapamycin.

11. A method of any one of claims 9 or 10, wherein an mTRO inihibor is 40-O-(2-hydroxyethyl)-rapamycin.

12. A method of any one of claims 1 to 11, wherein the mTOR inhibitor is administered intermittently.

13. A method of any one of claims 1 to 12 for treating neuroendocrine tumors.

14. A method of any one of claims 1 to 12 for treating carcinoid tumors.

15. An mTOR inhibitor for use in a method of any one of claims 1 to 12.

16. An mTOR inhibtor for use in the preparation of a pharmaceutical composition for use in a method of any one of claims 1 to 15.

17. A pharmaceutical combination for use in a method of any one of claims 1 to 15,. comprising an mTRO inhibitor together with one or more pharmaceutically acceptable diluents or carriers therefor.

18. A pharmaceutical combination for use in a method of any one of claims 1 to 15, comprising

a) a first agent which is an mTOR inhibitor and

b) a second drug substance as a co-agent which is a chemotherapeutic agent.

19. A method of any one of claims 1 to 15. comprising co-administration, concomitantly or in sequence, of a therapeutically effective amount an mTOR inhibitor and a second drug substance, said second drug substance being a chemotherapeutic agent.

20. A method of claim 19, wherein the mTOR inhibitor is 40-O-(hydroxyethyl)rapamycin and the second drug is somatostatin or a somatostatin analog.

21. A pharmaceutical combination, comprising as a first agent an mTOR inhibitor and as a second agent 5-fluorouracil, dacarbazine, streptozotocin , a receptor tyrosine kinase inhibitor that has a spectrum of activity that includes PDGFR, C-kit, and the VEGF receptor, e.g. SU011248, growth Hormone–Receptor Antagonists, such as pegvisomant, filgrastim or pegfilgrastim, or interferon alpha.

SC/10-Feb-06

Abstract

A method for treating endocrine tumors by adminstration of an mTOR inhibitor, optionally in combination with another drug.

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
12/094,173	05/19/2008	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	05/19/2008	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 12	Minus	** 20	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	* 7	Minus	***7	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1)		(Column 2)	(Column 3)					
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KATRINA S. TURNER/

DO/ EO WORKSHEET

Virginia Irby, Patent Application Specialist/ National Stage Division

U.S. Appl. No. 12 / 094173 International Appl. No. PCT/EP06/68656

Application filed by : ☐ 20 months ☑ 30 months

WIPO PUBLICATION INFORMATION :

Publication No.: WO2007/57457 Publication Language : ☑ English (IA used as specification) ☐ German ☐ Japanese ☐ Chinese ☐ Korean ☐ French ☐ Spanish ☐ Russian ☐ Other : ____

Publication Date : 24 May 2007 Not Published : ☐ U.S. only designated ☐ EP Request Published : ☐ EP Request

INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE :

- ☑ International Application *(RECORD COPY)*
- ☐ Article 19 Amendments
- ☐ PCT/IPEA/409 IPER (check Examination Authority) : ☐ EP ☐ JP ☐ SE ☐ AU ☐ US ☐ FR ☐ CN ☐ ES ☐ RU ☐ AT ☐ KR ☐ ___
- ☐ Annexes to 409
- ☐ PCT/ISA/237 : ☐ EP ☐ JP ☐ SE ☐ AU ☐ US ☐ FR ☐ CN ☐ ES ☐ RU ☐ AT ☐ KR ☐ ___
- ☐ Other : _____
- ☐ PCT/IB/306
- ☐ Request form PCT/RO/101
- ☑ PCT/ISA/210 - Search Report (check Searching Authority) : ☑ EP ☐ JP ☐ SE ☐ AU ☐ US ☐ FR ☐ CN ☐ ES ☐ RU ☐ AT ☐ KR ☐ ___ ☐ NONE
- ☐ Search Report References
- ☑ Priority Document (s) No. 7
  - ☐ N/A
  - ☐ Priority Document was NOT AVAILABLE at the time of paralegal review

RECEIPTS FROM THE APPLICANT *(filed with the application unless noted otherwise)* :

- ☑ Basic National Fee *(or authorization to charge)*
- ☑ Description 37 ☑ Claims 2 ☑ Abstract
- ☐ Drawing Figure(s) - (# of drwgs. 0)
- ☐ Translation of Article 19 Amendments ☐ entered ☐ not entered : ☐ not a page for page substitution ☐ replaced by Article 34 Amendment
- ☐ Annexes to 409 ☐ entered ☐ not entered : ☐ not a page for page substitution ☐ no translation ☐ other : _____
- ☐ Application Data Sheet
- ☐ Power of Attorney
- ☐ Change of Address
- ☐ Express Request to Begin Nat'l Examination Procedures
- ☑ Preliminary Amendment(s) Filed on : 1. ☑ same as 371 request date 2. _____ 3. _____
- ☑ Information Disclosure Statement(s) Filed on : 1. ☑ same as 371 request date 2. _____ 3. _____
- ☐ Assignee Statement Under 37 CFR 3.73(b)
- ☐ Assignee PG Publication Notice
- ☐ Substitute Specification Filed on : 1. ☐ same as 371 request date 2. _____ 3. _____
- ☐ Verified Small Status Statement
- ☑ Oath/ Declaration (executed)
- ☐ Oath/ Declaration ☐ unsigned ☐ no citizenship ☐ other
- ☐ DNA Diskette ☐ Sequence Listing
- ☐ Other : _____

NOTES :

35 U.S.C. 371 - Receipt of Request (PTO-1390)	19 May 2008
Date Acceptable Oath/ Declaration Received	19 May 2008
Date of Completion of requirements under 35 U.S.C. 371	21 May 2008
Date of Completion of DO/ EO 903 - Notification of Acceptance	08 July 2008
Date of Completion of DO/ EO 905 - Notification of Missing Requirements	
Date of Completion of DO/ EO 909 - Notification of Abandonment	
Date of Completion of DO/ EO 916 - Notification of Defective Response	
Date of Completion of DO/ EO 922 - Notification to Comply w/ Requirements for Patent Applications Containing Nucleotide and/or Amino Acid Sequence Disclosures	
Date of Completion of DO/ EO 923	

PATENT APPLICATION FEE DETERMINATION RECORD

Effective September 30, 2007

Application or Docket Number: 12/094,173

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)	SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
			RATE	FEE		RATE	FEE
U.S. NATIONAL STAGE FEES							
BASIC FEE	SMALL ENT. = $ 155	LARGE ENT. = $ 310	BASIC FEE		OR	BASIC FEE	310
EXAMINATION FEE	Satisfies PCT Article 33(1)-(4) = $ 50 / $ 100	All other situations = $ 105 / $ 210	EXAM. FEE			EXAM. FEE	210
SEARCH FEE	U.S. is ISA = $ 50 / $ 100 ALL other countries = $ 205 / $ 410	ALL other situations = $ 255 / $ 510	SEARCH FEE			SEARCH FEE	410
FEE FOR EXTRA SPEC. PGS.	minus 100 =	/ 50 =	X $ 130 =			X $ 260 =	
TOTAL CHARGEABLE CLAIMS	12 minus 20 =	*	X $ 25 =		OR	X $ 50 =	
INDEPENDENT CLAIMS	7 minus 3 =	* 4	X $ 105 =		OR	X $ 210 =	840
MULTIPLE DEPENDENT CLAIM PRESENT ☐			+ $ 185 =		OR	+ $ 370 =	
			TOTAL		OR	TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
AMENDMENT A	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐				

SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
X $ 25 =		OR	X $ 50 =	
X $ 105 =		OR	X $ 210 =	
+ $ 185 =		OR	+ $ 370 =	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
AMENDMENT B	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐				

SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
X $ 25 =		OR	X $ 50 =	
X $ 105 =		OR	X $ 210 =	
+ $ 185 =		OR	+ $ 370 =	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '20', enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '3', enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

(FOR USE WITH FORM PTO-875)

SERIAL NO. 12/094173

FILING DATE

APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1			1			
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TOTAL IND.		⬇	7	⬇		⬇
TOTAL DEP.		⬅	5	⬅		⬅
TOTAL CLAIMS			12			

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
51						
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TOTAL IND.		⬇		⬇		⬇
TOTAL DEP.		⬅		⬅		⬅
TOTAL CLAIMS						

PTO - 1360 (REV. 11/04)

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Patent and Trademark Office

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/094,173	05/19/2008		1770	34678-US-PCT	12	7

CONFIRMATION NO. 9572

1095
NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 104/3
EAST HANOVER, NJ 07936-1080

FILING RECEIPT

OC000000030860999

Date Mailed: 07/10/2008

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Peter Wayne Marks, Woodbridge, CT;
David Lebwohl, Madison, NJ;

Assignment For Published Patent Application

NOVARTIS AG, Basel, SWITZERLAND

Power of Attorney: The patent practitioners associated with Customer Number 1095

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/EP2006/068656 11/20/2006

Foreign Applications

UNITED KINGDOM 0523658.3 11/21/2005
UNITED KINGDOM 0601082.1 01/19/2006
UNITED KINGDOM 0602747.8 02/10/2006
UNITED KINGDOM 0607942.0 04/21/2006
UNITED KINGDOM 0609272.0 05/10/2006
UNITED KINGDOM 0609912.1 05/18/2006
UNITED KINGDOM 06120660.3 09/14/2006

If Required, Foreign Filing License Granted: 07/08/2008

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/094,173**

Projected Publication Date: 10/16/2008

Non-Publication Request: No

Early Publication Request: No

Title

Neuroendocrine Tumor Treatment

Preliminary Class

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

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For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/094,173	Peter Wayne Marks	34678-US-PCT

1095
NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 104/3
EAST HANOVER, NJ 07936-1080

INTERNATIONAL APPLICATION NO.	
PCT/EP2006/068656	
I.A. FILING DATE	PRIORITY DATE
11/20/2006	11/21/2005

CONFIRMATION NO. 9572
371 ACCEPTANCE LETTER

OC000000030861000

Date Mailed: 07/10/2008

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as a Designated / Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is ACCEPTED for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above and the relevant dates are:

05/19/2008	05/21/2008
DATE OF RECEIPT OF 35 U.S.C. 371(c)(1), (c)(2) and (c)(4) REQUIREMENTS	DATE OF COMPLETION OF ALL 35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE " FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1), (c)(2) and (c)(4) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** *The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363).* Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

The following items have been received:

- Copy of the International Application filed on 05/19/2008
- Copy of the International Search Report filed on 05/19/2008
- Preliminary Amendments filed on 05/19/2008
- Information Disclosure Statements filed on 05/19/2008
- Oath or Declaration filed on 05/19/2008
- U.S. Basic National Fees filed on 05/19/2008
- Priority Documents filed on 05/19/2008

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

VIRGINIA L IRBY

Telephone: (703) 308-9140 EXT 229

FORM PCT/DO/EO/903 (371 Acceptance Notice)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/094,173	05/19/2008	Peter Wayne Marks	34678-US-PCT

CONFIRMATION NO. 9572

1095
NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 104/3
EAST HANOVER, NJ 07936-1080

PUBLICATION NOTICE

CC00000003262451 2

Title: Neuroendocrine Tumor Treatment

Publication No.US-2008-0255029-A1
Publication Date:10/16/2008

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

[CANCER RESEARCH 64, 252–261, January 1, 2004]

Antitumor Efficacy of Intermittent Treatment Schedules with the Rapamycin Derivative RAD001 Correlates with Prolonged Inactivation of Ribosomal Protein S6 Kinase 1 in Peripheral Blood Mononuclear Cells

Anne Boulay,[1] Sabine Zumstein-Mecker,[1] Christine Stephan,[1] Iwan Beuvink,[2] Frederic Zilbermann,[2] Roland Haller,[1] Sonja Tobler,[1] Christoph Heusser,[1] Terence O'Reilly,[1] Barbara Stolz,[1] Andreas Marti,[1] George Thomas,[2] and Heidi A. Lane[1]

[1]*Novartis Institutes for BioMedical Research Basel, Novartis Pharma AG, Basel, Switzerland, and* [2]*Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland*

ABSTRACT

The orally bioavailable rapamycin derivative RAD001 (everolimus) targets the mammalian target of rapamycin pathway and possesses potent immunosuppressive and anticancer activities. Here, the antitumor activity of RAD001 was evaluated in the CA20948 syngeneic rat pancreatic tumor model. RAD001 demonstrated dose-dependent antitumor activity with daily and weekly administration schedules; statistically significant antitumor effects were observed with 2.5 and 0.5 mg/kg RAD001 administered daily [treated tumor *versus* control tumor size (T/C), 23% and 23–30%, respectively], with 3–5 mg/kg RAD001 administered once weekly (T/C, 14–36%), or with 5 mg/kg RAD001 administered twice weekly (T/C, 36%). These schedules were well tolerated and exhibited antitumor potency similar to that of the cytotoxic agent 5-fluorouracil (T/C, 23%). Moreover, the efficacy of intermittent treatment schedules suggests a therapeutic window allowing differentiation of antitumor activity from the immunosuppressive properties of this agent. Detailed biochemical profiling of mammalian target of rapamycin signaling in tumors, skin, and peripheral blood mononuclear cells (PBMCs), after a single administration of 5 mg/kg RAD001, indicated that RAD001 treatment blocked phosphorylation of the translational repressor eukaryotic initiation factor 4E-binding protein 1 and inactivated the translational activator ribosomal protein S6 kinase 1 (S6K1). The efficacy of intermittent treatment schedules was associated with prolonged inactivation of S6K1 in tumors and surrogate tissues (≥72 h). Furthermore, detailed analysis of the dose dependency of weekly treatment schedules demonstrated a correlation between antitumor efficacy and prolonged effects (≥7 days) on PBMC-derived S6K1 activity. Analysis of human PBMCs revealed that S6K1 also underwent a concentration-dependent inactivation after RAD001 treatment *ex vivo* (>95% inactivation with 20 nM RAD001). In contrast, human PBMC-derived eukaryotic initiation factor 4E-binding protein 1 was present predominantly in the hypophosphorylated form and was unaffected by RAD001 treatment. Taken together, these results demonstrate a correlation between the antitumor efficacy of intermittent RAD001 treatment schedules and prolonged S6K1 inactivation in PBMCs and suggest that long-term monitoring of PBMC-derived S6K1 activity levels could be used for assessing RAD001 treatment schedules in cancer patients.

INTRODUCTION

RAD001 (everolimus), an orally bioavailable derivative of rapamycin, is a macrolide antifungal antibiotic that demonstrates potent antiproliferative effects against a variety of mammalian cell types. Specifically, RAD001 inhibits cytokine-driven lymphocyte proliferation (1), as well as the proliferation of human tumor-derived cells grown either in culture or as tumors in animal models (2, 3). As a result of these properties, RAD001 is being clinically developed both as an immunosuppressant for prevention of allograft rejection (Certican; Ref. 1) and as a novel therapeutic in the fight against human cancer (2–4).

RAD001, like rapamycin, binds with high affinity to a ubiquitous intracellular receptor, the immunophilin FKBP12. This complex specifically interacts with the mammalian target of rapamycin (mTOR) protein kinase; inhibiting downstream signaling events (5). The mTOR kinase is a member of the phosphoinositide kinase-related kinase family, which consists of high molecular weight serine/threonine kinases involved in cell cycle checkpoint control (6). Several lines of evidence suggest that mTOR acts as a sensor for stress (7) and the availability of amino acids (8–10) or intracellular ATP (11). In the presence of mitogens and sufficient nutrients, mTOR relays a signal to translational regulators, specifically enhancing the translation of mRNAs encoding proteins essential for cell growth (12) and progression through the G_1 to S transition (13, 14). Consistent with targeting the mTOR pathway, treatment of mammalian cells with rapamycin has been shown to inhibit these signaling events, mimicking a starvation phenotype (15) and leading to growth retardation and accumulation of cells in G_1 phase (16). The mechanism of growth stimulus and nutrient level integration by mTOR is, as yet, not fully understood. However, an increasing body of evidence suggests the involvement of the phosphatidylinositol 3′-kinase/Akt/TSC/Rheb pathway (12, 17–23). Indeed, it has been suggested that, in tumor cells, the activation status of the Akt pathway may be indicative of responsiveness to rapamycin or its derivatives (24–27).

mTOR is part of a multisubunit complex that contains the regulatory proteins raptor (28, 29) and GβL (30). The mTOR complex signals to at least two downstream effectors, the translational repressor protein eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). These share an evolutionary conserved amino acid motif, the TOS motif, that functions as a docking site for raptor (31–33). Binding of 4E-BP1 to the translational activator eIF-4E is modulated by mTOR-dependent phosphorylation of specific serine and threonine residues (5). Ser37 and Ser46 are constitutively phosphorylated, acting as priming sites for the mitogen-induced, rapamycin-sensitive phosphorylation of Thr70 and Ser65 (34). After a final phosphorylation event at Ser65, 4E-BP1 dissociates from eIF-4E (35), thereby allowing the reconstitution of a translationally competent initiation factor complex (eIF-4F; Ref. 5). eIF-4F activation results in the translation of a subset of capped mRNA containing highly structured 5′-untranslated regions and encoding proteins involved in G_1- to S-phase progression (13, 14). Mitogen-induced activation of the S6K1 is also dependent on mTOR function and has been implicated in the translational regulation of mRNAs possessing a 5′-terminal oligopyrimidine tract (36–38). 5′-Terminal oligopyrimidine tract mRNAs are characterized by a stretch of 4–14 pyrimidines located at their extreme 5′ terminus and typically encode ribosomal proteins as well as components of the

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translational machinery. Activation of S6K1 itself is also tightly regulated by hierarchical phosphorylation events, which are dependent on the activation of various signal transduction pathways and culminate in the phosphorylation of the rapamycin-sensitive site Thr389, an event closely paralleling kinase activation (12, 39). Immunopurified mTOR has been shown to autophosphorylate on Ser2481 (40) and to phosphorylate Ser37, Ser46, and Ser65 on 4E-BP1 *in vitro* (11, 34, 41, 42). However, some of these events have been demonstrated to be resistant to antiproliferative concentrations of rapamycin (40–42). It is therefore unclear what role mTOR kinase activity plays *per se* in rapamycin-sensitive signaling events.

Because mTOR couples nutrient/growth factor availability to cell growth and proliferation in a variety of cell types, there is a potential for developing rapamycin derivatives such as RAD001 as novel inhibitors of the deregulated cell growth characteristic of human cancers. Consistent with this, RAD001 inhibits the proliferation of a wide variety of human solid tumor cell lines both *in vitro* in cell culture and *in vivo* in animal xenograft models (2, 3, 27, 43, 44). Furthermore, antiproliferative effects of RAD001 in posttransplant lymphoproliferative disorder-like B cell lines have been observed *in vitro* and *in vivo* (45, 46). In the present study, we have demonstrated that RAD001 displays significant antitumor activity in the syngeneic CA20948 rat pancreatic tumor model. Equivalent activity was observed with daily and intermittent treatment schedules, suggesting the possibility of a therapeutic window allowing differentiation of antitumor activity from the immunosuppressive properties of this agent. Detailed biochemical analysis of the mTOR effectors 4E-BP1 and S6K1 in tumor, skin, and peripheral blood mononuclear cell (PBMC) extracts obtained from RAD001-treated rats suggests that modulation of 4E-BP1 activity and significant inactivation of S6K1 are associated with antitumor activity. Furthermore, the efficacy observed using intermittent treatment schedules is paralleled by long-term down-regulation of S6K1 activity in all three tissues. We also provide evidence that the duration of S6K1 inactivation in PBMCs correlates with the dose-dependent suppression of tumor growth observed with weekly regimens. Moreover, unlike 4E-BP1 phosphorylation, S6K1 activity can be reproducibly measured in human PBMCs and represents a potentially valuable pharmacodynamic biomarker by which to monitor RAD001 treatment schedules in cancer patients.

MATERIALS AND METHODS

Drug Preparation. RAD001 (everolimus) is a derivative of rapamycin [40-*O*-(2-hydroxyethyl)-rapamycin; Ref. 47]. For animal studies, RAD001 was formulated at 2% (w/v) in a microemulsion vehicle, which was diluted to the appropriate concentration in 5% (w/v) glucose solution just before administration by gavage. For *in vitro* and *ex vivo* analyses, RAD001 was prepared in DMSO before addition to cell culture or human volunteer blood samples.

Antitumor Efficacy Studies and Statistical Analyses. Male Lewis rats were purchased from Iffa Credo (L'Abresque, France) and allowed food and water *ad libitum*. A suspension of CA20948 tumor cells (obtained from donor rats because this line is nonculturable *in vitro*) in Ham's F-12 medium supplemented with 10% FCS, 0.1 g/100 ml $NaHCO_3$, 1% penicillin, and 1% fungizone was injected s.c. into the left flank of rats. Treatment of randomized rats started when the tumors reached about 100 mm^3. RAD001 was administered p.o. daily at 0.5 or 2.5 mg/kg (×6/week), twice weekly at 5 mg/kg, or weekly at 0.5, 1, 2, 3, or 5 mg/kg. A volume of vehicle equivalent to the highest dose of RAD001 administered in the experiment was used as a negative control. As a positive control, the cytotoxic agent 5-fluorouracil (5-FU; ICN Pharmaceuticals Inc., Costa Mesa, CA) was administered at a near maximum tolerated dose (15 mg/kg, i.v., 4×/week, 2 days treatment/2 days rest), which gives maximal antitumor effect. Tumors were measured every day or every other day with a caliper, and the volumes were calculated by using the formula of an ellipsoid [$V = \pi/6\ (d_1 \times d_2 \times d_3)$, where d_1, d_2, and d_3 represent the three largest diameters]. Animals were also weighed the same day tumors were measured. The animals were sacrificed when either their tumor burden exceeded 25,000 mm^3 or when skin overlaying the tumor exhibited evidence of necrosis. All protocols involving animals were approved by the Veterinäramt of Baselstadt, Switzerland.

Results are presented as mean ± 1 SEM or as percentage of T/C (mean increase of tumor volumes of treated animals divided by the mean increase of tumor volumes of control animals multiplied by 100). The statistical significance of differences between treatment and control groups were determined by ANOVA followed by the Dunnett test. Statistical analyses on body weight were performed by ANOVA followed by Tukey's test, and for comparison between weight at start and end of the experiment for individual animals, the paired *t* test was used. The level of significance was set at $P < 0.05$. Statistical calculations were performed using SigmaStat 2.03 (Jandel Scientific).

Rat-Derived and Human Volunteer-Derived Tissue/PBMC Protein Extract Preparation. CA20948 tumor-bearing rats were given 0.5, 1, 2, or 5 mg/kg RAD001 or an equivalent volume of vehicle. At the indicated times after administration, rats were sacrificed, and tumor and shaved skin samples (for 0.5 and 5 mg/kg RAD001 doses) were dissected and weighed. Samples were rinsed in ice-cold PBS and immediately extracted in ice-cold extraction buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM PP_i, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% NP40] with a constant ratio of 45 mg tumor/ml extraction buffer and 90 mg skin/ml extraction buffer, using a PT3000 Polytron (probe PT-DA 3012/2S; Kinematica AG) or a hand-held PT2100 Polytron (probe PT-DA 2112/2EC), respectively. Lysates were cleared by centrifugation for 30 min at 12,000 × *g* at 4°C. Supernatants were subsequently aliquoted, snap frozen on dry ice, and stored at −80°C. In the case of skin samples, before further analysis, samples were centrifuged for 20 min at 436,000 × *g* at 4°C to remove the fat fraction.

Blood (for 0.5, 1, 2, and 5 mg/kg RAD001 doses) from tumor-bearing and non-tumor-bearing rats was withdrawn into syringes containing EDTA [0.5% (w/v) final] and then placed into an ice-cold tube and mixed. Unless otherwise stated, the blood from individual animals within the same treatment group was analyzed separately. The blood was immediately centrifuged for 20 min at 430 × *g* at 4°C. The PBMCs, deposited at the interface between the RBCs and the plasma, were collected and pelleted by centrifugation for 5 min at 3000 × *g* at 4°C. PBMCs were washed with 10 ml of ice-cold PBS and then repelleted by centrifugation for 5 min at 3000 × *g* at 4°C. Cell pellets were resuspended in ice-cold extraction buffer containing 1% NP40 at the fixed ratio of 500 μl extraction buffer/10 ml initial blood volume. The cells were sheared by vigorous pipetting and then centrifuged for 30 min at 12,000 × *g* at 4°C. Supernatants were aliquoted, snap frozen on dry ice, and stored at −80°C.

Human blood from healthy volunteers was collected under medical supervision into tubes containing either sodium-citrate (BD Vacutainer 9NC; BD Vacutainer Systems, Plymouth, United Kingdom) or EDTA (BD Vacutainer K3E) as an anticoagulant. The blood was either immediately processed or, for *ex vivo* treatments, treated with 2, 20, and 200 nM RAD001 or DMSO vehicle for 30 min at room temperature. Human PBMCs were isolated and extracted as described for rat PBMCs.

A549 Cell Culture and Protein Extract Preparation. A549 human lung carcinoma cells (CCL185) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Amimed, Allschwil, Switzerland) supplemented with 10% FCS, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin at 37°C and 5% CO_2. Cell lysates were prepared as described previously (48).

Immunoblot Analysis. Cell lysates (30–40 μg) were electrophoretically resolved on denaturing SDS polyacrylamide gels (SDS-PAGE), transferred to polyvinylidene difluoride (Millipore Corp., Bedford, MA), and probed with the following primary antibodies: anti-S6 (provided by J. Mestan; Oncology Research, Novartis Pharma AG, Basel, Switzerland); anti-4E-BP1 (kindly provided by N. Sonenberg; McGill University, Montreal, Quebec, Canada); anti-eIF-4E (kindly provided by S. J. Morley; University of Sussex, Brighton, United Kingdom); anti-phospho-4E-BP1 Thr70, anti-S6K1, and anti-phospho-S6 Ser240/Ser244 (all from Cell Signaling Technology Inc., Beverly, MA); and anti-β-tubulin (Tub2.1; Sigma, St. Louis, MO). "Decorated" proteins were revealed using horseradish peroxidase-conjugated antimouse or antirabbit immunoglobulins in conjunction with the enhanced chemiluminescence procedure (Amersham Pharmacia Biotech Inc., Buckinghamshire, United Kingdom).

Affinity Purification of 4E-BP1-eIF-4E Complexes with 7-Methyl-GTP-Sepharose. Rat tumor (1 mg), skin (0.7 mg), or PBMC (0.25 mg) extracts were diluted to a final volume of 500 μl in ice-cold extraction buffer and adjusted to a final NP40 concentration of 0.1%. The 4E-BP1-eIF-4E complexes were affinity purified with 20 μl of 7-methyl-GTP-Sepharose beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) by gentle rotation for 2.5 h at 4°C. Proteins retained on the beads were washed twice with extraction buffer in the absence of NP40 and resuspended in 15 μl of Laemmli buffer. Denatured samples were subjected to 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were first immunoblotted for 4E-BP1 protein, followed by stripping as described previously (49) and reprobing for eIF-4E protein (see above).

40S Ribosomal S6 Kinase Assay. Rat tumor (1 mg), skin (0.7 mg), or PBMC (0.25 mg) extracts were diluted to a final volume of 1 ml (tumor and skin) or 500 μl (PBMC) with ice-cold extraction buffer and adjusted to a final NP40 concentration of 1%. Human-derived PBMC extracts (0.8–1 mg) were diluted to a final volume of 750 μl with ice-cold extraction buffer (final NP40 concentration, 1%). In some experiments, human-derived PBMC extracts were first precleared with 20 μl of 50% protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) by rotating for 20 min at 4°C. S6K1 was immunoprecipitated from all extracts by addition of 2.5 μl of the M5 S6K1-specific polyclonal antibody and incubation on ice for 1 h, followed by retrieval of immunocomplexes with 20 μl of 50% protein A-Sepharose. S6K1 activity was measured using rat liver 40S ribosomal subunits as a specific substrate, as described previously (50), except that *p*-nitrophenyl phosphate was omitted in the reaction mixture. Phosphorylated S6 was resolved by 12.5% SDS-PAGE and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). [γ-^{32}P]phosphate incorporation into S6 was quantified using ImageQuant (Molecular Dynamics). Where appropriate, the statistical significance of differences between treatment groups and untreated control groups was determined using ANOVA or ANOVA on ranks followed by the Dunnett test. The level of significance was set at $P < 0.05$. Statistical calculations were performed using SigmaStat 2.03 (Jandel Scientific). Coefficient of variation is defined as SD divided by the mean and multiplied by 100.

RESULTS

Intermittent RAD001 Treatment Schedules Display Antitumor Efficacy. Short-term exposure to rapamycin *in vitro* has long-term antiproliferative effects on tumor cell lines (51), suggesting that intermittent treatment schedules may retain antitumor activity. Furthermore, daily oral administration of RAD001 is effective in rat models of autoimmune disease and allotransplantation (47, 52), whereas we have found that weekly (5 mg/kg) RAD001 dosing schedules have reduced immunosuppressive properties in rats as compared with daily treatment (2.5 mg/kg): 66 ± 18% and 98 ± 1% inhibition of IgG antibody response after dinitrophenol-coupled keyhole limpet hemocyanogen immunization, respectively.[3] With these observations in mind, we evaluated whether RAD001 treatment schedules, with potentially reduced immunosuppressive properties, could elicit antitumor responses. Daily *versus* intermittent RAD001 administration schedules were compared using the s.c. CA20948 rat pancreatic tumor model. Vehicle was used as a negative control, and the cytotoxic agent 5-FU was used as a positive control (Fig. 1; Table 1, Experiment 1). RAD001 treatment at 0.5 or 2.5 mg/kg/day, six times a week, resulted in antitumor activity characterized by statistically significant inhibition of tumor growth as compared with vehicle controls [treated tumor *versus* control tumor size (T/C), 30% and 23%, respectively; $P < 0.05$ after 10 days of treatment; Fig. 1*A*; Table 1, Experiment 1]. Statistically significant tumor growth suppression was also observed after intermittent administration of 5 mg/kg RAD001 twice a week (T/C, 36%) or once a week (T/C, 36%). Moreover, all RAD001 treatment schedules suppressed tumor growth to a similar extent as the cytotoxic 5-FU (T/C, 23%). Continued treatment with RAD001 after vehicle controls were sacrificed due to tumor burden led to a prolonged low tumor growth rate with all treatment schedules, resulting in similar tumor burden after 17 days of treatment as compared with 5-FU (Fig. 1*A*). For all treatment schedules, RAD001 was well tolerated, with no significant body weight loss or mortalities observed (Fig. 1*B*; Table 1, Experiment 1). These results demonstrate that RAD001 is a well-tolerated antitumor agent in a rat model of pancreatic cancer and indicate a potential for intermittent administration schedules that may allow dissociation of antitumor from immunosuppressive effects.

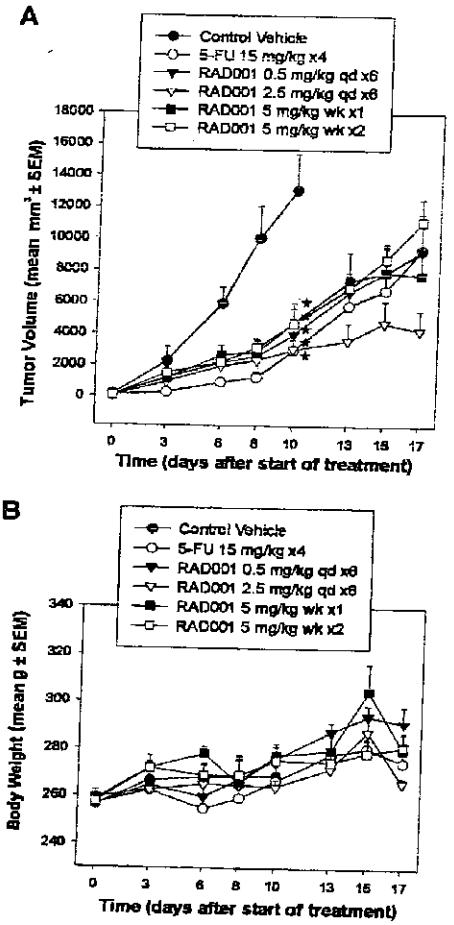

Fig. 1. Suppression of tumor growth by daily and intermittent dosing schedules of RAD001. Tumors were established in male Lewis rats by s.c. injection of CA20948 tumor suspension obtained from donor rats. Treatments started on day 4 after inoculation. Formulated RAD001 was diluted in a 5% glucose solution and administered p.o. daily at a dose of 0.5 or 2.5 mg/kg (*qd* ×6, 6 times/week) or once (*wk* ×1) or twice (*wk* ×2) weekly at 5 mg/kg RAD001. Vehicle and 5-fluorouracil (*5-FU* ×4; 4 times/week) were administered as negative and positive controls, respectively. Tumor volumes were measured (*A*), and rats were weighed (*B*) as described in "Materials and Methods." Vehicle control-treated rats were sacrificed on day 10 due to tumor burden. Data are means ± SEM (n = 7–8 animals/group). *Stars* represent $P < 0.05$ *versus* vehicle controls.

RAD001 Modulates 4E-BP1 and S6K1 Activity in Tumor, Skin, and PBMCs Obtained from CA20948 Pancreatic Tumor-Bearing Rats. To investigate RAD001-specific effects on mTOR signaling *in vivo*, three CA20948 tumor-bearing rats were treated with vehicle or a single efficacious dose of RAD001 (5 mg/kg). Rats were sacrificed

[3] T. O'Reilly, H. A. Lane, and C. Heusser, unpublished data.

Table 1 *Effect of daily and intermittent RAD001 administration on CA20948 rat pancreatic tumor-bearing rats*

Compound	Schedule	Tumor response		Host response		
		% T/C[a]	Δ Tumor volume (mm^3)	Δ Body weight (g)	%Δ Body weight	Survival (alive/total)
Experiment 1						
Vehicle	2 ml/kg p.o. daily	100	12972 ± 2188	12 ± 8	5	8/8
5-FU	15 mg/kg i.v. 4× weekly	23	2863 ± 764[b]	18 ± 4	7	7/7
RAD001	0.5 mg/kg p.o. daily	30	3904 ± 856[b]	35 ± 7	14	7/7
RAD001	2.5 mg/kg p.o. daily	23	2959 ± 624[b]	7 ± 2	3	7/7
RAD001	5 mg/kg p.o. weekly	36	4652 ± 1220[b]	22 ± 5	8	7/7
RAD001	5 mg/kg p.o. twice weekly	36	4604 ± 928[b]	21 ± 3	8	7/7
Experiment 2						
Vehicle	2 ml/kg p.o. daily	100	12331 ± 1410	29 ± 2	14	8/8
RAD001	0.5 mg/kg p.o. daily	23	2894 ± 567[b]	30 ± 5	17	8/8
RAD001	0.5 mg/kg p.o. weekly	48	5951 ± 1739	36 ± 2	15	8/8
RAD001	5 mg/kg p.o. weekly	14	1708 ± 339[b]	32 ± 2	15	8/8
Experiment 3						
Vehicle	2 ml/kg p.o. weekly	100	19270 ± 3918	28.3 ± 2.1	10	8/8
RAD001	0.5 mg/kg p.o. weekly	48	9275 ± 1926	21 ± 2.4	8	8/8
RAD001	1 mg/kg p.o. weekly	45	8617 ± 1704	32.8 ± 2.7	12	8/8
RAD001	2 mg/kg p.o. weekly	32	6161 ± 1079[b]	24.9 ± 1.9	9	8/8
RAD001	3 mg/kg p.o. weekly	36	6869 ± 611[b]	24.3 ± 3.3	9	6/6
RAD001	5 mg/kg p.o. weekly	24	4680 ± 1595[b]	22.8 ± 1.7	8	8/8

[a] T/C, treated tumor *versus* control tumor size.
[b] $P < 0.05$ *versus* control. Dunnett test.

24 h later, and protein extracts were prepared from tumors, skin, and PBMCs. By immunoblot analysis, mTOR could be detected in tumor and PBMC extracts; however, neither mTOR expression nor phosphorylation on Ser2448 was modified on RAD001 treatment.[4] In contrast, 4E-BP1 exhibited a decrease in Thr70 phosphorylation in tumor, skin, and PBMC extracts (Fig. 2*A*), a phenomenon associated with changes in 4E-BP1 electrophoretic mobility, particularly striking in PBMCs. This observation is consistent with previous work demonstrating dephosphorylation of 4E-BP1 on Thr70 in tumors derived from mouse xenograft models after five daily treatments with an ester of rapamycin CCI-779 (1 h after last administration; Ref. 53). Interestingly, the phosphorylation of another rapamycin-sensitive residue (Ser65; Refs. 5, 34, and 35) was unaffected by RAD001 treatment,[4] indicating that RAD001-insensitive phosphorylation of this site can occur as reported previously (54).

To determine whether the decreased phosphorylation state of 4E-BP1 resulted in a change in functionality, the eIF-4E binding activity of 4E-BP1 was assessed using an *in vitro* 7-methyl-GTP-binding assay (Fig. 2*B*). Whereas similar levels of eIF-4E were recovered in the control- and RAD001-treated extracts, in two animals increased eIF-4E·4E-BP1 complex formation was clearly observed in skin and PBMC samples after RAD001 treatment. In tumor samples, two electrophoretically distinct forms of 4E-BP1 protein were bound to eIF-4E in vehicle control-treated rats (Fig. 2*B*). After RAD001 treatment, only the lower migrating form was found bound to eIF-4E, with an associated loss of the upper band consistent with reduced 4E-BP1 phosphorylation levels (Fig. 2*A*). A similar 4E-BP1 doublet with eIF-4E binding activity has been observed previously in proliferating cells/tissue (29, 54) and presumably reflects differential 4E-BP1 phosphorylation states within the proliferating tumor.

To further assess the effect of RAD001 administration on the mTOR pathway, S6K1 protein and activity levels were also analyzed (Fig. 2, *C* and *D*). Whereas S6K1 protein levels were unaffected by RAD001 treatment (Fig. 2*C*), *in vitro* kinase assay using 40*S* ribosomal subunits as a substrate revealed a statistically significant reduction in S6K1 activity in all extracts [Fig. 2*D*; 83% (tumors), 80% (skin), and 75% (PBMC); all $P < 0.05$ *versus* vehicle-treated controls]. This reduction in S6K1 activity was associated with the dramatic dephosphorylation of its physiological substrate, 40*S* ribosomal protein S6, in tumor extracts (Fig. 2*C*). A similar reduction was not observed in skin and PBMC extracts because these tissues exhibited no detectable S6 phosphorylation in control animals. Interestingly, a

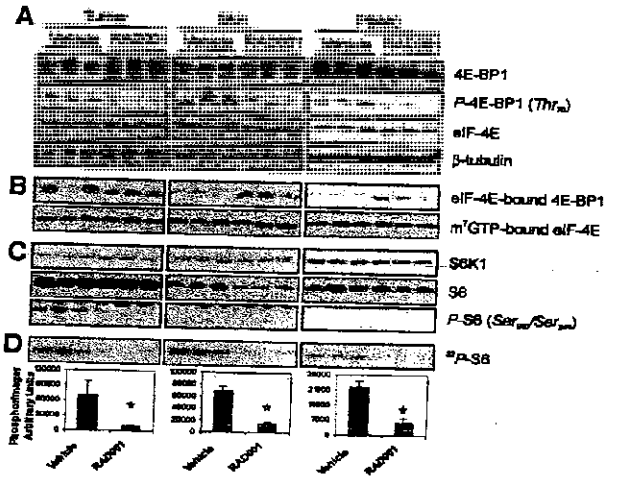

Fig. 2. RAD001 administration inhibits mammalian target of rapamycin signaling in CA20948 tumor-bearing rats. S.c. CA20948 tumor-bearing rats received a single administration of an efficacious dose of RAD001 (5 mg/kg) or vehicle and were sacrificed 24 h after administration (3 rats/group). Tumors, skin, and PBMCs were individually prepared and extracted as described in "Materials and Methods." Results from individual rats are presented. *A* and *C*, total protein was subjected to electrophoresis followed by immunoblot analysis. Membranes were probed for eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and phospho-threonine 70 4E-BP1 [*P-4E-BP1 (Thr70)*] levels, with eukaryotic initiation factor 4E (eIF-4E) and β-tubulin levels acting as loading controls (*A*) or ribosomal protein S6 kinase 1 protein, S6 40*S* ribosomal protein, and phospho-serine 240/244 S6 [*P-S6 (Ser240/Ser244)*] levels (*C*). *B*, the level of 4E-BP1 bound to eIF-4E was measured by purification of 4E-BP1·eIF-4E complexes on 7-methyl-GTP-Sepharose, as described in "Materials and Methods," followed by immunoblot analysis. *D*, ribosomal protein S6 kinase 1 was immunoprecipitated from equal amounts of total protein extract, and activity was measured by *in vitro* kinase assay using 40*S* ribosomal subunits as a specific substrate, as described in "Materials and Methods." Phosphorimages (^{32}P-*S6*) and PhosphorImager quantifications of the kinase assay are presented. Data are means ± SD of $n = 3$ animals/group. *Stars* represent $P < 0.05$ *versus* vehicle-treated controls (Dunnett test).

[4] A. Boulay and H. A. Lane, unpublished data.

reduction in S6 protein expression was observed in RAD001-treated skin, but not in tumor or PBMC extracts. A similar phenomenon has been reported previously in tumors after treatment of mice bearing human prostate cancer xenografts with CCI-779 (24). Moreover, the translation of S6 (as a 5′-terminal oligopyrimidine tract mRNA) has been shown to be specifically inhibited by rapamycin in 3T3 cells (36). It is not known why, in this model, RAD001 treatment only has effects on S6 expression in skin; however, differential downstream effects of mTOR pathway inhibition, depending on the tissue source, are a plausible possibility (54). Taken together, these data demonstrate that both 4E-BP1 and S6K1 pathways are affected in tumors, skin, and PBMC samples obtained from CA20948 tumor-bearing rats after a single administration of an efficacious dose of RAD001.

Prolonged Inactivation of S6K1 in Tumors, Skin, and PBMCs Correlates with the Efficacy of Intermittent RAD001 Treatment Schedules. To investigate whether the antitumor efficacy of intermittent RAD001 treatment schedules is associated with prolonged effects on the mTOR pathway, CA20948 tumor-bearing rats were treated with a single dose of RAD001 (5 mg/kg) or vehicle, and tumor, skin, and PBMC extracts were prepared 12, 24, 48, or 72 h after administration. Because S6K1 was significantly inactivated 24 h after a single RAD001 administration in all tissues analyzed (Fig. 2*D*), long-term effects on mTOR function were assessed using the 40*S* kinase assay (Fig. 3). Tumor and skin extracts were obtained from each of 3 rats/treatment group, whereas PBMC extracts were obtained from pooled blood from each treatment group. A dramatic reduction in S6K1 activity was already observed in tumors, skin, and PBMCs 12 h after RAD001 administration (91%, 91%, and 82% inhibition, respectively; all $P < 0.05$ *versus* untreated controls; Fig. 3). In contrast, treatment with vehicle did not significantly modulate S6K1 activity as compared with untreated controls (Fig. 3). Moreover, RAD001 treatment resulted in the sustained inactivation of S6K1 in all tissues. In tumors, statistically significant inhibition of S6K1 was maintained up to 48 h after administration, with some evidence of recovery after 72 h (80% and 62% inhibition at 48 and 72 h, respectively; Fig. 3*A*). In comparison, S6K1 derived from skin samples remained significantly inhibited for at least 72 h (72% inhibition at 72 h; Fig. 3*B*). Although a statistical analysis could not be performed on the pooled PBMC samples, S6K1 activity was also dramatically inhibited for up to 72 h in these samples (82% inhibition at 72 h; Fig. 3*C*). Thus, consistent with the antitumor efficacy of intermittent 5 mg/kg RAD001 treatment schedules in CA20948 tumor-bearing rats, a single administration of 5 mg/kg RAD001 resulted in long-term inactivation of S6K1 in tumors, skin, and PBMCs.

The Antitumor Efficacy of Intermittent RAD001 Treatment Schedules Is Dose Dependent: Correlation Between Efficacy and Prolonged Effects on mTOR Effectors in Rat PBMCs. Following the observation that intermittent RAD001 (5 mg/kg) treatment schedules significantly inhibited tumor growth, we explored the effect of RAD001 dose on the efficacy of weekly treatment schedules (Table 1, Experiments 2 and 3). As expected, 5 mg/kg/week RAD001 significantly suppressed CA20948 tumor growth as compared with vehicle controls (T/C, 14% and 24% at 7 and 8 days, respectively; $P < 0.05$). In contrast, although 0.5 mg/kg RAD001 caused a significant inhibition of tumor growth when administered daily (T/C, 23%), weekly administration of the same dose did not significantly affect tumor growth (T/C, 48%; $P > 0.05$). This apparent dose dependency of weekly RAD001 schedules was confirmed by a more stringent analysis comprising doses between 5 and 0.5 mg/kg (Table 1, Experiment 3). Statistically significant antitumor responses were observed with 3 and 2 mg/kg RAD001 (T/C, 36% and 32%, respectively), but not with 1 mg/kg (T/C, 45%). Interestingly, 3 mg/week elicited a similar antitumor response (T/C, 36%) as 0.5 mg/kg/day (×6/week; T/C, 30% and 23%). Because both these schedules involve administration of 3 mg/kg RAD001 per week, these data indicate that, with the same total RAD001 exposure, intermittent dosing schedules can elicit equivalent antitumor responses as daily schedules.

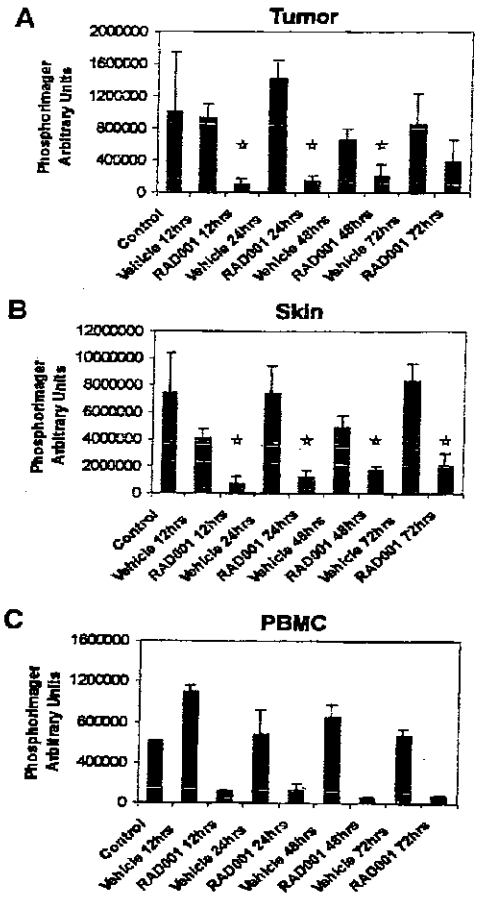

Fig. 3. RAD001 administration (5 mg/kg) causes prolonged inactivation of ribosomal protein S6 kinase 1 in tumors, skin, and PBMCs derived from CA20948 tumor-bearing rats. CA20948 tumor-bearing rats were treated once with 5 mg/kg RAD001 or vehicle (3 rats/group). After 12, 24, 48, and 72 h, tumor and skin samples were individually extracted. Blood obtained from rats within each treatment group was pooled, and peripheral blood mononuclear cells (PBMCs) were isolated and extracted. Assay of ribosomal protein S6 kinase 1 activity was performed using 40S ribosomal subunits as *in vitro* substrate. PhosphorImager quantifications of the S6 kinase assays are presented. *A* (*Tumor*) and *B* (*Skin*): data are means ± SD of $n = 3$ animals/group. *Stars* represent $P < 0.05$ *versus* untreated controls (Dunnett test). *C* (*PBMC*): data are means; *error bars* represent the range of duplicate assays.

To further investigate the dose dependency of weekly schedules in terms of effects on mTOR signaling in a surrogate tissue, the duration of S6K1 inactivation in response to a single administration of 0.5 *versus* 5 mg/kg RAD001 was determined in PBMCs derived from three non-tumor-bearing rats (Fig. 4, *A* and *B*). Whereas in vehicle controls, no effect on S6K1 activity could be observed (24 h after administration), a single administration of 5 mg/kg RAD001 resulted in statistically significant, prolonged inactivation of the S6K1 for up to 7 days (99% and 86% inhibition after 24 h and 7 days, respectively; $P < 0.05$). In comparison, 0.5 mg/kg RAD001 caused a significant inhibition of PBMC-derived S6K1 activity 24 h after administration

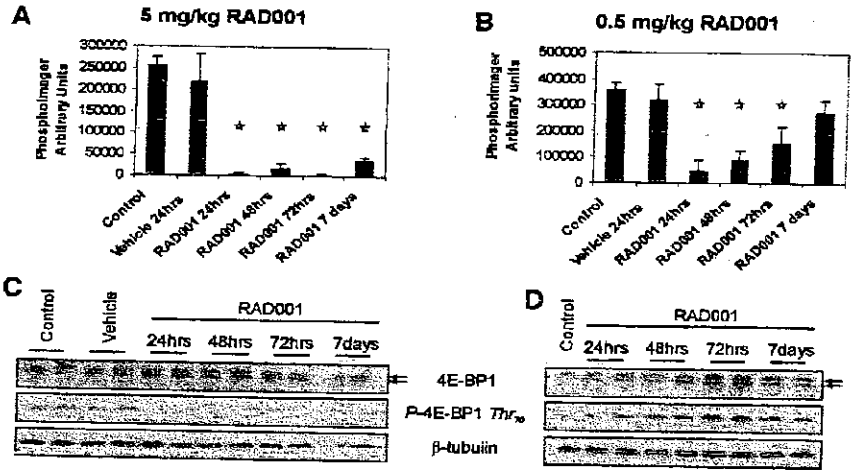

Fig. 4. Dose-dependent effects of RAD001 on ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) in peripheral blood mononuclear cells (PBMCs) obtained from non-tumor-bearing rats. Rats were treated with a single optimal (*A* and *C*) *versus* suboptimal (*B* and *D*) RAD001 dose (5 and 0.5 mg/kg, respectively) or vehicle (3 rats/group). At the times indicated, PBMC samples were collected and individually extracted. *A* and *B*, S6K1 was immunoprecipitated from equal amounts of protein extract, and S6K1 activity was assayed using 40S ribosomal subunits as a substrate. Phosphorimager quantification of the kinase assays are presented (means ± SD of $n = 3$ animals/group). *Stars* represent $P < 0.05$ *versus* untreated controls (Dunnett test). *C* and *D*, equal amounts of PBMC extracts were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed for 4E-BP1 protein, phospho-threonine 70 4E-BP1 (*P-4E-BP1 Thr*$_{70}$), or β-tubulin as a loading control. *Arrows* denote hypophosphorylated (*bottom arrow*) and hyperphosphorylated (*top arrow*) forms of 4E-BP1 protein.

(88% inhibition); however, kinase activity began to recover after 48 h (75% inhibition) and was almost totally recovered after 7 days [23% inhibition; not significant ($P > 0.05$ *versus* controls)]. In contrast to effects on S6K1 activity, no effect on Thr70 phosphorylation or the electrophoretic mobility of 4E-BP1 was observed with the 0.5 mg/kg RAD001 dose, whereas decreased Thr70 phosphorylation and a shift to a lower migrating form were observed with the 5 mg/kg RAD001 dose (Fig. 4, *C* and *D*). The latter effect was maintained for 72 h, with evidence of recovery by 7 days.

The above observations indicate that RAD001 has dose-dependent effects on the mTOR pathway in rat PBMCs. Moreover, long-term effects are associated with a RAD001 dose shown to have significant antitumor efficacy with intermittent treatment schedules. To confirm this hypothesis, a more stringent RAD001 titration was also performed to analyze effects on PBMC-derived S6K1 activity after a single administration of 0.5, 1, 2, or 5 mg/kg RAD001 (Fig. 5). In all cases, inactivation of S6K1 was observed 24 h after RAD001 administration. However, at RAD001 doses that do not elicit a significant antitumor response with weekly schedules (0.5 and 1 mg/kg; see Table 1), evidence of recovery of S6K1 activity was already observed

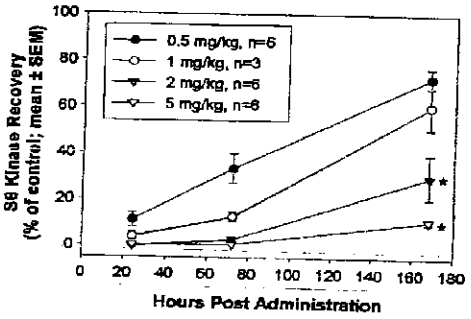

Fig. 5. Association between antitumor efficacy of weekly RAD001 schedules and long-term down-regulation of ribosomal protein S6 kinase 1 (S6K1) activity in rat peripheral blood mononuclear cells (PBMCs). Non-tumor-bearing rats were treated with a single RAD001 dose (0.5, 1, 2, or 5 mg/kg). At the times indicated, PBMC samples were collected and individually extracted and assayed for S6K1 activity. Data are presented as mean percentage recovery of S6K1 activity *versus* untreated control animals ± SEM of $n = 3$ or 6 S6K1 assays from different rat PBMC extracts. All data are derived from two separate experiments, except in the case of 1 mg/kg, where data from a single experiment are presented. *Stars* represent $P < 0.05$ *versus* untreated controls (ANOVA on ranks test).

at 72 h (34% and 13% recovery *versus* untreated controls, respectively) and was dramatic at 7 days [73% and 61%, respectively; no significant inhibition of S6K1 ($P > 0.05$ *versus* controls)]. In contrast, at RAD001 doses that do elicit a significant antitumor response with weekly schedules (2 and 5 mg/kg; see Table 1), minimal recovery was observed at 72 h (3% and 1%, respectively) or 7 days [30% and 12%, respectively; significant inhibition of S6K1 ($P < 0.05$ *versus* controls)]. These data confirm that long-term inactivation of PBMC-derived S6K1 correlates with the antitumor efficacy of weekly RAD001 treatment schedules.

S6K1 Activity Can Be Reproducibly Detected in Human PBMC Extracts: RAD001 Induces Concentration-Dependent S6K1 Inactivation *Ex Vivo*. To evaluate the potential of using mTOR effectors as biomarkers to evaluate RAD001 treatment schedules, we assessed whether basal S6K1 activity could also be measured in human PBMC extracts obtained from healthy volunteers. Human blood was collected into tubes containing either sodium citrate or EDTA as an anticoagulant, and PBMC extracts were prepared. Subsequent assay of S6K1 activity demonstrated that activity could indeed be detected in non-challenged human PBMCs derived from unrelated donors (Fig. 6*A*). Interestingly, S6K1 activity was reproducibly higher when the blood was initially collected in EDTA as compared with sodium citrate, a phenomenon potentially related to the different chelating properties of these anticoagulants. Using EDTA, a coefficient of variation of 10% was obtained among six assays on PBMC extracts prepared separately from the same blood donor, indicating good reproducibility of preparation (Fig. 6*B*). Accordingly, equivalent S6K1 protein levels were detected in the same extracts by immunoblot analysis (Fig. 6*B*). These results demonstrate that, in analogy with the rat PBMC data, basal S6K1 activity can be detected in human PBMCs. However, unlike control rat PBMC extracts (see Fig. 2*A*), there was no evidence of Thr70 phosphorylation in any of the human PBMC extracts analyzed,[5] an observation correlating with the fact that most of the 4E-BP1 protein was present in the hypophosphorylated/fast migrating state (when compared with 4E-BP1 derived from proliferating human tumor cells; Fig. 7*A*, *DMSO*). *Ex vivo* treatment of whole blood with 20 nM RAD001 for 30 min did not further increase protein mobility (Fig. 7*A*, *RAD001*), suggesting that 4E-BP1 is largely active as a translational repressor in human PBMCs. Hence, unlike the situation

[5] A. Boulay, S. Zumstein-Mecker, and H. A. Lane, unpublished data.

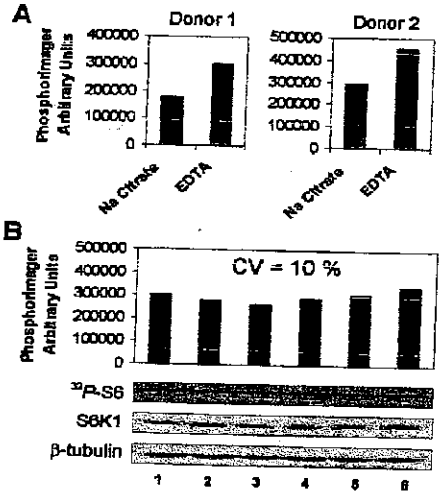

Fig. 6. Detection of ribosomal protein S6 kinase 1 (S6K1) activity in human peripheral blood mononuclear cells (PBMCs). Blood from healthy volunteers was withdrawn at the same time into tubes containing either sodium citrate or EDTA as an anticoagulant. PBMCs were immediately isolated and extracted. *A*, S6K1 was immunoprecipitated from equal amounts of PBMC protein extracts, and activity was assessed using 40S ribosomal subunits as a substrate. PhosphorImager quantifications are presented and represent basal S6K1 activity (means of duplicate assays of a single sample) from two unrelated donors. *B*, blood from a single volunteer was withdrawn into tubes containing EDTA as an anticoagulant and was split into six equal fractions. PBMCs were prepared separately and extracted from each blood fraction, and extracts were simultaneously assayed for S6K1 kinase activity. Phosphorimages (*^{32}P-S6*) and PhosphorImager quantifications (*graph*) of duplicate kinase assays are presented. As internal controls, equal amounts of PBMC protein extracts were analyzed by immunoblot for S6K1 and β-tubulin protein levels.

in rat, this protein may not be applicable as a biomarker for monitoring RAD001-specific effects on mTOR signaling in human PBMCs.

To assess whether human PBMC-derived S6K1 is inactivated in the presence of RAD001, whole blood from two unrelated healthy volunteers was treated *ex vivo* with either DMSO vehicle or increasing concentrations of RAD001 for 30 min, followed by isolation, extraction, and assay of PBMC-derived S6K1 activity (Fig. 7*B*). Treatment with 2 nM RAD001 diminished PBMC-derived S6K1 activity as compared with DMSO vehicle controls (44% and 63% inhibition in donor 1 and 2, respectively). Furthermore, increasing RAD001 concentrations led to almost complete inactivation of S6K1 (≥95% inhibition with ≥20 nM RAD001 in donor 1 and 2). These results demonstrate that RAD001 treatment of human blood *ex vivo* results in a concentration-dependent inactivation of PBMC-derived S6K1, supporting the notion that changes in PBMC-derived S6K1 activity could serve as a biomarker when assessing treatment schedules with rapamycin derivatives such as RAD001 in clinical trials for cancer.

DISCUSSION

The mTOR pathway plays a major role in cell proliferation by coupling cell growth with G_1-S progression. Compounds targeting the mTOR pathway have potential, therefore, for application in cancer treatment modalities (2, 3, 4). In this context, RAD001 potently inhibits the proliferation of numerous tumor cell lines *in vitro* and inhibits the growth of a range of human xenografts in nude mice (2, 27, 43–46).[6] Rapamycin and the rapamycin ester CCI-779 also present antitumor activity in a number of animal models of cancer (2, 24–26, 53, 55–59). However, although human pancreatic tumors have been reported in abstract form to be sensitive to CCI-779 (reviewed in Ref. 2), and mTOR/S6K1 signaling appears to be required for pancreatic cancer cell proliferation (60, 61), the work presented here is the first full publication demonstrating significant antitumor efficacy of a rapamycin derivative in an animal model of pancreatic cancer. Orally administered RAD001 was found to be well tolerated and to elicit antitumor potency equivalent to that of the cytotoxic agent 5-FU. Moreover, similar responses were achieved with daily or weekly RAD001 administrations, indicating that frequent drug administration is unnecessary to maintain an antitumor response. Although weekly rapamycin dosing schedules have been used previously (55, 56), a comparative analysis addressing the efficacy of daily *versus* weekly administration had not been performed. The fact that weekly RAD001 administration produces statistically significant antitumor responses in the CA20948 model is supported by a number of experimental observations. First, *in vitro* pulse treatment with either RAD001 (43) or rapamycin (51) causes prolonged down-regulation of the mTOR pathway in tumor cell lines. Indeed, Hosoi *et al.* (51) postulated that this phenomenon was due to the slow dissociation rate of the rapamycin-FKBP12 complex. Second, prolonged effects of CCI-779 on xenograft tumor growth were evident after cessation of daily treatment schedules (24, 53, 57), and antitumor responses have been

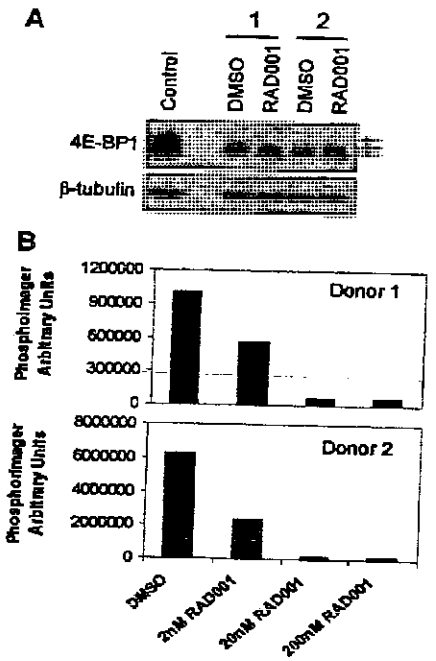

Fig. 7. Effects of *ex vivo* RAD001 treatment on ribosomal protein S6 kinase 1 activity and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) mobility in human PBMCs. *A*, blood was collected from two unrelated donors using EDTA as an anticoagulant and treated *ex vivo* with DMSO vehicle or 20 nM RAD001 for 30 min at room temperature, followed by PBMC isolation and extraction. Equal amounts of PBMC protein extracts were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was probed for 4E-BP1 protein, with β-tubulin as a loading control. Human lung adenocarcinoma tumor cell lines lysates (*Control*; A549) were included as an example of 4E-BP1 mobility in a highly proliferative human cell population. *B*, blood was collected from two unrelated donors using EDTA as an anticoagulant and treated *ex vivo* with 2, 20, or 200 nM RAD001 or DMSO vehicle for 30 min at room temperature, followed by PBMC isolation and extraction. Ribosomal protein S6 kinase 1 was immunoprecipitated from equal amounts of PBMC protein extract, and activity was assessed *in vitro* using 40S ribosomal subunits as a substrate. PhosphorImager quantifications of the kinase assay are presented and represent means of duplicate assays of a single sample.

[6] A. Boulay, T. O'Reilly, and H. A. Lane, unpublished data.

reported in Phase I clinical trials with weekly CCI-779 administration (2, 4).

One advantage of administering RAD001 intermittently in oncology is the avoidance of prolonged immunosuppression (1). In this context, the minimal effective dose of RAD001 in stringent rat kidney and heart allotransplantation models is ≥5 mg/kg administered daily (47, 52). Moreover, the immunosuppressive capacity of RAD001 (everolimus in combination with cyclosporin) in transplant patients has been related to maintenance of blood drug trough levels (1, 62), suggesting that constant drug exposure is required to provide clinically relevant immunosuppression. The demonstration that weekly administration of RAD001 (at doses of 2–5 mg/kg) is sufficient to elicit a significant antitumor response indicates that the above premise does not apply to oncology. Indeed, in support of this notion, as compared with daily RAD001 administration (2.5 mg/kg), a 5 mg/kg weekly RAD001 regimen allows a 20-fold higher T-cell-dependent antibody response, as measured by serum IgG antibody titers after immunization of rats with dinitrophenol-coupled keyhole limpet hemocyanogen.[3] Hence, intermittent dosing allows for differentiation between immunosuppressive and antitumor effects, a possibility also suggested from preliminary clinical data (2, 4). The basis of this is presumably related to the biology of T cells as compared with tumor cells. In this respect, rapamycin potently prevents resting T cells from entering the cell cycle in response to interleukin 2 but has little effect on proliferating T cells (63, 64). This may explain why constant drug exposure is required in the immunosuppression setting, as opposed to the antitumor setting where the proliferation of cycling tumor cells is potently inhibited (2, 4) for long periods (51). This possibility is worthy of further investigation.

A limited analysis of the effects of rapamycin derivatives on mTOR effectors in tumor material derived from xenograft models was reported previously (24, 53). Until now, however, a comprehensive analysis had not been performed. Similarly, the possibility that the efficacy of intermittent treatment schedules correlates with long-term effects on the mTOR pathway in tumors and surrogate tissues had not been addressed. This prompted us to profile RAD001-mediated effects on mTOR signaling in CA20948 tumors and normal rat tissues. Mitogen-induced, multisite phosphorylation of the translational suppressor protein 4E-BP1 is known to cause its release from the initiation factor eIF-4E, thereby facilitating formation of the eIF-4F initiation complex and derepression of cap-dependent mRNA translation (2, 5). Indeed, the 4E-BP1 protein has been proposed to be a direct substrate for the mTOR kinase (34, 41, 42). Moreover, rapamycin treatment of cell lines decreases 4E-BP1 phosphorylation, resulting in increased affinity for eIF-4E *in vitro* (2, 5). Consistent with these observations, a single administration of 5 mg/kg RAD001 to three tumor-bearing rats reproducibly inhibited 4E-BP1 phosphorylation in tumors, skin, and PBMCs at 24 h, in accordance with changes in 4E-BP1 electrophoretic mobility and increased 4E-BP1-eIF-4E association. In the same animals, S6K1 signaling was virtually abolished in all tissues. The physiological downstream target of the S6K1 is the S6 40S ribosomal protein (12, 65). Hence, reductions in S6 phosphorylation are expected to parallel S6K1 inactivation, as observed in CA20948 tumor extracts. However, because S6 phosphorylation could not be detected in either skin or PBMC control extracts, no such correlation could be made in these tissues. This failure to detect S6 phosphorylation could reflect a reduced proliferation index as compared with the aggressively growing CA20948 tumors. Strikingly, and in agreement with previous *in vitro* analyses (43, 51), tumors, skin, and PBMC extracts derived from rats treated with a single 5 mg/kg RAD001 dose demonstrated prolonged inactivation of the S6K1 for ≥72 h. Taken together, these data suggest that RAD001-specific effects on 4E-BP1 and S6K1 activity can be reproducibly observed in tumors and surrogate tissues. Moreover, long-term effects of RAD001 on S6K1 activity occur with a dose of RAD001 known to elicit significant antitumor responses with intermittent treatment schedules.

The observation that the mTOR pathway is affected for long periods of time in tumors and PBMCs is consistent with preliminary pharmacokinetic studies performed in CA20948 tumor-bearing rats. Pharmacokinetic measurements after a single RAD001 administration (5 mg/kg, over a 72 h period) demonstrated good bioavailability/efficient tumor penetration (maximal concentrations in blood and tumor, ~200 and ~700 nM, respectively) and prolonged residency (RAD001 half-life, ~20–22 h.[7] Unfortunately, a precise correlation of pharmacokinetic parameters with antiproliferative effects in tumors is difficult in this model because of the inability to determine *in vitro* IC_{50} values with the nonculturable CA20948 line. However, the efficient tumor accumulation and relatively long half-life of RAD001 provide further rationale for the long-term effects observed in this model.

Sequential tumor sampling is difficult in the clinical setting, necessitating some reliance on surrogate tissue to assess pharmacodynamic effects of antitumor agents. For this reason, the possibility of using PBMCs as a source for biomarker analysis when assessing RAD001 treatment schedules was evaluated. Detailed efficacy experiments demonstrated that antitumor response to weekly administration of RAD001 was dose dependent. Moreover, significant antitumor responses were associated with long-term effects on the mTOR pathway in PBMCs. Interestingly, PBMC-derived 4E-BP1 was unaffected by a suboptimal RAD001 dose (0.5 mg/kg), despite transient effects on S6K1 activity. This suggests that S6K1 is a more sensitive marker of RAD001 exposure in PBMCs than 4E-BP1. Indeed, all doses of RAD001 evaluated elicited a dramatic inhibition of PBMC-derived S6K1 after 24 h. However, the rate at which S6K1 activity subsequently recovered differed, with RAD001 doses that were efficacious with weekly schedules causing more profound long-term effects on S6K1 activity (≥7 days). The demonstration that the mTOR pathway is affected in PBMCs for a week after administration of 5 mg/kg RAD001 may be interpreted as being contrary to our observations that weekly treatment with this dose is suboptimal in terms of suppression of T-cell-dependent antigen responses. To reconcile these observations, one has to consider that T- and B-cell proliferative responses to foreign antigen presentation occur mainly in the secondary lymphoid organs (64). Here we assayed S6K1 derived from PBMCs, a source that does not reflect the situation in these organs. We therefore speculate that, using weekly schedules, there is a possibility to recover T-cell responses, a phenomenon that may also reflect the pharmacokinetic characteristics of RAD001.

To most efficiently exploit the pharmacological profile of targeted agents such as RAD001, it is important to carefully monitor the dose given to a cancer patient, especially considering the observation that rapamycin can be less effective as an antitumor agent in animal models if overdosed (59). The ease of human PBMC preparation suggests that this could be a valuable surrogate tissue when establishing treatment regimens for RAD001 in clinical trials for oncology. Based on this premise, S6K1 activity could be reproducibly assayed in PBMC extracts prepared from healthy volunteers, and RAD001 treatment of whole blood *ex vivo* resulted in concentration-dependent inactivation of the kinase. In contrast, despite promising results in tumor extracts derived from xenograft models (53) and suggestions that 4E-BP1 phosphorylation could be used as a confirmatory measure of mTOR inhibition in PBMCs (66), we have shown that 4E-BP1 phosphorylation cannot be detected in human PBMCs. During the

[7] T. O'Reilly and L. McMahon, unpublished data.

revision of this manuscript, others (66, 67) also reported on the potential for PBMC-derived S6K1 activity measurements to aid pharmacodynamic evaluation of rapamycin derivatives. Analysis of cancer patient-derived PBMCs after i.v. administration of 25, 75, and 250 mg CCI-779 demonstrated inactivation of PBMC-derived S6K1 for up to 8 days, with no evidence of dose dependency at the doses used (66–68). Although a limited feasibility study in nine patients indicated an association between time to disease progression and the degree of inhibition of S6K1 24 h after CCI-779 administration, no conclusions were drawn regarding the predictive nature of this biomarker or associated implications of the long-term S6K1 inactivation observed in patients (67). Our data provide a strong experimental rationale for analyzing long-term effects on PBMC-derived S6K1 activity when establishing weekly administration schedules. Indeed, recent Phase I trials with weekly administration of RAD001 in patients with advanced cancer have demonstrated a clear association between RAD001 dose and the recovery of PBMC-derived S6K1 activity over a ≥7-day period (69). The value of these observations in terms of prediction of patient response is now being pursued in clinical trials of RAD001 in oncology.

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The rapamycin analog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation ☆

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Abstract

We present immunohistochemical evidence that the mTOR/p70s6k pathway is activated in pancreatic tumors and show that the mTOR inhibitor and rapamycin analog CCI-779 potently suppresses the proliferation of pancreatic cancer cells. Consistent with a recent study, CCI-779 increased c-Jun phosphorylation (Ser63) in a dose- and time-dependent manner, and induced apoptosis in p53-defective BxPC-3 cells. In contrast to the study, however, we observed that CCI-779 concomitantly increased c-Jun protein levels and that its ability to induce apoptosis might not require the activated c-Jun. Furthermore, CCI-779 neither induced c-Jun phosphorylation in other p53-defective pancreatic cancer cells (MiaPaCa-2) nor inhibited their proliferation. c-Jun, in fact, appeared to be partly responsible for the resistance of MiaPaCa-2 cells to CCI-779. Together, these results indicate a complex role for c-Jun in cellular responses to CCI-779 and provide an important basis for investigating CCI-779 further as a potential therapeutic agent for pancreatic tumors.

Keywords: CCI-779; Rapamycin; Pancreatic cancer; p70s6k; Akt; PI 3-kinase; c-Jun; Drug resistance

Pancreatic cancer is one of the leading causes of cancer-related mortality in the western world. Conventional strategies such as chemotherapy and radiotherapy have not improved the median survival time of patients with metastatic disease for the last 30 years [1]. Given this dismal record, attention has turned to molecular therapeutics as a powerful new approach for targeting proteins implicated in pancreatic cancer initiation and progression. The goal is to accelerate the discovery of novel drugs to use in pancreatic cancer therapy [2,3].

Rapamycin is a macrolide fungicide that has demonstrated impressive anti-tumor activity [4]. It also possesses potent anti-microbial and immunosuppressant properties, and inhibits the translation of proteins required for cell-cycle progression from G_1 to S phase. A rapamycin analog (ester) known as CCI-779 has been developed in an effort to obtain more favorable pharmaceutical, toxicologic, and anti-tumor profiles in preclinical evaluations than those achieved by rapamycin [4,5]. Inhibition of rapamycin-sensitive signaling pathways by CCI-779 appears to be the basis for its potent activity against a wide range of human tumors in tissue culture and xenograft models [4,5]. Like rapamycin, it binds intracellularly to the immunophilin FK506 binding protein (FKBP12) and forms a complex that inhibits the

☆ *Abbreviations:* mTOR, mammalian target of rapamycin; p70s6k, p70 ribosomal S6 protein kinase, PI 3-kinase, phosphoinositide 3-kinase.

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activity of mammalian target of rapamycin (mTOR) or FRAP, a protein kinase that belongs to the phosphoinositide 3-kinase (PI 3-kinase) super-family [4,6]. When mTOR is inhibited, its signals such as those that induce the phosphorylation of the 70 kDa, 40S ribosomal protein kinase (p70s6k), and the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) are blocked, leading to G1 arrest in most cell types [7,8] and to p53-independent apoptosis in some others [9–11]. The mechanism by which rapamycin induces apoptosis in cells lacking functional p53 under serum-free conditions was recently shown to involve 4E-BP1 and the ASK/JNK/Jun pathway [11].

There is considerable evidence that mTOR functions in the PI 3-kinase pathway downstream of Akt [12–16]. Consistent with a role for mTOR in tumorigenesis, both PI 3-kinase and Akt as well as the PTEN gene, a lipid phosphatase [17] which functions as their natural antagonist in normal cells, have been strongly implicated in human cancer [18–20]. It has been shown that tumors that depend on activated PI 3-kinase/Akt such as those with defective PTEN function are highly sensitive to mTOR inhibitors [21–23]. Various studies, including our own, have demonstrated that the PI 3-kinase pathway is activated in pancreatic adenocarcinoma and that it is important for the survival, proliferation, and drug resistance of pancreatic cancer cells [24–31]. We also showed that PTEN expression was significantly reduced in over 60% of the pancreatic tumor tissues and cell lines we examined [31]. In addition to PTEN, a large proportion of pancreatic tumor tissues and cell lines also lack functional p53 [32,33]. We therefore investigated the activation status of mTOR in pancreatic normal and tumor tissue specimens, and the effects of CCI-779 on specific cell lines. Our results indicate that mTOR is activated in pancreatic tumors and that CCI-779 is a potent inhibitor of growth for many if not all pancreatic cancer cell lines that contained defective p53. However, while CCI-779 induced c-Jun phosphorylation in a sensitive cell line consistent with a previous study [11], its ability to induce apoptosis did not seem to require the activated c-Jun. Furthermore, c-Jun appeared to partly account for the resistance of at least one pancreatic cancer cell line to CCI-779. Together, these data suggest that CCI-779 deserves further investigation as a potential therapeutic agent for pancreatic cancer treatment.

Materials and methods

Materials. LY294002 (PI 3-kinase inhibitor) and SP600125 (c-Jun N-terminal kinase or JNK inhibitor) were obtained from Biomol Research Laboratories, and rapamycin (mTOR inhibitor) was purchased from Calbiochem. Akt1/2, JNK, p65RelA, c-Jun, and anti-phospho-c-Jun (serine 63) antibodies were obtained from Santa Cruz Biotechnology whereas phospho-Akt (Ser473), p70s6k, and phospho-p70s6k (Thr389) antibodies were from Cell Signaling Technology. Anti-PARP antibodies were purchased from BD Pharmingen. Tissue sections were obtained from the NCI Cooperative Human Tissue Network.

Cell culture. AsPC-1, Panc-1, Capan-1, MIA PaCa-2, and BxPC-3 cells were purchased from the American Type Culture Collection and cultured as described in their product information sheets. Panc-3, Panc-28, and Panc-48 cells were provided by Drs. Paul Chiao and Keping Xie (M.D. Anderson Cancer Center), and maintained in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum under standard culture conditions.

Immunoblotting analysis. Serum-starved (16 h) pancreatic cancer cells were lysed and whole-cell extracts (WCE) were prepared as described previously [34]. Where indicated, cells were treated with vehicle (control), the PI 3-kinase inhibitor LY294002 or the mTOR inhibitors rapamycin or CCI-779, and washed with ice-cold phosphate-buffered saline before WCE preparation. WCE were clarified by centrifugation and proteins were resolved by SDS–PAGE. Following protein transfer, nitrocellulose membranes were probed for total and phosphorylated p70s6k, total and phosphorylated Akt, and actin. Specific bands were detected by ECL (Amersham-Pharmacia Biotech). For the detection of poly(ADP)-ribose polymerase (PARP) cleavage, total c-Jun, phospho-c-Jun, JNK, or p65RelA, the pellet that remained after WCE preparation was further extracted and protein was combined with WCE as described previously [34]. This protein mixture was resolved by SDS–PAGE and probed by Western blotting as described above.

Immunohistochemistry. The phosphorylation status of p70s6k was investigated in paraffin sections of residual surgical tissues from four different patients with ductal pancreatic adenocarcinoma. These sections were heated at 65 °C overnight, deparaffinized in xylene, and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. Sections were then blocked with 3% bovine serum albumin and normal horse serum at 37 °C for 30 min, and then overnight with phospho-p70s6k (Thr389) antibody at 4 °C in a humidified chamber, and finally with a biotinylated secondary antibody for 30 min at 37 °C. The antibody complex was detected by avidin–biotin–peroxidase complex solution and visualized by 3,3′-diaminobenzidine (Zymed Laboratories). Sections were counterstained with hematoxylin for 5 min and mounted with Eukit (Calibrated Instrument). Immunostaining was observed under a light microscope, and a semiquantitative score for intensity was given to the different samples: strong, moderate, or weak, relative to the staining observed for smooth muscle cells in the vascular wall and the intestinal wall.

Proliferation assays. Pancreatic cancer cells were plated at a density of 5×10^3 cells per well in 96-well culture plates. After 16 h, the medium was removed, and the cells were cultured in fresh serum-containing medium in the presence or absence of CCI-779, TGFβ, and SP600125. At various times, cells were stained with crystal violet to determine the absorbance at 540 nm in a Packard plate reader. Each data point was obtained in triplicate, and averages and standard deviation were estimated.

Soft-agar assays. Cells (1×10^4) were mixed with 1 ml of a 0.33% Noble agar solution and added on top of 1 ml of a solidified layer of 0.5% agarose in 12-well culture plates. Fresh inhibitors were added every 3 days, and colony formation was monitored biweekly for 3 weeks. Assays were performed in triplicate and colonies were photographed using a Nikon Eclipse TE2000S Inverted microscope and a Hamamatsu digital camera.

Stable expression of TAM67 in MiaPaCa-2 cells. MiaPaCa-2 cells were cultured in DMEM containing 10% FCS, 1000 U/ml penicillin–streptomycin, and 2 mM glutamine, and transfected with the empty pcDNA3.1 vector or with the pcDNA3.1-TAM67 plasmid using the FuGene reagent (Roche). After 24 h, transfected cells were incubated with 400 μg/ml G418. Drug-resistant colonies were expanded and employed for proliferation assays to test the effects of TGFβ and CCI-779.

Results

mTOR is activated in pancreatic tumor tissues and cell lines

We investigated the phosphorylation status of p70s6k as a measure of mTOR activation in eight different serum-starved pancreatic cancer cell lines. Immunoblotting analysis showed that p70s6k was phosphorylated on Thr389, an mTOR-modified residue that is critical for its kinase activity, and that the levels of phosphorylated enzyme varied substantially between cell lines (Fig. 1A). These data indicate that pancreatic cancer cells contain activated mTOR and p70s6k, consistent with previous studies on Panc-1, BxPC-3, and MiaPaCa-2 cells [29,30]. For confirmation, we next examined normal and tumor tissue specimens from pancreatic cancer patients. Immunohistochemical analysis indicated that pancreatic tumor tissues indeed contained higher levels of phosphorylated p70s6k (p-p70s6k) than normal controls (Fig. 1B) and that three of the four pancreatic, adenocarcinoma samples examined were strongly positive for p-p70s6k.

mTOR inhibitor CCI-779 blocks anchorage-dependent and -independent growth of pancreatic cancer cells

A number of important studies have shown that p70s6k is regulated in response to multiple signaling inputs [35]. There is also compelling evidence that it is an important target of the PI 3-kinase pathway and that PI 3-kinase/Akt-induced p70s6k activation is mediated by mTOR [13–16]. Thus, the mechanism of p70s6k activation in this signaling cascade is sensitive to both mTOR (rapamycin) and PI 3-kinase (wortmannin, LY294002) inhibitors and involves p70s6k phosphorylation on Thr389 [35]. Confirming the essential role of PI 3-kinase and mTOR in pancreatic cancer cells, the levels of p-p70s6k were potently inhibited by LY294002, rapamycin, and the rapamycin analog CCI-779 (Fig. 2A). Akt-Ser473 phosphorylation was inhibited by LY294002 but not by CCI-779 or rapamycin consistent with the notion that mTOR is downstream of Akt in the PI 3-kinase signaling pathway (Fig. 2A). The inhibition of p70s6k phosphorylation was not due to a reduction in p70s6k protein levels which were unaffected by inhibitor treatment.

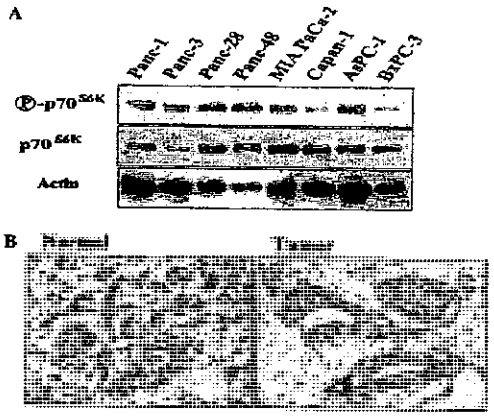

Fig. 1. p70s6k is phosphorylated in pancreatic tumor cell lines and tissues. (A) Whole-cell extracts were prepared from various serum-starved (16 h) pancreatic cancer cell lines and analyzed by immunoblotting with antibodies that specifically recognized total p70s6k or its phosphorylated form (Thr389). (B) Immunohistochemical staining. A representative section from the four different pancreatic adenocarcinoma tissue specimens (tumor) examined is shown. Residual normal adjacent tissues were present in the sample and pancreatic epithelial cells showed weak to moderate staining (normal).

We next investigated if the pharmacological inactivation of mTOR by CCI-779 would affect the proliferation of pancreatic cancer cells under two different conditions. The ability of different pancreatic cancer cell lines to proliferate under serum-induced, anchorage-dependent conditions was investigated over a period of 4 days. CCI-779 potently inhibited the proliferation of four different cell lines, with Capan-1 and BxPC-3 being slightly more sensitive (over 90% inhibition, Fig. 2B) than Panc-1 and AsPC-1.

Since transformed cells and cells derived from tumors grow in anchorage-independent conditions, we investigated if CCI-779 also affected the ability of pancreatic cancer cells to form colonies in agarose medium. Compared to vehicle-treated controls, the ability of cells to grow in soft agar was potently inhibited when cultured in medium containing CCI-779 (Fig. 2C). Consistent with previous studies, colony formation was strongly inhibited by rapamycin and LY294002 as well.

CCI-779 induces c-Jun expression and apoptosis in BxPC-3 cells

Huang et al. [11] recently demonstrated that rapamycin increases c-Jun phosphorylation, without altering its protein levels, in p53-defective, serum-deprived cells and thus induces apoptosis. We, therefore, investigated the effect of CCI-779 on pancreatic cancer cell lines many of which lack functional p53. Indeed, CCI-779 at concentrations as low as 2 pM was able to potently induce the phosphorylation/activation of c-Jun in BxPC-3 cells (Fig. 3A). However, in contrast to the observations of Huang et al. [11], CCI-779 was concomitantly also able to increase c-Jun protein levels over a wide range of concentrations (Fig. 3A). These effects of CCI-779 were observed both in the absence and presence of serum (Fig. 3A) within 2 h of treatment (Fig. 3B), and appeared to be specific because no changes were observed for other proteins such as p65RelA and JNK.

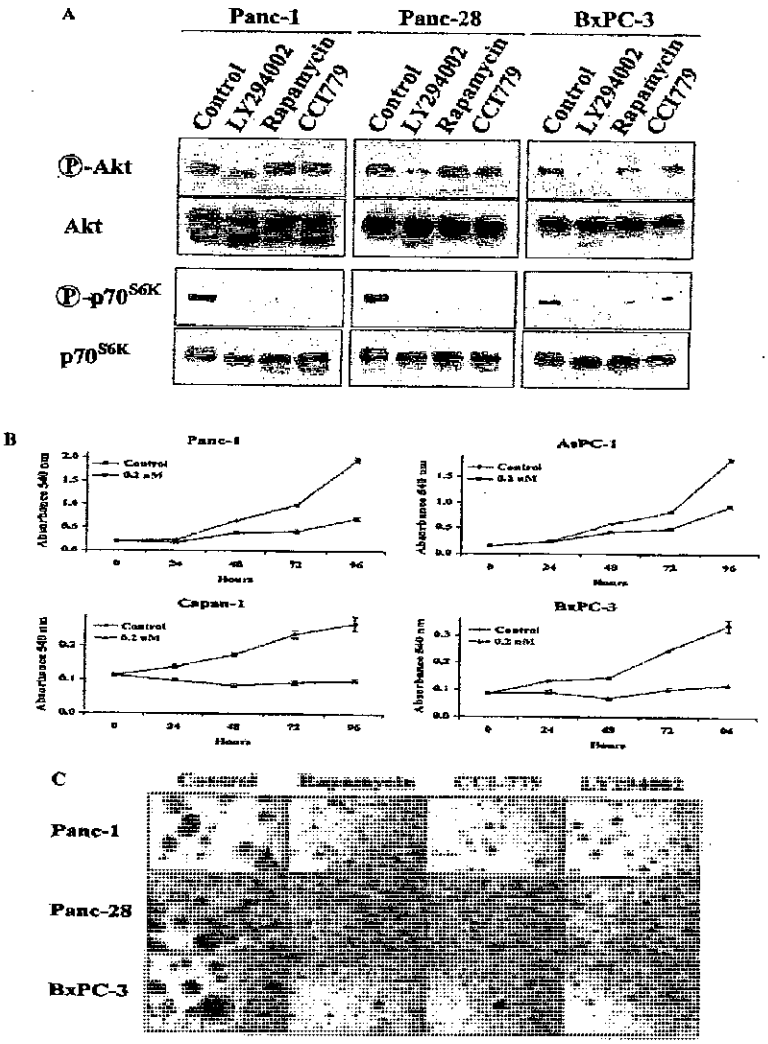

Fig. 2. CCI-779 is a potent inhibitor of mTOR and pancreatic cancer cell growth. (A) Serum-starved cells were either treated with LY294002 (20 μM), rapamycin (0.25 μM), or CCI-779 (0.2 nM) for 2 h or left untreated (control). Whole-cell extracts were then prepared and analyzed by immunoblotting analysis for Akt, p70s6k, and their phosphorylated forms. (B) The effect of CCI-779 on the proliferation (anchorage-dependent) of pancreatic cancer cells was determined by crystal-violet staining (540 nm). (C) Cells were treated with CCI-779, rapamycin, or LY294002 to test their ability to grow in soft agar (anchorage-independent conditions).

Since rapamycin induces apoptosis in p53-defective cells, we investigated the effect of CCI-779 on serum-starved BxPC-3 cells using PARP ((poly)ADP-ribose polymerase) as a marker. PARP is typically cleaved from a 116 kDa protein to a smaller 85 kDa form in cells undergoing apoptosis. Indeed, PARP was completely cleaved in BxPC-3 cells within 8–24 h of treatment (Fig. 3C), indicating that CCI-779, like rapamycin, triggers apoptosis in p53-deficient serum-deprived cells. Surprisingly, however, pre-treatment of BxPC-3 cells with an inhibitor (SP600125) that blocked c-Jun phosphorylation/activation (data not shown) neither induced PARP cleavage on its own nor appeared to interfere with the ability of CCI-779 to trigger apoptosis (Fig. 3C).

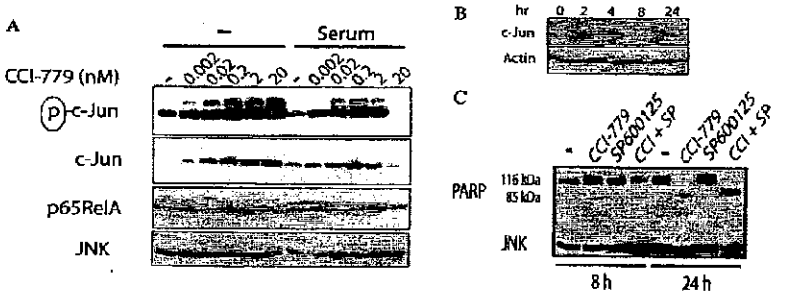

Fig. 3. CCI-779 induces apoptosis independently of c-Jun in BxPC-3 cells. (A) BxPC-3 cells cultured in the absence (16 h) or presence of serum were treated, as indicated, with various concentrations of CCI-779. Whole-cell extracts were prepared, subjected to SDS–PAGE, and analyzed by immunoblotting with antibodies that recognized phospho-c-Jun (Ser63), c-Jun, p65RelA or JNK. (B) Whole-cell extracts from BxPC-3 cells treated with 0.2 nM CCI-779 for the indicated time points were analyzed by Western blotting for c-Jun and actin. (C) BxPC-3 cells were treated with or without 2 nM CCI-779. Where indicated, the JNK inhibitor SP600125 (10 μM) was added to cells 30 min prior to the addition of CCI-779. After the addition of CCI-779, cells were harvested after 8 or 24 h and whole-cell extracts were prepared for immunoblotting analysis of PARP and JNK expression. PARP is a 116 kDa polypeptide that is typically degraded in apoptotic cells to an 85 kDa form.

Resistance of MiaPaCa-2 cells to CCI-779 treatment is reduced by a c-Jun transactivation mutant (TAM67)

CCI-779 did not induce the phosphorylation or expression of c-Jun in MiaPaCa-2 pancreatic cancer cells (Fig. 4A). Significantly, MiaPaCa-2 was also resistant to CCI-779 treatment unlike the BxPC-3 cells (Fig. 4B). However, because MiaPaCa-2 expressed much higher levels of c-Jun than BxPC-3 cells (data not shown), we investigated the possibility that c-Jun was, in fact, the cause of their resistance to CCI-779. MiaPaca-2 cells stably expressing a c-Jun transactivation mutant (TAM67) were therefore generated to test the possibility and, to our surprise, found to be more sensitive to CCI-779 treatment than those that had been transfected with empty vector (Fig. 4C). In sharp contrast, TAM67 expression did not sensitize MiaPaCa-2 cells to TGF-β treatment.

Discussion

Using cell lines and tumor tissue specimens, we show that the Akt/mTOR/p70s6k pathway is constitutively activated in pancreatic cancer. Our data also indicated that CCI-779 blocked this pathway and that it potently inhibited the serum-induced proliferation of various pancreatic cancer cell lines in anchorage-dependent and -independent conditions. Trypan blue exclusion assays and immunoblotting analysis of PARP cleavage showed that greater than 95% of pancreatic cancer cells were viable under these conditions suggesting that CCI-779 did not induce apoptosis in the presence of serum (data not shown). Previous studies utilized fluorescence-activated cell sorting analysis to show that rapamycin induced a G_0–G_1 cell-cycle arrest and inhibited the serum-induced proliferation of a fraction of Panc-1 and BxPC-3 cells [30]. Our data suggest, in comparison, that CCI-779 is likely to be a far more effective inhibitor of proliferation and that it induces apoptosis in serum-deprived, p53-defective BxPC-3 cells. Huang et al. [11] recently showed that rapamycin-induced apoptosis that is observed in p53-deficient cells was dependent on the ability of rapamycin to induce c-Jun phosphorylation/activation. Our data indicate that CCI-779 was capable of inducing c-Jun phosphorylation in BxPC-3 cells but that it also

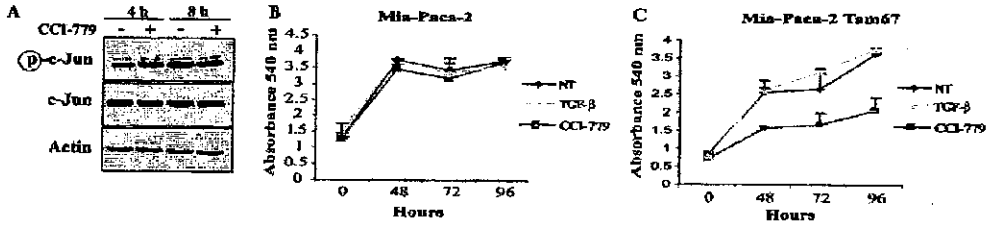

Fig. 4. MiaPaCa-2 cells are resistant to CCI-779 due to a c-Jun-regulated mechanism. (A) Whole-cell extracts of MiaPaCa-2 cells treated in the absence or presence of 2 nM CCI-779 for the indicated time points were analyzed by Western blotting for phospho-c-Jun (Ser63), c-Jun or actin. MiaPaCa-2 cells stably transfected with empty vector (B) or the c-Jun TAM67 mutant (C) were treated without (NT) or with CCI-779 (0.2 nM) or TGF-β. At the indicated time points, crystal violet assays were performed and the absorbance was determined at 540 nm in a Packard plate reader.

increased c-Jun protein levels. Furthermore, we showed that phospho-c-Jun might not mediate CCI-779-induced apoptosis raising questions about its role in CCI-779 action on BxPC-3 cells. Not all pancreatic cancer cells might be sensitive, since the serum-induced proliferation of MiaPaCa-2 cells was unaffected by a similar dose of CCI-779. To our surprise, the resistance of MiaPaCA-2 cells to CCI-779 was significantly lowered by a Jun mutant. The role of Jun in the resistance of cancer cells to drugs is well documented [36].

CCI-779 is currently being investigated in various clinical studies in patients with solid tumors and appears to be well tolerated at doses that exhibit potent anti-tumor activity against different types of refractory neoplasms [4,5,37,38]. Recent studies have indicated that tumors with certain characteristics would be more responsive to CCI-779 than others [21–23]. Indeed, CCI-779 reduced neoplastic proliferation and tumor size in PTEN $^{+/-}$ mice and preferentially blocked growth of PTEN-deficient cancer cells in vitro and in vivo. In contrast to its effects on PTEN-deficient cells, CCI-779 treatment did not affect the growth of mouse embryonic fibroblasts that contained wild-type PTEN. Although PTEN may not be mutated or deleted [39,40], we [31], and others [41], have observed that its expression is frequently either reduced or lost in pancreatic cancer. It is unclear nonetheless, if PTEN is a factor in the sensitivity of pancreatic cancer cells to CCI-779, because even though Panc-1, Capan-1, and AsPC-1 expressed PTEN at a substantially higher level than the BxPC-3 [31], they were all equally sensitive to 0.2 nM CCI-779. A more detailed study is underway to test if these cell lines are differentially sensitive to CCI-779 in a PTEN-dependent manner.

There are indications that a wider variety of tumors than those lacking functional PTEN respond to CCI-779 treatment and that cancer cells overexpressing PI 3-kinase or Akt, for example, are also sensitive [2]. In addition to differences in PTEN expression, the four cell lines we examined differed in the extent to which p70s6k was phosphorylated (Fig. 1A) and Cheng et al. [42], have reported that the AKT2 gene is amplified and overexpressed in the Panc-1 and AsPC-1 cell lines, and that it contributes to the malignant phenotype in 10% of pancreatic carcinomas. Compelling evidence has been presented elsewhere that Akt-mediated sensitivity of glioblastoma and prostate cancer cells to rapamycin and CCI-779 is due to the ability of both inhibitors to downregulate cyclin D1 and c-Myc [43]. Surprisingly, rapamycin did not affect cyclin D1 and c-Myc levels in AsPC-1 and BxPC-3 cells in our previous study [31], and yet both cell lines were highly sensitive to CCI-779 treatment (Fig. 2B). It is pertinent to note that activated Ki-Ras does not appear to be influencing the sensitivity of pancreatic cancer cells to CCI-779 because cells expressing wild-type Ki-Ras (BxPC-3) were inhibited almost as potently as the others that harbored the oncogenic form. In conclusion, the responsiveness of pancreatic cancer cells to CCI-779 could be due to the interplay of a complex set of factors that remain to be identified.

Together, these data indicate that CCI-779 potently blocks the growth of pancreatic cancer cells and establish the basis for evaluating it further as a potential therapeutic agent for treating pancreatic cancer patients.

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(54) Title: ANTINEOPLASTIC COMBINATIONS

(57) Abstract: This invention provides the use of a combination of CCI-779 and an aromatase inhibitor in the treatment of neoplasms.

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ANTINEOPLASTIC COMBINATIONS

BACKGROUND OF THE INVENTION

This invention relates to the treatment of neoplasms.

Rapamycin is a macrocyclic triene antibiotic produced by *Streptomyces hygroscopicus*, which was found to have antifungal activity, particularly against *Candida albicans*, both *in vitro* and *in vivo* [C. Vezina *et al.*, *J. Antibiot.* **28**, 721 (1975); S.N. Sehgal *et al.*, *J. Antibiot.* **28**, 727 (1975); H. A. Baker *et al.*, *J. Antibiot.* **31**, 539 (1978); US Patent 3,929,992; and US Patent 3,993,749]. Additionally, rapamycin alone [US Patent 4,885,171] or in combination with picibanil [US Patent 4,401,653] has been shown to have antitumor activity.

The immunosuppressive effects of rapamycin have been disclosed in FASEB 3, 3411 (1989). Cyclosporin A and FK-506, other macrocyclic molecules, also have been shown to be effective as immunosuppressive agents, therefore useful in preventing transplant rejection [FASEB 3, 3411 (1989); FASEB 3, 5256 (1989); R. Y. Calne *et al.*, Lancet 1183 (1978); and US Patent 5,100,899]. R. Martel et al. [Can. J. Physiol. Pharmacol. 55, 48 (1977)] disclosed that rapamycin is effective in the experimental allergic encephalomyelitis model, a model for multiple sclerosis; in the adjuvant arthritis model, a model for rheumatoid arthritis; and effectively inhibited the formation of IgE-like antibodies.

Rapamycin is also useful in preventing or treating systemic lupus erythematosus [US Patent 5,078,999], pulmonary inflammation [US Patent 5,080,899], insulin dependent diabetes mellitus [US Patent 5,321,009], skin disorders, such as psoriasis [US Patent 5,286,730], bowel disorders [US Patent 5,286,731], smooth muscle cell proliferation and intimal thickening following vascular injury [US Patents 5,288,711 and 5,516,781], adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1], ocular inflammation [US Patent 5,387,589], malignant carcinomas [US Patent 5,206,018], cardiac inflammatory disease [US Patent 5,496,832], and anemia [US Patent 5,561,138].

Rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid (CCI-779) is an ester of rapamycin which has demonstrated significant inhibitory effects on tumor growth in both in vitro and in vivo models. The preparation and use of hydroxyesters of rapamycin, including CCI-779, are disclosed in US Patents 5,362,718 and 6,277,983.

CCI-779 exhibits cytostatic, as opposed to cytotoxic properties, and may delay the time to progression of tumors or time to tumor recurrence. CCI-779 is considered to have a mechanism of action that is similar to that of sirolimus. CCI-779 binds to and forms a complex with the cytoplasmic protein FKBP, which inhibits an enzyme, mTOR (mammalian target of rapamycin, also known as FKBP12-rapamycin associated protein [FRAP]). Inhibition of mTOR's kinase activity inhibits a variety of signal transduction pathways, including cytokine-stimulated cell proliferation, translation of mRNAs for several key proteins that regulate the G1 phase of the cell cycle, and IL-2-induced transcription, leading to inhibition of progression of the cell cycle from G1 to S. The mechanism of action of CCI-779 that results in the G1-S phase block is novel for an anticancer drug.

In vitro, CCI-779 has been shown to inhibit the growth of a number of histologically diverse tumor cells. Central nervous system (CNS) cancer, leukemia (T-cell), breast cancer, prostate cancer, and melanoma lines were among the most sensitive to CCI-779. The compound arrested cells in the G1 phase of the cell cycle.

In vivo studies in nude mice have demonstrated that CCI-779 has activity against human tumor xenografts of diverse histological types. Gliomas were particularly sensitive to CCI-779 and the compound was active in an orthotopic glioma model in nude mice. Growth factor (platelet-derived)-induced stimulation of a human glioblastoma cell line in vitro was markedly suppressed by CCI-779. The growth of several human pancreatic tumors in nude mice as well as one of two breast cancer lines studied in vivo also was inhibited by CCI-779.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides the use of combinations of CCI-779 and an aromatase inhibitor as antineoplastic combination chemotherapy. In particular, these combinations are useful in the treatment of renal cancer, soft tissue cancer, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small lung cell cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer. This invention also provides combinations of CCI-779 and an aromatase inhibitor for use as antineoplastic combination chemotherapy, in which the dosage of either CCI-779 or the aromatase inhibitor or both are used in subtherapeutically effective dosages. Letrozole is the preferred aromatase inhibitor.

This invention also provides use of combinations of 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor as antineoplastic combination chemotherapy. The preparation of 42-O-(2-hydroxy)ethyl rapamycin is described in US Patent 5,665,772, which is hereby incorporated by reference.

As used in accordance with this invention, the term "treatment" means treating a mammal having a neoplastic disease by providing said mammal an effective amount of a combination of CCI-779 and an aromatase inhibitor with the purpose of inhibiting growth of the neoplasm in such mammal, eradication of the neoplasm, or palliation of the mammal.

As used in accordance with this invention, the term "providing," with respect to providing the combination (including simultaneous, separate or sequential administration of the components of the combination), means either directly administering the combination, or administering a prodrug, derivative, or analog of one or both of the components of the combination which will form an effective amount of the combination within the body.

Aromatase is an enzyme which converts androgens to estrone. Estrone can subsequently be converted to estradiol, which has been linked to increased growth or proliferation of estrogen receptor positive carcinoma. As used in accordance with this invention, the term "aromatase inhibitor" means compounds or substances which

inhibit the activity of the enzyme aromatase. Thus the goal of using aromatase inhibitors in chemotherapy is typically to reduce the levels of circulating estradiol, to ultimately inhibit the growth of neoplasms that are estrogen receptor positive. There are two types of aromatase inhibitors; steroidal (type I inhibitors) and non-steroidal inhibitors (type II inhibitors). Examples of steroidal aromatase inhibitors include exemestane, formestane, and atamestane, and the like. Examples of non-steroidal aromatase inhibitors include fadrozole, letrozole, vorozole, anastrozole, YM511 [Susaki *et al, J. Steroid Biochem Molec Biol,* **58**:89-194 (1996) and the like. When used with CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin, letrozole is the preferred aromatase inhibitor.

It is also preferred that the combination of CCI-779 and an aromatase inhibitor be used in an treating estrogen receptor positive carcinoma, particularly estrogen receptor positive breast or ovarian cancer.

The preparation of CCI-779 is described in US Patent 5,362,718, which is hereby incorporated by reference. A regiospecific synthesis of CCI-779 is described in US Patent 6,277,983, which is hereby incorporated by reference. Letrozole is commercially available [*e.g.,* as Femara® (Novartis), CGS 20267].

As used in this invention, the combination regimen can be given simultaneously or can be given in a staggered regimen, with CCI-779 being given at a different time during the course of chemotherapy than an aromatase inhibitor. This time differential may range from several minutes, hours, days, weeks, or longer between administration of the two agents. Therefore, the term combination does not necessarily mean administered at the same time or as a unitary dose, but that each of the components are administered during a desired treatment period. The agents may be administered by the same or different routes. For example, one component may be administered orally, while the other parenterally. These combination can be administered daily, weekly, or even once monthly. As typical for chemotherapeutic regimens, a course of chemotherapy may be repeated several weeks later, and may follow the same timeframe for administration of the two agents, or may be modified based on patient response.

The combinations of the invention may be in the form of a kit of parts. The invention therefore includes a product containing (a) CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin and (b) an aromatase inhibitor as a combined preparation for simultaneous, separate or sequential use in treating a neoplasm in a mammal in need thereof. The invention also includes a pharmaceutical pack containing a course of treatment of a neoplasm for one individual mammal, wherein the pack contains (a) units of CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin in unit dosage form and (b) units of an aromatase inhibitor in unit dosage form.

For the combination of CCI-779 and letrozole, it is preferred that both components are provided orally, and that the initial oral dosage of CCI-779 will in the range of about 2 to about 100 mg/day, 5 mg/day to 75 mg/day, 10 mg/day to 50 mg/day, 15 mg/day to 35 mg/day, or about 20 mg/day to 25 mg/day (on days that it is provided) and the initial oral dose of letrozole will be about 0.1 to 10 mg daily, 0.5 mg to 5 mg, or 1 to 3 mg, or about 2.5 mg (on days that it is provided).

When the combination of CCI-779 and letrozole are provided orally, it is preferred that the CCI-779 and letrozole are provided daily, or that the CCI-779 is provided 5 times every two weeks, while the letrozole is provided daily.

As typical with chemotherapy, dosage regimens are closely monitored by the treating physician, based on numerous factors including the severity of the disease, response to the disease, any treatment related toxicities, age, health of the patient, and other concomitant disorders or treatments. After one or more treatment cycles, the dosages can be adjusted upwards or downwards depending on the results obtained and the side effects observed.

In providing chemotherapy, multiple agents having different modalities of action are typically used as part of a chemotherapy "cocktail." It is anticipated that the combinations of this invention will be used as part of a chemotherapy cocktail that may contain one or more additional antineoplastic agents depending on the nature of the neoplasia to be treated.

Oral formulations containing the active compounds of this invention may comprise any conventionally used oral forms, including tablets, capsules, buccal forms, troches, lozenges and oral liquids, suspensions or solutions. Capsules may

contain mixtures of the active compound(s) with inert fillers and/or diluents such as the pharmaceutically acceptable starches (e.g. corn, potato or tapioca starch), sugars, artificial sweetening agents, powdered celluloses, such as crystalline and microcrystalline celluloses, flours, gelatins, gums, etc. Useful tablet formulations may be made by conventional compression, wet granulation or dry granulation methods and utilize pharmaceutically acceptable diluents, binding agents, lubricants, disintegrants, surface modifying agents (including surfactants), suspending or stabilizing agents, including, but not limited to, magnesium stearate, stearic acid, talc, sodium lauryl sulfate, microcrystalline cellulose, carboxymethylcellulose calcium, polyvinylpyrrolidone, gelatin, alginic acid, acacia gum, xanthan gum, sodium citrate, complex silicates, calcium carbonate, glycine, dextrin, sucrose, sorbitol, dicalcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, talc, dry starches and powdered sugar. Preferred surface modifying agents include nonionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. Oral formulations herein may utilize standard delay or time release formulations to alter the absorption of the active compound(s). The oral formulation may also consist of administering the active ingredient in water or a fruit juice, containing appropriate solubilizers or emulsifiers as needed.

Particularly suitable oral formulations for rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid are disclosed in USSN 60/411,264 and PCT/US03/29228, which are hereby incorporated by reference. Such an oral formulation contains a granulation prepared using a wet granulation process. The granulation contains CCI-779, a water soluble polymer, a pH modifying agent, a surfactant, and an antioxidant. In one embodiment, the formulation contains from 0.1 to 30%, from 0.5 to 25%, from 1 to 20%, from 5 to 15%, or from 7 to 12% (wt/wt) CCI-779, from 0.5 to 50%, from 1 to 40%, from 5 to 35%, from 10 to 25%, or from 15 to 20% (wt/wt) water soluble polymer, from 0.5 to 10%, 1 to 8%, or 3 to 5% (wt/wt) surfactant, and from 0.001% to 1%, 0.01% to 1%, or 0.1% to 0.5% (wt/wt)

antioxidant. However, other embodiments may contain more, or less, of these components.

The oral formulation may also contain suitable chelating agents, fillers, binders, surfactants, and the like to facilitate the granulation and tableting process. It is preferred that the wet granulation be performed with a hydroalcoholic solvent system comprising water and an alcohol, with ethanol being the preferred alcoholic component.

Typical water soluble polymers include, but are not limited to, polyvinylpyrrolidone (PVP), hydroxypropylmethylcellulose (HPMC), polyethylene glycol (PEG), and cyclodextrin or mixtures thereof. It is preferred that the water-soluble polymer is PVP, and having a molecular weight of between 2.5 and 60 kilodaltons. Any given oral formulation useful in the invention may contain multiple ingredients of each class of component. For example, an oral formulation containing an antioxidant may contain one or more antioxidants as the antioxidant component.

Acceptable pH modifying agents include, but are not limited to citric acid, sodium citrate, dilute HCl, and other mild acids or bases capable of buffering a solution containing CCI-779 to a pH in the range of about 4 to about 6. Acceptable antioxidants include, but are not limited to, citric acid, d,l-α-tocopherol, BHA, BHT, monothioglycerol, ascorbic acid, and propyl gallate. It is expected that the antioxidants of the oral formulations used in this invention will be used in concentrations ranging from 0.001% to 3% wt/wt. Chelating agents, and other materials capable of binding metal ions, such as ethylene diamine tetra acetic acid (EDTA) and its salts are capable of enhancing the stability of CCI-779. Surfactants may include polysorbate 80, sodium lauryl sulfate, sodium dodecyl sulfate, salts of bile acids (taurocholate, glycocholate, cholate, deoxycholate, etc.) that may be combined with lecithin. Alternatively, ethoxylated vegetable oils, such as Cremophor EL, vitamin E tocopherol propylene glycol succinate (Vitamin E TGPS), polyoxyethylene-polyoxypropylene block copolymers, and poloxamers. Binders, fillers, and disintegrants such as sucrose, lactose, microcrystalline cellulose, croscarmellose sodium, magnesium stearate, gum acacia, cholesterol, tragacanth, stearic acid, gelatin, casein, lecithin (phosphatides), carboxymethylcellulose calcium,

carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethycellulose phthalate, noncrystalline cellulose, cetostearyl alcohol, cetyl alcohol, cetyl esters wax, dextrates, dextrin, lactose, dextrose, glyceryl monooleate, glyceryl monostearate, glyceryl palmitostearate, polyoxyethylene alkyl ethers, polyethylene glycols, polyoxyethylene castor oil derivatives, polyoxyethylene stearates, and polyvinyl alcohol, and the like may also be incorporated into the oral formulation.

The oral formulation useful in the method of the invention can be prepared by preparing an alcoholic solution comprising CCI-779 and an antioxidant, and an aqueous solution comprising a water-soluble polymer, a surfactant, and a pH modifier, in sufficient quantity to adjust the pH of the aqueous solution to 4 to 6. Suitable alcohols include methanol, ethanol, isopropanol, and the like, where ethanol is the preferred alcohol. The solutions were mixed and added to a mixer containing intragranular excipients. Alternatively, the alcoholic and aqueous solutions can be added separately without mixing with each other. Such intragranular excipients comprise binders and fillers to promote dissolution enhancement. Typical intragranular excipients may include, but are not limited to, microcrystalline cellulose, lactose, and croscarmellose sodium. The solid intragranular excipients are granulated with the solutions in the mixer until a uniform granulation is achieved. The mixer can be a blender with intensifying bar, a low shear granulator or a high shear granulator. The granulation is dried in a fluid bed dryer at approximately 50°C, and milled using a suitable milling device, such as a Fitz mill. The wet granulation and drying can be done in a fluid bed granulator/dryer. The wet granulation can be dried using a tray drying oven. If desired, the dried granulation can be further blended with extragranular fillers and binders, such as microcrystalline cellulose, croscarmellose sodium, and magnesium stearate in a blender, such as a V-blender, before compression into tablets.

Alternatively, some of the water-soluble polymer can be contained in the intragranular excipients, and the aqueous and alcoholic solutions added to the mixer containing the intragranular excipients stepwise. For example, the order of addition to the mixer may be one half of the aqueous solution, followed by the entire alcoholic

solution, and then the remainder of the aqueous solution. Other sequences of addition are possible and permissible in these solid oral formulations.

In some cases it may be desirable to administer the compounds directly to the airways in the form of an aerosol.

The compounds may also be administered parenterally or intraperitoneally. Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

Particularly suitable injectable formulations for rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid are disclosed in US Patent Application No. 10/626,943 and PCT/US03/223276, which are hereby incorporated by reference. In this embodiment, the injectable formulation useful in the invention provides a CCI-779 cosolvent concentrate containing an parenterally acceptable solvent and an antioxidant as described above and a parenteral formulation containing CCI-779, composed of CCI-779, an parenterally acceptable cosolvent, an antioxidant, a diluent solvent, and a surfactant. Any given formulation useful in this invention may contain multiple ingredients of each class of component. For example, a parenterally acceptable solvent can include a non-alcoholic solvent, an alcoholic solvent, or mixtures thereof. Examples of suitable non-alcoholic solvents include,

e.g., dimethylacetamide, dimethylsulfoxide or acetonitrile, or mixtures thereof. "An alcoholic solvent," may contain one or more alcohols as the alcoholic solvent component of the formulation. Examples of solvents useful in the formulations invention include, without limitation, ethanol, propylene glycol, polyethylene glycol 300, polyethylene glycol 400, polyethylene glycol 600, polyethylene glycol 1000, or mixtures thereof. These cosolvents are particularly desirable because degradation via oxidation and lactone cleavage occurs to a lower extent for these cosolvents. Further, ethanol and propylene glycol can be combined to produce a less flammable product, but larger amounts of ethanol in the mixture generally result in better chemical stability. A concentration of 30 to 100%v/v of ethanol in the mixture is preferred.

In this embodiment, the stability of CCI-779 in parenterally acceptable alcoholic cosolvents is enhanced by addition of an antioxidant to the formulation. Acceptable antioxidants include, but are not limited to, citric acid, d,l-α-tocopherol, BHA, BHT, monothioglycerol, ascorbic acid, propyl gallate, and mixtures thereof. Generally, the parenteral formulations useful in this embodiment of the invention will contain an antioxidant component(s) in a concentration ranging from 0.001% to 1% w/v, or 0.01% to 0.5% w/v, of the cosolvent concentrate, although lower or higher concentrations may be desired. Of the antioxidants, d,l-α-tocopherol is particularly desirable and is used at a concentration of 0.01 to 0.1% w/v with a preferred concentration of 0.075% w/v of the cosolvent concentrate.

In certain embodiments, the antioxidant component of the formulation of the invention also exhibits chelating activity. Examples of such chelating agents include, e.g., citric acid, acetic acid, and ascorbic acid (which may function as both a classic antioxidant and a chelating agent in the present formulations). Other chelating agents include such materials as are capable of binding metal ions in solution, such as ethylene diamine tetra acetic acid (EDTA), its salts, or amino acids such as glycine are capable of enhancing the stability of CCI-779. In some embodiments, components with chelating activity are included in the formulations of the invention as the sole "antioxidant component". Typically, such metal-binding components, when acting as chelating agents are used in the lower end of the range of concentrations for the antioxidant component provided herein. In one example, citric

acid enhanced the stability of CCI-779 when used at a concentration of less than 0.01% w/v. Higher concentrations are less stable solutions and thus, less desirable for products to be subject to long-term storage in liquid form. Additionally, such chelating agents may be used in combination with other antioxidants as part of the antioxidant component of the invention. For example, an acceptable formulation may contain both citric acid and d,l-α-tocopherol. Optimal concentrations for the selected antioxidant(s) can be readily determined by one of skill in the art, based upon the information provided herein.

Advantageously, in certain embodiments of the parenteral formulations useful in the invention, precipitation of CCI-779 upon dilution with aqueous infusion solutions or blood is prevented through the use of a surfactant contained in the diluent solution. The most important component of the diluent is a parenterally acceptable surfactant. One particularly desirable surfactant is polysorbate 20 or polysorbate 80. However, one of skill in the art may readily select other suitable surfactants from among salts of bile acids (taurocholate, glycocholate, cholate, deoxycholate, etc.) which are optionally combined with lecithin. Alternatively, ethoxylated vegetable oils, such as a pegylated castor oil [e.g., such as PEG-35 castor oil which is sold, e.g., under the name Cremophor EL, BASF], vitamin E tocopherol propylene glycol succinate (Vitamin E TGPS), and polyoxyethylene-polyoxypropylene block copolymers can be used in the diluent as a surfactant, as well as other members of the polysorbate family such as polysorbate 20 or 60 Other components of the diluent may include water, ethanol, polyethylene glycol 300, polyethylene 400, polyethylene 600, polyethylene 1000, or blends containing one or more of these polyethylene glycols, propylene glycol and other parenterally acceptable cosolvents or agents to adjust solution osmolarity such as sodium chloride, lactose, mannitol or other parenterally acceptable sugars, polyols and electrolytes. It is expected that the surfactant will comprise 2 to 100% w/v of the diluent solution, 5 to 80% w/v, 10 to 75% w/v, 15 to 60 % w/v, and preferably, at least 5% w/v, or at least 10% w/v, of the diluent solution.

A parenteral formulation useful in the invention can be prepared as a single solution, or preferably can be prepared as a cosolvent concentrate containing CCI-

779, an alcoholic solvent, and an antioxidant, which is subsequently combined with a diluent that contains a diluent solvent and suitable surfactant. Prior to use, the cosolvent concentrate is mixed with a diluent comprising a diluent solvent, and a surfactant. When CCI-779 is prepared as a cosolvent concentrate according to this invention, the concentrate can contain concentrations of CCI-779 from 0.05 mg/mL, from 2.5 mg/mL, from 5 mg/mL, from 10 mg/mL or from 25 mg/mL up to approximately 50 mg/ml. The concentrate can be mixed with the diluent up to approximately 1 part concentrate to 1 part diluent, to give parenteral formulations having concentrations of CCI-779 from 1mg/mL, from 5 mg/mL, from 10 mg/mL, from 20 mg/mL, up to approximately 25 mg/ml. For example the concentration of CCI-779 in the parenteral formulation may be from about 2.5 to 10 mg/mL. This invention also covers the use of formulations having lesser concentrations of CCI-779 in the cosolvent concentrate, and formulations in which one part of the concentrate is mixed with greater than 1 part of the diluent, e.g., concentrate: diluent in a ratio of about 1:1.5, 1:2, 1:3, 1:4 ,1:5, or 1:9 v/v and so on, to CCI-779 parenteral formulations having a CCI-779 concentration down to the lowest levels of detection.

Typically the antioxidant may comprise from about 0.0005 to 0.5% w/v of the formulation. The surfactant may for example comprise from about 0.5% to about 10% w/v of the formulation. The alcoholic solvent may for example comprise from about 10% to about 90% w/v of the formulation.

The parenteral formulations useful in this invention can be used to produce a dosage form that is suitable for administration by either direct injection or by addition to sterile infusion fluids for intravenous infusion.

For the purposes of this disclosure, transdermal administrations are understood to include all administrations across the surface of the body and the inner linings of bodily passages including epithelial and mucosal tissues. Such administrations may be carried out using the present compounds, or pharmaceutically acceptable salts thereof, in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing the active compound and a carrier that is inert to the

active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semi-permeable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerin. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

The following examples are illustrative of the present invention, but are not a limitation thereof.

Example 1 - CCI-779 in combination with an aromatase inhibitor in a neoplasm

The combination of CCI-779 and letrozole in postmenopausal women with locally advanced or metastatic breast cancer is being evaluated in this clinical trial.

Fifty-five patients (pts) were enrolled. Randomization is in a 1:1:1 ratio (~30 evaluable pts/arm), letrozole alone: letrozole with CCI daily (CCI daily arm): letrozole with CCI daily for 5 days every 2 weeks (CCI intermittent arm). All pts receive 2.5 mg letrozole daily.

Initially, 6 patients each were enrolled at high dose (HD) schedules, 25 mg CCI daily and 75 mg CCI intermittent; 3 patients in each arm had toxicity that resulted in dose delay/reduction or discontinuation. Thus, the protocol was amended and doses were reduced to low dose (LD) schedules, 10 mg CCI daily and 30 mg CCI intermittent. As of 01 Dec 2003, 12 and 23 patients were enrolled in the HD and LD schedules, respectively. The median age was 60 yrs (range, 42-81). Safety data are

available for 12 pts treated with the HD schedules (25 mg, 6 pts; 75 mg, 6 pts), 11 pts treated with the LD schedules (10 mg, 4 pts; 30 mg, 7 pts), and 12 pts treated with letrozole alone. The most frequently occurring grade 3-4 CCI related toxicity was stomatitis for the HD schedules (2/6 pts, 1/6 pts) and diarrhea for the LD schedules (0 pts, 1/7 pts). No grade 3-4 toxicities were reported for pts treated with letrozole alone. Of 55 pts, 7 have been on study for 40+ wk. Preliminary tumor responses (RECIST) are available for 19 evaluable pts. CCI pts (n=13) had 1 complete response (HD schedule), 3 partial responses (HD schedules), 9 stable disease (6 pts on HD schedules, 3 on LD schedules, incl 4 pts on HD schedules with SD ≥24 wk). Letrozole-alone pts (n=6) had 2 PR and 4 SD (including 1 pt with SD ≥24 wk)

The combination of 10 mg CCI daily or 30 mg CCI intermittent with letrozole showed favorable results for tolerability.

Example 2

Tablets each containg 2.5 mg of letrozole and also tablets each containing a dose of CCI-779 as mentioned in Example 1 are packaged in a container to provide a course of treatment for a patient.

All patents, patent applications, articles, and other documents referenced herein are incorporated by reference. It will be clear to one of skill in the art that modifications can be made to the specific embodiments described herein without departing from the scope of the invention.

What is claimed is:

1. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising CCI-779 and an aromatase inhibitor.

2. The method according to claim 1, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestaue, fadrozole, letrozole, vorozole, and anastrozole.

3. The method according to claim 2, wherein the aromatase inhibitor is letrozole.

4. The method according to claim 1, 2 or 3, wherein the neoplasm is selected from the group consisting of renal cancer,

5. The method according to claim 1, 2 or 3, wherein the neoplasm is soft tissue sarcoma.

6. The method according to claim 1, 2 or 3, wherein the neoplasm is breast cancer.

7. The method according to claim 1, 2 or 3, wherein the neoplasm is a neuroendocrine tumor of the lung.

8. The method according to claim 1, 2 or 3, wherein the neoplasm is cervical cancer.

9. The method according to claim 1, 2 or 3, wherein the neoplasm is uterine cancer.

10. The method according to claim 1, 2 or 3, wherein the neoplasm is a head and neck cancer.

11. The method according to claim 1, 2 or 3, wherein the neoplasm is glioma.

12. The method according to claim 1, 2 or 3, wherein the neoplasm is non-small cell lung cancer.

13. The method according to claim 1, 2 or 3, wherein the neoplasm is prostate cancer.

14. The method according to claim 1, 2 or 3, wherein the neoplasm is pancreatic cancer.

15. The method according to claim 1, 2 or 3, wherein the neoplasm is lymphoma.

16. The method according to claim 1, 2 or 3, wherein the neoplasm is melanoma.

17. The method according to claim 1, 2 or 3, wherein the neoplasm is small cell lung cancer.

18. The method according to claim 1, 2 or 3, wherein the neoplasm is ovarian cancer.

19. The method according to claim 1, 2 or 3, wherein the neoplasm is colon cancer.

20. The method according to claim 1, 2 or 3, wherein the neoplasm is esophageal cancer.

21. The method according to claim 1, 2 or 3, wherein the neoplasm is gastric cancer.

22. The method according to claim 1, 2 or 3, wherein the neoplasm is leukemia.

23. The method according to claim 1, 2 or 3, wherein the neoplasm is colorectal cancer.

24. The method according to claim 1, 2 or 3, wherein the neoplasm is unknown primary cancer.

25. A method of treating a neoplasm in a mammal in need thereof, which comprises providing to said mammal an effective amount of a combination comprising CCI-779 and an aromatase inhibitor, wherein either CCI-779, the aromatase inhibitor, or both are provided in subtherapeutically effective amounts.

26. The method according to claim 25 in which CCI-779 is provided in a subtherapeutically effective amount.

27. The method according to claim 25 in which the aromatase inhibitor is provided in a subtherapeutically effective amount.

28. The method according to claim 25 in which both CCI-779 and the aromatase inhibitor are provided in subtherapeutically effective amounts.

29. The method according to any one of claims 25 to 28, wherein the aromatase inhibitor is letrozole.

30. An antineoplastic combination comprising an antineoplastic effective amount of a combination of CCI-779 and an aromatase inhibitor.

31. A method of treating a neoplasm in a mammal in need thereof, comprising providing to said mammal an effective amount of a combination comprising 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor.

32. A method of treating an estrogen receptor positive carcinoma in a mammal in need thereof, comprising providing to said mammal an effective amount of a combination comprising CCI-779 and an aromatase inhibitor.

33. The method according to claim 32, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

34. The method according to claim 33, wherein the aromatase inhibitor is letrozole.

35. The method according to claim 32 or 33, wherein the estrogen receptor positive carcinoma is of the breast cancer or ovarian cancer.

36. The method according to claim 35, wherein the aromatase inhibitor is letrozole.

37. The method according to any one of claims 32 to 36, wherein the CCI-779 or the aromatase inhibitor, or both are provided in subtherapeutically effective amounts.

38. A method of treating an estrogen receptor positive carcinoma in a mammal in need thereof, comprising providing to said mammal an effective amount

of a combination comprising 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor.

39. Use of CCI-779 and an aromatase inhibitor in preparing a medicament for treating a neoplasm in a mammal in need thereof.

40. Use according to claim 39, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

41. Use according to claim 40, wherein the aromatase inhibitor is letrozole.

42. Use according to claim 39, wherein the neoplasm is selected from the group consisting of renal cancer, soft tissue sarcoma, breast cancer, neuroendocrine tumor of the lung, cervical cancer, uterine cancer, head and neck cancer, glioma, non-small cell lung cancer, prostate cancer, pancreatic cancer, lymphoma, melanoma, small cell lung cancer, ovarian cancer, colon cancer, esophageal cancer, gastric cancer, leukemia, colorectal cancer, and unknown primary cancer.

43. Use according to any of claims 39 to 42, wherein either CCI-779, the aromatase inhibitor, or both are provided in subtherapeutically effective amounts.

44. Use of 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor in preparing a medicament for treating a neoplasm in a mammal in need thereof.

45. Use of CCI-779 and an aromatase inhibitor in preparing a medicament for treating an estrogen receptor positive carcinoma in a mammal in need thereof.

46. Use according to claim 45, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

47. Use according to claim 45 or 46, wherein the aromatase inhibitor is letrozole.

48. Use according to claim 45 or 46, wherein the estrogen receptor positive carcinoma is of the breast cancer or ovarian cancer.

49. Use according to claim 48, wherein the aromatase inhibitor is letrozole.

50. Use according to any of claims 45 to 49, wherein the CCI-779 or the aromatase inhibitor, or both are provided in subtherapeutically effective amounts.

51. Use of 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor in preparing a medicament for treating an estrogen receptor positive carcinoma in a mammal in need thereof.

52. A product containing (a) CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin and (b) an aromatase inhibitor as a combined preparation for simultaneous, separate or sequential use in treating a neoplasm in a mammal in need thereof.

53. The product according to claim 52, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

54. The product according to claim 53, wherein the aromatase inhibitor is letrozole.

55. Use of CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin in in the manufacture of a medicament for treating a neoplasm in a mammal with an aromatase inhibitor.

56. Use of an aromatase inhibitor in the manufacture of a medicament for treating a neoplasm in a mammal with CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin.

57. The use according to claim 55 or 56, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

58. The use according to claim 57, wherein the aromatase inhibitor is letrozole.

59. A pharmaceutical pack containing a course of treatment of a neoplasm for one individual mammal, wherein the pack contains (a) units of CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin in unit dosage form and (b) units of an aromatase inhibitor in unit dosage form.

60. A pharmaceutical pack according to claim 59, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

61. A pharmaceutical pack according to claim 59, wherein the aromatase inhibitor is letrozole.

62. A pharmaceutical composition useful in treating a neoplasm in a mammal in need thereof, the composition comprising (a) CCI-779 or 42-O-(2-hydroxy)ethyl rapamycin and (b) an aromatase inhibitor in combination or association with a pharmaceutically acceptable carrier.

63. The pharmaceutical composition according to claim 62, wherein the aromatase inhibitor is selected from the group consisting of exemestane, formestane, atamestane, fadrozole, letrozole, vorozole, and anastrozole.

64. The pharmaceutical composition according to claim 63, wherein the aromatase inhibitor is letrozole.

65. An antineoplastic combination comprising an antineoplastic effective amount of a combination of 42-O-(2-hydroxy)ethyl rapamycin and an aromatase inhibitor.

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(54) Title: ANTINEOPLASTIC COMBINATIONS COMPRISING A RAPAMYCIN DDERIOVATIVE AND AN AROMATSE INIIIBITOR

(57) Abstract: This invention provides the use of a combination of CCI-779 or 42-0-(2-hydroxy) ethylrapanycin and an aromatase inhibitor and the use thereof for the manufacture of a medicament for the treatment of neoplasms.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/436 A61P35/00 A61K31/4196 A61K31/5685 A61K31/4188 A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/066019 A (NOVARTIS ERFIND VERWALT GMBH ; NOVARTIS AG (CH); LANE HEIDI (CH); O'RE) 29 August 2002 (2002-08-29) page 1, line 16 - line 17 the whole document in particular page 2, last paragraph - page 3, paragraph 1 page 4, line 3 - line 5 page 4, line 5.1 - line 5.2 page 5, paragraph 2 - paragraph 3 p. 5, 'chemotherapeutic agent', 'aromatase inhibitor' p. 6, 'aromatase inhibitor' page 18 page 19, paragraph 1 page 21, last paragraph - page 22, paragraph 1 claims	1-65
X	WO 02/098416 A (WYETH CORP) 12 December 2002 (2002-12-12) the whole document in particular page 13, last paragraph - page 14, paragraph 1 claims	1-65
X	WO 02/080975 A (WYETH CORP) 17 October 2002 (2002-10-17) in particular the whole document page 13, line 33 claims	1-65
A	WO 02/40000 A (WYETH CORP) 23 May 2002 (2002-05-23) the whole document	
P,X	WO 03/020266 A (WYETH CORP) 13 March 2003 (2003-03-13) the whole document in particular page 9, line 5	1-65

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/006354

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-29 and 31-38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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(71) Applicant *(for all designated States except US)*: **WYETH** [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).

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(54) Title: ANTINEOPLASTIC COMBINATIONS COMPRISING A RAPAMYCIN DERIVATIVE AND AN AROMATASE INHIBITOR

(57) Abstract: This invention provides a combination of CCI-779 or 42-0-(2-hydroxy) ethylrapanycin and an aromatase inhibitor and the use thereof for the manufacture of a medicament for the treatment of neoplasms.

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Rapamycin-Induced Endothelial Cell Death and Tumor Vessel Thrombosis Potentiate Cytotoxic Therapy against Pancreatic Cancer

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ABSTRACT

Purpose: **Despite current chemotherapies, pancreatic cancer remains an uncontrollable, rapidly progressive disease. Here, we tested an approach combining a recently described antiangiogenic drug, rapamycin, with standard gemcitabine cytotoxic therapy on human pancreatic tumor growth.**

Experimental Design: **Tumor growth was assessed in rapamycin and gemcitabine-treated nude mice orthotopically injected with metastatic L3.6pl human pancreatic cancer cells. H&E staining was performed on tumors, along with Ki67 staining for cell proliferation and immunohistochemical terminal deoxynucleotidyl transferase-mediated nick end labeling and CD31 analysis. Rapamycin-treated tumor vessels were also directly examined in dorsal skin-fold chambers for blood flow after thrombosis induction. Cell death in human umbilical vein endothelial cells was assessed by flow cytometry after annexin-V staining.**

Results: **Rapamycin therapy alone inhibited tumor growth and metastasis more than gemcitabine, with remarkable long-term tumor control when the drugs were combined. Mechanistically, H&E analysis revealed tumor vessel endothelium damage and thrombosis with rapamycin treatment. Indeed, dorsal skin-fold chamber analysis of rapamycin-treated tumors showed an increased susceptibility of tumor-specific vessels to thrombosis. Furthermore, terminal deoxynucleotidyl transferase-mediated nick end labeling/CD31 double staining of orthotopic tumors demonstrated apoptotic endothelial cells with rapamycin treatment, which also occurred with human umbilical vein endothelial cells *in vitro*. In contrast, gemcitabine was not antiangiogenic and, despite its known cytotoxicity, did not reduce proliferation in orthotopic tumors; nevertheless, rapamycin did reduce tumor proliferation.**

Conclusions: **Our data suggest a novel mechanism whereby rapamycin targets pancreatic tumor endothelium for destruction and thrombosis. We propose that rapamycin-based vascular targeting not only reduces tumor vascularization, it decreases the number of proliferating tumor cells to be destroyed by gemcitabine, thus introducing a new, clinically feasible strategy against pancreatic cancer.**

INTRODUCTION

Pancreatic cancer remains a major unsolved health problem with an estimated overall 5-year survival rate of only 1–4%, making it one of the leading causes of cancer-related mortality. Presently, over 80% of these patients have locally advanced or metastatic disease at the time of diagnosis, which excludes even the possibility of curative surgery (1–3). Moreover, tumor control in these cases is not normally successful with currently available systemic chemotherapy. In fact, a response rate of one-quarter or less can be expected with standard chemotherapy, with a dismal median survival of <6 months (4, 5). With this background, the question is what different approach, besides standard cytotoxic therapy, could be used to attack this aggressive, highly resistant form of cancer. One key to this question could lie in the emerging realization that pancreatic tumors may be susceptible to antiangiogenic therapy (6–9).

Indeed, recent clinical studies suggest that pancreatic cancer is highly angiogenesis dependent. More specifically, clinical prognostic data indicate that expression of proangiogenic factors such as vascular endothelial growth factor (VEGF), epidermal growth factor, and thymidine phosphorylase positively correlates with a higher relapse rate and shorter patient survival (10, 11). Furthermore, a high density of microvessels within pancreatic tumors is a prognostic factor for early disease progression (10, 12, 13). Therefore, we hypothesized in the current study that pancreatic cancer progression may be sensitive to antiangiogenic therapy, particularly when combined with a cytotoxic agent. With regard to antiangiogenic therapy, we chose to test whether the mammalian target of rapamycin inhibitor rapamycin could be effective against metastasizing pancreatic cancer. This choice was based on our recent study showing that rapamycin is a potent antiangiogenic substance, working most effectively at noncytotoxic, nanomolar concentrations (14). The antiangiogenic activity of rapamycin is due, at least in part, to

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Note: C. Bruns and G. Koehl contributed equally to this work. Present address for C. Bruns, M. Guba, M. Yezhelyev, H. Seeliger, and K. Jauch is the Department of Surgery, Ludwig-Maximilians University, Munich, Germany.

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inhibition of VEGF production and blockage of VEGF-mediated stimulation of endothelial cells. However, a clinically relevant corollary to this initial study was that nests of tumor cells not requiring angiogenesis continued to exist and eventually progressed into larger masses once the rapamycin therapy was discontinued. Therefore, in the present study, we tested the possibility that the combination of cytotoxic chemotherapy with rapamycin could better control or reduce these nests of tumor cells over a long-term period. In the situation of pancreatic carcinoma, our approach combines daily rapamycin treatment with repeated use of the best available cytotoxic drug for this disease, gemcitabine. Mechanistically, intracellular phosphorylation of gemcitabine produces di- and triphosphate molecular forms capable of acting as a fraudulent base in DNA and inhibiting DNA synthesis-dependent ribonucleotide reductase (15), together producing a strong cytotoxic effect.

Using a model of metastatic human pancreatic cancer in nude mice, our present study shows that antiangiogenic therapy with rapamycin alone has an antitumor effect exceeding that of gemcitabine and that the combination of rapamycin and gemcitabine dramatically reduces long-term tumor growth and the development of metastases. Mechanistically, our data suggest that rapamycin affects tumor vascularization and decreases the number of proliferating tumor cells, thereby enhancing the effectiveness of gemcitabine's cytotoxic activity against tumor growth. Moreover, this study provides the first evidence that tumor control achieved with rapamycin is associated with tumor vessel thrombosis related to the death of endothelial cells. Therefore, rapamycin promotion of thrombosis in new pancreatic tumor vessels introduces a novel mechanism potentially contributing to its anticancer action.

MATERIALS AND METHODS

Pancreatic Cancer Model and Treatment Regimens. The highly metastatic human pancreatic cancer cell line L3.6pl was maintained in cultures supplemented as described previously (16). Using animal procedures approved by the local authorities, 1×10^6 L3.6pl tumor cells were orthotopically implanted in the subcapsular region of the pancreas of male athymic 8–12-week-old nude mice (BALB/c nu/nu; Charles River, Sulzfeld, Germany), as detailed previously (16). After implantation, tumors were allowed to grow for 7 days before treatment initiation. At the start of treatment, the median tumor volume in sacrificed mice is typically 18 mm^3 (17). Tumor-bearing mice were randomized and subjected to the following treatment: (*a*) 1.5 mg/kg/day rapamycin (5 mg/ml stock solution; Wyeth Pharma, Münster, Germany) by i.p. injection; (*b*) biweekly 50 or 100 mg/kg gemcitabine (Gemzar 1000 powder dissolved in 0.9% saline; Lilly, Giessen, Germany) by i.p. injection; (*c*) i.p. combination of 1.5 mg/kg/day rapamycin with either 50 or 100 mg/kg gemcitabine biweekly; or (*d*) i.p. injections of 0.9% saline control solution at corresponding time points (rapamycin and gemcitabine were diluted for injection with 0.9% saline).

Mice were sacrificed on day 28 after tumor cell injection in experiments aimed at measuring tumor growth at a fixed point. Excised pancreatic tumors were weighed and measured. The tumor volume was then calculated using the formula $V = \pi/6(a \times b \times c)$, where a, b, and c represent the length, width, and height of the mass. For H&E staining and immunohistochemical analysis, half of the primary tumor was fixed in formalin for paraffin embedding, and the other half was prepared for frozen sectioning. Metastatic L3.6pl tumor growth was also evaluated. For metastases in the liver, macroscopically visible tumor nodules (>1 mm) were noted on the liver surface. Furthermore, enlarged regional (celiac and para-aortic) lymph nodes were recorded. Liver and lymph node tissue were excised and processed to confirm metastases by H&E staining.

In one experiment, all mice in the control group and 6 of 10 mice from each treatment group were sacrificed as usual on day 28 after orthotopic tumor cell injection. The pancreatic tumor and metastases were analyzed as described above. However, the remaining four mice in each treatment group were kept alive to obtain long-term data, and drug therapy was continued. Those mice in good condition were kept alive until day 60; any mice showing progressive tumor growth, signs of tumor burden, drug toxicity (weight loss $\geq$ 20%), or reduction in mobility to easily access food and water were sacrificed. To monitor cancer progression, the tumor mass was held between the fingers and moved to the abdominal surface, where its size could be measured using a caliper. Tumor volume was estimated by the formula $V = \pi/6(a^2 \times b)$, where a is the width of the tumor, and b is the length of the tumor.

Immunohistochemical Staining for Ki67, Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL), and CD31. Cell proliferation analysis was performed on paraffin-embedded tissues with standard Ki67 staining techniques (18, 19). Briefly, a mouse antihuman Ki67 monoclonal antibody (DAKO A/S, Glostrup, Denmark) was used in the primary reaction. The DAKO EnVision System, containing a secondary horseradish peroxidase-conjugated antimouse antibody complex, was used with 3,3′-diaminobenzidine to detect Ki67. Sections were counterstained with Gill's hematoxylin. To quantify the amount of proliferation, all Ki67-positive and -negative cells were counted in 10 random high-power fields (0.159 mm^2 at ×100 magnification) per slide.

Colorimetric immunohistochemical staining for apoptotic cell death (TUNEL) was performed on paraffin-embedded tissue sections using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) and the AEC substrate pack (Biogenex, Hamburg, Germany), according to the manufacturers' instructions.

Analysis of apoptotic endothelial cells was performed on frozen tissue sections using a previously described immunofluorescent CD31/TUNEL double-labeling technique (17). Briefly, sections were first incubated with a rat antimouse CD31/platelet/endothelial-cell adhesion molecule-1-monoclonal-antibody (PharMingen, San Diego, CA), followed by staining with Texas red-conjugated goat antirat IgG (Jackson ImmunoResearch Laboratories, West Grove, CA). A TUNEL procedure was subsequently performed using the Fluorescein Apoptosis Detection System (Promega, Madison, WI).

Dorsal Skin-Fold Chamber (DSFC) Analysis. Tumor angiogenesis was analyzed *in vivo* via the transparent DSFC model, as described previously (20, 21). Chambers were inoculated with 1×10^5 L3.6pl cells. The day after tumor inoculation, mice were treated i.p. with saline or 1.5 mg/kg/day rapa-

Table 1 Effect of rapamycin and gemcitabine on established human L3.6pl pancreatic tumors in nude mice
All values were obtained from mice sacrificed 28 days after orthotopic tumor cell injection.

Treatment (started 1 week after tumor cell injection)	Orthotopic pancreatic tumor		Metastases (incidence)	
	Incidence	Volume (mm^3)	Liver	Lymph node
Saline (control)	8/8	1672 ± 144	4/8	8/8
Gemcitabine (100 mg/kg, 2×/week)	9/9	773 ± 117[a]	2/9	9/9
Rapamycin (1.5 mg/kg/day)	10/10	388 ± 5[a,b]	2/10	5/10
Rapamycin + gemcitabine	9/9	147 ± 23[a,b,c]	1/9	0/9

[a] $P < 0.00002$ *versus* saline-injected controls.
[b] $P < 0.01$ *versus* gemcitabine.
[c] $P < 0.001$ comparing rapamycin + gemcitabine treatment *versus* rapamycin alone.

mycin. On day 7, intravital microscopy (Zeiss Axiotech Vario microscope; Göttingen, Germany) was performed on DSFCs to examine tumor blood vessels. The entire tumor was examined, and these images (7–15 images/tumor) were recorded on video for analysis (modified Sony 3CCD Color Video Camera; AVT Horn, Aalen, Germany). Vessel diameter was measured using Image J software (from Wayne Rasband; Version 1.25s; NIH, Bethesda, MD) by generating horizontal grid lines every 50 pixels. Tumor vessels crossing the grid lines were individually measured, whereas vertically aligned vessels were not included in the analysis.

Blood flow in tumor vessels in DSFCs was measured directly using a modified thrombosis induction technique (22). In principle, i.v. injected FITC-dextran (M_r 464,000; Sigma-Aldrich Chemicals, St. Louis, MO), when activated by prolonged UV light irradiation, causes oxidative stress by free-radical production as well as activation of the thrombosis cascade (22). In our experiments, L3.6pl tumors were allowed to grow in DSFCs of nude mice for 7 days, with or without rapamycin treatment (1.5 mg/kg/day). Mice then received injection via the tail vein with 0.5 ml of 5% FITC-dextran dissolved in PBS. At the same time, mice also received i.v. injection with 8×10^7 red blood cells that had been labeled with a red fluorescent stain (Red Fluorescent Cell Linker Kit; Sigma-Aldrich Chemicals). The fluorescent red blood cells could be easily seen flowing through blood vessels in the tumors of the DSFCs by intravital microscopy. Phototoxic UV (Zeiss filter set EX BP 450–490, BSFT 510, EM BP 515–565) light was directly applied to a vascular area of the tumor through a ×20 objective lens for 1 min, resulting in a dose of 1010 mW/cm^2. Then, the vascular architecture was observed for 30 s under normal bright-field light, followed by 30 s of RBC flow observation under filtered light for red fluorescence (Zeiss filter set EX BP 546/12, BS FT 580, EM LP 590). The cycle of phototoxic, bright-field, and red fluorescent light was repeated up to a maximum of 20 times. When all of the blood vessels within the area showed total occlusion (no blood flow), this time point was recorded, and light cycles were discontinued. In addition, normal vascular areas clearly outside the tumor region were analyzed in the same way.

***In Vitro* Cell Proliferation Assay.** L3.6pl cells were cultured for 48 h in 96-well microtiter plates in medium with or without rapamycin or gemcitabine. Proliferation was assessed by adding bromodeoxyuridine (bromodeoxyuridine proliferation kit; Roche Diagnostics GmbH, Mannheim, Germany) to individual wells 4 h before completion of the 48-h incubation period and then measuring absorbance at 450 nm.

Fluorescence-Activated Cell-Sorting Analysis for Cell Death. Human umbilical vein endothelial cells (HUVECs) were cultured under normal conditions with endothelial cell basal medium (PromoCell, Heidelberg, Germany) supplemented with growth factors (PromoCell) and 2% fetal bovine serum, or they were placed under minimal culture conditions, where cells were deprived of fetal bovine serum and other supplements. Under supplement and serum-deprived conditions, recombinant human $VEGF_{165}$ (R&D Systems, Wiesbaden, Germany) was added at a concentration of 50 ng/ml in the presence of increasing concentrations of rapamycin. After 8 h, HUVECs were removed from the culture dishes with gentle trypsinization, labeled with annexin V-FITC (R&D Systems), and analyzed by flow cytometry.

Statistical Analysis. Data are given as the mean ± SEM in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student's t test was performed with InStat 3.0 Statistical Software (Graphpad Software, San Diego, CA).

RESULTS

Growth and Metastasis of Established Pancreatic Tumors. To determine the potential for rapamycin treatment in a pancreatic cancer situation, athymic nude mice received orthotopic injection with metastatic human L3.6pl cancer cells. Pancreatic tumors were allowed to become established for 7 days before initiation of rapamycin or gemcitabine treatment. Standard doses of rapamycin (1.5 mg/kg/day) and gemcitabine (100 mg/kg, 2×/week) were used in the first group of experiments, and all animals were sacrificed 28 days after tumor cell injection. All control mice and treated animals did develop primary pancreatic tumors, but the growth and extent of tumor progression depended on the treatment regimen. Standard pancreatic cancer treatment with gemcitabine alone resulted in a significant reduction in the pancreatic tumor volume, compared with control mice (Table 1). Interestingly, and unexpectedly, rapamycin treatment alone reduced tumor volume 2-fold more than standard gemcitabine therapy. Furthermore, when rapamycin and gemcitabine treatment were combined, tumors were very small, growing to only 19% of the size observed with gemcitabine treatment alone. Mice tolerated rapamycin well (0.8 ± 1.6% weight gain), with animals treated with gemcitabine alone or

rapamycin + gemcitabine experiencing some weight loss during therapy (7.7 ± 1.6% and 12.7 ± 1.9%, respectively).

Metastasis of pancreatic tumors was also affected by the different treatment regimens. Lymph node metastases were reduced by rapamycin treatment and completely eliminated by combination therapy with rapamycin and gemcitabine, but treatment with gemcitabine alone did not reduce the incidence of these metastases (Table 1). On day 28, macroscopically visible liver metastases were present in 50% of controls, and this tended to be reduced in frequency by all three treatment regimens, with combination therapy giving the lowest incidence.

A second group of similar experiments was performed to test whether a lower dose of gemcitabine could be effective and sustained long-term. Results showed that in mice sacrificed at 28 days, low-dose gemcitabine (50 mg/kg) inhibited tumor growth to the same degree as the higher dose (100 mg/kg; Fig. 1*A*). However, combination therapy using high-dose gemcitabine combined with rapamycin did lead to a slightly greater reduction in tumor volume, compared with the rapamycin combination with low-dose gemcitabine ($P < 0.001$). In these same experiments, all controls were sacrificed on day 28 because of their deteriorating condition, but 4 of 10 drug-treated mice were continued on therapy for as long as 60 days to determine long-term effects (Fig. 1*B*). All mice on single-agent therapy or high-dose rapamycin + gemcitabine had to be sacrificed by day 53 because of either tumor progression or therapy side effects. In contrast, all mice on low-dose gemcitabine + rapamycin therapy tolerated the treatment well and survived throughout the observation period. Moreover, this treatment group showed an average total weight loss of <10% at day 60; between day 28 and day 60, animal weight remained quite stable in this group (weight loss < 5%). Importantly, tumor growth estimations made by *in vivo* palpation measurements showed that the pancreatic tumor volume remained stable in these mice between day 40 (211 ± 49 mm^3) and day 60 (218 ± 54 mm^3), which is also nearly identical to measurements made in sacrificed animals from the same group on day 28 (Fig. 1*A*).

Analysis of Pancreatic Tumors for Proliferation. Ki67 staining for cell proliferation was performed in the tumors removed from the animals on day 28. Results from this analysis show that the relative number of Ki67-positive tumor cells was substantially less in tumors from mice treated with rapamycin or rapamycin + gemcitabine, when compared with control tumors (Fig. 2, *A* and *B*). In contrast, gemcitabine had no significant effect on tumor cell proliferation compared with controls. Interestingly, results from *in vitro* pancreatic tumor cell proliferation assays did not completely reflect what was observed directly in the tumors. More specifically, rapamycin at concentrations relevant *in vivo* did show some antiproliferative effect on cultured L3.6pl tumor cells, but gemcitabine also demonstrated an antiproliferative effect, albeit at relatively high concentrations (Fig. 2*C*). Notably, cytotoxic effects with gemcitabine are typically seen in the micromolar range with pancreatic cancer cells (23); therefore, the decrease in proliferation in this assay may be due at least in part to a reduction in cell numbers. In contrast, rapamycin in the concentrations tested is not cytotoxic to L3.6pl cells (data not shown), suggesting some direct antiproliferative effect.

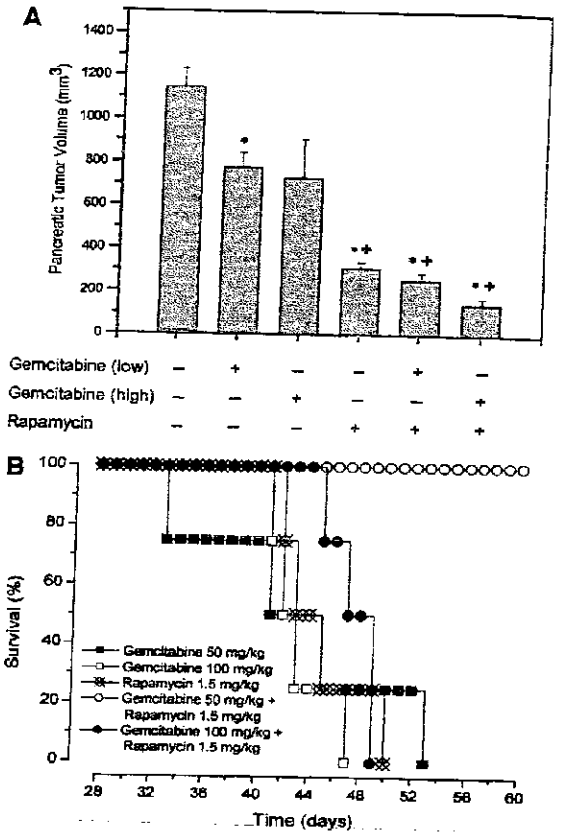

Fig. 1 Effect of rapamycin and different gemcitabine doses on tumor growth and long-term survival of mice with pancreatic tumors. *A*, 6 of 10 mice treated with rapamycin (1.5 mg/kg/day) and/or biweekly low-dose (50 mg/kg) or high-dose (100 mg/kg) gemcitabine were sacrificed 28 days after orthotopic tumor implantation, and tumor volume was measured. Results shown are the mean ± SEM from mice in each treatment group. All 10 controls (*left bar*) receiving daily saline injections were sacrificed because of tumor burden. *, $P < 0.004$ *versus* saline controls; +, $P < 0.0004$ *versus* gemcitabine (low). *B*, the remaining four mice in each drug treatment group were continued on the indicated protocol for up to 60 days to determine long-term effects, and these results are shown.

Histomorphological Analysis of Pancreatic Tumors after Therapy. Standard H&E and TUNEL staining of tumors removed after 28 days revealed some striking features with regard to blood vessel formation in rapamycin-treated mice. In rapamycin-treated or rapamycin + gemcitabine-treated tumors, we consistently observed the presence of dilated tumor vessels containing organized thrombi (Fig. 3*A*). Furthermore, in many tumor vessels with thrombosis, destruction and detachment of the endothelial cell layer were observed. The pathological effects of the thrombosis could be seen by the death of tumor cells in the areas surrounding the incapacitated vessels (Fig. 3*B*).

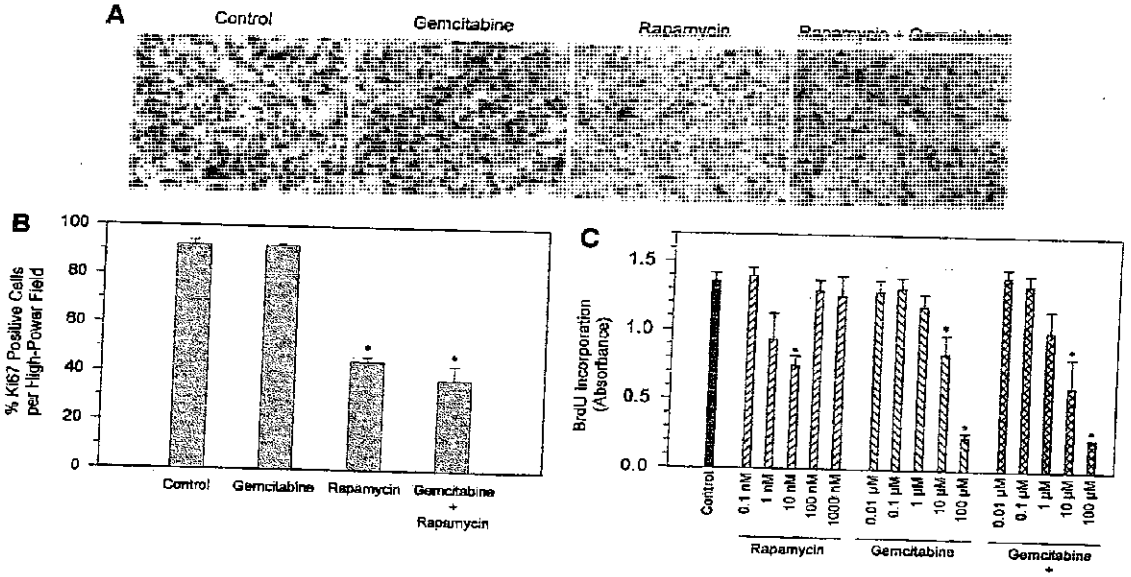

Fig. 2 Effect of rapamycin and gemcitabine (100 mg/kg dose) on pancreatic tumor cell proliferation. *A*, pancreatic tumors from treated mice were removed on day 28 after L3.6pl cell injection and stained for the proliferation marker Ki67 (*bar* = 100 μm). *B*, Ki67-positive cells from 10 high-power fields/tumor were counted, and the mean percentage ± SEM of positive cells was calculated from the total cell number (*, $P < 0.004$ *versus* control). *C*, cultures of L3.6pl pancreatic cancer cells were tested for proliferation by determining bromodeoxyuridine incorporation in the presence or absence of increasing rapamycin or gemcitabine concentrations. In one experimental group, a therapeutically relevant rapamycin concentration (10 nM) was combined with increasing amounts of gemcitabine. Results are shown as the mean absorbance value ± SEM and are representative of one of three experiments (*, $P < 0.05$ *versus* control).

These perivascular tumor cells exhibited signs of apoptosis evident from the accumulation of apoptotic bodies within the dying cells. Importantly, neither tumors from controls nor tumors from mice treated with gemcitabine alone showed any of the same signs of vascular thrombosis (Fig. 3*A*). Furthermore, there were no signs of thrombosis within vessels of adjacent normal pancreatic tissue in rapamycin-treated mice, suggesting that the thrombotic effect was localized to the tumor (Fig. 3*C*).

To directly test the effect of rapamycin on tumor blood vessel flow dynamics, L3.6pl tumor cells were implanted into DSFCs, and vessels were examined by intravital microscopy on day 7. Results showed that tumor vessels in rapamycin-treated mice were dilated in size compared with tumor vessels in control mice (Fig. 4, *A* and *B*). Furthermore, when we photodynamically promoted vascular thrombosis in tumors, blood flow was rapidly blocked by thrombosis in rapamycin-treated mice, compared with tumor vessels in controls (Fig. 4*C*). Other comparisons indicate that tumor vessels in two of three control mice did show blood flow blockade slightly before that seen in nontumor vessels (area outside the tumor) of the same mice. Analysis of blood flow and thrombosis in nontumor vessels also revealed that normal vessels in rapamycin-treated mice were less susceptible to thrombosis than tumor vessels, suggesting that rapamycin preferentially provokes clotting in tumor vessels. When comparing thrombosis only between nontumor vessels in controls and those in rapamycin-treated mice, flow continued in all controls for >20 min, whereas blood flow in nontumor areas in rapamycin-treated mice stopped between 17 and 19 min, indicating only a slightly increased rate of induced thrombosis in nontumor vessels with rapamycin treatment. Furthermore, to exclude the influence of tumor on thrombosis, thrombosis induction was performed in DSFCs without L3.6pl tumor implantation. In both the control and rapamycin-treated mice ($n = 4$ mice/group), thrombosis time exceeded 20 min (data not shown), suggesting that clot formation in blood vessels completely unassociated with tumor is not affected by rapamycin. Therefore, in general, tumor blood vessels in rapamycin-treated mice showed a pattern of development and function that was consistent with our histological vascular findings in the orthotopic pancreatic tumors.

Effects on Endothelial Cell Survival and Proliferation. Disruption of the tumor vessel endothelium observed in the earlier histological studies suggested that endothelial cells may be susceptible to cell death in the presence of rapamycin. To test this hypothesis, we first examined tumor vessels from rapamycin-treated mice for endothelial cell death by colorimetric TUNEL staining. Results showed that endothelial cells in these rapamycin-treated tumors, particularly in damaged and clotted vessels, were indeed positive for TUNEL (Fig. 5*A*). Tumor vessels from mice treated with rapamycin + gemcitabine also demonstrated a high rate of endothelial cell death. Next we performed a fluorescence double-labeling procedure for endo-

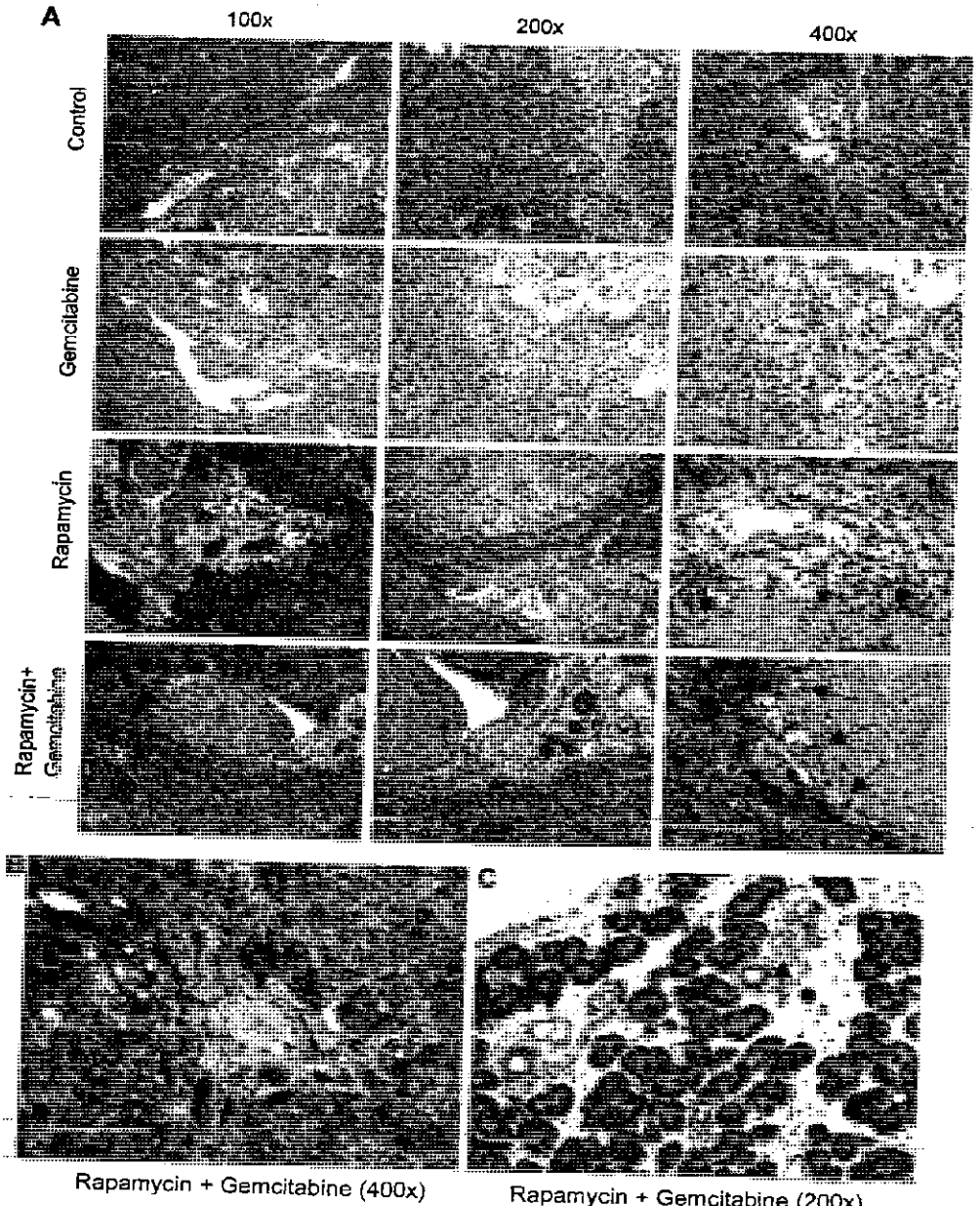

Fig. 3 Rapamycin treatment results in the development of thrombi in pancreatic tumors. *A*, increasing magnification views of an area of pancreatic tumor from control, rapamycin, gemcitabine, and combination treatment mice are shown. No thrombi were found in either control or gemcitabine-treated mice; however, the presence of thrombi was a predominant feature in tumors of mice treated with rapamycin alone or with rapamycin in combination with gemcitabine (×100, ×200). At ×400 magnification, normal blood vessels can be seen in control and gemcitabine-treated mouse tumors, whereas clotted tumor vessels in rapamycin-treated mice show disruption of the endothelial layer (*arrows*). *B*, this photomicrograph shows a clotted vessel in a rapamycin + gemcitabine-treated tumor (*arrow*). Note the signs of tumor cell death (apoptotic bodies) in the area surrounding the thrombosed vessel. *C*, areas of normal pancreatic tissue outside tumors treated with rapamycin (+gemcitabine) showed no evidence of vessel thrombosis (*arrows*). All *bars* in this figure = 50 μm.

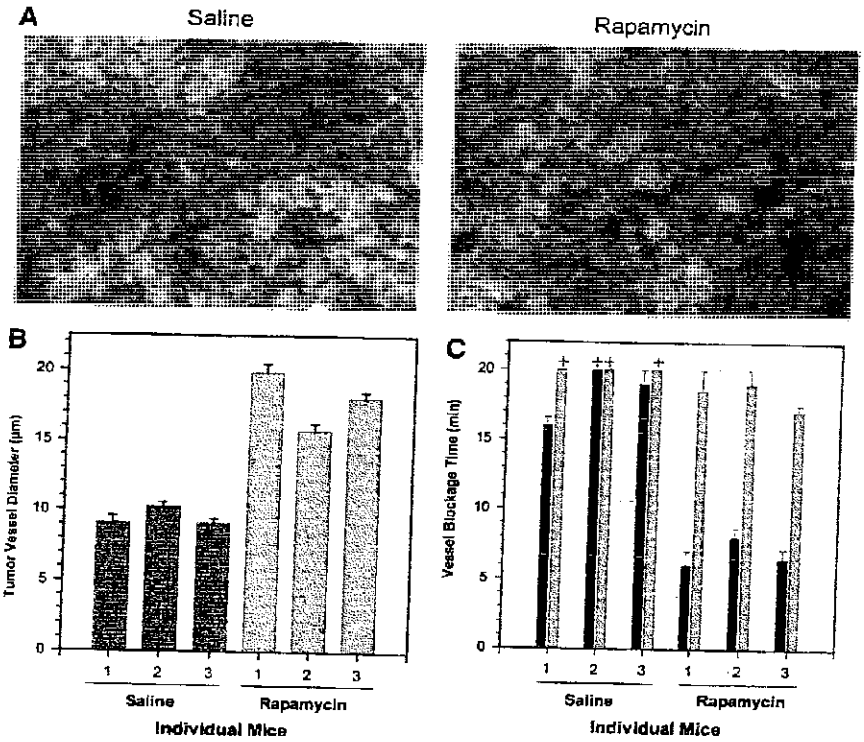

Fig. 4 Effect of rapamycin on vessel development and thrombus formation. To dynamically observe angiogenesis in pancreatic tumors, L3.6pl cells were inoculated into dorsal skin-fold chambers (DSFCs), and blood vessels were allowed to develop for 7 days before analysis by intravital microscopy. In mice receiving rapamycin, 1.5 mg/kg/day treatment was initiated 1 day after tumor cell inoculation. *A*, photomicrographs of DSFC tumors from saline (control) and rapamycin-treated mice are shown. Note the presence of enlarged, irregularly formed blood vessels in the rapamycin-treated tumor (*bars* = 100 μm). *B*, analysis of blood vessel diameter demonstrates a significant increase in vessel diameter with rapamycin treatment, as compared with controls ($P = 0.003$). Mean results ($n = 7$–9 fields of view) from individual mice tested in each group are shown. *C*, to directly assess whether tumor vessels in rapamycin-treated mice are susceptible to thrombosis, clotting was promoted in DSFCs by i.v. injected FITC-dextran and UV light. Blood flow was monitored by observing the movement of injected, fluorescence-labeled RBCs. *Bars* indicate the time required for complete stagnation of blood flow in areas either inside (■) or outside (▨) the clearly demarcated tumor region in DSFCs. Results are shown from three individual mice in each treatment group, and values are the mean of two or more light-treated vessel fields. When blood flow continued at the 20 min time point, a + is indicated above the respective bar.

thelial cells (CD31) and TUNEL, and we observed that structures in pancreatic tumors composed of endothelial cells could also be shown to be TUNEL positive (Fig. 5*B*). In contrast, endothelial cells in gemcitabine-treated tumors were not found to be TUNEL positive. To further test whether rapamycin causes endothelial cell death, we cultured HUVECs in the presence or absence of increasing concentrations of drug and performed flow cytometric analysis on annexin-FITC-labeled cells. Results showed that compared with full stimulation of HUVECs, serum and supplement-deprived conditions caused the cells to die (Fig. 6). Addition of VEGF to the serum and supplement-deprived environment rescued the cells from death. Interestingly, 5 nM rapamycin completely blocked the rescue effect of VEGF on HUVECs; lower drug levels had a lesser effect. Treatment of HUVECs with gemcitabine had no effect on annexin staining (data not shown). Together, these results suggest that endothelial cell survival pathways are blocked by rapamycin, but not by gemcitabine, leading to cell death and subsequent vascular endothelium disruption in tumor vessels.

DISCUSSION

Pancreatic cancer remains one of the most uncontrollable neoplasms encountered in clinical oncology. In this study, we present data that suggest human pancreatic cancer may be particularly responsive to a new therapeutic approach involving conventional use of the cytotoxic agent gemcitabine with a recently discovered antiangiogenic drug, rapamycin (14). Our study indicates that rapamycin use alone can inhibit human pancreatic tumor growth in nude mice to a greater degree than gemcitabine, a standard first-line chemotherapeutic agent available for the treatment of human pancreatic cancer. Moreover,

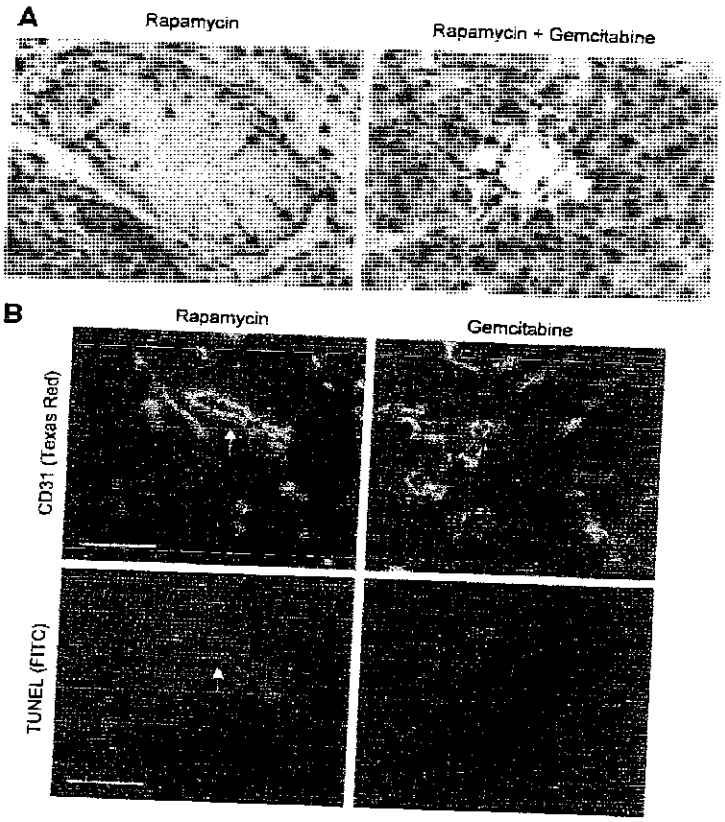

Fig. 5 Effect of rapamycin on the viability of endothelial cells in orthotopic pancreatic tumors. *A*, tissue sections from pancreatic tumors of rapamycin-treated mice were colorimetrically stained for terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). The *left panel* shows TUNEL staining of apoptotic endothelial cells (*arrows*) in a rapamycin-treated tumor vessel containing a thrombus. In the *right panel*, a tumor vessel from a rapamycin + gemcitabine-treated tumor shows extensive death of endothelial cells (*arrows*). *B*, fluorescent CD31 and TUNEL double labeling of a rapamycin-treated tumor cryosection (*left panels*) identifies a tumor blood vessel undergoing apoptosis (*arrow*). *Right panels*, tumor vessels after gemcitabine treatment did not show double labeling for CD31 and TUNEL. All *bars* in this figure = 50 μm.

when gemcitabine was combined therapeutically with rapamycin, growth of established pancreatic tumors was severely compromised, and importantly, metastases to the liver and local lymph nodes were reduced or eliminated. The clinical promise and scope of the combined effects of rapamycin and gemcitabine were shown by the lack of tumor progression in long-term surviving nude mice bearing the human pancreatic cancer.

One of the most intriguing features of rapamycin treatment was the presence of damaged, dilated vessels containing thromboses in orthotopic pancreatic tumors. These areas of thrombosis were clearly associated with tumor attrition in regions surrounding the damaged vessels, thus restraining pancreatic cancer advancement. A closer look at the tumor vessel endothelium in rapamycin-treated mice revealed a potential cause for the thrombosis. More specifically, histological analysis showed damaged and sometimes detached endothelial cell layers that could also be shown to contain a high number of endothelial cells that had undergone cell death. Under more well-defined *in vitro* experimental conditions using HUVECs, we could confirm that endothelial cells maintained with VEGF did not survive in the presence of rapamycin in the 0.1 nM range, with a maximal effect reached at 5 nM. Interestingly, data in the recent literature indirectly support the hypothesis that rapamycin treatment can induce apoptosis of VEGF-stimulated endothelial cells, potentially leading to tumor vessel thrombosis. The evidence begins with data indicating that VEGF induction of the phosphatidylinositol 3′-kinase/Akt intracellular signaling pathway is important for endothelial cell survival (24, 25). Phosphatidylinositol 3′-kinase/Akt up-regulation of FLICE-inhibitory protein protects endothelial cells from Fas-mediated apoptosis (25), which is critical because Fas is constitutively expressed on endothelial cells. It has also been shown that phosphatidylinositol 3′-kinase/Akt signaling inhibits endothelial cell death by down-regulating p38 mitogen-activated protein kinase-dependent apoptosis pathways (26). Therefore, it is logical to suggest that rapamycin inhibition of mammalian target of rapamycin, which is downstream of phosphatidylinositol 3′-kinase/Akt (27), could indeed be effective at inducing apoptosis of endothelial cells. Clinical observations also correlate with this hypothesis. For example, abnormal thrombus formation in microvascular thrombotic diseases such as idiopathic thrombotic thrombocytopenia purpura has been linked to induction of endothelial cell apoptosis by soluble serum factors (28–30), and interestingly, thrombocytopenia and hemolytic uremic syndrome have been reported as

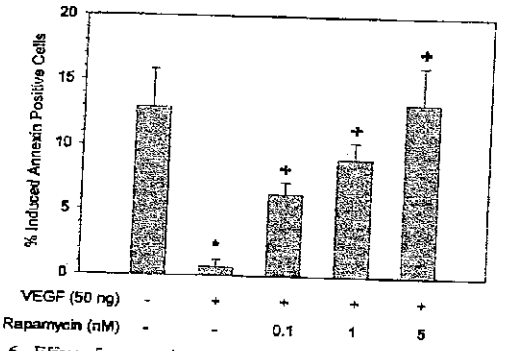

Fig. 6 Effect of rapamycin on endothelial cell survival *in vitro*. Human umbilical vein endothelial cells (HUVECs) were cultured for 8 h under fully stimulated conditions (baseline) or under deprived conditions ± vascular endothelial growth factor $(VEGF)_{165}$ or rapamycin; cells were then analyzed for annexin-FITC by flow cytometry. With full stimulation, HUVECs were cultured with exogenous growth factor supplemented medium + 2% fetal bovine serum; under deprived conditions, supplemented growth factors and fetal bovine serum were withheld. Results are shown as the percentage of induced annexin-positive cells, which is defined as the percentage of annexin-positive cells under deprived conditions (±VEGF/rapamycin), after subtracting baseline annexin-FITC positivity (baseline annexin-FITC positivity was ≤7%). *Bars* represent the mean ± SEM of three separate experiments. These data indicate that VEGF stimulation "rescues" HUVECs from death, whereas rapamycin blocks VEGF-induced rescue of HUVECs in a dose-dependent manner. *, $P = 0.015$ *versus* cultures with no VEGF or rapamycin; +, $P < 0.013$ *versus* cultures with VEGF, but no rapamycin.

common side effects when rapamycin is used for immunosuppressive treatment of acute graft-*versus*-host disease (31). From another perspective, research on microangiopathic hemolytic diseases has revealed that endothelial cells derived from various tissues have different susceptibilities to apoptosis and thus to thrombosis. Because this variability has been linked to the relative tissue expression of several apoptosis survival genes, including *Bcl-2*-family genes and VEGF (28), the histological presence of thrombi in our study in the pancreatic tumors, but not in the surrounding normal pancreas, could be attributed to a differential expression pattern between the normal and cancerous tissue. Another contributing factor to the specificity of the thrombosis in the pancreatic tumors could be related to observations that pancreatic cancer patients tend to develop regional blood clots, reportedly due to thrombin activation (32). Indeed, our thrombus induction experiments in DSFCs support this observation but, most importantly, are the first to show that tumor vessels are particularly susceptible to thrombus formation with rapamycin treatment. Thus, rapamycin's negative effect on the survival of rapidly-expanding tumor-associated endothelial cells, combined with the reported local activation of thrombin via cancer cells, may favorably concentrate thrombotic events within pancreatic tumors.

Interestingly, from a completely different clinical perspective, our thrombosis induction experiments caution that nontumor vessels may exhibit some risk for clotting with rapamycin treatment (Fig. 4*C*). This could be of considerable significance because of the widespread use of rapamycin as an immunosuppressant in organ transplant patients. As mentioned previously, microangiopathic thrombosis can lead to severe side effects that have necessitated the discontinuation of this immunosuppressive treatment in some bone marrow transplant patients (31). The development of potentially fatal hepatic vein or artery thrombosis after rapamycin treatment following liver transplantation (33) further emphasizes possible effects of this drug on blood vessels and coagulation. In these instances, however, it is possible that blood vessel damage resulting from graft-*versus*-host reactions, immunological rejection, or surgical trauma raises the likelihood that rapamycin treatment could contribute to a thrombotic event. Therefore, there is a broad-based clinical need to understand the potential local specificity and mechanism of the prothrombotic effect of rapamycin in noncancerous tissue, as well as in tumors.

Nothwithstanding the potential importance of thrombosis, other mechanistic issues from our study relate to the question of how combined rapamycin and gemcitabine treatment keeps aggressive pancreatic tumors from advancing. Although the mechanisms are not clear, it is reasonable to speculate that the different activities of the two drugs strike at multiple critical aspects of tumor growth. One logical explanation for their combined potency could relate first to the ability of rapamycin to prevent vascular expansion and to promote tumor vessel thrombosis. However, whereas the present study suggests that these rapamycin effects alone clearly inhibit tumor growth, tumors do continue to expand slowly, leading to only a moderate improvement in long-term results (Fig. 1). Therefore, we reason that because rapamycin is not generally cytotoxic to tumor cells at the doses we used (34) and has only a moderate direct antiproliferative effect on pancreatic tumor cells *in vitro*, pockets of cells with at least some rudimentary angiogenesis can proliferate. The role of gemcitabine at this phase could then be to destroy tumor cells that do enter the S-phase of cell proliferation, which is one its primary anticancer activities (35). Interestingly, under circumstances where rapamycin is not present, gemcitabine's cytotoxic effect alone is not able to completely counterbalance the concomitant high proliferation rate we observed in pancreatic tumors (Fig. 2*A*). Indeed, a lowering of the proliferation rate in pancreatic tumors was only associated with rapamycin treatment. As discussed previously, rapamycin did have some direct antiproliferative effect on L3.6pl cells *in vitro*, as has been reported with other pancreatic cell lines (36), but this may not be the only explanation for its exceptional antiproliferative activity in tumors. We suggest that there is an indirect antiproliferative effect of rapamycin correlating with its antiangiogenic activity and the previously reported observation that proliferation rate is inversely proportional to the distance of tumor cells from the nearest blood vessel (37, 38). Considering these data together, we propose that whereas rapamycin's antiangiogenic, prothrombotic, and antiproliferative effects can reduce pancreatic tumor growth, an equally important "trap" must be set (*i.e.*, gemcitabine) for those tumor cells that do acquire adequate resources to proliferate and advance tumor growth. Consistent with this strategy, long-term control of pancreatic cancer progression in our experiments could only be achieved by combining rapamycin and gemcitabine.

Finally, clinical use of rapamycin in a gemcitabine-based protocol to treat human cancer is highly feasible. Currently,

rapamycin is approved for use in human organ transplantation as an immunosuppressive agent to prevent allograft rejection. The drug is maintained on a daily basis in patients for several years or indefinitely. An important corollary to this issue from our study is that rapamycin exerts its most potent effect on endothelial cells near 5 nM, which coincides with serum drug levels targeted in transplant patients. Therefore, it is reasonable to suggest that long-term, continuous inhibition of tumor neoangiogenesis is possible by incorporating these already thoroughly tested rapamycin treatment protocols into cancer treatment regimens. Another positive aspect of combining rapamycin with gemcitabine is that the latter agent can also be effectively and safely administered over an extended period at a reduced dose (39), lending credibility to the potential of a clinical protocol for long-term tumor control, as we were able to achieve in mice with low-dose gemcitabine + rapamycin treatment. Therefore, our study suggests that rapamycin and gemcitabine could offer a novel, clinically feasible drug therapy to control pancreatic cancer disease progression, and the general strategy of combining rapamycin with other cytotoxic drugs may also prove to be effective for a broader range of cancers for which drug cytotoxicity alone is not curative or does not provide tumor control with a favorable quality of life.

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#2981 Suppression of Akt and MAPK with activation of p38 and JNK during depsipeptide-mediated apoptosis in lung cancer cells. Xiaodan Yu, Zong-Sheng Guo, G. Aaron Chen, Dao M. Nguyen, and David S. Schrump. *National Cancer Institute/NIH, Bethesda, MD.*

Recent data indicate that the antiproliferative effects of histone deacetylase (HDAC) inhibitors in cancer cells cannot be attributed solely to histone acetylation. Previously, we have demonstrated that low dose Depsipeptide FR901228 (DP) induces apoptosis in lung cancer cells but not cultured normal human bronchial epithelia. Furthermore, we have shown that DP depletes mutant p53, erbB1, erbB2, and raf-1 protein levels resulting in diminished MAP kinase activity in lung cancer cells. The present study was undertaken to examine if DP might alter signal transduction by additional pathways known to modulate apoptosis in cancer cells. H-322 lung cancer cells overexpressing erbB1 and erbB2, and expressing activated *ras* and mt p53 were exposed to normal media or DP (25ng/ml) for either 6 hr or 24hr. Proliferation and apoptosis were assessed with MTT and ApoBrdU techniques, respectively. PI3K/AKT, extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and c-JUN N-terminal kinase (JNK) activities were examined using commercially available kinase assay kits, or western blot techniques using phosphorylation-specific antibodies. DP mediated time dependent growth arrest and apoptosis in H322 lung cancer cells coinciding with suppression of Akt and MAPK, and simultaneous activation of JNK and p38 kinase. DP-mediated apoptosis in H322 cells could be potentiated by the PI3K inhibitor, LY294002, as well as the MEK inhibitor, PD98059. In contrast, DP-mediated apoptosis was partially abrogated by the p38 inhibitor, SB203580. Collectively these data indicate that DP modulates multiple signal pathways regulating apoptosis in lung cancer cells. The ability of DP to simultaneously suppress Akt and MAPK mediated survival pathways, while enhancing p38 and JNK mediated apoptotic signaling contributes to the cytotoxic effects of this novel HDAC inhibitor in lung cancer cells.

#2982 Antisense inhibition of PIK3CA reverses cancer phenotype. Su Dao, Pornchai O-Charoenrat, Iven Ngai, Pabbathi Reddy, and Bhuvanesh Singh. *Memorial Sloan-Kettering Cance Center, New York, NY.*

PIK3CA, encoding for the p110alpha subunit of PI3K, has been identified as a candidate oncogene associated with 3q amplification in several human malignancies. However, its oncogenic potential and inclusive functional role remains to be elucidated. In this study, we analyze the transformation ability of PIK3CA by stable transfection of a constituently active (myrPIK3CA) form of the gene into 3T3 cells. Stable expression of the myr-PIK3CA gene in 3T3 cells induced an aggressive tumorigenic phenotype, promoting morphological changes, increased growth rate, resistance to serum deficient conditions, anchorage independent growth, and tumorigenic potential in nude mice. Conversely, blocking PI3K p110alpha expression with antisense PIK3CA (asPIK3CA) in 3T3 cells results in rapid cell death. Gene expression profiling of myrPIK3CA and asPIK3CA transfected 3T3 cells revealed several genes, which are alternatively regulated and may represent novel PI3K/AKT pathway targets. Examples of genes up regulated in myrPIK3CA transfected cells and down regulated in asPIK3CA transfected cells include prostaglandin E receptor EP4 subtype, Peg1/MEST, Kreisler leucine zipper protein, lysosome M, laminin a-4 and Xist. In addition, reversion of PIK3CA-expressing MDA886 cells (derived for head and neck squamous cell carcinoma) to a more normal cellular morphology, with enhanced cell death, sensitivity to serum deficient conditions, suppression anchorage-independent growth and diminished tumorigenic potential in nude mice, results from asPIK3CA transfection. These findings confirm that PIK3CA is indeed an oncogene, and direct abrogation of PIK3CA expression can nullify the cancer phenotype. PIK3CA represents a gene that may serve as a target for the gene-based therapy of squamous cell carcinomas.

#2983 Pharmacologic PI3 kinase inhibitors interact in a highly synergistic manner with the cyclin-dependent kinase flavopiridol to induce mitochondrial damage, caspase activation, and apoptosis in human leukemia cells. Chunrong Yu, Paul Dent, and Steven Grant. *Medical College of Virginia, Richmond, VA.*

Interactions between the cyclin-dependent kinase flavopiridol(FP) and PI3 Kinase inhibitors (e.g., LY294002; LY, Wortmannin;Wtn) have been examined in human leukemia cells. Exposure of U937 cells to 75 nM FP for 6 hr only minimally induce apoptosis in these cells (e.g. <5%). However, when cells were co-incubated with FP in conjunction with LY (15 μM), a striking and rapid (i.e., within 6 hr) increase in mitochondrial damage was noted, reflected by loss of $\Delta\Psi m$ and cytochrome c/Smac/DIABLO release into the cytosolic S-100 fraction, accompanied by a marked increase procaspase 9, 3, 8, Bid, PARP cleavage, and the morphologic features of apoptosis(i.e., in >75% of cells). However, no changes were observed in levels of Bcl-2, Bcl-xL, XIAP, phospho-GSK3, phospho-Bad, phospho-Erk1/2, or phospho-JNK. FP induced phosphorylation of Akt in U937 cells, an effect that was abrogated by LY. Synergistic induction of apoptosis by FP and LY was also observed in multiple other leukemic cell lines, including HL-60, NB-4, CCRF-CEM, and Jurkat. Wtn (300 nM) also markedly potentiated FP-mediated lethality. Overexpression of Bcl-2 or a phosphorylation loop-deleted mutant Bcl-2 effectively blocked apoptosis induced by FP/LY. Both the loss of $\Delta\Psi m$ and the morphologic features of apoptosis in FP/LY-treated cells were blocked by caspase inhibitors (e.g., Boc-fmk, DEVD-fmk), but not release of cytochrome c. Finally, co-administration of FP and LY was associated with a marked decrease in expression of Mcl-1 and an increase in phospho-p38 MAPK. Together, these findings indicate that the CDK inhibitor FP, administered at a pharmacologically relevant concentration, interacts in a highly synergistic manner with the PI3K inhibitor LY to induce mitochondrial damage and apoptosis in human leukemia cells, and raise the possibility that the PI3K cascade exerts critical cytoprotective effects that attenuate FP-mediated lethality.

#2984 Prolonged effect of the rapamycin derivative RAD001 on p70S6 Kinase activity in tumors, skin and peripheral blood lymphocytes derived from a syngeneic rat pancreatic tumor model: Correlation with efficacy of intermittent dosing schedules. Anne Boulay, Sabine Zumstein-Mecker, Iwan Beuvink, Frederic Zilbermann, Christine Stephen, Roland Haller, Sonja Tobler, Marc Hattenberger, Barbara Stolz, Terence O'Reilly, George Thomas, and Heidi A. Lane. *Novartis Pharma AG, Basel, Switzerland, and Friedrich Miescher Institute, Basel, Switzerland.*

RAD001 is a hydroxyethyl ether derivative of rapamycin that is orally bioavailable. RAD001 has demonstrated *in vitro* and *in vivo* anti-proliferative activity against a number of human tumor cell lines. In this context, RAD001 demonstrates a dose-dependent antitumor activity in the syngeneic CA20948 rat pancreas carcinoma model, at well tolerated doses. Significant tumor growth suppression was observed consistently with a dosing regimen of 2.5 mg/kg p.o. q.d. x6 (% T/C values: 37 %, 23 % and 30 %) and with 5 mg/kg twice weekly (% T/C values: 36 % and 32 %). The antitumor effect with these schedules was similar to that of 5-Fluorouracil (% T/C values: 23 % and 35 %). Antitumor effects were also observed with a 5 mg/kg once weekly dosing schedule (% T/C values: 36 % and 64 %). These data indicate that intermittent RAD001 dosing schedules do have antitumor efficacy in this model. Treatment of CA20948 tumor-bearing rats with a single administration of RAD001 (5 mg/kg) resulted in significant inactivation of p70S6 kinase in tumors, skin and peripheral lymphocytes, as compared to untreated controls. Vehicle alone had no significant effects. In tumors, significant inhibition of p70S6 kinase activity was maintained for up to 48 hrs, with some evidence of recovery after 72 hrs. In contrast, p70S6 kinase derived from skin and peripheral lymphocytes remained significantly inhibited for at least 72 hrs. Additionally, evaluation of the effects of RAD001 dose on p70S6 kinase activity in skin and peripheral lymphocytes, indicated dose-dependent effects on the duration of p70S6 kinase inactivation. Taken together, these observations provide mechanistic support for the observed antitumor potency of intermittent dosing schedules, and point to the potential of using peripheral blood lymphocytes or skin sampling as a source of material for biomarker/surrogate marker evaluation when establishing RAD001 dosing regimens in the clinic. Consistent with this statement, p70S6 kinase activity can reproducibly be detected in human peripheral lymphocytes obtained from volunteers.

#2985 Myogenic differentiation is dependent on both the kinase function of mTOR and the N-terminal HEAT sequence. Lili Shu, Xiangwen Zhang, and Peter Houghton. *St. Jude Children's Research Hospital, Memphis, TN.*

mTOR is a S/T protein kinase known to control translation initiation through two downstream pathways: 4E-BP1/eIF4E and ribosomal $p70^{S6K}$. Using C2C12 murine myoblasts we have previously reported that rapamycin arrests cells in G1 phase, and completely inhibits terminal myogenesis. To understand the pathways that regulate myogenesis, we have established stable C2C12 cell lines expressing a rapamycin-resistant mTORrr (S2035I) with deletions in the N-terminus (Δ10 or Δ91) or a full-length kinase dead mTORrr mutant (D2338A). Our results show that Δ10mTORrr signals to both $p70^{S6K}$ and 4E-BP1 and permits myogenesis in rapamycin-treated myoblasts. This confirms that inhibition of myogenesis by rapamycin is mTOR-dependent. However, C2C12 cells expressing either Δ91mTOR-rr or the kinase-dead mutant could not signal to either $p70^{S6K}$ or 4E-BP1, and could not abrogate the inhibition of myogenesis by rapamycin. Taken together, our results indicate that the N-terminus of mTOR (residues 11-91 containing the first HEAT domain), and kinase function of mTOR are both essential for myogenic differentiation. A more detailed analysis of the N-terminus of mTOR and the role of first HEAT domain in myogenic differentiation is under investigation. Supported by CA23099, CA77776 and American Lebanese Syrian Associated Charities.

#2986 Rapamycin-induced apoptosis is associated with sustained phosphorylation of c-Jun. Shile Huang, Lili Shu, Michael B. Dilling, Glen S. Germain, and Peter J. Houghton. *St. Jude Children's Research Hospital, Memphis, TN.*

Rapamycin analogues (CCI-779, RAD001) are being developed as novel cancer therapeutic agents. Rapamycins inhibit mTOR/FRAP, a serine/threonine kinase downstream of PI3' kinase causing G1 cell cycle arrest. Cells lacking functional p53 progress through G1, initiate replication, and undergo apoptosis. Restoring functional p53 or expressing p21Cip1 completely protects cells from death (Huang et al. Cancer Res.61:3373, 2001). In this work, we have investigated whether activation of the JNK cascade is involved in rapamycin-induced apoptosis using p53 mutant Rh30 rhabdomyosarcoma cells and wild type and nullizygous mouse embryo fibroblasts (MEFs). In Rh30 cells rapamycin rapidly increased JNK activity and phospho-c-Jun (Ser65) in a concentration-dependent manner, but did not affect their protein levels. c-Jun phosphoryation was sustained in Rh30 cells, but only transient in Rh30 cells expressing wild type p53 or p21Cip1. Similarly, under serum-free conditions, rapamycin induced sustained phosphorylation of c-Jun in MEFs with p53-/- or p21-/- genotype, but only

differentiation of myeloid leukemia cell lines (NB4, HL-60 and U937). SS was identified in our recent studies as a potent inhibitor of PTPases. Herein, we present data demonstrating that SS (250 μg/ml, 6 days) induced 87% of NB4 cells to reduce nitroblue tetrazolium (NBT), in comparison to the 90% induced by ATRA (1 μM, 6 days). SS-induced NB4 cell differentiation was confirmed by increased CD11b expression and associated with growth arrest at S phase and increased cell death. Our results showed further that SS-induced NB4 differentiation was irreversible and required continuous drug exposure for optimal induction. Moreover, SS (400 μg/ml, 6 days) induced 60% and 55% of NBT-positive cells in HL-60 and U937 cell lines, which were augmented in the presence of GM-CSF (2 ng/ml) to levels (85% and 81%, respectively) comparable to those induced by ATRA. These results provide the first evidence of a differentiation induction activity of PTPase inhibitor SS in myeloid leukemia cell lines and ssuggest its potential therapeutic use in myeloid leukemia. Since SS induces differentiation via targeting PTPases, a mechanism distinct from that of ATRA, it may be particularly useful in AML cases unresponsive or developed resistance to ATRA.

#356 NAD(P)H:quinone oxidoreductase (NQO1)-dependent and -independent cytotoxicity of potent quinone cdc25 phosphatase inhibitors. Yusheng Han, Hongmei Shen, Brian Carr, John S. Lazo, and Su-Shu Pan. *University of Pittsburgh Cancer Institute, Pittsburgh, PA, and University of Pittsburgh, Pittsburgh, PA.*

A vitamin K analogue, compound 5 (Cpd5, a thioethanol naphthoquinone), inhibits oncogenic Cdc25 phosphatases, and arrests cell cycle progression at both G1 and G2/M. Recently, we evaluated >10,000 compounds in the NCI chemical repository for *in vitro* inhibition against recombinant human Cdc25B phosphatase and identified a quinone substructure in many of the active compounds. Bioreductive enzymes in cells, however, are known to reduce various quinones resulting in either detoxification or activation. Therefore, we used an isogenic set of human colon cancer cell lines to evaluate the effect of NQO1 on the cytotoxic activity of Cpd5 and the two most potent phosphatase inhibitors from the repository: NSC 95397 (a bis-thioethanol naphthoquinone) and NSC 663284 (a quinolinedione). The two cancer cell lines used were HCT116, which has intermediate NQO1 activity, and its mitomycin C-resistant sub-line HCT116-R30A (R30A), which has minimum NQO1 activity. Cell survival was measured by colony formation after 7 days drug exposure. Cell cycle arrest was evaluated by flow cytometry after 6 hr drug exposure. Cpd5 had an IC_{50} of 2.2±0.3 μM for HCT116 and 0.23±0.05 μM for R30A, i.e. a 10-fold difference. Inclusion of dicoumarol (10 μM), an inhibitor of NQO1, decreased the IC_{50} of Cpd5 for HCT116 to 0.24±0.04 μM, but had no effect on R30A. In contrast, HCT116 and R30A cells were equally inhibited by NSC 95375 with IC_{50}s of 1.4±0.3 μM and 1.3±0.2 μM, respectively. Similarly, HCT116 and R30A cells were equally inhibited by NSC 663284 with IC_{50}s of 2.4±0.3 μM and 2.6±0.5 μM, respectively. All three compounds blocked the two cell lines at the G2/M phase transition, consistent with cdc25 inhibition. Cpd5 at 2.5 μM arrested R30A cells at G2/M but 7.5 μM Cpd5 was needed to arrest HCT116 cells to a similar degree. NSC 95375 and 663284 arrested cell cycle progression at G2/M of HCT116 and R30A cells similarly, and did so in a concentration-dependent manner between 2.5 and 7.5 μM. Our data imply that NQO1 in HCT116 cells protected cells from the action of Cpd5, probably by the reduction of Cpd5 to a less active hydroxylquinone. In contrast, both NSC 95397 and 663284 displayed cytotoxicity that was independent of NQO1 levels. (Support: NCI CA81882 and CA78039)

#357 Antitumor and anticarcinogenic action of Cpd 5: A new class of protein phosphatase inhibitor. Siddhartha Kar, Meifang Wang, Zhenggang Ren, Xiangbai Chen, and Brian I. Carr. *University of Pittsburgh, Pittsburgh, PA.*

Background: We have chemically synthesized a new class of inhibitors of dual specificity phosphatases (DSP), which play an important role in cell cycle and signal transduction. Cpd 5 or 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone is one of the most potent. it inhibits DSPs (especially the Cdc25 family) in tissue culture cells and induces tyrosine phosphorylation of various DSP substrates, including Cdks and inhibits cell growth both *in vitro* and *in vivo* (JBC 270:28304, 1995; Proc. AACR 39:224, 1998). Purpose: In this study we evaluated (a) the antitumor and (b) the anticarcinogenic activity of Cpd 5 for the first time. Methods: (a) JM1 hepatomas were grown in 2 month old Fischer male rats by subcutaneous injection on the back or intra-portally in the liver. Rats were treated with Cpd 5 by intratumor, subcutaneous (nearby site), intramuscular (distant site), or intraperitoneal injection, either as a single high acute dose or chronically as several low doses. (b) Rats were injected intraperitoneally with a single dose of the carcinogen N-Nitrosodiethylamine (DEN). Immunostained liver sections for glutathione-S-transferase-pi (GST-pi) detected pre-neoplastic foci after 3 weeks. Cpd 5 was injected subcutaneously or intraperitoneally two weeks after DEN as a single high acute dose or chronically as several low doses. Results: (a) Cpd 5 had significant inhibitory effect on both intrahepatic (14% of control, p<0.00000008) and subcutaneous (33% of control, p<0.00008) tumor growth and also had significant inhibitory effect when injected intramuscularly at a site distant from the tumor (50% of control, p<0.002). There was no significant difference between the effects after acute or chronic injections. However, toxicity was much lower with chronic treatment. (b) The number of enzyme altered foci was also significantly reduced when rats were treated with acute (40% of control, p<0.00002) or chronic (50% of control, p<0.02) Cpd 5. Conclusions: Cpd 5 had significant inhibitory effect on growth of tumors and foci.

#358 Bosentan, a novel endothelin-A and -B receptor antagonist inhibits proliferation of malignant melanoma cells. Aleksandar Sekulic, Padma Suresh, Mark R. Pittelkow, and Svetomir N. Markovic. *Mayo Foundation, Rochester, MN.*

Here we tested a feasibility of endothelin (ET) receptor blockade with a dual endothelin-A and -B receptor (ETR-A and ETR-B) antagonist, Bosentan, as a novel therapeutic approach for malignant melanoma. ETs are 21aa peptides primarily produced by endothelial cells and implicated in a variety of physiological functions. Binding of ET to ETR-A on vascular structures potently stimulates angiogenesis and, thus, likely plays an important role in growth of multiple cancers. Activation of ETR-Bs, among other things, regulates melanocyte development and function. We first examined patterns of ETR subtype expression on six established melanoma cell lines using flow cytometry and immunocytochemistry. Follcwing this sections of primary and metastatic melanoma tissues were evaluated for ETRA/ETRB expression by immunohistochemistry. To test functional effects Bosentan on melanoma cell proliferation, six melanoma cell lines were subjected to standard 3H-thymidine incorporation assays in presence or absence of various concentrations of Bosentan. All examined melanoma tissues (2 primary, 9 metastatic) express ETRB, albeit to different levels, whereas ETRA was expressed to low levels in only 3 metastatic tumors. In functional assays Bosentan inhibited proliferation of all examined cell lines with the IC50 ranging between 7 and 40 μg/ml. Our results suggest that malignant melanocytes express functional ETRs, and their treatment with Bosentan leads to significant growth inhibition. Concurrent inhibition of ETR-A and ETR-B *in vivo* by low toxicity, orally available inhibitor Bosentan might therefore prove useful as a novel mode of anti-melanoma therapy through simultaneous inhibition of cancer cell growth and process of angiogenesis.

#359 *In vivo* activity of RAD001, an orally active rapamycin derivative, in experimental tumor models. Terence O'Reilly, Juliane Vaxelaire, Melanie Muller, Heinz-Herbert Fiebig, Marc Hattenberger, and Heidi A. Lane. *Business Unit Oncology, Novartis Pharma AG, Basel, Switzerland, and Oncotest gmbH, Freiberg, Germany.*

RAD001 is a hydroxyethyl ether derivative of rapamycin that is orally bioavailable. RAD001 has demonstrated *in vitro* anti-proliferative activity against a panel of human tumor lines. For *in vivo* testing, tumor-bearing nude mice were administered RAD001 in a variety of doses and schedules. Tumors were established by transplantation of fragments generated from injection of cells, or by transplantation of fragments from stabilized tumors originating from surgically removed human tumors. When administered once daily p.o., at doses ranging from 0.5-5.0 mg/kg/day, RAD001 was a potent inhibitor of tumor growth in 10 different xenograft models of human tumors (including pancreatic, colon, epidermoid, lung and melanoma). In general, RAD001 was well tolerated and better tolerated in mouse xenograft models than standard cytotoxic agents (i.e. doxorubicin and 5-fluorouracil), while possessing similar antitumor activity. Only one instance of *in vivo* resistance has been observed (MAXF 401 mammary xenograft model), otherwise the activity of RAD001 was generally inhibition of tumor growth (persistent regressions in one tumor line, T/C values of 9 to 45 % in 8 tumor lines). Xenograft models sensitive to RAD001 treatment included tumors exhibiting comparative resistance *in vitro* (KB-31 and HCT116). Persistent tumor regressions (41-%) were observed in a line displaying sensitivity to RAD001 *in vitro* (A549). Pharmacokinetic analyses, following a 5 mg/kg administration, indicated rapid uptake into plasma (Cmax 2513 ng/ml; Tmax 1 h), but the time to Cmax was delayed in tumors (Cmax 102 ng/g; Tmax 2 h). Elimination from the tumor (t1/2, 16 hr) was apparently slower than for plasma (t1/2, 7.5 hr). RAD001 levels were above the IC50 of A549 cells for a 72 h period. Interestingly, tumor RAD001 levels, following a single 5 mg/kg administration, never exceeded the *in vitro* antiproliferative IC50 for either KB-31 or HCT116 cells; despite the sensitivity of these lines *in vivo*. From these observations, and given the extreme sensitivity of endothelial cells to RAD001, it is plausable that RAD001 may not only act on tumor cells but may also affect angiogenesis. Taken together, these data support the application of RAD001 as an antitumor agent.

#360 Discovery of anticancer agents from sponge-associated fungi. Frederick A. Valeriote, Karen Tenney, Charles Griesbaber, Halina Pietraszkiewicz, Akiko Amagata, Taro Amagata, Jeff Gautschi, Joseph Media, Joseph Steyancff, Richard Wiegand, and Phil Crews. *Henry Ford Health System, Detroit, MI, and University of California Santa Cruz, Santa Cruz, CA.*

We have evaluated 1,112 extracts (from 660 sponge-associated fungi) for assessment of potential anticancer activity. Both broth and mycelia extracts were assayed in most cases. Each sample was assayed *in vitro* against up to 8 cell types (murine and human) in a disk diffusion/ clonogenic assay. From these results, the samples were assigned into one of 4 categories: Inactive (79% of the extracts), Equally active across cell types (16% of the extracts), Equally active and potent (9 extracts or 1%), and Solid tumor selective (42 extracts or 3.8%). The equally active and potent category is studied further since solid tumor selective compounds might exist in the extract but be concealed by one or more potent, cytotoxic compounds. Further, a novel, potent compound could form the basis for analog synthesis in an attempt to develop an active anticancer agent. One

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF Art Unit:

Marks, Peter Wayne et al. Examiner:

INTERNATIONAL APPLICATION NO: PCT/EP06/068656

FILED: November 20, 2006

U.S. APPLICATION NO: 12/094173

35 USC §371 DATE: May 19, 2008

FOR: Neuroendocrine Tumor Treatment

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Sir:

This paper is being filed:

☒ supplemental to the Information Disclosure Statement filed May 19, 2008.

☐ within three months of the date of entry of the national stage as set forth in 37 C.F.R. §1.491 of the international application. Therefore, no fees are required.

☒ before the mailing date of a first Office action on the merits, and so under 37 C.F.R. §1.97(b)(3) no fees are required.

If a fee is deemed to be required, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 19-0134 in the name of Novartis.

☐ This Information Disclosure Statement is being filed in accordance with 37 C.F.R. §1.97(c) or 37 C.F.R. §1.97(d).

☐ A letter for payment of fee set forth in 37 C.F.R. §1.17(p) is enclosed.

In accordance with 37 C.F.R. §1.56, applicants wish to call the Examiner's attention to the references cited on the attached form(s) PTO-1449.

☐ The listed references were cited in the international stage search report. Since these references are of record in the instant PCT application PCT/EP06/068656, copies are not enclosed herewith.

☐ Copies of the non-asterisked references are enclosed herewith.

☒ The references were cited in a search report in a corresponding United Kingdom and European applications. Copies of these references and the search reports are enclosed herewith.

The Examiner is requested to consider the foregoing information in relation to this application and indicate that each reference was considered by returning a copy of the initialed PTO 1449 form(s).

Respectfully submitted,

Gregory Houghton
Attorney for Applicant
Reg. No. 47,666

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-2614

Date: 12-3-09

The Patent Office

For Innovation

Application No:	GB0523658.3	**Examiner:**	Dr Carol Davies
Claims searched:	1-21	**Date of search:**	10 April 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-9, 12-19, 21	US2005/0187184 A1 (GIBBONS) See for example, [0002], [0006], [0009], [0010], [0016], [0032] and the claims.
X	1-9, 12-19 & 21	US2002/0183239 A1 (GIBBONS) See for example, [0007], [0009], [0010], [0039], [0055], [0056], [0063], [0064] & claims
X	1-9, 12-19 & 21	WO03/020266 A1 (GIBBONS) See for example, p.2 lines 6-27; p.3 lines 26-30; p.4 lines 14-23; p.8 line 27-p.9 line 11 & claims
X	1-9, 12-17	Biochemical and Biophysical Research Communications, 331, 2005-05-27, Asano Takayuki et al, "The Rapamycin analog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation", 295-302.
X	1-9, 12-19 & 21	Clinical cancer research: an official Journal of the American Association for Cancer Research; 10, 2004-10-15, Stephan Susann et al, "Effect of rapamycin alone and in combination with antiangiogenesis therapy in an orthotopic model of human pancreatic cancer", 6993-7000
X	1-9, 12-19 & 21	Clinical Cancer Research: an official Journal of the American Association for Cancer Research, 10, 2004-03-15, Bruns Christiane et al, "Rapamycin-induced endothelial cell death and tumor vessel thrombosis potentiate cytotoxic therapy aganist pancreatic cancer", 2109-2119.
X	1-9, 12-17 at least	Cancer Research, 64, 2004-01-01, Boulay Anne et al, "Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 Kinase 1 in peripheral blood mononuclear cells", 252-261.
X	1-9, 12-17 at least	Proceedings of the American Association for Cancer Research Annual Meeting, 43, 2002, Boulay Anne et al, "Prolonged effect of the rapamycin derivative RAD001 on p70S6 Kinase activity in tumors ... dosing schedules", 602.

The Patent Office

For innovation

X	1-9, 12-17 at least	Proceedings of the American Association for Cancer Research Annual Meeting, 43, 2002-03, O'Reilly Terence et al, "In vivo activity of RAD001, an orally active rapamycin derivative in experimental tumor models", 71

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

The following online and other databases have been used in the preparation of this search report

ONLINE: EPODOC, WPI, MEDLINE, BIOSIS

The Patent Office

For Innovation

Your ref :	TX/4-34678P1/NFI 8102	**Examiner :**	Dr Carol Davies
Application No:	GB0523658.3	**Tel :**	01633 81 4673
Applicant :	Novartis AG	**Date of report :**	11 April 2006

Patents Act 1977
Examination Opinion
Invention not patentable

1. Claims 1-8, 9-11 (insofar as they relate to claims 1-8), 12-14 (insofar as they relate to claims 1-11) & claims 19 & 20 (insofar as they relate to claims 1-14) are method of treatment claims which contravene Sections 1(1)(c) and 4(2) of the Patents Act 1977.

Novelty

2. The invention, as defined in claims 1-9, 12-19 & 21 at least, is not new because it has already been disclosed in each of the documents which have been cited in the search report in category X. The pharmaceutical combination of claim 21 need not necessarily be used for the treatment of endocrine tumors. The novelty of this claim has been destroyed by several of the documents which show combinations of rapamycin and eg. 5-fluorouracil in therapy.

The Patent Office

For innovation

Application No:	GB0609912.1	**Examiner:**	Dr Stephen Evans
Claims searched:	1-21	**Date of search:**	4 September 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-21	WO 02/098416 A ✓ (WYETH) see page 3 lines 1-5, page 13 line 15-19, in vivo tests and claims 5, 38
X	1-21	WO 02/080975 A ✓ (WYETH) see page 2 lines 30-33, page 13 lines 10-14, in vivo tests, claims 1, 5 & 26
X	1-21	WO 03/020266 A ✓ (WYETH) see page 4 lines 14-17, page 8 lines 14-18, claims 1, 5, 31-34 and in vivo tests
X	1-21	WO 2004/078133 A ✓ (WYETH) see page 3 lines 1-5, examples and claims 1 & 7
X	1-21	US 2003/0008923 A ✓ (DUKART et al) see paragraphs 10 & 63, in vivo tests and claims 1 & 5

Categories:

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&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

A61K; A61P

The following online and other databases have been used in the preparation of this search report

CAS ONLINE, WPI, EPODOC, TXTE

The Patent Office

For innovation

Your ref :	TX/4-34678P6/NPI 8102	**Examiner :**	Dr Stephen Evans
Application No:	GB0609912.1	**Tel :**	01633 81 3742
Applicant :	Novartis AG	**Date of report :**	5 September 2006

Patents Act 1977
Examination Opinion

Novelty

1. The invention, as defined in claims 15-18at least, is not new because it has already been disclosed in each of the documents which have been cited in the search report in category X, which are only selected examples.

Inventive Step

2. The invention, as defined in claims 1-14, 19-21 at least, is obvious in view of what has already been disclosed in the documents which have been cited in the search report in category X, which are only selected examples and in the light of the common general knowledge in this technical field.

Methods of treatment

3. Claims 1-14, 19, 20 clearly relate to methods of treatment and will conflict with section 4(2) of the Act. However, those claims were searched.

The Patent Office

For Innovation

Application No:	GB0602747.8	**Examiner:**	Dr Rowena Dinham
Claims searched:	1-21	**Date of search:**	26 June 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-21	WO 2004/078133 A3 (WYETH) See entire document, especially page 3 line 2-12, Examples and Claim 7
X	1-21	WO 03/020266 A1 (WYETH) See entire document, especially page 4 line 14-23, Examples and Claim 5
X	1-21	WO 02/098416 A3 (WYETH) See entire document, especially page 3 line 2-27, Examples and Claim 5

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

A61K

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, JAPIO, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS

The Patent Office

For innovation

Your ref :	TX/4-34678P3/NFL8102	**Examiner :**	Dr Rowena Dinham
Application No:	GB0602747.8	**Tel :**	01633 81 4995
Applicant :	Novartis AG	**Date of report :**	27 June 2006

Patents Act 1977
Examination Opinion

Non-patentable subject matter

1. Claims 1-14 relate to the treatment of the human or animal body and as such are non-patentable according to section 4(2).

Novelty

2. The invention, as defined in claims 1-8 & 13-19 at least, is not new because it has already been disclosed in each of the documents which have been cited in the search report in category X.

Inventive Step

3. The invention, as defined in claims 9-12 & 20-21 at least, is obvious in view of what has already been disclosed in the documents which have been cited in the search report in category X.

The Patent Office

For innovation

Application No:	GB0609272.0	**Examiner:**	Dr Stephen Evans
Claims searched:	1-21	**Date of search:**	4 September 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-21	WO 02/098416 A (WYETH) see page 3 lines 1-5, page 13 line 15-19, in vivo tests and claims 5, 38
X	1-21	WO 02/080975 A (WYETH) see page 2 lines 30-33, page 13 lines 10-14, in vivo tests, claims 1, 5 & 26
X	1-21	WO 03/020266 A (WYETH) see page 4 lines 14-17, page 8 lines 14-18, claims 1, 5, 31-34 and in vivo tests
X	1-21	WO 2004/078133 A (WYETH) see page 3 lines 1-5, examples and claims 1 & 7
X	1-21	US 2003/0008923 A (DUKART et al) see paragraphs 10 & 63, in vivo tests and claims 1 & 5

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&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

A61K; A61P

The following online and other databases have been used in the preparation of this search report

CAS ONLINE, WPI, EPODOC, TXTE

The Patent Office

For Innovation

Application No:	GB0607942.0	**Examiner:**	Dr Stephen Evans
Claims searched:	1-21	**Date of search:**	15 August 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-21	WO 02/098416 A (WYETH) see page 3 lines 1-5, page 13 line 15-19, in vivo tests and claims 5, 38
X	1-21	WO 02/080975 A (WYETH) see page 2 lines 30-33, page 13 lines 10-14, in vivo tests, claims 1, 5 & 26
X	1-21	WO 03/020266 A (WYETH) see page 4 lines 14-17, page 8 lines 14-18, claims 1, 5, 31-34 and in vivo tests
X	1-21	WO 2004/078133 A (WYETH) see page 3 lines 1-5, examples and claims 1 & 7
X	1-21	US 2003/0008923 A (DUKART et al) see paragraphs 10 & 63, in vivo tests and claims 1 & 5

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

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Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

A61K; A61P

The following online and other databases have been used in the preparation of this search report

CAS ONLINE, WPI, EPODOC, TXTE

Exceptions You do not have to supply details of a search report that (1) shows a nil response, or (2) has been published by WIPO or EPO, or (3) you have already supplied to us on a previous GB application.

Publication

I estimate that, provided you have met all the formal requirements, preparations for publication of your application will be completed soon after **11 September 2007**. At this time you will receive a letter confirming the exact date when the preparations for publication will be completed. This letter will also tell you the publication number and date of publication of your application.

Withdrawal/amendment

If you wish to withdraw your application before it is published you must do so before the preparations for publication are complete. **WARNING** – after preparations for publication are complete it will NOT be possible to withdraw your application from publication.

If you wish to file amended claims for inclusion with the published application you must do so before the preparations for publication are completed. If you write to the Office less than 3 weeks before 11 September 2007 please mark your letter prominently:

"URGENT - PUBLICATION IMMINENT".

Yours faithfully

Dr Stephen Evans
Examiner

The Patent Office

For Innovation

Application No:	GB0601082.1	**Examiner:**	Dr Stephen Evans
Claims searched:	1-21	**Date of search:**	22 May 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-21	WO 02/098416 A (WYETH) see page 3 lines 1-5, page 13 line 15-19, in vivo tests and claims 5, 38
X	1-21	WO 02/080975 A (WYETH) see page 2 lines 30-33, page 13 lines 10-14, in vivo tests, claims 1, 5 & 26
X	1-21	WO 03/020266 A (WYETH) see page 4 lines 14-17, page 8 lines 14-18, claims 1, 5, 31-34 and in vivo tests
X	1-21	WO 2004/078133 A (WYETH) see page 3 lines 1-5, examples and claims 1 & 7
X	1-21	US 2003/0008923 A1 (DUKART et al) see paragraphs 10 & 63, in vivo tests and claims 1 & 5

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X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

A61K; A61P

The following online and other databases have been used in the preparation of this search report

CAS ONLINE, WPI, EPODOC, TXTE

Exceptions You do not have to supply details of a search report that (1) shows a nil response, or (2) has been published by WIPO or EPO, or (3) you have already supplied to us on a previous GB application.

Publication

I estimate that, provided you have met all the formal requirements, preparations for publication of your application will be completed soon after **12 June 2007**. At this time you will receive a letter confirming the exact date when the preparations for publication will be completed. This letter will also tell you the publication number and date of publication of your application.

Withdrawal/amendment

If you wish to withdraw your application before it is published you must do so before the preparations for publication are complete. **WARNING** – after preparations for publication are complete it will NOT be possible to withdraw your application from publication.

If you wish to file amended claims for inclusion with the published application you must do so before the preparations for publication are completed. If you write to the Office less than 3 weeks before 12 June 2007 please mark your letter prominently:

"URGENT - PUBLICATION IMMINENT".

Yours faithfully

Dr Stephen Evans
Examiner

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21. Feb. 2007
F/F | MD | Inv. | RF | LE | PA

EPO Customer Services
Tel.: +31 (0)70 340 45 00

Date: 19.02.07

Reference	Application No./Patent No.
34678-EP-EPA	06120660.3 - 1216

Applicant/Proprietor: Novartis AG

Communication

The partial European search report (R. 46 EPC) is enclosed.

The applicant is informed that if the European search report is also to cover inventions other than the invention(s) already searched and for which a meaningful search can be carried out, a further search fee must be paid for each of these inventions, in the present instance

8 search fees

within **one month** after notification of this communication.

The amount payable for a search fee is EUR 1000,-- (OJ EPO 2006, 495).

If applicable, a European search opinion covering those invention(s) for which a search fee has been paid will then be sent together with the European search report.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☐ Abstract ☐ Title

☑ the Abstract was modified and the definitive text is attached to this communication.

The following figure will be published together with the abstract:

Note to the users of the automatic debiting procedure

Unless the EPO receives prior instructions to the contrary, the search fee(s) will be debited on the last day of the period for payment. For further details see the Arrangements for the automatic debiting procedure, Supplement No.2 to Official Journal No.01/2005.

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

under Rule 46, paragraph 1 of the European Patent Convention

Application Number EP 06 12 0660

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	TOWNSLEY, CAROL A. ET AL: "Evaluation of Adverse Events Experienced by Older Patients Participating in Studies of Molecularly Targeted Agents Alone or in Combination" CLINICAL CANCER RESEARCH, CODEN: CCREF4; ISSN: 1078-0432, vol. 12, no. 7, 1 April 2006 (2006-04-01), pages 2141-2149, XP002417742 * page 2143; table 1 *	1	INV. A61K31/436 A61P35/00 A61P35/04
X	US 2004/176339 A1 (SHERMAN MATTHEW L [US] ET AL) 9 September 2004 (2004-09-09) * claim 7 *	1	
X	US 2002/183240 A1 (GIBBONS JAMES J [US] ET AL) 5 December 2002 (2002-12-05) * claim 5 * * claim 32 *	1	
X	WO 03/020266 A (WYETH CORP [US]) 13 March 2003 (2003-03-13) * claim 5 * * claim 23 *	1	TECHNICAL FIELDS SEARCHED (IPC) A61K

-/--

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

The present partial European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims.

Place of search	Date of completion of the search	Examiner
The Hague	31 January 2007	Langer, Oliver

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 06 12 0660

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (IPC)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	WO 02/080975 A (WYETH CORP [US]) 17 October 2002 (2002-10-17) * claim 2 * * claim 5 * * claim 21 *	2	
X	WO 2005/082411 A (CHRISTENSEN JAMES G [US]; SALGIA RAVI [US]; SUGEN INC [US]; DANA FARBE) 9 September 2005 (2005-09-09) * page 2, paragraph 2 * * abstract * * claim 9 *	2	
X	WO 02/098416 A2 (WYETH CORP [US]) 12 December 2002 (2002-12-12) * claim 5 * * claim 29 *	2	TECHNICAL FIELDS SEARCHED (IPC)

2

(P04C19) FORM 1503 03.82

Although claims 1-9, 12-14 and 19 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 06 12 0660

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is rapamycin.

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2. claims: 11, 20, and partially 1-10, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is 40-O-(2-hydroxyethyl)-rapamycin.

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3. claims: 1-10, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is selected form the group of compounds consisting of 32-deoxorapamycin and 16-pent-2-ynyloxy-32-deoxorapamycin.

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4. claims: 1-10, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is selected form the group of compounds consisting of 16-pent-2-ynyloxy-32-(S or R)-dihydro-rapamycin and 16-pent-2-ynyloxy-32-(S or R)-dihydro-40-O-(2-hydroxyethyl)-rapamycin.

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5. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is 40-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin.

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

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6. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is 40-epi-(tetrazolyl)-rapamycin.

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7. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is "a compound disclosed under the name TAFA-93".

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8. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is "a compound disclosed under the name" "biolimus".

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9. claims: 1-9, 12-19, 21 (all partially)

Use of an mToR inhibitor in the manufacture of a medicament for treating endocrine tumors; a pharmaceutical composition comprising an mToR inhibitor; a pharmaceutical combination of an mToR inhibitor with a chemotherapeutic agent; wherein in each embodiment the mToR inhibitor is a "rapalog", as far as not comprised within one or more of inventions 1 to 8.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 06 12 0660

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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31-01-2007

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ABSTRACT / ZUSAMMENFASSUNG / ABRÉGÉ

06120660.3

Treatment of endocrine tumors by administration of an mTOR inhibitor, optionally in combination with another drug.

Effect of Rapamycin Alone and in Combination with Antiangiogenesis Therapy in an Orthotopic Model of Human Pancreatic Cancer

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ABSTRACT

Purpose: **The overall 5-year survival of patients with pancreatic cancer remains <5%. Novel therapeutic strategies are needed. We examined the effect of rapamycin, alone and in combination with antiangiogenesis therapy, on pancreatic cancer *in vivo*.**

Experimental Design: **Human pancreatic cancer AsPC-1 cells were orthotopically injected into severe combined immunodeficient/beige mice to evaluate primary tumor growth and liver metastasis after treatment with rapamycin alone or in combination with anti-vascular endothelial growth factor antibody 2C3. Tumor cell proliferation was determined by bromodeoxyuridine incorporation. To detect tumor cell apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling assay was used. Tumor angiogenesis was investigated by using a monoclonal anti-CD31 antibody. All statistical tests were two-sided.**

Results: **Rapamycin, alone and in combination with 2C3, strongly inhibited primary and metastatic tumor growth in an orthotopic pancreatic cancer animal model. Furthermore, the combination therapy significantly improved the effect on liver metastasis compared with single treatment with either rapamycin ($P = 0.0128$) or 2C3 ($P = 0.0099$). Rapamycin alone inhibited pancreatic tumor cell proliferation, induced apoptosis, and decreased tumor angiogenesis. Nevertheless, the combination therapy showed a significant, stronger inhibition of tumor cell proliferation ($P = 0.0002$ *versus* rapamycin alone and $P < 0.0001$ *versus* 2C3 alone). The induction of apoptosis was significantly higher than in the rapamycin-treated group ($P = 0.0039$). Additionally, the combination therapy further improved suppression of tumor cell angiogenesis compared with rapamycin treatment ($P = 0.029$)**

Conclusions: **Our studies propose new therapeutic strategies to inhibit both primary and metastatic tumor growth in pancreatic cancer. Considering the fact that liver metastasis is a crucial problem in advanced stages of pancreatic cancer, the combination therapy of rapamycin plus anti-vascular endothelial growth factor antibody 2C3 is a significant advantage compared with single treatment with rapamycin.**

INTRODUCTION

Adenocarcinomas of the pancreas are the fourth-leading cause of death in North America among men and women of all ages and the third cause of death among men between the ages of 40 and 59 years. More than 50% of the patients have advanced-stage disease at diagnosis. The 5-year survival rate among those patients remains about 2% (1). Although there have been encouraging results with the use of gemcitabine as a standard first-line agent for the treatment of advanced pancreatic cancer, a recent phase III trial has shown an overall median survival of 5.4 months for treatment with gemcitabine alone. A combination therapy of gemcitabine plus 5-fluorouracil did not improve the attained results (2). Therefore, novel therapeutic strategies are required to improve the prognosis of patients with pancreatic cancer. Among these strategies are drugs that target signal transduction pathways involved in tumor cell proliferation, invasion, or tumor angiogenesis.

One of the most promising drugs may be rapamycin, a bacterial macrolide with antifungal, immunosuppressant, and antitumor activities. Rapamycin is known to target the atypical Ser/Thr kinase mammalian target of rapamycin (mTOR) and inhibits the translation of key mRNAs of proteins required for cell cycle progression. Rapamycin binds with high affinity to the cytosolic 12-kDa FK506-binding protein (FKBP12). The rapamycin/FKBP12 complex inhibits interleukin-2–stimulated signal transduction and causes a partial dephosphorylation and deactivation of p70S6 kinase, an enzyme critical for the G_1 to S transition (3). Furthermore, rapamycin inhibits mitogen-stimulated phosphorylation of 4E-BP-1 (4). Dephosphorylated 4E-BP-1 interacts with the translation initiation factor eIF-4E and thereby inhibits cap structure-dependent protein synthesis

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Requests for reprints: Debabrata Mukhopadhyay, Mayo Clinic Cancer Center and Department of Biochemistry and Molecular Biology; Gugg 1401A, 200 First Street SW, Rochester, MN 55905. Phone: 507-538-3581; Fax: 507-284-1767; E-mail: mukhopadhyay.debabrata@mayo.edu.

and cell growth (5). Due to the inhibitory effect of rapamycin on interleukin-2–stimulated T-cell division, rapamycin is approved as an immunosuppressive drug for prevention of allograft rejection after renal transplantation. Interestingly, recent experiments with immunosuppressant doses of rapamycin indicated an inhibition of primary and metastatic tumor growth *in vivo* (6).

Another strategy involves targeting angiogenic factors that are important for the growth of new blood vessels from a preexisting microvascular bed. The blood supply delivers oxygen and nutrients to the tumor cells and contributes to metastatic spread. Vascular permeability factor/vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors described thus far. An increased expression of vascular permeability factor/VEGF in adenopancreatic carcinoma cells is associated with liver metastasis and a poor prognosis for the patient (7). Thus, antiangiogenic drugs may represent a promising therapeutic option.

Because tumorigenesis has been shown to be a multifaceted process involving a variety of potential therapeutic targets, further improvement may be achieved by combining different therapeutic concepts. In this study, we describe experiments indicating that immunosuppressive doses of rapamycin inhibit primary and metastatic tumor growth in pancreatic cancer *in vivo*. Furthermore, we show that a combination therapy of rapamycin, administered in conjunction with an angiogenesis inhibitor, improves the results achieved in the single-treatment groups.

MATERIALS AND METHODS

Drugs. Rapamycin isolated from *Streptomyces hygroscopicus* was obtained from Sigma-Aldrich (St. Louis, MO). A carrier solution was produced by using a diluent containing Tween 80 (10%), *N-N*-dimethylacetamide (20%), and polyethylene glycol 400 (70%; all from Sigma-Aldrich).

The VEGF antibody 2C3 is a mouse IgG2a monoclonal antibody developed to target recombinant human VEGF as described previously (8). 2C3 prevents VEGF from binding to VEGF receptor (VEGFR) 2 (KDR/Flk-1), but not VEGFR1 (Flt-1), in enzyme-linked immunosorbent and coprecipitation assays performed in solution. 2C3 is specific to human VEGF (both $VEGF_{121}$ and $VEGF_{165}$).

Human Pancreatic Adenocarcinoma Cell Line. The human pancreatic adenocarcinoma cell line AsPC-1 was purchased from American Type Culture Collection (Manassas, VA). AsPC-1 cells were cultured in RPMI 1640 with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

Orthotopic Tumor Model. Female 6-week–old severe combined immunodeficient (SCID)/beige mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in the institutional animal facilities. All animal work was performed under protocols approved by the Beth Israel Deaconess Medical Care Institutional Animal Care and Use Committee. To establish tumor growth in mice, animals were anesthetized by subcutaneously administering Avertin [2,2,2 tribromethanol; Sigma-Aldrich), 2-methyl-2-butanol (Fisher-Scientific, Pittsburgh, PA), and tert-amyl alcohol (Fisher Scientific) at doses of 0.2 mL per 10 g of body weight. Then 2×10^6 AsPC-1 cells, resuspended in 200 μL of RPMI 1640, were injected directly into the pancreas. Tumors were allowed to grow for 3 days without treatment. On day 4 after tumor cell injection, mice were randomized into four groups (eight animals per group), and treatment was initiated. One group was treated with rapamycin alone, administered at doses of 1.5 mg/kg/d intraperitoneally. A second group was treated with anti-VEGF antibody 2C3 alone, given at 100 μg per injection intraperitoneally three times per week during the first week and twice during the second and third week. In a third group, both rapamycin and anti-VEGF antibody 2C3 were administered by using the same schedule for each drug as described for the single treatments. The control group received daily injections of the carrier solution intraperitoneally. All mice were sacrificed by asphyxiation with CO_2 on day 20, and tumors were removed, measured, and prepared for immunochemistry. Primary tumor volumes were calculated by the formula $V = 1/2a \times b^2$, where a is the longest tumor axis, and b is the shortest tumor axis.

Cell Proliferation Assay. To perform the tumor cell proliferation assay *in vivo*, we used a technique that incorporates bromodeoxyuridine (BrdUrd) into proliferating cells (S-phase). All animals received intraperitoneal injection with 1 ml of BrdUrd labeling reagent (Zymed Laboratories, South San Francisco, CA) per 100 g of body weight 2 hours before sacrifice. Then tumor samples were removed, fixed in neutral buffered 10% formalin overnight, and embedded in paraffin. Tissue sections were cut and mounted on slides. After slides were pretreated by heating at 60°C, tissue samples were deparaffinized and rehydrated by washing in xylene and a graded series of ethanol. Endogenous peroxidase was blocked with 30% hydrogen peroxide. Slides were then rinsed with PBS and digested with trypsin (Zymed Laboratories). Nonspecific binding was blocked (blocking solution; Zymed Laboratories). BrdUrd was stained with a biotinylated mouse monoclonal anti-BrdUrd antibody (Zymed Laboratories). Slides were then incubated with streptavidin-peroxidase, and peroxidase activity was visualized with 3,3′-diaminobenzidine (Zymed Laboratories). Counterstaining was done with hematoxylin. The stained sections of five tumors from each group were reviewed, and BrdUrd-positive nuclei were scored in 10 randomly selected high-power fields (magnification, ×40).

Detection and Quantitation of Apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Intergen Co., Purchase, NY) was used for proving apoptosis in individual cells. The procedure was carried out following the manufacturer's directions using an ApopTaq Plus peroxidase *in situ* detection kit (Intergen). Briefly, after deparaffinization and rehydration in xylene and a graded series of ethanol, tissue sections were incubated with proteinase K (20 μg/mL) for 15 minutes at room temperature. Endogenous peroxidase was inhibited with 3% hydrogen peroxide for 5 minutes at room temperature. Terminal deoxynucleotidyl transferase was then applied to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of the fragmented DNA for 1 hour at 37°C. Subsequently, the slides were incubated with a horseradish peroxidase-conjugated antidigoxigenin antibody. To visualize peroxidase activity, 3,3′-diaminobenzidine substrate was added. Sections were counterstained with methyl green. Sections from normal female rat mammary gland (pro-

vided by Intergen), in which extensive apoptosis occurs, served as a positive control. Negative controls were run in which terminal deoxynucleotidyl transferase was omitted. The stained sections of three tumors of each group were reviewed, and TUNEL-positive cells were scored in 10 randomly selected high-power fields (magnification, ×40).

To detect the human Fas receptor (CD95), we used formalin-fixed, paraffin-embedded tumor tissue. The Fas mouse monoclonal antibody was obtained from NovoCastra (Newcastle upon Tyne, United Kingdom). Cells were counted in 10 randomly selected high-power fields (magnification, ×40) in 3 of 8 animals per group, and the average was calculated.

Quantitation of Blood Vessels in Solid Tumors. We determined the total number of blood vessels in cross-sections of three solid pancreatic tumors of each group with a rat monoclonal antibody to mouse CD31 (PharMingen, San Diego, CA). Briefly, tumor tissues were immersed in freshly prepared 4% paraformaldehyde in 0.02 mol/L phosphate buffer (pH 7.4). After 4 hours at room temperature, tissues were transferred to 30% sucrose in PBS (pH 7.4) and incubated overnight at 4°C before embedding in OCT compound (Ted Pella, Inc., CA). Frozen 5-μm to 10-μm cryostat sections were collected on slides, fixed in ice-cold acetone and 80% methanol, and rehydrated in PBS. Nonspecific binding was blocked in 20% fetal calf serum and 10% normal goat serum for 20 minutes. Primary antibody was added, and slides were incubated for 60 minutes at room temperature. Endogeneous peroxide was blocked with 30% hydrogen peroxide. Secondary antibody (biotinylated anti-rat IgG; Vector Laboratories, United Kingdom) was added at 1:200. ABC Elite kit solution (Peterborough; Vector Laboratories) was added at 1:200, and slides were incubated for 30 minutes at 4°C. Slides were then placed in staining solution [3-amino-9-ethyl-carbazole in *N-N*-dimethyl formamide, 0.1 mol/L acetate buffer (pH 5), and 30% hydrogen peroxide] for 5 minutes. To stop the staining reaction, slides were placed in 4% formaldehyde-acetate buffer (pH 5) for 10 minutes. Counterstaining was done with hematoxylin.

Liver Metastasis. Intrahepatic tumor growth was evaluated in tissue sections from six animals of the control group, five animals of the combination therapy-treated group, and four animals from the single-treatment groups. Briefly, the entire liver of each animal was fixed in neutral-buffered 10% formalin overnight and embedded in paraffin. Tissue sections were then mounted on slides and processed for staining with hematoxylin and eosin following standard protocols. Histologic views were digitalized, metastases were outlined, and the hepatic replacement area was calculated from the ratio of tumor area to total liver area (IP Lab software).

Statistical Analysis. Tumor volumes were summarized by means and 95% confidence intervals (CIs). BrdUrd-labeled nuclei, CD31-stained blood vessels, and TUNEL-positive cells were counted as described above, and means and 95% CIs were calculated. The hepatic replacement area was summarized as described above; data are given as mean and 95% CIs. To determine the statistical difference among the groups, Gaussian distribution was tested by using the method of Kolmogorov and Smirnov. Groups that followed the Gaussian distribution were tested by parametric tests (analysis of variance for differences among all four groups; unpaired Student's *t* test for differences among two groups). Groups that did not follow Gaussian distribution were log-transformed or tested by nonparametric tests [Kruskal-Wallis test (nonparametric analysis of variance) for differences among all four groups; Mann-Whitney test for differences among two groups]. Two-tailed *P* values of <0.05 were considered statistically significant.

RESULTS

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Primary Tumor Growth in Pancreatic Cancer *In vivo*. To investigate the effect of rapamycin on tumor growth in pancreatic cancer *in vivo*, we injected AsPC-1 human pancreatic adenocarcinoma cells into the pancreas of SCID/beige mice, simulating orthotopic primary tumor growth. Rapamycin was administered at doses of 1.5 mg/kg/d intraperitoneally, beginning on day 4 after tumor cell implantation. All control mice received an equal volume of carrier solution intraperitoneally. By day 20 relative to tumor cell injection, we found a significant suppression in primary tumor growth among mice treated with rapamycin alone in comparison to the control group. The average tumor size was 0.36 cm^3 (95% CI, 0.25–0.46) in the control group and 0.074 cm^3 (95% CI, 0.042–0.11) in the rapamycin-treated group ($P = 0.0002$, Mann-Whitney test *versus* control group; Fig. 1*A* and *B*).

To evaluate whether the effect of rapamycin on pancreatic primary tumor growth can be improved by a combination of rapamycin with anti-angiogenesis therapy, we used a mouse IgG2a monoclonal VEGF antibody (2C3) that prevents VEGF from binding to its receptor, VEGFR-2/KDR. The treatment was initiated on day-4-after-tumor-cell-injection. To monitor the effect of anti-VEGF antibody alone, 2C3 was administered as a single agent intraperitoneally three times within the first week and twice within the second and third week. The combination therapy of rapamycin + 2C3 was administered by using the same schedule for each drug as described for the single treatment. On day 20 relative to tumor cell injection, mice treated with 2C3 showed a significant suppression of primary tumor growth. The observed average tumor size was 0.16 cm^3 (95% CI, 0.13–0.19; $P = 0.0002$, Mann-Whitney test *versus* control group). The highest suppression of primary tumor growth was found in mice treated with the combination therapy. The average tumor size was 0.057 cm^3 (95% CI, 0.033–0.081; $P = 0.0002$, Mann-Whitney test *versus* control group). The combination therapy significantly improved the results achieved in the 2C3-treated group ($P = 0.0002$, Mann-Whitney test for comparison between combination therapy-treated group and 2C3-treated group). Although animals receiving the combination therapy showed an 18.6% smaller tumor volume than animals treated with rapamycin alone, this difference did not reach statistical significance ($P = 0.3282$, Mann-Whitney test for comparison between combination therapy-treated group and rapamycin-treated group, Fig. 1*A* and *B*).

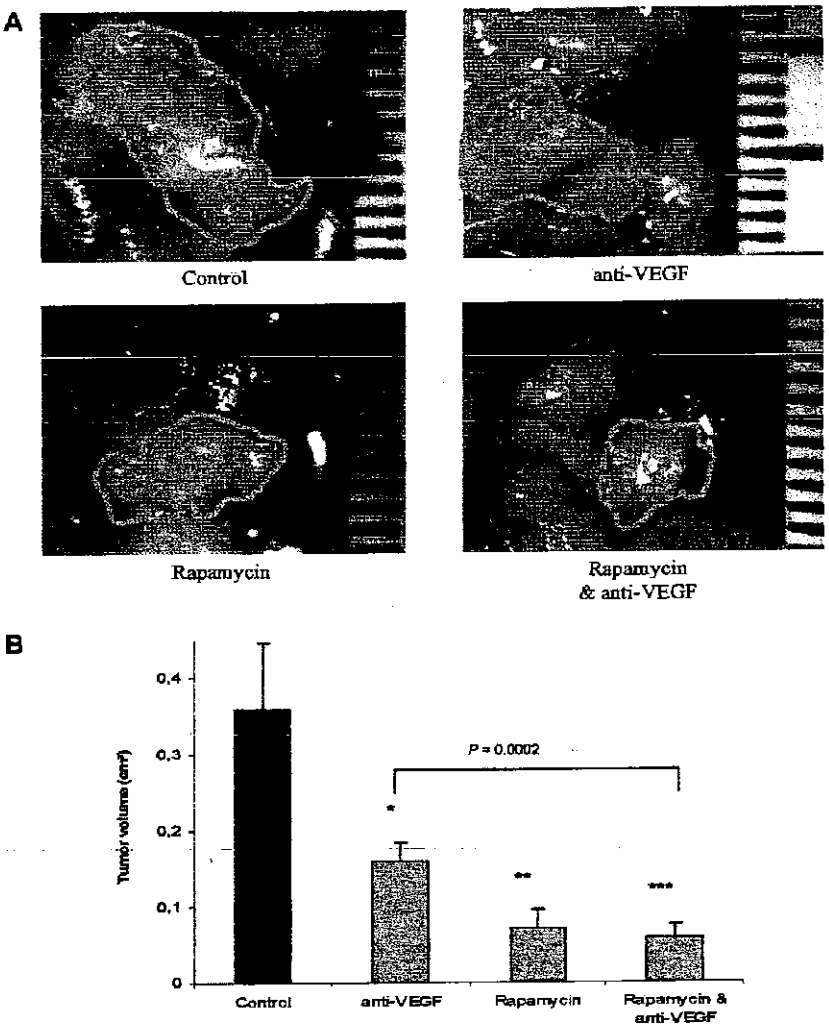

Fig. 1 Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on primary tumor growth in pancreatic cancer *in vivo*. Mice received orthotopic injection with AsPC-1 cells, and tumors were allowed to grow for 3 days before initiation of single-agent treatment with 2C3, single-agent treatment with rapamycin, and combination treatment with rapamycin and 2C3. The control group received only carrier solution. *A*, orthotopic pancreatic tumor growth *in situ* on day 20 after tumor cell injection. Tumor foci are *outlined*. *B*, average tumor volume on day 20 after tumor cell injection. *, $P = 0.0002$, Mann-Whitney test *versus* control group; **, $P = 0.0002$, Mann-Whitney test *versus* control group; ***, $P = 0.0002$, Mann-Whitney test *versus* control group. Although animals receiving the combination therapy showed an 18.6% smaller tumor volume than animals treated with rapamycin alone, this difference did not reach statistical significance ($P = 0.3282$, Mann-Whitney test for comparison between combination therapy-treated group and rapamycin-treated group). All statistical tests were two-sided. *Error bars* show 95% CIs.

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Metastatic Tumor Growth in Pancreatic Cancer *In vivo*. The selected orthotopic tumor model also mimics the metastatic progression of pancreatic cancer as observed in human pancreatic tumor patients. Histologic analysis of the entire liver tissue showed an average hepatic tumor replacement area of 0.47% (95% CI, 0.24–0.70) measured in the control group 20 days after tumor cell injection. The animals treated with single-agent rapamycin therapy showed a significant suppression of metastatic tumor growth in comparison with the control group. The calculated hepatic replacement area was 0.16% (95% CI, 0.1–0.22; $P = 0.018$, Student's t test *versus* control group; Fig. 2*A* and *B*). Although single-agent 2C3 treatment caused a reduction of the hepatic tumor replacement area of 22.3%, this difference did not reach statistical significance. The calculated average was 0.37% (95% CI, 0.2–0.54; $P = 0.348$, Student's t-test *versus* control group). The highest suppression of metastatic tumor growth was observed in mice receiving the combination therapy. The average hepatic replacement area was 0.05% (95% CI, 0.04–0.06; $P = 0.0053$, Student's t test *versus* control group). Furthermore, the combination therapy significantly improved the results achieved in both single agent-treated groups ($P = 0.0099$, Student's t test for comparison between 2C3-treated

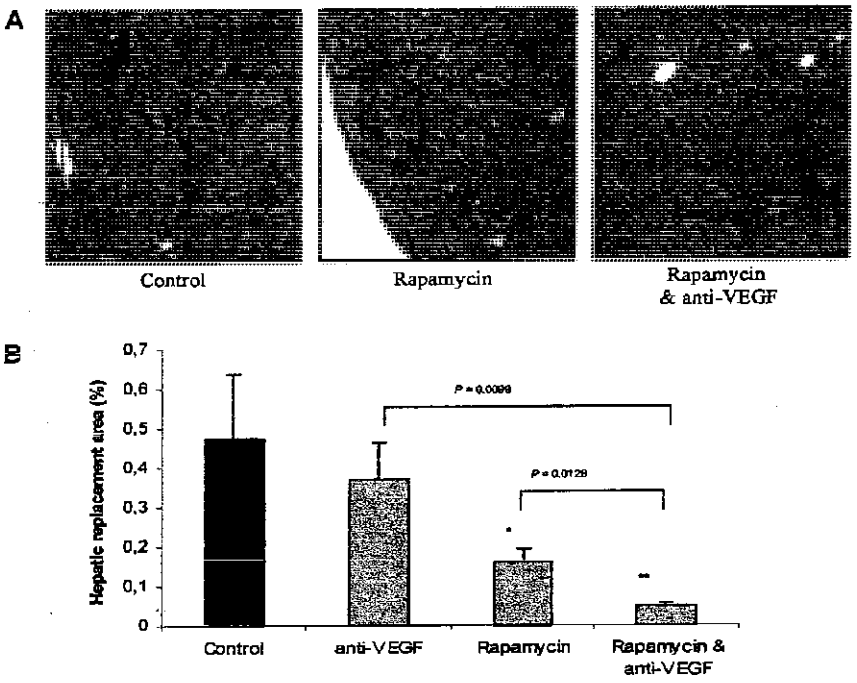

Fig. 2 Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on metastatic tumor growth in pancreatic cancer *in vivo*. Hematoxylin and eosin-stained liver sections were evaluated for metastatic tumor growth 20 days after injection of AsPC-1 cells into the pancreas of SCID/beige mice treated with 2C3, rapamycin, or rapamycin + 2C3 as combination therapy. Control mice received carrier solution. *A*, histologic sections of tumor-bearing livers from mice treated with carrier solution (*left panel*), rapamycin (*middle panel*), or combination therapy (*right panel*). *B*, intrahepatic metastases were measured, and the hepatic replacement area was determined. *, $P = 0.018$, Student's *t* test *versus* control group; **, $P = 0.0053$, Student's *t* test *versus* control group. All statistical tests were two-sided. *Error bars* show 95% CIs.

group and combination therapy-treated group; $P = 0.0128$, Student's *t* test for comparison between rapamycin-treated group and combination therapy-treated group; Fig. 2*A* and *B*).

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Tumor Cell Proliferation in Pancreatic Cancer. To determine whether the observed tumor growth suppression was caused by inhibition of cell proliferation, we investigated the effect of rapamycin alone and in combination with 2C3 on tumor cell proliferation as measured by BrdUrd incorporation with a BrdUrd-specific antibody. As shown in Fig. 3, the average amount of BrdUrd-labeled nuclei in 10 randomly selected microscopic fields was 493 (95% CI, 453.9–546.1) observed in the control group. A significant inhibition of tumor cell proliferation was found in the rapamycin-treated group. The average amount of proliferating cells was 380.2 (95% CI, 353.3–417.3; $P = 0.0018$, Student's *t* test *versus* control group). No significant differences were observed in the 2C3-treated group by scoring 457.8 proliferating cells (95% CI, 432.4–497.9; $P = 0.179$, Student's *t* test *versus* control group). However, the highest inhibition of tumor cell proliferation was seen in the combination therapy-treated group. An average of 270.8 (95% CI, 251.4–303.2) proliferating cells were counted ($P < 0.0001$, Student's *t* test *versus* control group). The combination therapy showed a significantly stronger suppression of tumor cell proliferation in comparison with both single agent-treated groups ($P < 0.0001$, Student's *t* test for comparison between combination therapy-treated group and 2C3-treated group; $P = 0.0002$, Student's *t* test for comparison between combination therapy-treated group and rapamycin-treated group).

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Apoptosis in Pancreatic Cancer. To further investigate the mechanism of the observed tumor-suppressive activities, we examined the effect of rapamycin alone and in combination with 2C3 on tumor cell apoptosis by TUNEL. The average number of TUNEL-positive cells measured in 10 randomly selected microscopic fields in the control group was 9.6 (95% CI, 8.2–11.1). The rapamycin-treated group showed a significant increase in the number of apoptotic cells. The calculated average was 18.7 (95% CI, 17.2–20.1; $P < 0.0001$, Student's *t* test *versus* control group). A significant increase was also observed in the 2C3-treated group with an average of 24.7 apoptotic cells (95% CI, 20.8–28.5; $P < 0.0001$, Student's *t* test *versus* control group) and in the combination therapy-treated group with 25 apoptotic cells (95% CI, 20.7–29.3; $P = 0.0001$, Student's *t* test *versus* control group). No significant difference was observed between the 2C3-treated group and the group receiving the combination therapy ($P = 0.815$, Student's *t* test for comparison between the 2C3-treated group and combination therapy-treated group). The combination therapy did improve the results achieved in the rapamycin-treated group ($P = 0.0039$, Student's *t* test for comparison between the rapamycin-treated group and the combination therapy-treated group; Fig. 4*A*). To confirm these results,

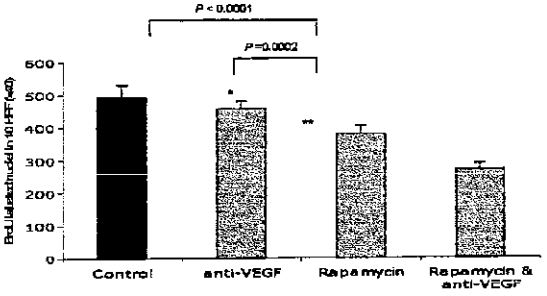

Fig. 3 Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on tumor cell proliferation in pancreatic cancer *in vivo*. Cell proliferation in tumor tissue of SCID/beige mice that received BrdUrd by intraperitoneal injections. Mice were treated with 2C3, rapamycin, or rapamycin in combination with 2C3 after orthotopic injection of AsPC-1 human pancreatic cancer cells. The control group received carrier solution. *, $P = 0.0018$, Student's t test *versus* control group; **, $P < 0.0001$, Student's t test *versus* control group. No significant differences were observed in the 2C3-treated group ($P = 0.179$, Student's t test *versus* control group). All statistical tests were two-sided. *Error bars* show 95% CIs.

we also examined the distribution of apoptosis-inducing Fas receptor CD95. The average number of CD95-positive cells measured in 10 randomly selected microscopic fields was 74.3 (95% CI, 47.1–101.6) in the control group. A significant increase in the number of apoptotic cells was observed for the rapamycin-treated group with an average of 182.3 (95% CI, 157.3–207.3; $P = 0.00023$, Student's t test *versus* control group) and the 2C3-treated group with an average of 184 (95% CI, 120.6–247.4; $P = 0.0024$, Student's t test *versus* control group). The combination therapy-treated group also showed a significant increase in the number of apoptotic cells with an average of 229 (95% CI, 207.5–250.5; $P < 0.0001$, Student's t test *versus* control group). The combination therapy improved the results achieved in the 2C3-treated group ($P = 0.044$, Student's t test for comparison between the 2C3-treated group and the combination therapy-treated group). A very significant increase in apoptosis was found after combination therapy in comparison with the rapamycin-treated group ($P = 0.0037$, Student's t test for comparison between the rapamycin-treated group and the combination therapy-treated group; Fig. 4*B*).

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Tumor Angiogenesis in Pancreatic Cancer. Because recent investigations suggest that rapamycin may have antiangiogenic activities linked to a decrease in production of VEGF and to a markedly inhibited response of vascular endothelial cells to stimulation by VEGF (6), we were interested in determining the effect of rapamycin alone and in combination with 2C3 on tumor angiogenesis. Therefore, we stained tumor sections with a rat antimouse monoclonal CD31 antibody. The average number of CD31-positive vessels measured in the control group was 626 (95% CI, 554.3–697). We found a significant decrease of tumor vessels measured in the rapamycin-treated group. The average number of stained vessels was 211.3 (95% CI, 188.3–234.4; $P < 0.0001$, Student's t test *versus* control group). As expected, we also found a significant inhibition of tumor angiogenesis in the group treated with only 2C3. The average number of stained vessels was 252 (95% CI, −16.6 to 520.6; $P = 0.0044$, Student's t test *versus* control group). An additional effect on tumor angiogenesis was observed in the group that received both drugs. The average number of stained blood vessels was 144.5 (100.03–188.9; $P = 0.0002$, Student's t test *versus* control group). The combination therapy-treated group showed significantly fewer tumor vessels than the group treated with rapamycin alone ($P = 0.0029$, Student's t test for comparison between the combination therapy-treated group and the rapamycin-treated group). No significant difference was found between the group treated with 2C3 and the group treated with combination therapy ($P = 0.2747$; Fig. 5).

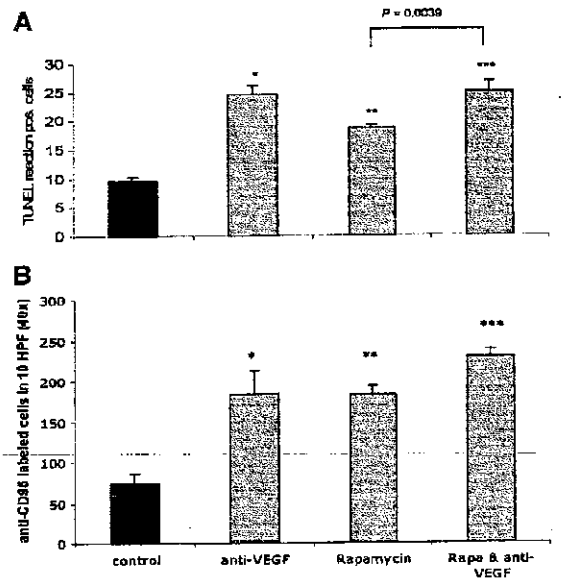

Fig. 4 Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on apoptosis in pancreatic cancer *in vivo*. SCID/beige mice were treated with 2C3, rapamycin, or rapamycin in combination with 2C3 after orthotopic injection of AsPC-1 human pancreatic cancer cells. The control group received only carrier solution. *A*. To detect tumor cell apoptosis, TUNEL was used. The average number of TUNEL-positive cells was scored in 10 randomly selected microscopic fields. *, $P < 0.0001$, Student's t test *versus* control group; **, $P < 0.0001$, Student's t test *versus* control group; ***, $P = 0.0001$, Student's t test *versus* control group. No significant difference was observed between the 2C3-treated group and the group receiving the combination therapy ($P = 0.815$, Student's t test). All statistical tests were two-sided. *Error bars* show 95% CIs. *B*. To determine the distribution of apoptosis inducing Fas receptor CD95, a monoclonal anti-Fas antibody was used. The average number of CD95-positive cells in 10 randomly selected microscopic fields was calculated. *, $P = 0.0024$, Student's t test *versus* control group; **, $P = 0.00023$, Student's t test *versus* control group; ***, $P < 0.0001$, Student's t test *versus* control group. No significant difference was observed between the single-agent treatment groups and the group receiving the combination therapy. All statistical tests were two-sided. *Error bars* show 95% CIs.

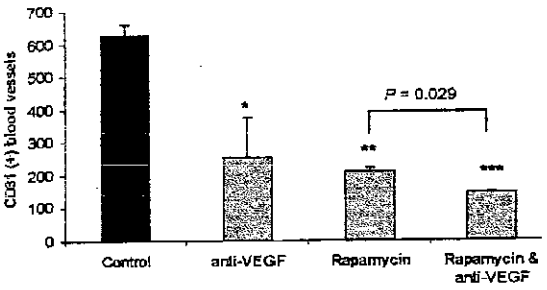

Fig. 5 Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on tumor angiogenesis in pancreatic cancer *in vivo*. SCID/beige mice were treated with 2C3, rapamycin, or rapamycin in combination with 2C3 after orthotopic injection of AsPC-1 human pancreatic cancer cells. The control group received carrier solution. To determine the effect of rapamycin alone and in combination with 2C3 on tumor angiogeaesis, tumor sections were stained with an antimouse monoclonal CD31 antibody. *, $P = 0.0044$, Student's t test *versus* control group; **, $P < 0.0001$, Student's t test *versus* control group; ***, $P = 0.0002$, Student's t test *versus* control group. All statistical tests were two-sided. *Error bars* show 95% CIs.

DISCUSSION

Our data indicate that rapamycin, alone and in combination with antiangiogenesis therapy, strongly inhibits primary and metastatic tumor growth in pancreatic cancer *in vivo*. Furthermore, we could show that the combination therapy inhibits liver metastasis more assertively than treatment with a single agent. Although the difference between the primary tumor volume of mice treated with combination therapy *versus* rapamycin alone did not reach a statistical significance, we found an approximately 19% smaller pancreatic tumor volume in the combination therapy-treated group. However, to our knowledge, there are no *in vivo* studies thus far using rapamycin as a single treatment as well as rapamycin in combination with antiangiogenic therapy in this highly aggressive tumor model of pancreatic cancer.

To explain the potential antitumor effect of rapamycin alone and in combination with anti-VEGF antibody 2C3, we considered three basic theories. First, rapamycin may directly inhibit tumor cell proliferation. Grewe *et al.* (9) found evidence that rapamycin inhibits basal p70s6K activity and induced dephosphorylation of p70s6K and 4E-BP-1 in human pancreatic cancer lines MiaPaCa-2 and Panc-1. Rapamycin also inhibited DNA synthesis and anchorage-dependent and -independent proliferation. Finally, rapamycin strikingly inhibited cyclin D1 expression in pancreatic cancer cells (9). Additionally, Shah *et al.* (10) have shown that rapamycin significantly inhibited serum-induced proliferation in two human pancreatic cancer cell lines, BxPC3 and Panc-1. The inhibition observed in both cell lines was about 30%. Our *in vivo* results also strongly support this hypothesis because we found an inhibition of tumor cell proliferation of approximately 23% after single-agent rapamycin treatment. The single-agent treatment with 2C3 caused no significant inhibition of tumor cell proliferation. Surprisingly, the combination therapy did improve the results achieved with single-agent rapamycin treatment. The precise contribution of 2C3 to the observed improvement of the therapeutic effect of rapamycin is difficult to determine and is not yet clear to us. From a rather mechanistic perspective, a possible explanation for this phenomenon could be the fact that VEGF-A induces not only angiogenesis but also lymphangiogenesis. Nagy *et al.* (11) could show that VEGF-A ($VEGF_{164/165}$) induces proliferation of lymphatic endothelium, resulting in the formation of greatly enlarged and poorly functioning lymphatic channels. They described "giant vessels" with incompetent valves, sluggish flow, and delayed lymph clearance (11). Consequently, the lymphatic malformation enhanced the VEGF-A–driven plasma extravasation and edema (11). This mechanism, in turn, could cause a poor biological availability of additional administered drugs. Using anti-VEGF antibody 2C3 might have improved the biological availability of rapamycin. Other authors support the hypothesis that after antiangiogenic treatment, the blood vessels that are still there will carry more blood to the tumor and in that way enhance the delivery of drugs (12). Further investigations will be necessary to elucidate the mechanism by which antiangiogenesis therapy improves drug effects when administered in a combination therapy.

Secondly, the observed antitumor effect of rapamycin alone and in combination with 2C3 may be a result of drug-induced apoptosis. Nave *et al.* (13) found that Akt/protein kinase B regulates the phosphorylation state of mTOR directly. Akt, an apoptotic regulator, is frequently activated in pancreatic cancer (14). Although rapamycin is not considered to be an antiapoptotic factor, a recent publication established Akt signaling through mTOR and eIF-4E as an important mechanism of oncogenesis and drug resistance *in vivo* by proving that rapamycin can restore apoptotic sensitivity to lymphomas expressing Akt *in vivo* (15). Our studies also indicate that rapamycin might have an effect on regulation of programmed cell death. We found an almost 2-fold induction of tumor cell apoptosis after single-agent rapamycin treatment. The observed induction of apoptosis by disrupting VEGFR2 signaling was not unexpected. It is known that targeting VEGF (16) or VEGFR (17) in pancreatic cancer *in vivo* causes a higher apoptosis index. Our results suggest that 2C3 as a single-agent treatment induces more apoptosis in pancreatic cancer than rapamycin as a single agent and that those effects are not additive.

The third theory about the observed antitumor effect of rapamycin alone and in combination with 2C3 is based on the fact that rapamycin showed antiangiogenic activities *in vivo* not only by decreasing the production of VEGF but also by inhibiting the response of vascular endothelial cells on stimulation by VEGF (6). Our findings strongly support this theory because we found a significant inhibition of tumor angiogenesis after single-agent rapamycin treatment. The effect of 2C3 on tumor angiogenesis was as expected. 2C3 is known to decrease the mean microvascular density in an orthotopic model of human breast cancer in nude mice (18). The combination therapy of rapamycin + anti-VEGF antibody 2C3 amplified the inhibition of tumor angiogenesis from 66% after single-agent rapamycin treatment to 77% compared with the control.

One of the major problems (besides the rapid primary tumor progression in pancreatic cancer) is an early and wide-

spread metastatic tumor growth. To address this issue, we were also interested in determining whether rapamycin treatment, alone or in combination with anti-VEGF antibody, has an impact on metastasis. We found significant suppression of metastatic tumor growth in both groups treated with rapamycin, either alone or in combination with anti-VEGF antibody 2C3. Furthermore, the combination therapy improved the results achieved in both single agent–treated groups remarkably. To our knowledge, there are no single or combination therapies for pancreatic cancer thus far achieving comparable results.

Hence, the combination therapy of rapamycin and anti-VEGF antibody 2C3 could be a new and promising therapeutic approach to the treatment of pancreatic cancer. The suppression of primary tumor volume and the significant reduction of metastasis in the combination therapy-treated group was caused by a combinatorial effect of both rapamycin and 2C3. Tumor cell proliferation and metastasis were predominantly regulated by rapamycin, although there were additive effects resulting from treatment with anti-VEGF antibody 2C3. On the other hand, 2C3 antibody plays a major role in regulating programmed cell death in the combination therapy-treated group. Both rapamycin and 2C3 antibody are important for inhibiting tumor angiogenesis.

Taken together, our studies propose new therapeutic strategies to inhibit both primary and metastatic tumor growth in pancreatic cancer. Rapamycin administered as a single agent as well as in combination with anti-VEGF antibody 2C3 strongly inhibits pancreatic tumor growth and liver metastasis. Nevertheless, the combination therapy has significant advantages compared with the single-agent treatment with rapamycin, especially when considering the fact that early metastatic tumor growth is a crucial problem in advanced stages of pancreatic cancer.

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Evaluation of Adverse Events Experienced by Older Patients Participating in Studies of Molecularly Targeted Agents Alone or in Combination

Carol A. Townsley, Gregory R. Pond, Amit M. Oza, Hal W. Hirte, Eric Winquist, Glenwood Goss, Pamela Degendorfer, Malcolm J. Moore, and Lillian L. Siu

Abstract

Background: The tolerability of molecularly targeted agents in older patients has not been specifically examined. Adverse event data from clinical trials in the Princess Margaret Hospital Phase II Consortium database were analyzed to address this question.

Methods: The Consortium database collects trial information on all patients treated with either a molecularly targeted agent alone or in combination since 2001. The frequency of adverse events was determined and analyzed by two different age groups, <65 years and ≥65 years. Toxicity indices (TI) and frequencies of dose-limiting toxicities (DLT), based on adverse events of all causalities (TI_{ALL} and DLT_{ALL}), and on adverse events that were at least possibly related to the molecularly targeted agent (TI_{MTA} and DLT_{MTA}), were calculated for both age groups.

Results: Four hundred and one patients who received 1,252 treatment cycles were analyzed from 19 different studies. Baseline performance status was similar between both age groups, but fewer older patients have had multiple prior regimens of chemotherapy or prior radiation therapy. A comparison of the proportions of younger and older patients experiencing DLT_{ALL} and DLT_{MTA} showed similar results. The TI_{MTA} values were comparable between the two age groups in both single agent (3.25 versus 3.00, for <65 versus ≥65 years) and multi-agent (3.65 versus 3.00, for <65 versus ≥65 years) trials.

Conclusions: Older patients seem to tolerate molecularly targeted therapies either alone or in combination with chemotherapy as well as younger patients. Age alone should not be a barrier in the administration of targeted agents.

Individuals ages 65 years or over constitute the fastest growing segment of the North American population (1). In 2001, >50% of new cases of cancer and 67% of all cancer deaths occurred in people over the age of 65 years, thus making the effective care of the older patient with cancer an imperative goal (2). This geriatric population presents a significant challenge to the medical system, not only because of increasing numbers but also because of the complex health issues that often develop with increasing age (3). Attitudes toward the older patient affect their cancer management. Many health professionals associate chronological age with poor prognosis, cognitive impairment, decreased quality of life, limited life expectancy, and decreased social worth (4). Thus, the older patient receives less screening for cancer, fewer staging tests, less aggressive therapy, and more often, no treatment at all (5). Older patients themselves may attribute cancer symptoms to the aging process, resulting in delayed diagnosis. Preconceived impressions of the toxicities from cancer therapies may also make older patients less likely to accept or request more aggressive treatments (6).

Unfortunately, the current literature is not helpful in educating physicians who accept a stereotype that all older patients have poor tolerance to chemotherapy or radiation therapy. Many studies have investigated this phenomenon, but reports are contradictory. Several trials using cytotoxic therapy have shown an increased risk of toxicity including myelotoxicity in the elderly (7–13), although other studies have shown almost equivalent toxicity profiles between older and younger patients (14–18).

Molecularly targeted agents are currently emerging as a new cancer treatment strategy. Theoretically, they are more specific against cancer targets than conventional cytotoxic chemotherapy, and as such, may be better tolerated by all patients, including those of more advanced age. As yet, no age-specific analysis evaluating the tolerability of molecularly targeted agents in this population have been reported.

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This study uses the Princess Margaret Hospital Phase II Consortium database to examine the tolerability of molecularly targeted agents alone or in combination with chemotherapy in two different age groups, those <65 years and those ≥65 years old. If evidence of good tolerability of molecularly targeted agents in older patients can be provided to clinicians, their enrollment on clinical trials may be increased, more treatment options may be offered, and care may be improved in this population.

Materials and Methods

The Princess Margaret Hospital Phase II Consortium database collects data on all clinical trials done through the Consortium from the year 2001 to the present. The Consortium is contracted to conduct early phase clinical trials under the auspices of the Cancer Therapy and Evaluation Program of the U.S. National Cancer Institute. The majority of the clinical trials done by the Consortium are phase II disease-specific trials to determine therapeutic efficacy, and a minority are dose- and toxicity-finding phase I or phase I/II trials. All trials done by the Consortium to date have involved molecularly targeted agents, either given alone or in combination with a cytotoxic chemotherapeutic agent. The data collected by the Consortium database include baseline demographic information, baseline comorbidities as reported by the patients, response evaluations based on Response Evaluation Criteria in Solid Tumors criteria for solid tumors or standard response criteria for hematologic malignancies for each patient, toxicity and adverse event reports on all trial patients which are captured at the cycle level. Furthermore, the doses of study drug(s) received by patients are recorded on a per cycle basis into the database.

For this project, the Consortium database was used to compare adverse events experienced by patients participating in Consortium studies as stratified by age. Patients were divided into two age groups: <65 years and ≥65 years. These cutoffs were chosen in order to be consistent with the current literature examining the recruitment of older patients with cancer into clinical trials which has typically used 65 years to define the elderly population (19). A recent study examining the tolerability of bevacizumab in an elderly population has also used 65 as the cutoff age (20, 21). Results were grouped into studies evaluating molecularly targeted anticancer agents given as monotherapy, and studies comprised of a combination of a molecularly targeted agent and chemotherapy. Baseline demographics such as age, performance status and prior treatments were obtained from the database. Comorbidities were taken from baseline case report forms and were quantified based on the Charlson Comorbidity Index (22). Best responses to treatment on the Consortium studies were recorded for all patients. For each patient, dose intensity was calculated by taking the actual dose received divided by the expected dose × 100%. The expected dose was calculated by taking the total dose a patient should have received as per protocol for the length of time they were on the study and assuming no dose reductions, delays, or omissions occurred. When dosing was based on body surface area or weight, the baseline values of height and weight were used in the calculation of the expected dose. Thus, if a patient had an increase in weight, which led to a greater body surface area while on study treatment, it is possible that >100% dose intensity can be achieved. For patients treated with a combination of chemotherapy and molecularly targeted agent, the dose intensities were calculated for both agents.

A modified toxicity index (TI) was calculated using the methods of Rogatko et al. (23). Depending on the protocol, adverse events were graded on Consortium studies by the National Cancer Institute Common Toxicity Criteria versions 2.0 or 3.0. All adverse events for each patient are ordered from most severe to least severe using National Cancer Institute Common Toxicity Criteria versions 2.0 or 3.0: $x_1, x_2, \ldots, x_n$, where x_i is the grade of the ith most severe adverse event for each patient. Then, the TI is calculated as:

$$x_1 + \frac{x_2}{(1+x_1)} + \frac{x_3}{(1+x_1)(1+x_2)} + \cdots + \frac{x_n}{(1+x_1)(1+x_2)\ldots(1+x_{n-1})}.$$

The TI is a patient level statistic between 0 and 6 describing the cumulative toxicity experienced by each patient where the integer number indicates the grade of the highest grade adverse event experienced for each patient. All other adverse events are accounted for in the final TI score. However, lower grade adverse events contribute less to the final score as a large number of similarly graded adverse events create a TI score slightly lower than a single adverse event of the next highest grade. For our analysis, hematologic adverse events for the leukemia or lymphoma studies were excluded as these patients often have abnormal pretreatment hematologic values due to their disease, but grade 5 adverse events (e.g., on-study deaths) were included.

The causality of adverse events that occur on Consortium studies are attributed based on the treating physician's opinion regarding their relationship to the study drug(s). The existing categories include adverse events that are definitely related, probably related, possibly related, unlikely related, or unrelated to the study drug(s). For the purpose of this project, the TI was calculated in two ways. First, inclusion of adverse events into the TI_{ALL} was based on incorporating adverse events of all attributions, regardless of the perceived causal relationship between the study drug(s) and the development of the adverse event. A second calculation of the TI, TI_{MTA}, was done by including only adverse events which were at least possibly related (i.e., possibly, probably, and/or definitely related) to the molecularly targeted agent. For studies which were comprised of a combination of a molecularly targeted agent and a cytotoxic chemotherapeutic agent, only the molecularly targeted agent was considered in the derivation of the TI_{MTA}. Adverse events related only to the chemotherapeutic agent, in the combination trials, were not included when calculating the TI_{MTA}. Adverse events related at least possibly to either the molecularly targeted agent, or the chemotherapeutic agent, in the combination trials, would be included in the calculation of the TI_{MTA}. If a patient experienced the same adverse event multiple times in the same cycle, only the worst grade adverse event was included in the calculation of the TI.

In addition to the TI, the frequencies of dose-limiting toxicities (DLT) were compared between age groups. A DLT was defined as any ≥grade 3 nonhematologic adverse event, or any grade ≥4 hematologic adverse event, except for the leukemia or lymphoma studies, in which only the nonhematologic and grade 5 adverse events were included. Analyses were done based on the number of patients who experienced at least one DLT, as well as the number of cycles in which at least one DLT occurred, were calculated. Similar to the abovementioned derivations of TI_{ALL} and TI_{MTA}, the frequencies of DLT were reported in two ways. DLT_{ALL} incorporated DLT of all attributions, regardless of the perceived causal relationship between the study drug(s) and the development of the adverse event. DLT_{MTA} included only DLT, which were at least possibly related (i.e., possibly, probably, and/or definitely related) to the molecularly targeted agent, in monotherapy or combination studies.

Generalized estimating equations with a compound symmetry correlation matrix for patients within the same trial were used to test whether the TI was different for patients <65 versus patients 65 and older. A similar analysis was used to test whether the dose intensity was different between patients from different age groups. Logistic regression with a compound symmetry correlation matrix was used to test for differences in the frequency of DLT between patients from different age groups. When examining at the frequency of DLT per cycle, an association was assumed to exist between patients from the same trial and between cycles from the same patient. Cycles closer to one another were assumed more likely to be associated, thus, a first-order autoregressive correlation matrix was assumed between cycles for the

same patient with patients nested within the same trial. The correlation matrix is included as it is likely that the TI and the probability of having a DLT for patients within the same trial, and the probability of having a DLT for cycles from the same patient, are associated with one another. In other words, it is likely that patients given the same treatment will have similar levels of adverse events. Each analysis was done separately for monotherapy and combination trials. Statistical significance was defined as $P \leq 0.05$ and all tests were two-sided. As a check of the results, a secondary analysis was done with age defined as a continuous variable, however, the results were similar, thus only the categorical results are shown for simplicity. Approval for this retrospective data analysis was obtained from the Princess Margaret Hospital Research Ethics Board.

Results

Molecularly targeted agents evaluated. All patients accrued to a Consortium trial that included a molecularly targeted drug, alone or in combination with a cytotoxic chemotherapeutic agent, were included in this study. A total of 401 patients who received 1,252 treatment cycles on 19 studies were analyzed (Table 1). Most studies were phase II, but three phase I and three phase I/II studies were also included. Only 32% of all patients were age 65 years or older, however, all studies had accrued at least one patient in both the older and younger age groups. The molecularly targeted agents evaluated in these studies have diverse mechanisms of anticancer activities by acting on a variety of cellular pathways. Examples include inhibitors of the epidermal growth factor receptor tyrosine kinase, mTOR pathway, and proteasome-ubiquitin pathway. A listing of the class of each molecularly targeted agent, based on its proposed mechanism(s) of anticancer activity, is included in Table 2. The Consortium studies accrued patients from many different tumor sites, with gastrointestinal, gynecologic, genitourinary, head and neck cancers, and leukemia/lymphoma studies enrolling the greatest numbers of patients (Table 3).

Patient characteristics. The baseline characteristics of all patients were compared by age group, in both monotherapy and multi-agent combination drug trials (Table 4). The median ages were comparable between the single and multi-drug trials for the younger patient groups, and likewise for the older patient groups. More than half of the patients in each age group were male, except for the group <65 years old in multi-drug combination trials in which 53% were female. The range of Eastern Cooperative Oncology Group performance status was similar between the two age groups in the monotherapy trials. However, the percentage of older patients who had a poorer performance status (i.e., Eastern Cooperative Oncology Group status 2) was higher than younger patients in the multi-agent drug trials. Older patients had more comorbidities, based on the Charlson Index, than the younger patients, but the difference was not statistically significant. In general, older patients were less heavily pretreated than younger patients both in the number of prior systemic therapeutic regimens received, and in the frequency of prior radiation. Baseline adverse events were similar between the younger and older age groups, in both single and multi-agent trials. The majority of grade 3 or 4 baseline adverse events were due to disease or other intercurrent comorbid conditions, including biochemical (e.g., hyperglycemia, elevated alkaline phosphatase) and hematologic abnormalities (e.g., lymphopenia, anemia requiring blood

Table 1. Number of patients enrolled by study and age group ($N = 401$)

Phase of study	Agents used	Tumor types	Single drug trials		Multi-drug trials	
			<65	≥65	<65	≥65
I/II	Erlotinib and Cisplatin	Head and neck	0	0	42	9
II	Erlotinib	Colorectal	20	18	0	0
I	Decitabine	Leukemia	21	19	0	0
I	UCN-01 and topotecan	Solid tumor	0	0	30	4
I	UCN-01 and fludarabine	Leukemia/lymphoma	0	0	3	1
II	Tipifamib	Bladder	2	9	0	0
I	Imatinib, cisplatin, irinotecan	Small cell lung cancer	0	0	5	4
II	Imatinib	Salivary gland	13	3	0	0
I/II	Oblimersen sodium and doxorubicin	Hepatocellular	0	0	18	9
II	Bortezomib	Colorectal	13	6	0	0
II	Perifosine	Breast	16	2	0	0
II	Perifosine	Pancreas	13	6	0	0
I/II	GTI-2040 and docetaxel	Non – small cell lung cancer	0	0	13	6
II	Bortezomib	Bladder	6	13	0	0
II	UCN-01 and topotecan	Ovarian	0	0	13	5
II	Temsirolimus	Neuroendocrine	20	5	0	0
II	Triapine and gemcitabine	Pancreas	0	0	11	9
II	Sorafenib and gemcitabine	Ovarian	0	0	10	1
II	Lapatinib	Salivary gland	2	1	0	0
I			21	19	35	8
I/II			0	0	73	24
II			105	63	37	16
Total (%)			126 (31.4)	82 (20.4)	145 (36.2)	48 (12.0)

Table 2. Listing of molecularly targeted drugs under evaluation on Consortium trials

Molecularly targeted agent	Class of drug based on proposed mechanism(s) of anticancer activity
Erlotinib	Epidermal growth factor tyrosine kinase inhibitor
Decitabine	Inhibitor of DNA methylation
UCN-01	Protein kinase C inhibitor
Tipifarnib	Farnesyltransferase inhibitor
Imatinib	Protein tyrosine kinase inhibitor of ABL, c-kit, and platelet-derived growth factor receptor
Oblimersen sodium	Oligonucleotide antisense molecule that inactivates the *Bcl-2* gene
Bortezomib	Proteasome inhibitor
Perifosine	Alkylphospholipid: cell cycle inhibitor
GTI-2040	Ribonucleotide reductase inhibitor
Temsirolimus	mTOR inhibitor
Triapine	Ribonucleotide reductase inhibitor
Sorafenib	Raf kinase inhibitor and vascular endothelial growth factor receptor 2 inhibitor
Lapatinib	Epidermal growth factor and erbB2 inhibitor

transfusions especially in leukemic patients), or tumor-related symptoms (e.g., myalgia, fatigue, tumor pain, and dysphagia).

Protocol treatment and dose intensity. The amount of treatment received, based on the number of cycles delivered, was compared between age groups (Table 5). Over 90% of patients received at least one cycle of treatment and the mean number of cycles received per patient was >3 in all groups, with the patients in multi-drug trials getting slightly more cycles per patient. The mean number of cycles received by younger patients in multi-drug trials was higher than their older counterparts (3.8 versus 3.2). This disparity may be due to an artifact of enrollment, as almost 50% of younger patients, treated with combination therapy, were entered in one of two specific trials in which patients stayed on treatment for multiple cycles, whereas only 27% of older patients treated with combination therapy were entered in these two trials. Thus, the difference in number of cycles is possibly due to disproportional accrual of patients into different studies.

In order to ensure that any differences in toxicity observed between patients of different age groups were not based on discrepancies in the amount of drug received, a dose intensity calculation was done (Table 5). For both single drug and multi-drug trials, dose intensity of the molecularly targeted agent was calculated based on the percentage of the actual total dose received compared with the expected total dose. Patients in all groups received close to or >90% of their total planned dose. No statistically significant difference in dose intensity was observed between age groups for either monotherapy ($P = 0.26$) or combination ($P = 0.39$) trials. In multi-drug trials, dose intensities of the cytotoxic chemotherapeutic agents achieved by the younger and older age groups were also not different (82.8% versus 83.8%, respectively; $P = 0.53$).

Best response. The best objective responses achieved by the 401 patients are listed in Table 5, based on monotherapy versus multi-agent studies, and by age groups. The range of outcomes observed was similar between the two age groups. A comparison of treatment outcome by study type showed higher objective response and stabilization rates for the multi-drug trials than the single agent trials. This is an expected finding because the addition of a cytotoxic agent to a molecularly targeted agent in combination trials is likely to increase their response and stable disease rates.

Toxicity index and dose-limiting toxicities. The median TI_{ALL} observed by age group including adverse events of all causalities is shown in Table 6. In all groups, the median TI_{ALL} was between 3 and 3.9, demonstrating that more than half of

Table 3. Number of patients enrolled by tumor type

Tumor type	Single drug trials		Multi-drug trials		Total no. of patients
	<65	≥65	<65	≥65	
Breast	16	2	0	0	18
Endocrine	18	4	0	0	22
Gastrointestinal	57	33	23	10	123
Head and neck	2	1	35	9	47
Hepatobiliary	0	0	17	9	26
Leukemia/lymphoma	21	19	3	1	44
Genitourinary	7	17	0	1	25
Gynecological	1	6	46	11	64
Lung	3	0	20	7	30
Other/not listed	1	0	1	0	2

Table 4. Baseline demographics, prior therapy, and baseline adverse events ($N = 401$)

Age group	Single drug trials		Multi-drug trials	
	<65	≥65	<65	≥65
No. of patients	126	82	145	48
Age, median (range)	52.7 (22.8-65.0)	71.1 (65.5-83.1)	55.0 (23.3-64.8)	68.7 (65.1-81.0)
No. (%) of female patients	61 (48)	30 (37)	77 (53)	19 (40)
Eastern Cooperative Oncology Group performance status				
0	59 (47)	35 (43)	52 (36)	20 (42)
1	56 (44)	43 (52)	82 (57)	20 (42)
2	11 (9)	4 (5)	11 (8)	8 (17)
Comorbidity by Charlson Index (%)				
0	109 (87)	65 (79)	117 (81)	34 (71)
1	9 (7)	10 (12)	13 (9)	10 (21)
2	6 (4)	7 (9)	11 (8)	4 (8)
3 or more	2 (2)	0 (0)	4 (3)	0 (0)
No. of prior regimens				
0	26 (21)	23 (28)	66 (46)	30 (63)
1	49 (39)	38 (46)	35 (24)	14 (29)
2	29 (23)	11 (13)	30 (21)	2 (4)
3 or more	22 (18)	10 (12)	14 (9)	2 (4)
No. (%) of patients who had prior radiation	42 (33)	19 (23)	60 (41)	15 (31)
Worst grade baseline adverse event				
0	3 (2)	3 (4)	0 (0)	0 (0)
1	41 (33)	25 (30)	27 (19)	13 (27)
2	56 (44)	41 (50)	92 (63)	24 (50)
3	23 (18)	13 (16)	24 (17)	9 (19)
4	3 (2)	0 (0)	2 (1)	2 (4)

patients had at least one grade 3 adverse event while on study. No statistically significant difference was found for any comparison between age groups. When the TI_{MTA} is calculated based on adverse events that are at least possibly related to the molecularly targeted agent (Table 7), the results show a slightly lower TI but still consist of values between 3 and 3.9. This is an expected finding, as the TI should decrease when some adverse events are excluded from the total calculation; however, adverse events of a high grade are not likely to be attributed as unrelated to the molecularly targeted drug under evaluation, and therefore, the overall TI_{MTA} value still remains in the 3 to 3.9 range.

The number of patients and number of cycles with at least one DLT were also calculated (Tables 6 and 7). For DLT_{ALL}, which incorporated DLT of all attributions, about two-thirds to three-quarters of patients among the younger and older age groups in both single and multi-drug trials experienced at least one DLT_{ALL}, as defined by the criteria used for this analysis. At least one DLT_{ALL} occurred in ~50% of treatment cycles among all age groups in both trial types. For DLT_{MTA}, which included only DLT that were at least possibly related to the molecularly targeted agent, 40% to 50% of patients in both age groups and in both single and multi-drug trials experienced at least one DLT_{MTA}. At least one DLT_{MTA} occurred in 20% to 35% of treatment cycles among all age groups in both trial types. No clinically significant differences were apparent between the younger and the older age groups for DLT_{ALL} and for DLT_{MTA}, and none of the comparisons showed differences of statistical significance.

The detailed listing of TI_{ALL} and TI_{MTA} values by individual study for both age groups is provided in Table 8. In general, the values of TI_{ALL} between the younger and older age groups were very similar, regardless of study type. The TI_{MTA} values in the older age group were slightly higher than those in the younger age groups in some studies, but the small number of older patients in these studies preclude any meaningful comparisons.

Finally, further analyses were undertaken in order to appreciate whether there were substantial differences in adverse events occurring during the earlier treatment cycles compared with the later treatment cycles. Of patients receiving at least six cycles of treatment, there was no difference in the number of adverse events experienced in cycle 1 compared with cycle 6 ($P = 0.14$), or in the number of DLT_{ALL} ($P = 0.35$) or DLT_{MTA} ($P = 0.16$) experienced in cycle 1 compared with cycle 6. These findings were valid for both the younger and older age groups.

Discussion

With an aging population, there is an urgent need to optimize how we manage older patients with cancer. There are data suggesting that this portion of the population may be undertreated due to misconceptions of both clinicians and the patients themselves, about their ability to tolerate cancer treatment. Novel biologically targeted agents that aim to disrupt specific key properties of a tumor's neoplastic activities, whereas interfering with host functions to a relatively minor degree, promise a better therapeutic index than many conventional drug therapies. This study was undertaken based on the

Table 5. Treatment records, dose intensity, and best response observed on Consortium trials

Age group	Single drug trials		Multi-drug trials	
	<65	≥65	<65	≥65
No. of patients	126	82	145	48
No. (%) of patients with ≥1 cycle	119 (94.4%)	77 (93.9%)	134 (92.4%)	44 (91.7%)
Total no. of cycles delivered	369	238	503	142
Mean (SD) cycles per patient	3.1 (2.5)	3.1 (2.8)	3.8 (2.8)	3.2 (2.5)
Median (IQR) cycles per patient	2 (2-4)	2 (2-3)	3 (2-6)	2 (2-4)
Mean (SD) targeted agent dose intensity*	88.8% (14.4%)	87.0% (16.4%)	91.1% (14.8%)	89.2% (11.0%)
Median (IQR) target agent dose intensity	94.1% (83.3-100)	94.9% (76.6-100)	97.5% (86.6-100)	91.5% (81.8-99.9)
Mean (SD) chemotherapy dose intensity†	NA	NA	82.8% (19.2%)	83.8% (18.9%)
Median (IQR) chemotherapy dose intensity	NA	NA	86.6% (75.2-97.5)	87.5% (78.7-94.8)
Best response				
Complete response + partial response	2 (1.6)	1 (1.2)	14 (9.7)	3 (6.3)
Stable disease	41 (32.5)	15 (18.3)	61 (42.1)	21 (43.8)
Progressive disease	64 (50.8)	45 (54.9)	45 (31.0)	14 (29.2)
Not evaluable	13 (10.3)	17 (20.7)	10 (6.9)	5 (10.4)
Too early	6 (4.8)	4 (4.9)	15 (10.3)	5 (10.4)

Abbreviation: IQR, interquartile range.
*Differences in the mean dose intensities of the molecularly targeted agents between age groups were not statistically significant ($P = 0.26$ for single drug trials and 0.39 for multi-drug trials).
†Differences in the mean dose intensities of chemotherapeutic agents between age groups were not statistically significant ($P = 0.53$).

hypothesis that better evidence of the ability of older cancer patients to tolerate anticancer drug therapy, and molecularly targeted agents in particular, might result in greater clinical trial enrollment, and ultimately more and better anticancer treatment in this group.

Although these results are preliminary, our analyses show no clear differences in the frequency, type, or severity of toxicities between patients under or over 65 years of age treated with molecular-based therapies in the setting of clinical trials by our group. The baseline pretreatment characteristics of the two age groups were comparable, although older patients had more comorbidities than the younger group, and a higher proportion of the younger patients had received prior chemotherapy and radiotherapy. Dose intensities were calculated to ensure that older patients were not receiving less intensive therapy. Our results revealed that older patients were receiving dose intensities of molecularly targeted and chemotherapeutic agents comparable to their younger counterparts, and that there were no statistically significant differences between the frequencies and intensities of adverse events experienced by the different age groups. The use of generalized estimating equation modeling is necessary to allow for dependence between patients from the same trial (for patient-level outcomes), and between cycles of treatment received by the same patient. As a result of the modeling, statistical tests are not unduly influenced by one or two agents/trials with excess toxicity and variable age distributions compared with other agents/trials. These hierarchical-type statistical methods, although more complicated, are necessary to reduce the chance of observing an artificial relationship between age and toxicity. One further caveat that warrants cautious interpretation are the comparisons of median TI values and frequencies of DLT occurrences between age groups in Tables 6 and 7. It is possible that small differences in the frequency or intensity of adverse events experienced by the two age groups may exist, and the power to detect these differences was limited in this study. However, it is

Table 6. Median toxicity index (TI_{ALL}) and frequency of DLT (DLT_{ALL}) of all adverse events

	Single drug trials <65	Single drug trials ≥65	Multi-drug trials <65	Multi-drug trials ≥65
No. of patients	126	82	145	48
No. of patients with ≥1 cycle	119	77	134	44
Median (IQR) TI_{ALL}	3.94 (3.00-4.00)	3.75 (3.00-4.00)	3.99 (3.75-4.79)	3.98 (3.75-4.77)
P	0.97		0.91	
No. of (%) patients with ≥1 DLT_{ALL}	88 (73.9)	52 (67.5)	101 (75.4)	34 (77.3)
P	0.12		0.82	
No. of (%) cycles with ≥1 DLT_{ALL}	208 (56.4)	107 (45.0)	224 (44.5)	70 (49.3)
P	0.16		0.54	

Table 7. Median toxicity index (TI_{MTA}) and frequency of DLT (DLT_{MTA}) of possibly, probably, or definitely related adverse events to molecularly targeted agents

	Single drug trials <65		Single drug trials ≥65		Multi-drug trials <65		Multi-drug trials ≥65	
No. of patients	126		82		145		48	
No. of patients with ≥1 cycle	119		77		134		44	
Median (IQR) TI_{MTA}	3.25 (2.66-3.87)		3.00 (2.50-3.81)		3.65 (2.66-3.99)		3.00 (2.00-4.00)	
P		0.94				0.29		
No. of (%) patients with ≥1 DLT_{MTA}	61 (51.3)		36 (46.8)		56 (41.8)		17 (38.6)	
P		0.36				0.61		
No. of (%) cycles with ≥1 DLT_{MTA}	130 (35.2)		54 (22.7)		97 (19.3)		36 (25.4)	
P		0.10				0.45		

evident from the tables that these variables are similar between the older and younger patients. We estimated the differences in the median TI values and in the rates of DLT between age groups (data not shown), and no significant pattern was observed. Thus, although we cannot rule out the possibility of a small difference, we believe any difference is not likely to be clinically important.

The results can be considered internally valid, as patients entered into studies in which a molecularly targeted agent was combined with chemotherapy were consistently observed to have experienced more adverse events, regardless of age, than the patients entered into monotherapy studies involving a molecularly targeted agent alone. In particular, hematologic adverse events were more severe in these combination studies as expected.

Our analyses are limited in several ways. First, although a comparison of the patients' comorbidities was done, our database only captures those factors reported by the patients and subsequently recorded in source documents by the clinical trials team. As such, the capture of comorbid conditions

Table 8. Toxicity index (TI_{ALL} and TI_{MTA}) by study

Agent(s)	Disease site	Median (IQR) TI_{ALL} (all adverse events)		Median (IQR) TI_{MTA} (possibly, probably or definitely related adverse events to molecularly targeted agents)	
		<65	≥65	<65	≥65
No. of patients		126	82	145	48
Single agent studies					
Erlotinib	Colorectal	3.72 (2.78-3.94)	3.00 (2.89-3.75)	2.88 (2.50-3.66)	2.63 (1.98-2.96)
Decitabine	Leukemia	4.00 (3.94-4.00)	4.00 (3.98-4.00)	3.87 (3.44-3.98)	3.92 (3.75-3.97)
Tipifarnib	Bladder	4.10 (3.66-4.53)	3.74 (2.96-3.93)	3.88 (3.38-4.39)	2.66 (1.97-3.91)
Imatinib	Salivary gland	3.98 (3.94-4.00)	3.94 (3.75-4.79)	3.75 (2.99-3.94)	3.74 (1.50-4.00)
Bortezomib	Colorectal	3.72 (3.00-3.81)	3.73 (3.72-3.75)	3.00 (1.99-3.67)	3.72 (2.65-3.74)
Perifosine	Breast	3.66 (2.89-3.98)	2.99 (2.99-3.00)	2.88 (2.65-3.72)	2.98 (2.96-2.99)
Perifosine	Pancreas	3.98 (3.75-4.00)	3.87 (2.99-5.62)	2.86 (1.88-3.50)	3.58 (2.88-3.69)
Bortezomib	Bladder	3.94 (2.98-3.98)	3.84 (3.69-3.98)	2.89 (2.83-3.98)	2.83 (2.50-3.72)
Temsirolimus	Neuroendocrine	3.74 (2.98-3.94)	3.72 (3.00-4.00)	3.70 (2.98-3.84)	3.00 (2.98-3.72)
Lapatinib	Salivary gland	2.89 (2.89-2.89)	—	2.88 (2.88-2.88)	—
Multiple agent studies					
Erlotinib and cisplatin	Head and neck	3.98 (3.00-4.79)	4.00 (3.98-4.75)	2.99 (2.63-3.74)	1.98 (1.50-3.00)
UCN-01 and topetecan	Solid tumor	4.75 (3.98-4.96)	3.99 (3.87-4.39)	3.93 (2.89-4.80)	3.75 (2.31-4.20)
UCN-01 and fludarabine	Leukemia/lymphoma	3.00 (2.66-4.75)	2.50 (2.50-2.50)	2.99 (2.58-4.74)	0.00 (0.00-0.00)
Imatinib, cisplatin and irinotecan	Small cell lung	4.60 (2.99-4.80)	3.00 (2.99-4.80)	2.65 (1.50-4.00)	2.33 (2.00-4.79)
Oblimersen sodium and doxorubicin	Hepatocellular	3.99 (3.94-4.80)	3.98 (3.93-4.79)	3.94 (2.99-4.79)	3.98 (3.74-4.79)
GTI-2040 and docetaxel	Non – small cell lung	4.00 (3.73-4.79)	3.94 (3.93-4.74)	2.50 (1.50-3.50)	2.74 (2.06-3.43)
UCN-01 and topetecan	Ovarian	4.00 (3.74-4.00)	3.98 (3.94-4.80)	3.92 (2.99-3.98)	3.72 (3.38-3.72)
Triapine and gemcitabine	Pancreas	3.98 (3.94-4.00)	3.99 (3.83-4.65)	3.87 (3.74-3.98)	3.37 (2.91-4.30)
Sorafenib and gemcitabine	Ovarian	3.84 (3.75-3.98)	—	2.89 (2.67-3.74)	—

depends largely on the completeness of the patients' reporting and some information may be missing. Second, the proportion of older patients accrued into clinical trials has been consistently lower than younger patients in the literature (19), and this is the case with our protocols as well. Therefore, there were greater numbers of younger patients included in the analysis for both monotherapy and combination trials than older patients. In addition, older patients accrued to these studies were required to meet certain baseline physical and metabolic criteria to be eligible. These patients are selected and likely represent the "well" elderly and our results may not be generalizable to all older patients. In clinical practice, comorbid illnesses are more prevalent among elderly patients than younger patients (24). In our study, older patients had more comorbidities than the younger patients but the difference was not statistically significant. This finding could likely be explained by patient selection, although one may conversely conclude that, as long as protocol eligibility criteria such as those specifying organ functions, performance status, and other conditions are met, chronological age alone should not influence enrollment decisions. Finally, our study has evaluated multiple molecularly targeted drugs in various tumor types, thus, our results cannot necessarily be applicable to all such agents in a specific way in view of the variety of these agents, their heterogeneous mechanisms of action, and adverse event profiles in different patient populations. Of note, another group examining the effects of patient characteristics on acute treatment toxicity in early phase clinical trials has used a similar methodology (23).

Molecularly targeted agents present a unique challenge when attempting to identify possible predictors of drug-related toxicity. Because molecularly targeted agents differ greatly in their modes of action, it is difficult to generalize the risk factors for toxicities to such varied groups of compounds. As these agents gain increased clinical use, more can be learned about the predictive factors for toxicities that are encountered by patients. For example, baseline hypertension may predispose to difficulties in tolerating antiangiogenic compounds (20, 21). These caveats, in most instances, have come from practical experiences in early clinical studies rather than from preclinical models.

There are few trials of molecularly targeted therapies in older populations with which to draw comparisons to our study. Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, has been evaluated retrospectively in an elderly population (25–28). Cappuzzo et al. showed that disease control could be obtained in >50% of 40 elderly patients with a median age of 74 years (range, 70-88 years) treated with gefitinib at a daily oral dose of 250 mg for advanced non–small cell lung cancer. Although no direct comparison was made between older and younger age groups in this study, it was found that the patients in the older age group tolerated the treatment extremely well (25). Grades 1 to 2 diarrhea occurred in only 24% of patients and only one patient experienced a grade 4 diarrhea. Grades 1 to 2 skin toxicity, the most common toxicity observed with gefitinib, occurred in 20 (50%) patients in this retrospective series. Other small subpopulation studies have found similar results both in Europe and in the U.S. where elderly patients with non–small cell lung cancer have tolerated this molecularly targeted agent very well based on the low number of adverse events (26–28).

Another molecularly targeted agent, bevacizumab, a monoclonal antibody against the vascular endothelial growth factor, has been evaluated in a population at high risk (20, 21). In a randomized phase II trial, patients with metastatic colorectal cancer received bolus 5-fluorouracil and leucovorin with either bevacizumab or placebo. Patients accrued to this study had to be considered nonoptimal candidates for first-line irinotecan therapy based on age, performance status, previous treatment, or low baseline albumin levels. The median ages for patients on the bevacizumab and placebo arms were 70.7 and 71.3, respectively. Patients in the bevacizumab arm tolerated the regimen well and had a longer median survival than the control arm. The tolerability and efficacy of other molecularly targeted agents, however, has not been evaluated prospectively and specifically in older patients.

In order to accurately determine whether molecularly targeted treatments are similarly tolerated and effective in older cancer patients, prospective clinical trials need to be done enrolling these subjects in numbers sufficiently large to draw valid conclusions. Our study has provided some preliminary evidence suggesting that chronological age alone should not be a barrier to the consideration of treatment with these novel agents in an older patient population. With the increasing integration of molecularly targeted therapy in standard anticancer regimens, further research in this area is urgently needed.

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Electronic Acknowledgement Receipt

EFS ID:	6574292
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	01095
Filer:	Gregory Houghton./Linda Adams
Filer Authorized By:	Gregory Houghton.
Attorney Docket Number:	34678-US-PCT
Receipt Date:	04-DEC-2009
Filing Date:	19-MAY-2008
Time Stamp:	13:27:04
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678_Supp_IDS.pdf	4078885 1a8e4a10fe7fcc335529fae4e/88bc8/4f9611 0c6	yes	25

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	23	
	Information Disclosure Statement (IDS) Filed (SB/08)		24	25	
Warnings:					
Information:					
2	Foreign Reference	WO2004078133.pdf	1344546 ae3405556f396996de1c359cfa0dbb62fc16729d	no	29
Warnings:					
Information:					
3	NPL Documents	Asano_rapamycin.pdf	733647 d766004ad7d96039196f003eef1433b3f15e3cfaa	no	8
Warnings:					
Information:					
4	NPL Documents	Boulay_Antitumor.pdf	1564857 898d1c73d00bb55e0f185021dc6d05da85ab1f90f	no	10
Warnings:					
Information:					
5	NPL Documents	Boulay_Proceedings.pdf	213655 a88892e3087584697c87d987c054848733f418e2	no	1
Warnings:					
Information:					
6	NPL Documents	Bruns_Rapamycin.pdf	1859554 47097b61b024c06451cc998b9fd33ff44843d60	no	11
Warnings:					
Information:					
7	NPL Documents	O_Reilly_Proceedings.pdf	200034 4204db9aab1410bfdc7e647952948224b4b8e05a9	no	1
Warnings:					
Information:					
8	NPL Documents	Stephan_Effect.pdf	1237263 583cbb6e2cd6b1ad3b1543d2e87ab391c3dfdb9c	no	8
Warnings:					
Information:					

9	NPL Documents	Townsley_Evaluation.pdf	887842 9c75dfbb57f8f55cc80b651ea8414ccd5f0d4bd2	no	10

Warnings:	
Information:	
Total Files Size (in bytes):	12120283

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP S03.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 3S U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	34678-US-PCT	9572

1695 7590 11/30/2010

NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 101/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
JEAN-LOUIS, SAMIRA JM	
ART UNIT	PAPER NUMBER
1627	
MAIL DATE	DELIVERY MODE
11/30/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/094,173	MARKS ET AL.	
	Examiner	Art Unit	
	SAMIRA JEAN-LOUIS	1627	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-12 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) 1-12 are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Claim 10 provides for the use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to claim 1, but, since the claim does not set forth any steps involved in the method/process, it is unclear what method/process applicant is intending to claim. Given that the claim may have dual interpretation either as a method of preparation or as a method of treatment, the claim is being interpreted herein as optionally both a method of making and a method of treating.

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions, which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

I. Group I, claims 1-3 and 8-12 are drawn to a method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

II. Group II, claim 4 is drawn to a method for inducing endocrine tumor regression, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

III. Group III, claims 5-6 are drawn to a method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

IV. Group IV, claim 7 is drawn to a method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

V. Group V, claim 10 is drawn to the use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to claim 1.

The inventions listed as Groups I, II, III, IV, and V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features.

An international application should relate to only one invention or, if there is more than one invention, the inclusion of those inventions in one international application is only permitted if all inventions are so linked as to form a single general inventive concept (PCT Rule 13.1). With respect to a group of inventions claimed in an international application, unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features.

The expression "special technical features" is defined in PCT Rule 13.2 as meaning those technical features that define a contribution which each of the

inventions, considered as a whole, makes over the prior art. The determination is made on the contents of the claims as interpreted in light of the description and drawings. Whether or not any specific technical feature makes a "contribution" over the prior art, and therefore constitutes a "special technical feature", should be considered with respect to novelty and inventive step.

In this instant application, the common technical feature in all groups is the mTOR inhibitor. This compound cannot be said to be a special technical feature under PCT Rule 13.2 because it is shown in the prior art.

In this case, Gibbons et al. (U.S. 2002/0183239 A1, cited by applicant and filed on an IDS 1449) teaches the use of a combination of mTOR and an antimetabolite neoplastic agent in the treatment of neoplasms, i.e. tumors (see abstract). Specifically, Gibbons et al. teach the use of compounds such as rapamycin for the aforementioned treatments. Consequently, Gibbons et al. render obvious applicant's invention.

As a result, no special technical features exist among the different groups because the inventions in Groups I, II, III, IV, and V fail to make a contribution over the prior art with respect to novelty and inventive step. In conclusion, there is a lack of unity of inventions, and therefore restriction for examination purposes as indicated is proper.

Species Election

This application contains claims directed to more than one species of the generic invention. These species (i.e. mTOR inhibitors) possess divergent structures which would cause them to possess contrasting physical and chemical properties (see rapamycin vs. Temsirolimus). Thus, these species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

The species listed below do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same special technical feature among the different species.

The species are as follows:

For Group I, II, III, IV, & V:

1) Applicant is required to elect a particular mTOR inhibitor to be utilized in the aforementioned inventions. Alternatively, applicant may elect a particular mTOR inhibitor listed in claims 11 or 12.

Applicant is required, in reply to this action, to elect a single species to which the claims shall be restricted if no generic claim is finally held to be allowable. The reply must also identify the claims readable on the elected species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered non-responsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include

all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

The following claims 1-12 are generic.

Applicant is also reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

No telephone call was made due to the complexity of the election/restriction.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samira Jean-Louis whose telephone number is 571-270-3503. The examiner can normally be reached on 7:30-5 PM EST M-Th.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Samira Jean-Louis/

Examiner, Art Unit 1627

11/26/2010

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	11/26/2010								
	1	÷								
	2	÷								
	3	÷								
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	5	÷								
	6	÷								
	7	÷								
	8	÷								
	9	÷								
	10	÷								
	11	÷								
	12	÷								

Electronic Acknowledgement Receipt	
EFS ID:	9150441
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	01095
Filer:	Gregory Houghton./Linda Adams
Filer Authorized By:	Gregory Houghton.
Attorney Docket Number:	34678-US-PCT
Receipt Date:	03-JAN-2011
Filing Date:	19-MAY-2008
Time Stamp:	14:11:20
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$130
RAM confirmation Number	337
Deposit Account	190134
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1		34678_US_restriction.pdf	149386 31d8f888ce1316de400dfb42a6386c9a77d925c3	yes	5

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Extension of Time	1	1
Response to Election / Restriction Filed	2	2
Claims	3	4
Applicant Arguments/Remarks Made in an Amendment	5	5

Warnings:

Information:

2	Fee Worksheet (PTO-875)	fee-info.pdf	30303 bda8bd427e185eb363408379db212119012725 87	no	2

Warnings:

Information:

Total Files Size (in bytes):	179689

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory Houghton./Linda Adams
Attorney Docket Number:	34678-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	130	130

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**130**

REMARKS/ARGUMENTS

The Examiner has requested that a restriction is required under 35 U.S.C. 121 and 372. Further, the Examiner requests that Applicant elect from the following:

Group I, claims 1-3 and 8-12 are drawn to a method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Group II, claim 4 is drawn to a method for inducing endocrine tumor regression, comprising administering to a subject in need thereof a therapeutically affective amount of an mTOR inhibitor.

Group III, claims 5-6 are drawn to a method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Group IV, claim 7 is drawn to a method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

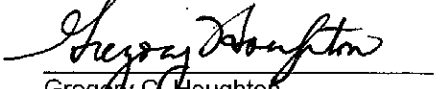
Group V, claim 10 is drawn to the use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to claim 1.

Without traverse, Applicants elect Group I. The Examiner also request that a particular mTOR inhibitor be elected. Applicants elect the mTOR inhibitor listed in claim 12.

Claims 1-3 and 8-12 are readable on the elected species. Claims 4-7 and 10 have been withdrawn based on the election required by the Restriction Requirement. Applicants respectfully maintain their right to file a divisional application toward any non-elected inventions.

Should the Examiner have any questions, please contact the undersigned attorney.

Respectfully submitted,



Gregory C. Houghton
Attorney for Applicants
Reg. No. 47,666

Novartis Pharmaceuticals Corp.
Patents Pharma
One Health Plaza, Building 104
East Hanover, NJ 07936-1080
(862) 778-2614
Date: January 3, 2011

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claim 1. (Original): A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 2. (Original) A method for inhibiting growth of endocrine tumors, comprising administering to a subject in need thereof a therapeutical effective amount of an mTOR inhibitor.

Claim 3. (Original) A method for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 4. (Withdrawn) A method for inducing endocrine tumor regression, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 5. (Withdrawn) A method for treating endocrine tumor invasiveness or symptoms associated with such tumor growth, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 6. (Withdrawn) A method for preventing metastatic spread of endocrine tumors or for preventing or inhibiting growth of micrometastasis, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 7. (Withdrawn) A method for the treatment of a disorder associated with endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor.

Claim 8. (Previously presented) A method according to claim 1, comprising administering in addition a therapeutically effective amount of at least one second drug substance.

Claim 9. (Original) A method according to claim 8, wherein a second drug substance is somastatin or a somastatin analogue.

Claim 10. (Previously presented) The use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to claim 1.

Claim 11. (Previously presented) A method according to claim 1, wherein an mTOR inhibitor is selected from rapamycin or a rapamycin derivative.

Claim 12. (Currently amended) A method according to claim 10, wherein an mTOR ~~inhibitor~~ inhibitor is 40-O-(2-hydroxyethyl)-rapamycin.

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF Art Unit: 1627

Marks, Peter Wayne et al. Examiner: Jean-Louis, Samira J

INTERNATIONAL APPLICATION NO: PCT/EP06/068656

FILED: November 20, 2006

U.S. APPLICATION NO: 12/094173

35 USC §371 DATE: May 19, 2008

FOR: Neuroendocrine Tumor Treatment

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

RESPONSE TO RESTRICTION REQUIREMENT

Sir:

In response to the Restriction Requirement under 35 USC 121 and 372 mailed November 30, 2010, response due within one month on December 30, 2010 and a petition for a one month extension of time is included with this response, thus extending the response due date to January 30, 2011, kindly enter the following response.

Amendments to the claims begin on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

PETITION FOR EXTENSION OF TIME

Sir:

The Office Action of November 30, 2010 has a shortened statutory time set to expire on December 30, 2010. A one-month extension is hereby requested pursuant to 37 CFR §1.136(a).

Please charge Deposit Account No. 19-0134 in the name of Novartis in the amount of $130 for payment of the extension fee. The Commissioner is hereby authorized to charge any additional fees under 37 CFR §1.17 which may be required, or credit any overpayment, to Account No. 19-0134 in the name of Novartis.

Respectfully submitted,

Gregory D. Houghton
Attorney for Applicant
Reg. No. 47,666

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-1422

Date: January 3, 2011

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 12/094,173	Filing Date 05/19/2008	☐ To be Mailed

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	01/03/2011	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 12	Minus	** 20	= 0	X $ =		OR	X $52=	0
	Independent (37 CFR 1.16(h))	* 7	Minus	***7	= 0	X $ =		OR	X $220=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)					
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/MARTHA NEWMAN/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	34678-US-PCT	9572

1695 7590 02/16/2011

NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 101/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
JEAN-LOUIS, SAMIRA JM	
ART UNIT	PAPER NUMBER
1627	
MAIL DATE	DELIVERY MODE
02/16/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/094,173	MARKS ET AL.	
	Examiner	Art Unit	
	SAMIRA JEAN-LOUIS	1627	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *03 January 2011*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-12* is/are pending in the application.

4a) Of the above claim(s) *4-7 and 11* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-3,8-10 and 12* is/are rejected.

7)☒ Claim(s) *9* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *05/19/08, 12/04/09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Priority

Acknowledgment is made of applicant's claim for foreign priority. Thus, the priority date of the instant invention is November 21rst, 2005 (the date of Foreign application 052 3658.3).

IDS

The information disclosure statements (IDS) submitted on 05/19/08 and 12/04/09 are acknowledged and have been entered. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statements have been considered by the examiner.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 10 and 12 are rejected under 35 U.S.C. 101 because the claimed recitation of a use, without setting forth any steps involved in the process, results in an improper definition of a process, i.e., results in a claim which is not a proper process claim under 35 U.S.C. 101. See for example *Ex parte Dunki*, 153 USPQ 678 (Bd.App. 1967) and *Clinical Products, Ltd.* v. *Brenner*, 255 F. Supp. 131, 149 USPQ 475 (D.D.C.

1966). Consequently, the subject matter claimed by claim 10 does not fall into a single statutory class of invention, as it claims both a method of making and a method of using, but rather encompasses or overlaps two different statutory classes of invention. The language of 35 U.S.C. 101 prohibits overlap between two different statutory classes in a single claim as it is drafted so as to set forth the statutory classes of invention in the alternative only. *See Ex parte Lyell, 17 USPQ2d 1551 (Bd. Pat. App. & inter.1990).*

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter, which the applicant regards as his invention.

Claims 10 and 12 provide for the use of an mTOR inhibitor for the manufacture of a medicament for use in a method according to claim 1, but, since the claims do not set forth any steps involved in the method/process, it is unclear what method/process applicant is intending to encompass. Such claim is indefinite as it merely recites a use without any active, positive steps delimiting how this use is actually practiced.

Claims 10 and 12 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Objections

Claim 9 is objected to because of the following informalities: Claim 9 recites the terms “somastatin” and “somastatin analogue” as opposed to the term "somatostatin" recited in the specification. Appropriate correction is required.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3 and 12 are rejected under 35 U.S.C. 102(b) as being anticipated by O’Reilly et al. (Proceedings of the American Association for Cancer Research Annual Meeting, 03/2002, Vol. 43, pg. 71, cited by applicant and filed on an IDS 1449) as evidenced by Merck Manuals (Merck Manuals, Pancreatic endocrine tumors, 2009, pgs. 1-4).

O’Reilly et al. teach the use of RAD001 (i.e. 40-O-(2-hydroxyethyl)-rapamycin; elected species; instant claim 12) as a bioavailable hydroxyethyl ether derivative of rapamycin that has demonstrated *in vitro* anti-proliferative activity against a panel of

human tumors (see pg. 71, #359). Importantly, O'Reilly et al. teach that RAD001 was found to be a potent inhibitor of tumor growth in 10 different cell lines and *in vivo* against pancreatic tumors (see pg. 71, #359). Persistent tumor regressions were observed and O'Reilly et al. suggest that RAD001 may not only be effective against tumor cells, it may also affect angiogenesis (see pg. 71, #359).

Merck Manual was provided to demonstrate that pancreatic cancer is characterized by endocrine tumors that tend to produce hormones that lead to aberrant functions (see pg. 1).

Accordingly, the teachings of O'Reilly et al. anticipate claims 1-3 and 12.

Claims 1-3, 8-9, and 12 are rejected under 35 U.S.C. 102(b) as being anticipated by Wecbecker (WO 97/47317, cited by applicant and filed on an IDS 1449).

Weckbecker teaches a combination of a somatostatin analogue and a rapamycin for the prevention and treatment of cell hyperproliferation (see abstract and pg. 1, paragraph 1). Additionally, Weckbecker teaches that rapamycin or derivatives thereof are desired given that such compounds are immunosuppressive and known to inhibit cancer (see pg. 10, last paragraph and pg. 12, last paragraph). A preferred rapamycin compound is 40-O-(2-hydroxy)ethyl-rapamycin (i.e. elected species; instant claim 12; see pg. 12, paragraph 3). According to Weckbecker, such combination can be used for preventing or treating cell hyperproliferation including GEP tumors (i.e. Gastroentero-

pancreatic neuroendocrine tumors: slow growing tumors of the pancreas and GI tract) and pituitary adenomas (another type of endocrine tumor; see pg. 13 and pg. 14, paragraph 2).

Accordingly, the teachings of Weckbecker anticipates claims 1-3, 8-9, and 12.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samira Jean-Louis whose telephone number is 571-270-3503. The examiner can normally be reached on 7:30-6 PM EST M-Th. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Samira Jean-Louis/

Examiner, Art Unit 1627

02/11/2011

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

SEARCHED			
Class	Subclass	Date	Examiner
None			

SEARCH NOTES		
Search Notes	Date	Examiner
Palm Inventor Name Search	2/10/2011	SJL
STN-see enclosed search history	2/10/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	2/11/2011	SJL

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

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=> d his

     (FILE 'HOME' ENTERED AT 19:00:05 ON 10 FEB 2011)

     FILE 'CAPLUS' ENTERED AT 19:00:22 ON 10 FEB 2011
L1             1 S US20080255029/PN

     FILE 'REGISTRY' ENTERED AT 19:00:44 ON 10 FEB 2011
L2             1 S 159351-69-6/RN
                 SET NOTICE 1 DISPLAY
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     FILE 'CAPLUS' ENTERED AT 19:01:38 ON 10 FEB 2011
L3          1657 S L2
                 E NEUROENDOCRINE TUMOR+ALL/CT
L4          2084 S (NEUROENDOCRINE TUMOR OR "ENDOCRINE SYSTEM, NEOPLASM" (L) "NE
L5            25 S L3 (W) L4
L6             6 S L5 AND (AY<=2005 OR PRY<=2005 OR PY<=2005)
L7            25 S L3 (A) L4
L8            25 S L3 (S) L4
L9            10 S L3 (L) L4
L10           25 S L7 OR L8 OR L9
L11            6 S L10 AND (AY<=2005 OR PRY<=2005 OR PY<=2005)
```

Doc code :IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-08)
Approved for use through 05/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	Peter Wayne Marks
Art Unit	
Examiner Name	
Attorney Docket Number	34768-US-PCT

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5538739		1996-07-23	BODMER DAVID, ET AL	

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20040176339		2004-09-09	SHERMAN MATTHEW L.	
	2	20020183240		2002-12-05	GIBBONS JAMES J.	

If you wish to add additional U.S. Published Application citation information please click the Add button.

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	02/080975	WO		2002-10-17	WYETH CORP		☐
	2	2005/082411	WO		2005-09-09	CHRISTENSEN JAMES G.		☐

EFS Web 2.1.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Peter Wayne Marks
	Art Unit	
	Examiner Name	
	Attorney Docket Number	34768-US-PCT

	3	02/098416	WO		2002-12-12	WYETH CORP		☐
	4	97/05167	WO		1997-02-13	DEGHENGHI ROMANO		☐
	5	0 462 071	EP		1991-12-18	SANDOZ LTD		☐
	6	03/020266	WO		2003-03-13	WYETH CORP		☐
	7	97/47317	WO		1997-12-18	CIBA GEIGY AG		☐

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1		☐

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EXAMINER SIGNATURE

Examiner Signature	/Samira Jean-louis/	Date Considered	02/14/2011

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

EFS Web 2.1.1

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BIB DATA SHEET

CONFIRMATION NO. 9572

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
12/094,173	05/19/2008 RULE	514	1627	34678-US-PCT

APPLICANTS

Peter Wayne Marks, Woodbridge, CT;
David Lebwohl, Madison, NJ;

**** CONTINUING DATA ***************************

This application is a 371 of PCT/EP2006/068656 11/20/2006

**** FOREIGN APPLICATIONS ***************************

UNITED KINGDOM 0523658.3 11/21/2005
UNITED KINGDOM 0601082.1 01/19/2006
UNITED KINGDOM 0602747.8 02/10/2006
UNITED KINGDOM 0607942.0 04/21/2006
UNITED KINGDOM 0609272.0 05/10/2006
UNITED KINGDOM 0609912.1 05/18/2006
UNITED KINGDOM 06120660.3 09/14/2006

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

07/08/2008

Foreign Priority claimed ☑ Yes ☐ No 35 USC 119(a-d) conditions met ☑ Yes ☐ No Verified and Acknowledged /SAMIRA JM JEAN-LOUIS/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CT	0	12	7

ADDRESS

NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 101/2
EAST HANOVER, NJ 07936-1080
UNITED STATES

TITLE

Neuroendocrine Tumor Treatment

FILING FEE RECEIVED	FEES: Authority has been given in Paper	☐ All Fees
1770	No._____ to charge/credit DEPOSIT ACCOUNT	☐ 1.16 Fees (Filing)
	No._____ for following:	☐ 1.17 Fees (Processing Ext. of time)
		☐ 1.18 Fees (Issue)
		☐ Other _____
		☐ Credit

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	2	WO-2005064343-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:02
S2	1	WO-02080975-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:49
S3	2	WO-2005082411-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:57
S4	1	WO-02098416-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:01
S5	1	("5538739").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/10 18:04
S6	2	WO-9705167-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:05
S7	0	EP-0462071-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:06
S8	2	EP-462071-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:06

S9	3	"2002257123"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:03
S10	7	"20020198137"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:05
S11	3	"20040176339"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:08
S12	2	"20040258662"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:09
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S14	4250	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:44
S15	26943	(endocrine tumor) or (neuroendocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic tumor) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (neuroendocrine carcinoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:51

S16	0	S14 near3 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S17	0	S14 near30 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S18	0	S14 near300 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S19	27	S14 same3 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S20	1	("5665772").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/11 10:34
S21	23453	(endocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:20
S22	4250	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:21
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S25	0	S21 near30 S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:22

S26	0	S21 near300 S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:22
S27	0	S21 with S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:23
S28	0	S21 adj S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:38
S29	5	S21 same S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:38
S30	237	S21 and S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:45
S31	3	"20070104721"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:21
S32	2	"20070185069"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:25
S33	7	"20020198137"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:47
S34	1	WO-2004004644-$.did.	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:55
S35	1	2004-091226.NRAN.	DERWENT	ADJ	ON	2011/02/11 18:57
S36	1	("6573285").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/11 22:00
S37	1	("5538739").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/13 23:25
S38	6	"20020183240"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 00:11
S39	1	"20050187184"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:19
S40	6	"20020183239"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:20
S41	6	"20030008923"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:21

EAST Search History (Interference)

< This search history is empty>

2/14/2011 11:53:10 AM
C:\Documents and Settings\sjeanlouis\My Documents\EAST\Workspaces\12094173-neuroendocrine tumor treatment.wsp

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	11/26/2010	02/11/2011							
	1	÷	✓							
	2	÷	✓							
	3	÷	✓							
	4	÷	N							
	5	÷	N							
	6	÷	N							
	7	÷	N							
	8	÷	✓							
	9	÷	✓							
	10	÷	✓							
	11	÷	N							
	12	÷	✓							

Notice of References Cited	Application/Control No. 12/094,173	Applicant(s)/Patent Under Reexamination MARKS ET AL.	
	Examiner SAMIRA JEAN-LOUIS	Art Unit 1627	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	O					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Merck Manuals, Pancreatic endocrine tumors, 2009, pgs. 1-4.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-08)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	12094173
Filing Date	2008-05-19
First Named Inventor	Peter Wayne Marks
Art Unit	
Examiner Name	
Attorney Docket Number	34768-US-PCT

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20050187184		2005-08-25	JAMES J. GIBBONS JR., ET AL	
	2	20020183239		2002-12-05	JAMES J. GIBBONS JR., ET AL	
	3	20030008923		2003-01-09	GARY DUKART, ET AL	

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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2004/078133	WO		2004-09-16	WYETH		☐

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	
	Examiner Name	
	Attorney Docket Number	34768-US-PCT

If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/S.J.L./	1	ASANO TAKAYUKI, et al: "The rapamycin analog CCI-779 is a potent inhibitor of pancreatic cancer cell proliferation", Biochemical and Biophysical Research Communications, 331, 2005-05-27, pages 295-302	☐
/S.J.L./	2	BOULAY ANNE, et al: "Antitumor efficacy of intermittent treatment sshedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells", Cancer Research, 64, 2004-01-01, pages 252-261	☐
/S.J.L./	3	BOULAY ANNE, et al: "Prolonged effect of the rapamycin derivative RAD001 on p70S6 kinase activity in tumors, skin and peripheral blood lymphocytes derived from a syngeneic rat pancreatic tumor model: correlation with efficacy of intermittent dosing schedules", Proceedings of the American Association for Cancer Research Annual Meeting, 43, 2002, page 602	☐
/S.J.L./	4	BURNS CHRISTIANE, et al: "Rapamycin-induced endothelial cell death and tumor vessel thrombosis potentiate cytotoxic therapy against pancreatic cancer", Clinical Cancer Research, Vol. 10, 2004-03-15, pages 2109-2199	☐
/S.J.L./	5	O'REILLY TERENCE, et al: "In vivo activity of RAD001, an orally active rapamycin derivative in experimental tumor models", Proceedings of the American Association for Cancer Research Annual Meeting, 43, 2002-03, page 71	☐
/S.J.L./	6	STEPHAN SUSANN, et al: "Effect of rapamycin alone and in combination with antiangiogenesis therapy in an orthotopic model of human pancreatic cancer", Clinical Cancer Research, Vol. 10, 200-10-15, pages 6993-7000 2004.	☐
/S.J.L./	7	TOWNSLEY CAROL A., et al: "Evaluation of adverse events experienced by older patients participating in studies of molecularly targeted agents alone or in combination", Cancer Therapy: Clinical, vol. 12, no. 7, 2006-04-01, pages 2141-2149	☐

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EXAMINER SIGNATURE

Examiner Signature	/Samira Jean-louis/	Date Considered	02/14/2011

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L./

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     FILE 'CAPLUS' ENTERED AT 17:51:12 ON 11 FEB 2011
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                E ENDOCRINE TUMOR+ALL/CT
L3         3223 S (ENDOCRINE TUMOR OR "ENDOCRINE SYSTEM, NEOPLASM")
L4           78 S ADRENAL GLAND TUMOR
                E GASTRINOMA+ALL/CT
                E INSULINOMA+ALL/CT
L5         3656 S ISLET CELL TUMOR OR (GASTRINOMA OR "NEOPLASM" (L) "GASTRINOMA
                E GLUCAGONOMA+ALL/CT
                E SOMATOSTATINOMA+ALL/CT
L6          464 S GLUCAGONOMA OR VIPOMA OR SOMATOSTATINOMA OR NONFUNCTIONING TU
                E NEUROENDOCRINE TUMOR+ALL/CT
                E PHEOCHROMOCYTOMA+ALL/CT
L7         9923 S (NEUROENDOCRINE TUMOR OR "ENDOCRINE SYSTEM, NEOPLASM" (L) "NE
L8          376 S NEUROENDOCRINE CARCINOMA
                E PARATHYROID TUMORS+ALL/CT
                E PITUITARY GLAND TUMOR+ALL/CT
                E THYROID CANCER+ALL/CT
L9        16233 S (PARATHYROID TUMOR OR "PARATHYROID GLAND, NEOPLASM") OR (PITU
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Pancreatic Endocrine Tumors

Pancreatic endocrine tumors are those that arise from the types of pancreatic cells that produce hormones. These tumors may or may not secrete hormones themselves and may or may not be cancerous (malignant). Even if they do not secrete hormones (nonfunctioning tumors) and are not cancerous, these tumors may cause symptoms by blocking the biliary tract or small intestine or by bleeding into the gastrointestinal tract. Functioning tumors secrete large amounts of a particular hormone, causing various syndromes.

INSULINOMA

An insulinoma is a rare type of pancreatic tumor that secretes insulin, a hormone that lowers the levels of sugar (glucose) in the blood.

Only 10% of insulinomas are cancerous.

Symptoms

Symptoms result from low levels of sugar (glucose) in the blood, which occur when the person does not eat for several hours (most often in the morning after an all-night fast). The symptoms include faintness, weakness, trembling, awareness of the heartbeat (palpitations), sweating, nervousness, and profound hunger. Other symptoms include headache, confusion, vision abnormalities, unsteadiness, and marked changes in personality. The low levels of sugar in the blood may even lead to a loss of consciousness, seizures, and coma.

Diagnosis and Treatment

Diagnosing an insulinoma can be difficult. Doctors try to perform blood tests while the person has symptoms. Blood tests include measurements of blood glucose levels and insulin levels. Very low levels of glucose and high levels of insulin in the blood indicate the presence of an insulinoma. Because many people have symptoms only occasionally, doctors may admit them to the hospital. In the hospital, the person fasts for at least 24 hours, sometimes up to 72 hours, and is closely monitored. During that time, the symptoms usually appear, and blood tests are performed to measure the levels of glucose and insulin.

If the blood tests suggest the person has an insulinoma, the location must then be pinpointed. Imaging tests, such as endoscopic ultrasonography (which shows the lining of the digestive tract more clearly because the ultrasound probe is placed on the tip of the endoscope) or positron emission tomography (PET—see Common Imaging Tests: Positron

Emission Tomography) scans, can be used to locate the tumor, but sometimes exploratory surgery is needed.

The primary treatment for an insulinoma is surgical removal, which has a cure rate of about 90%. When the insulinoma cannot be completely removed and symptoms continue, drugs such as diazoxide and octreotide can help keep blood glucose from falling too low. Chemotherapy drugs such as streptozotocin and 5-fluorouracil may help control the tumor.

GASTRINOMA

A gastrinoma is a tumor usually in the pancreas or duodenum (the first segment of the small intestine) that produces excessive levels of the hormone gastrin, which stimulates the stomach to secrete acid and enzymes, causing peptic ulcers.

Most people with gastrinomas have several tumors clustered in or near the pancreas. About half of the tumors are cancerous. Sometimes a gastrinoma occurs as part of multiple endocrine neoplasia, a hereditary disorder in which tumors arise from the cells of various endocrine glands, such as the insulin-producing cells of the pancreas.

Symptoms and Diagnosis

The excess gastrin secreted by the gastrinoma causes Zollinger-Ellison syndrome (see Zollinger-Ellison Syndrome: An Acid-Stimulating Cancer), in which a person suffers the symptoms of aggressive peptic ulcers in the stomach, duodenum, and elsewhere in the intestine. However, as many as 25% of people with Zollinger-Ellison syndrome may not have an ulcer when the diagnosis is made. Rupture, bleeding, and obstruction of the intestine can occur and are life threatening. For more than half of the people with a gastrinoma, symptoms are no worse than those experienced by people with ordinary peptic ulcer disease. In 25 to 40% of people, diarrhea is the first symptom.

A doctor suspects a gastrinoma when a person has frequent peptic ulcers or several peptic ulcers that do not respond to the usual ulcer treatments. Blood tests to detect abnormally high levels of gastrin are the most reliable diagnostic tests.

Once blood tests diagnose gastrinoma, doctors use several imaging techniques, such as computed tomography (CT), endoscopic ultrasonography, PET scans, and arteriography (an x-ray taken after a radiopaque dye is injected into an artery), to locate tumors. These tumors may be difficult to find, however, because usually they are small.

Treatment

High doses of proton pump inhibitors (see Peptic Disorders: Acid-reducing Drugs and Drugs Used to Treat Peptic Disorders) may be effective for reducing acid levels and relieving symptoms temporarily. About 20% of people who do not have multiple endocrine neoplasia can be cured with surgical removal of the gastrinoma. If these treatments do not work, an operation to remove the stomach completely (total gastrectomy) may be necessary. This operation does not remove the tumor, but the gastrin can no longer create ulcers after the acid-producing stomach is removed. If the stomach is removed, daily iron and calcium supplements taken by mouth and monthly injections of vitamin B_{12} are needed, because absorption of these nutrients is impaired when stomach juices that prepare these nutrients for absorption are no longer available.

If cancerous tumors have spread to other parts of the body, chemotherapy may help reduce the number of tumor cells and the levels of gastrin in the blood. However, such therapy does not cure the cancer, which is ultimately fatal.

VIPOMA

A vipoma is a rare type of pancreatic tumor that produces vasoactive intestinal peptide (VIP), a substance that causes severe watery diarrhea.

About 50 to 75% of these tumors are cancerous. In about 6% of people, vipoma occurs as

part of multiple endocrine neoplasia (see Multiple Endocrine Neoplasia Syndromes).

Symptoms

The major symptoms are prolonged massive watery diarrhea. People produce 1 to 3 quarts (1000 to 3000 mL) of stool per day, causing dehydration. In 50% of people, diarrhea is constant, and in the rest, the severity of the diarrhea varies over time.

Because the diarrhea removes many of the body's normal salts, people often develop low blood levels of potassium (hypokalemia), and excessively acidic blood (acidosis). These changes can cause lethargy, muscular weakness, nausea, vomiting, and crampy abdominal pain. Some people have flushing.

Diagnosis and Treatment

A doctor bases the diagnosis on the person's symptoms and finding elevated levels of VIP in the blood. People with elevated levels of VIP should also have an endoscopic ultrasound or PET scan to detect the location of the vipoma.

Initially fluids and electrolytes must be replaced. Bicarbonate must be given to replace that lost in the stool and avoid acidosis. Because water and electrolytes continue to be lost in the stool as rehydration is achieved, doctors may find it difficult to continually replace water and electrolytes.

The drug octreotide usually controls diarrhea, but large doses may be needed. Surgical removal of the vipoma cures about 50% of people whose tumor has not spread. Surgery may temporarily relieve symptoms in people whose tumor has spread. Chemotherapy does not cure the disease.

GLUCAGONOMA

A glucagonoma is a tumor of the pancreas that produces the hormone glucagon, which raises the level of sugar (glucose) in the blood and causes a distinctive rash.

About 80% of glucagonomas are cancerous. However, they grow slowly, and many people survive for 15 years or more after the diagnosis. The average age at which symptoms begin is 50. About 80% of people with glucagonomas are women.

Symptoms and Diagnosis

High levels of glucagon in the blood cause the symptoms of diabetes mellitus. Often, the person loses weight. In 90% of people, the most distinctive features are a chronic brownish red skin rash (necrolytic migratory erythema) and a smooth, shiny, bright red-orange tongue. The mouth also may have cracks at the corners. The rash, which causes scaling, starts in the groin and moves to the buttocks, forearms, and legs.

The diagnosis is made by identifying high levels of glucagon in the blood and then locating the tumor by performing an abdominal CT followed by an endoscopic ultrasound. An MRI or PET scan may be used if the CT scan does not show a tumor.

Treatment

Ideally, the tumor is surgically removed, which eliminates all symptoms. However, if removal is not possible or if the tumor has spread, chemotherapy may reduce the levels of glucagon and lessen the symptoms. However, chemotherapy does not improve survival.

The drug octreotide can be used to reduce glucagon levels, may clear up the rash, and may restore appetite, facilitating weight gain. But octreotide may elevate the levels of glucose in the blood even more. Zinc ointment may be used to treat the skin rash. Sometimes the rash is treated with intravenous amino acids or fatty acids.

Last full review/revision December 2007 by Elliot M. Livstone, MD

Back to Top

Previous: Pancreatic Cancer

Next: Small Intestine Cancer

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CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.:

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

This Reply is submitted in response to the Office Action mailed February 16, 2011. A three-month extension of time petition is included herewith. Reconsideration of the present rejections and withdrawal of the present rejections are respectfully requested.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. (Currently Amended) A method for treating endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of ~~an mTOR inhibitor~~ 40-O-(2-hydroxyethyl)-rapamycin.

2. (Currently Amended) A method for ~~inhibiting growth of~~ treating pancreatic neuroendrocine ~~endocrine~~ tumors, comprising administering to a subject in need thereof a therapeutically effective amount of ~~an mTOR inhibitor~~ 40-O-(2-hydroxyethyl)-rapamycin.

3. (Currently Amended) ~~A~~ The method of claim 2, ~~for inhibiting or controlling endocrine tumors, comprising administering to a subject in need thereof a therapeutically effective amount of an mTOR inhibitor~~ wherein the unit dose of 40-O-(2-hydroxyethyl)-rapamycin administered is from 0.1 mg to 15 mg.

4.-7. Cancelled

8. (Previously presented) A method according to claim 1, comprising administering in addition a therapeutically effective amount of at least one second drug substance.

9. (Currently Amended) A method according to claim 8, wherein a second drug substance is ~~somastatin~~ somatostatin or a ~~somastatin~~ somatostatin analogue.

10-12. Cancelled

Remarks/Arguments

Reconsideration of this application, as amended, is respectfully requested. Claims 1-12 were pending in the present application. Claims 4-7 and 11 were withdrawn form consideration as a result of a restriction requirement and Applicants have cancelled claims 4-7 and 11 without prejudice. Claims 1-3 and 9 were amended. Claims 10 and 12 were cancelled. Accordingly, claims 1-3, 8 and 9 are currently pending. No new matter has been added by the present amendments. All amendments are made without prejudice or disclaimer. Applicants reserve the right to prosecute any cancelled or otherwise unclaimed subject matter in this or another application. Applicants believe these amendments place claims 1-3, 8 and 9 in condition for allowance. Consideration and entry of these amendments is respectfully requested.

Rejection under 35 U.S.C. § 101

Claims 10 and 12 stand rejected under 35 U.S.C. § 101. Applicants traverse the rejection in view of Applicants cancelling claims 10 and 12, thereby obviating the Section 101 rejection. Applicants respectfully request the Office to withdraw the Section 101 rejection of claims 10 and 12.

Rejection under 35 U.S.C. § 112, 2nd paragraph

Claims 10 and 12 stand rejected under 35 U.S.C. § 112, 2nd paragraph as being indefinite. Applicants traverse the rejection in view of Applicants cancelling claims 10 and 12, thereby obviating the Section 112 rejection. Applicants respectfully request the Office to withdraw the Section 112 rejection of claims 10 and 12.

Claim Objection

Claim is objected to with regard to "somastatin" and "somastatin analogue". Applicants have corrected claim 9 to recite somatostatin and somatosstatin analogue, as disclosed at page 31, lines 28 and 29 of the specification. Applicants respectfully request the Office to withdraw the objection to claim 9 as amended.

Rejection under 35 U.S.C. § 102(b)

Claims 1-3 and 12 stand rejected under 35 U.S.C. § 102(b) as anticipated by a one page, non-patent publication by Terrence O'Reilly, et al in the Proceedings of the American Association for Cancer Research, 43, 2002, 0359, page 71 (O'Reilly, et al. reference). Applicants traverse the rejection in view of amendments to claims 1-3. The O'Reilly, et al. reference (Abstract 359) does not disclose or suggest Applicants' composition for treating endrocine tumors, as claimed in claim 1 or for treating pancreatic neuroendrocine tumors, as

claimed in claim 2. Applicants' amendments overcome the Section 102(b) rejection for claims 1 and 2 and corresponding dependent claim 3. Applicants respectfully request the Office to withdraw the Section 102(b) rejection.

Rejection under 35 U.S.C. § 102(b)

Claims 1-3, 8, 9 and 12 stand rejected under 35 U.S.C. § 102(b) as anticipated by international patent publication WO 97/47317 (Weckbecker, et al. reference). Applicants traverse the rejection in view of amendments to claims 1-3 and 9. The Weckbecker, et al. reference only refers to GEP tumors. It does not define which tumors GEP refers to at page 14 or anywhere in the specification. The Weckbecker, et al. reference does not disclose or suggest Applicants' composition for treating endrocine tumors, as claimed in claim 1 or for treating pancreatic neuroendrocine tumors, as claimed in claim 2. Applicants' amendments overcome the Section 102(b) rejection for claims 1 and 2 and corresponding dependent claim 3. Applicants respectfully request the Office to withdraw the Section 102(b) rejection.

CONCLUSIONS

Consideration and entry of these amendments and remarks are respectfully requested. Claims 1-3 and 9 are now in condition for allowance and Applicants respectfully request that a Notice of Allowance be issued as soon as possible. Should the Examiner have any questions, please contact the undersigned.

Respectfully submitted,

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-1422

Stephen Johnson
for Applicant
Reg. No. 45,916

Date: August 2, 2011

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Stephen E. Johnson/Monika van Houten
Attorney Docket Number:	34678-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1110	1110

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**1110**

CASE PAT034678-US-PCT

FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10	
Express Mail Label Number	Date of Deposit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF Art Unit: 1627

Marks, Peter Wayne et al. Examiner: Jean-Louis, Samira J

INTERNATIONAL APPLICATION NO: PCT/EP06/068656

FILED: November 20, 2006

U.S. APPLICATION NO: 12/094173

35 USC §371 DATE: May 19, 2008

FOR: Neuroendocrine Tumor Treatment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

PETITION FOR EXTENSION OF TIME

Sir:

The Office Action of February 16, 2011 has a shortened statutory time set to expire on May 16, 2011. . A three-month extension is hereby requested pursuant to 37 CFR §1.136(a).

Please charge Deposit Account No. 19-0134 in the name of Novartis in the amount of $1110 for payment of the extension fee. The Commissioner is hereby authorized to charge any additional fees under 37 CFR §1.17 which may be required, or credit any overpayment, to Account No. 19-0134 in the name of Novartis.

Respectfully submitted,

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-1422

Stephen Johnson
for Applicant
Reg. No. 45,916

Date: August 2, 2011

Electronic Acknowledgement Receipt	
EFS ID:	10644883
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	01095
Filer:	Stephen E. Johnson/Monika van Houten
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	02-AUG-2011
Filing Date:	19-MAY-2008
Time Stamp:	10:55:07
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1110
RAM confirmation Number	7280
Deposit Account	190134
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678-ROA.pdf	161333 2fc869f5af23118a9a75ec9698cadb1b142f6197	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Extension of Time	1	1
	Amendment/Req. Reconsideration-After Non-Final Reject	2	2
	Claims	3	3
	Applicant Arguments/Remarks Made in an Amendment	4	5

Warnings:

Information:

2	Fee Worksheet (SB06)	fee-info.pdf	30247 7fe0144090a917127768efca86df6d4033bd8ba5b	no	2

Warnings:

Information:

Total Files Size (in bytes):	191580

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

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PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
12/094,173	05/19/2008	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	08/02/2011	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 5	Minus	** 20	= 0	X $ =		OR	X $52=	0
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $220=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)					
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/SHARAIN MORELAND/

PTO/SB/08a (07-09)
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	1	of	2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO2005/080593 A2	09/01/2005	Novartis Pharma GmbH		☐
		WO2004/004644 A2	01/15/2004	Neel, Benjamin G		☐
		WO2006/065780	06-22-2006	Novartis AG		☐
		WO2002/066019	08-29-2002	Novartis AG		☐
						☐
						☐

Examiner Signature		Date Considered	

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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	2	of	2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Database Medline: Canobbio L. et al: "Use of long-acting somatostatin analog, lanreotide, in neuro-endocrine tumors." Oncology reports, vol. 1, no.1 Jan. 1994 p. 129- 131	☐
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Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw a line through citation if not in conformance and not considered. Include copy of this form with the next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: BIOMARKERS

(57) Abstract: Provided are biomarkers for determining the sensitivity of proliferative diseases such as cancer to therapeutic agents, in particular mTOR inhibitors in combination with a cytotoxic agent, in particular a cytotoxic agent which damages or affects the integrity of DNA.

WO 2005/080593 A2

Biomarkers

The present invention relates to biomarkers for determining the sensitivity of proliferative diseases such as cancer to therapeutic agents, in particular mTOR inhibitors in combination with a cytotoxic agent.

A number of mTOR inhibitors have potent antiproliferative properties which make them useful for cancer chemotherapy, particularly of solid tumors, especially of advanced solid tumors. mTOR inhibitors have also been combined with certain cytotoxic agents to further improve the efficiency of the treatment or to reduce the side-effects, e.g. as disclosed in WO 02/66019. However there is still a need for more targeted use of a combined therapy based on mTOR inhibitors, which requires identification of patients which are likely to respond to treatment with such combined agents. Accordingly there is a need for biomarkers useful in e.g. clinical tests, which are capable of predicting responsiveness of a benign or malignant proliferative disease, e.g. a tumor in a patient, to treatment with an mTOR inhibitor in association with a cytotoxic agent.

It has surprisingly been found that the presence of a wild-type p53 tumor suppressor gene (otherwise also known as the *TP53* gene) is a useful biomarker which is predictive of sensitivity of proliferative diseases to treatment with a combination of an mTOR inhibitor with a cytotoxic agent. In particular, it has been found that the presence of a wild-type p53 gene in human cancer cell lines correlates well with increased cell killing/programmed cell death/apoptosis resulting from treatment with an mTOR inhibitor in combination with a cytotoxic agent that damages or affects the integrity of DNA. Hence, mTOR inhibitors combined with a cytotoxic agent are more likely to show a more significant antiproliferative/cell killing effect when used to treat cancer cells which retain wild-type p53. The p53 protein (encoded by the *TP53* gene) is a tumor-suppressor which plays a major role in the regulation of cell cycle arrest, senescence, differentiation and programmed cell death/apoptosis in mammalian cells. In particular, the p53 pathway induces cell cycle arrest and/or apoptosis in mammalian cells exposed to stress (e.g. DNA damage, oncogenic stress, hypoxia, lack of survival signals). Mutations in *TP53* occur in about half of all human cancers, and the ability to induce a p53 response is compromised in many cancer cells (Vousden and Lu, Nature Reviews, 2002,2:594-604). The sequence of human p53 (mRNA [coding sequence; 1182 nucleotides] and protein [393 amino acids]) is available under GenBank accession numbers NM 000546 or P04637. The complete sequence of the human *TP53* gene is available under GenBank accession number U94788.

Accordingly, the present invention is based on the determination of the presence of a wild-type p53 (*TP53*) gene in cells which are prone to abnormal proliferation.

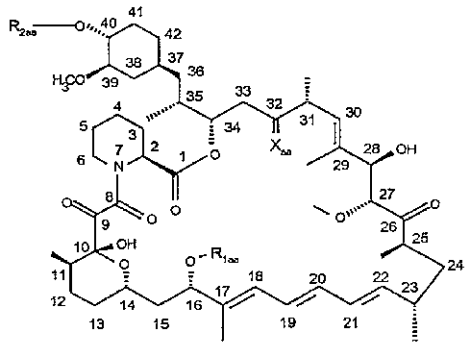
The present invention provides in one aspect the use of the presence of wild-type p53 (*TP53*) gene (as opposed to the absence, deficiency or deletion of the p53 [*TP53*] gene or the presence of a mutated p53 [*TP53*] gene) as a biomarker for determining the sensitivity of a proliferative disease to treatment with an mTOR inhibitor in combination with a cytotoxic agent.

By wild-type p53 (*TP53*) gene is meant not only the introns and exons but also regulatory regions associated with, and physically close to, the introns and exons, particularly those 5' to the 5'-most exon. It includes e.g. the full length DNA sequence of the natural gene and optionally nucleotide substitutions (including inversions), insertions and deletions of codons, provided that it expresses the wild-type p53 protein or a functional equivalent thereof, e.g. a functional p53 protein retaining its cell apoptosis-inducing properties. Conversely, absence, deficiency, deletion or mutation of the p53 *(TP53)* gene is meant for genetic and epigenetic changes e.g. amplification, methylation, polymorphisms, nucleotide mutations, deletions, inversions or translocations and loss of heterozygosity (LOH) which results in loss of p53 (*TP53*) gene expression or expression of a mutated gene which e.g. results in expression of a mutated p53 protein which no longer retains cell apoptosis-inducing properties.

In a further aspect the invention provides a method for determining the sensitivity of a proliferative disease in a subject to treatment with an mTOR inhibitor in combination with a cytotoxic agent, comprising determining p53 (*TP53*) status (wild-type versus mutant or deficient/absent) in a sample derived from the subject.

In another aspect the invention provides a method of selecting subjects suffering from a proliferative disease for treatment with an mTOR inhibitor in association with a cytotoxic agent, comprising determining the sensitivity of the proliferative disease to the combined treatment in each subject by a method as described above, and selecting those subjects retaining a wild-type p53 (*TP53*) gene for said combined treatment.

The term "mTOR inhibitor" as used herein includes, but is not limited to rapamycin (sirolimus) or a derivative thereof. Rapamycin is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Suitable derivatives of rapamycin include e.g. compounds of formula A



A

wherein

R_{1aa} is CH_3 or C_{3-6}alkynyl,

R_{2aa} is H or $-CH_2-CH_2-OH$, 3-hydroxy-2-(hydroxymethyl)-2-methyl-propanoyl or tetrazolyl, and

X_{aa} is =O, (H,H) or (H,OH)

provided that R_{2aa} is other than H when X_{aa} is =O and R_{1aa} is CH3.

or a prodrug thereof when R_{2aa} is $-CH_2-CH_2-OH$, e.g. a physiologically hydrolysable ether thereof.

Compounds of formula A are disclosed e.g. in WO 94/09010, WO 95/16691, WO 96/41807, USP 5,362,718 or WO 99/15530 which are incorporated herein by reference. They may be prepared as disclosed or by analogy to the procedures described in these references.

Representative rapamycin derivatives of formula I are e.g. 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32(S or R)-dihydro-rapamycin, 16-pent-2-ynyloxy-32(S or R)-dihydro-40-O-(2-hydroxyethyl)-rapamycin, 40-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin (also called CCI779) or 40-epi-(tetrazolyl)-rapamycin (also called ABT578). A preferred compound is e.g. 40-0-(2-hydroxyethyl)-rapamycin disclosed in Example 8 in WO 94/09010, or 32-deoxorapamycin or 16-pent-2-ynyloxy-32(S)-dihydro-rapamycin as disclosed in WO 96/41807. Rapamycin derivatives may also include the so-called rapalogs, e.g. as disclosed in WO 98/02441 and WO01/14387, e.g. AP23573, AP23464, AP23675 or AP23841. Further examples of a rapamycin derivative are those disclosed under the name TAFA-93 (a rapamycin prodrug), biolimus-7 or biolimus-9.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference. Comprised are likewise the pharmaceutically acceptable salts thereof, the

corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e.g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention can be prepared and administered as described in the cited documents, respectively.

The term "cytotoxic agent" as used herein is an agent which is harmful to cell structure and function, e.g. that damages or affects the DNA integrity, and may ultimately cause cell death, e.g. a antineoplastic drug, for instance a microtubule active agent or especially a drug which damages DNA, for example an antineoplastic antimetabolite, a platin compound, an alkylating agent or a topoisomerase I or II inhibitor. The term "cytotoxic agent" also includes an irradiation treatment which causes DNA damage, e.g ionizing radiation, e.g. radioactive iodine. Such irradiation treatment may also be combined with the cytotoxic agent therapy. The term "cytotoxic agent" also includes one, two or more cytotoxic agents which may be administered in the form of a "cocktail" therapy.

The term "topoisomerase I inhibitor" as used herein includes, but is not limited to topotecan, irinotecan, gimatecan, 9-nitrocamptothecin and the macromolecular camptothecin conjugate PNU-166148 (compound A1 in WO99/17804). Irinotecan can be administered, e.g. in the form as it is marketed, e.g. under the trademark CAMPTOSAR™. Topotecan can be administered, e.g., in the form as it is marketed, e.g. under the trademark HYCAMTIN™.

The term "topoisomerase II inhibitor" as used herein includes, but is not limited to the anthracyclines such as doxorubicin (including liposomal formulation, e.g. CAELYX™), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS™. Teniposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark VM 26-BRISTOL™. Doxorubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark ADRIBLASTIN™. Epirubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark FARMORUBICIN™. Idarubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark ZAVEDOS™. Mitoxantrone can be administered, e.g. in the form as it is marketed, e.g. under the trademark NOVANTRON™.

The term "microtubule active agent" relates to microtubule stabilizing and microtubule destabilizing agents including, but not limited to taxanes, e.g. paclitaxel and docetaxel, vinca

alkaloids, e.g., vinblastine, especially vinblastine sulfate, vincristine especially vincristine sulfate, and vinorelbine, discodermolides and epothilones and derivatives thereof, e.g. epothilone B or a derivative thereof. Paclitaxel may be administered e.g. in the form as it is marketed, e.g. TAXOL™. Docetaxel can be administered, e.g., in the form as it is marketed, e.g. under the trademark TAXOTERE™. Vinblastine sulfate can be administered, e.g., in the form as it is marketed, e.g. under the trademark VINBLASTIN R.P.™. Vincristine sulfate can be administered, e.g., in the form as it is marketed, e.g. under the trademark FARMISTIN™. Discodermolide can be obtained, e.g., as disclosed in US 5,010,099.

The term "alkylating agent" as used herein includes, but is not limited to cyclophosphamide, ifosfamide, melphalan or nitrosourea (BCNU or Gliadel™). Cyclophosphamide can be administered, e.g., in the form as it is marketed, e.g. under the trademark CYCLOSTIN™. Ifosfamide can be administered, e.g., in the form as it is marketed, e.g. under the trademark HOLOXAN™.

The term "antineoplastic antimetabolite" includes, but is not limited to 5-fluorouracil, tegafur, capecitabine, cladribine, cytaribine, fludarabine phosphate, fluorouridine, gemcitabine, 6-mercaptopurine, hydroxyurea, methotrexate, edatrexate and salts of such compounds, and furthermore ZD1694 (RALTITREXED™), LY231514 (ALIMTA™), LY264618 (LOMOTREXOL™) and OGT719. Capecitabine can be administered, e.g., in the form as it is marketed, e.g. under the trademark XELODA™. Gemcitabine can be administered, e.g., in the form as it is marketed, e.g. under the trademark GEMZAR™.

The term "platin compound" as used herein includes, but is not limited to carboplatin, cis-platin and oxaliplatin. Carboplatin can be administered, e.g., in the form as it is marketed, e.g. under the trademark CARBOPLAT™. Oxaliplatin can be administered, e.g., in the form as it is marketed, e.g. under the trademark ELOXATIN™.

The proliferative disease may be a benign or malignant proliferative disease, e.g. benign prostatic hyperplasia, or a neoplastic disease, preferably a malignant proliferative disease, e.g. a cancer, e.g. tumors and/or metastasis (where ever located), e.g. brain and other central nervous system tumors (eg. tumors of the meninges, brain, spinal cord, cranial nerves and other parts of central nervous system, e.g. glioblastomas or medulla blastomas); head and/or neck cancer; breast tumors; circulatory system tumors (e.g. heart, mediastinum and pleura, and other intrathoracic organs, vascular tumors and tumor-asso-ciated vascular tissue); excretory system tumors (e.g. kidney, renal pelvis, ureter, bladder, other and unspecified urinary organs); gastrointestinal tract tumors (e.g. oesophagus, stomach, small

intestine, colon, colorectal, rectosigmoid junction, rectum, anus and anal canal), tumors involving the liver and intrahepatic bile ducts, gall bladder, other and unspecified parts of biliary tract, pancreas, other and digestive organs); head and neck; oral cavity (lip, tongue, gum, floor of mouth, palate, and other parts of mouth, parotid gland, and other parts of the salivary glands, tonsil, oropharynx, nasopharynx, pyriform sinus, hypopharynx, and other sites in the lip, oral cavity and pharynx); reproductive system tumors (e.g. vulva, vagina, Cervix uteri, Corpus uteri, uterus, ovary, and other sites associated with female genital organs, placenta, penis, prostate, testis, and other sites associated with male genital organs); respiratory tract tumors (e.g. nasal cavity and middle ear, accessory sinuses, larynx, trachea, bronchus and lung, e.g. small cell lung cancer or non-small cell lung cancer); skeletal system tumors (e.g. bone and articular cartilage of limbs, bone articular cartilage and other sites); skin tumors (e.g. malignant melanoma of the skin, non-melanoma skin cancer, basal cell carcinoma of skin, squamous cell carcinoma of skin, mesothelioma, Kaposi's sarcoma); and tumors involving other tissues including peripheral nerves and autonomic nervous system, connective and soft tissue, retroperitoneum and peritoneum, eye and adnexa, thyroid, adrenal gland and other endocrine glands and related structures, secondary and unspecified malignant neoplasm of lymph nodes, secondary malignant neoplasm of respiratory and digestive systems and secondary malignant neoplasm of other sites, , tumors of blood and lymphatic system (e.g. Hodgkin's disease, Non-Hodgkin's lymphoma, Burkitt's lymphoma, AIDS-related lymphomas, malignant immunoproliferative diseases, multiple myeloma and malignant plasma cell neoplasms, lymphoid leukemia, acute or chronic myeloid leukemia, acute or chronic lymphocytic leukemia, monocytic leukemia, other leukemias of specified cell type, leukemia of unspecified cell type, other and unspecified malignant neoplasms of lymphoid, haematopoietic and related tissues, for example diffuse large cell lymphoma, T-cell lymphoma or cutaneous T-cell lymphoma). Myeloid cancer includes e.g. acute or chronic myeloid leukaemia.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

The term cytotoxic agent may also, in case of a lymphatic or myeloid cancer, be e.g. busulfan, cytarabine, 6-thioguanine, fludarabine, hydroxyurea, procarbazine, bleomycin or methotrexate. Topoisomerase II inhibitors e,g. daunorubicin or idarubicin or, particularly, compounds which target, decrease or inhibit the activity of PDGFR or of c-Abl family members and their gene fusion products, e.g. imatinib, farnesyltransferase inhibitors, Ara-C,

VP-16, Teniposide, Mitoxantrone, Carboplatin or midostaurine are preferred as cytotoxic agent in case of a lymphatic or myeloid cancer.

According to the method of the present invention, subjects suffering from such a proliferative disease can be screened in order to predict their sensitivity to a combined treatment of mTOR inhibitors with a cytotoxic agent. The method may be performed in vitro, e.g. on a sample of biological tissue derived from the subject. The sample may be any biological material separated from the mammalian body such as e.g. tissue, cell lines, plasma or serum, cell or tissue lysate, preferably tumor tissue.

The status of the p53 (*TP53*) gene is assayed in the biological sample by any technical means on the basis of e.g. DNA analysis for genetic and epigenetic changes e.g. DNA scanning for amplification, methylation, polymorphisms, nucleotide mutations (e.g. mutations of codons 175Arg, 245Gly, 248Arg, 249Arg, 273Arg, 282Arg and others) nucleotide deletions, inversions and/or translations and loss of heterozygosity (LOH). p53 (*TP53)* status is assayed in the biological samples by any technical means on the basis of e.g. RNA expression using for example the techniques of northern blotting or RT-PCR or on the basis of e.g. protein expression/modifications using for example the technique of Western blotting, immunohistochemistry or ELISA, including immunoassays, immunoprecipitation and electrophoresis assays.

For example, antibodies specific for p53 protein or p53 post-translational modifications such as phosphorylation (e.g. phosphorylation of Ser46), ubiquitination or acetylation may be used in a standard immunoassay format to measure p53 protein/phosphorylation/ubiquitination/ acetylation levels. ELISA (enzyme linked immunosorbent assay) type assays, immunoprecipitation type assays, conventional Western blotting assays and immunohistochemistry assays using e.g. monoclonal or polyclonal antibodies are also utilized to determine levels of p53 protein/post-translational modifications as a biomarker.

Polyclonal and monoclonal antibodies specific to p53 protein/post-translational modifications are produced in accordance with known immunization methods or are commercially available (e.g. Santa Cruz Biotechnology Inc catalogue #sc6253).

The p53 status may also be measured by two-dimensional (2-D) gel electrophoresis. 2-D gel electrophoresis is known in the art and typically involves isoelectric focusing (IEF) along a first dimension followed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) along a second dimension. The resulting electropherograms are analyzed, for example, by immunoblot analysis using antibodies.

The present invention thus provides a method of screening subjects suffering from a proliferative disease in order to predict their responsiveness to a combined treatment with an mTOR inhibitor and a cytotoxic agent, comprising determining the p53 (*TP53*) status by a method as defined above.

In a further aspect, the present invention provides a method of treating a proliferative disease in a subject in need thereof, comprising determining the status of the p53 (*TP53*) gene or the level of p53 expression and/or post-translational modifications in a sample derived from the subject, by a method as described above, and treating the subject with an mTOR inhibitor in combination with a cytotoxic agent accordingly.

In an alternative embodiment, the present invention provides a method for enhancing the activity of a cytotoxic agent or for overcoming resistance to a cytotoxic agent in a subject in need thereof, comprising determining the status of the p53 (*TP53*) gene/expression in a sample derived from the subject, by a method as described above, and administering to said subject a therapeutically effective amount of an mTOR inhibitor, either concomitantly or sequentially with said cytotoxic agent.

p53 (*TP53*) status in a particular tissue from a subject, e.g. a sample of tumor tissue, may be compared with a control sample, e.g. a sample of normal tissue from a subject not suffering from the disease, or a sample of normal (i.e non-tumor) tissue from the same subject. The p53 (*TP53*) wild-type status level at which use of an mTOR inhibitor in association with a cytotoxic agent is indicated, is predictive of a beneficial therapeutic effect (i.e. an antiproliferative and/or increased cell killing effect) of a combined treatment of an mTOR inhibitor with a cytotoxic agent.

Moreover, the method may be used to aid selection of an appropriate dose of a cytotoxic agent and/or an mTOR inhibitor in order to individually optimise therapy for each patient. Depending on the p53 wild-type status in a patient, lower doses of the active ingredients of the combination can be used; for example, the dosages need not only often be smaller but may also be applied less frequently, or can be used in order to diminish the incidence of side-effects, while controlling the undesired proliferation. Factors for consideration in this context include the particular condition being treated, the particular mammal being treated, the clinical condition of the individual patient, the site of delivery of the active compounds, the particular type of the active compounds, the method of administration, the scheduling of administration, the severity of the condition and other factors known to medical practitioners.

The terms "combined treatment" or "in combination with" or "in association with" or the like as utilized herein are meant to encompass administration of the selected mTOR inhibitor and cytotoxic agent to a single patient, and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time. For example, the mTOR inhibitor and the cytotoxic agent may be administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific time limits, wherein such administration provides therapeutically effective levels of the two compounds in the body.

The therapeutically effective amount of each active component of the combination to be administered will be governed by considerations as mentioned above, and is the minimum amount necessary to prevent, ameliorate, or treat the disease. Such amount is preferably below the amount that is toxic to the host or which renders the host significantly more susceptible to infections.

Appropriate doses of an mTOR inhibitor are e.g. as disclosed in WO 02/66019, e.g. daily dosage rates of the order of ca. 0.1 to 30 mg, e.g. from ca. 0.05 to 20 mg active ingredient p.o., as a single dose or in divided doses or intermittent, e.g. once a week. Rapamycin or a derivative thereof, e.g. a compound of formula A, may be administered by any conventional route, in particular enterally, e.g. orally, e.g. in the form of tablets, capsules, drink solutions or parenterally, e.g. in the form of injectable solutions or suspensions, containing, for example, from about 0.1 % to about 99.9%, preferably from about 1 % to about 60 %, of the active ingredient(s).

Topotecan may be administered to a human in a dosage range varying from about 1 to 5 mg/m^{2}day. Irinotecan may be administered to a human in a dosage range varying from about 50 to 350 mg/m^{2}day.

Paclitaxel may be administered to a human in a dosage range varying from about 50 to 300 mg/m^{2}day. Docetaxel may be administered to a human in a dosage range varying from about 25 to 100 mg/m^{2}day.

Cyclophosphamide may be administered to a human in a dosage range varying from about 50 to 1500 mg/m^{2}day. Melphalan may be administered to a human in a dosage range varying from about 0.5 to 10 mg/m^{2}day.

5-Fluorouracil may be administered to a human in a dosage range varying from about 50 to 1000 mg/m^{2}day, e.g. 500 mg/m^{2}day. Capecitabine may be administered to a human in a

dosage range varying from about 10 to 1000 mg/m^{2}day. Gemcitabine hydrochloride may be administered to a human in a dosage range varying from about 1000 mg/m^2/week.

Carboplatin may be administered to a human in a dosage range varying from about 200 to 400 mg/m^2 about every four weeks. Cisplatin may be administered to a human in a dosage range varying from about 25 to 75 mg/m^2 about every three weeks. Oxaliplatin may be administered to a human in a dosage range varying from about 50 to 85 mg/m^2 every two weeks.

Imatinib may be administered to a human in a dosage in the range of about 2.5 to 850 mg/day, more preferably 5 to 600 mg/day and most preferably 20 to 300 mg/day.

A preferred combination to be used in a method in accordance with the invention is e.g. a combination of rapamycin, 40- [3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin or 40-O-(2-hydroxyethyl) rapamycin with a cytotoxic agent such as gemcitabine or cisplatin. An alternative combination to be used in a method according to the invention is a combination in synergistic amounts of an mTOR inhibitor with a cytotoxic agent, e.g. gemcitabine or cisplatin, e.g. as disclosed above.

Preferably *TP53* is the human gene.

Preferably the methods of the invention are performed on tumor cells presenting a p53 (*TP53*) wild-type status.

In a further embodiment, it has surprisingly been found that the increased cell killing/programmed cell death/apoptosis resulting from treatment with an mTOR inhibitor in combination with a cytotoxic agent in p53 (*TP53*) wild-type cells is associated with a strong attenuation of cytotoxic-induced upregulation of $p21^{Waf1/Cip1}$ (also known as CDKN1A, WAF1, CIP1, SDI1, CAP20, MDA-6, p21) protein expression, referred to hereafter as p21.

p21 is a member of the cip/kip family of cyclin kinase "inhibitors", which plays a role in allowing cell cycle transit as well as preventing apoptosis. In the context of the present invention, the function of p21 to arrest cell growth in response to stress signals, e.g. DNA damage, in response to activated p53 is well established. Indeed, it is postulated that increased p21 protein expression allows such stressed cells to survive, e.g. allowing the cell to complete the DNA repair process. Hence, attenuation of increased p21 expression in response to treatment with cytotoxics may promote cell killing/programmed cell death/apoptosis (Weiss, Cancer Cell, 2003,4:425-429). The sequence of human p21 (mRNA [coding sequence: 495 nucleotides] and protein product [164 amino acids]) is available under

GenBank accession number NM 000389, NM 078467 or AAH01935. The complete sequence of the human p21 gene is available under GenBank accession number NM 078467.

Furthermore, some cancer patients have increased total or cytosolic tumor p21 expression which has been linked to poor prognosis and poor response to chemotherapy (Weiss, *supra*). The assessment of the basal p21 expression in a cancer patient may also allow to select the patients for a specific chemotherapeutic treatment, e.g. based on mTOR therapy in combination with one or more cytotoxic agents and optionally radiotherapy.

Accordingly, the present invention further provides:

i. use of p21 as a biomarker for determining the sensitivity or response of a proliferative disease in a subject to treatment with an mTOR inhibitor in combination with a cytotoxic agent;

ii. a method of selecting subjects suffering from a proliferative disease for treatment with an mTOR inhibitor in combination with a cytotoxic agent, comprising determining the sensitivity of the proliferative disease to treatment with an mTOR inhibitor in combination with a cytotoxic agent in each subject by a method as described above, and selecting those subjects showing increased basal p21 expression for combination treatment;

iii. a method for determining the sensitivity or response of a proliferative disease in a subject to a treatment with an mTOR inhibitor, in combination with a cytotoxic agent, comprising determining in a sample derived from the subject the level of p21 expression before and/or after treatment with the cytotoxic agent alone and in combination with an mTOR inhibitor;

iv. a method for enhancing the activity of a cytotoxic agent or for overcoming resistance to a cytotoxic agent in a subject treated with said cytotoxic agent, comprising

- determining the level of p21 expression in a sample derived from the subject, by a method as described above,
- if p21 expression is upregulated after administration of a cytotoxic agent, administering to said subject a therapeutically effective amount of an mTOR inhibitor in combination with the cytotoxic agent,
- determining again the level of p21 expression in a new sample derived from the subject after the treatment with the combination of the mTOR inhibitor and the cytotoxic agent, and

- if p21 expression is downregulated, further treating the subject with the mTOR inhibitor either concomitantly or sequentially with said cytotoxic agent.

As already mentioned above, p21 protein levels may be determined as disclosed above for p53; however, instead of using antibodies specific to p53, it is understood to use an antibody specific for p21, e.g. a monoclonal or polyclonal antibody, e.g. as commercially available (e.g. Oncogene Research products, Clone EA10, catalogue #OP64).

The level found in a particular tissue from a subject, e.g. a sample of tumor tissue, may be compared with a control sample, e.g. a sample of normal tissue from a subject not suffering from the disease, or a sample of normal (i.e non-tumor) tissue from the same subject. A lack of or attenuation of the induction of p21 expression (when treated with an mTOR inhibitor in combination with a cytotoxic agent as compared to the induction observed with the cytotoxic agent alone) is predictive of a beneficial therapeutic effect (i.e. an antiproliferative/cell killing effect) of an mTOR inhibitor in combination with a cytotoxic agent. The assessment of the induction of p21 expression by the cytotoxic agent and/or of the reversing effect by the mTOR inhibitor on p21 expression level may also be useful to adapt the doses of the cytotoxic agent, e.g. to reduce the cytotoxic dose.

The present Examples illustrate the invention without any limitation.

Example 1

p53 (*TP53)* wild-type human adenocarcinoma A549 (CCL-185) tumor cells (American Type Culture Collection, Rockville, MD.,USA) are seeded at a density of 2x 10^3 cells/100 µl per well in 96-well plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are incubated with suboptimal concentrations of gemcitabine (e.g. 5 to 17.5 nM) either in combination with 20nM 40-O-(2-hydroxyethyl) rapamycin or with the vehicle-control DMSO for an additional 72 hours. YO-PRO dye (YO-PRO^R-1 iodide [491/509], cat #Y3603, Molecular Probes) is added to the cells and a Cytofluor II Fluorescence plate reader is used to determine cell death or cytotoxicity and, after cell lysis, the relative cell proliferation. In this assay, the mTOR inhibitor, e.g. 40-O-(2-hydroxyethyl) rapamycin, causes a statistically significant potentiation of the cell killing effect of suboptimal concentrations of gemcitabine ($p<0.05$; ANOVA with Tukey test). Similar results as disclosed above are obtained when using a p53 (*PT53*) wild-type cell line other than the human lung adenocarcinoma A549, e.g. human MCF7 breast carcinoma cells (HTB-22; American Type Culture Collection).

This procedure is repeated however with the use of p53 (*TP53*) mutated/deficient tumor cell lines, e.g. PC3M human prostate carcinoma cells (seeded at a density of $0.8x10^3$ cells/100 µl) or MDA-MB231 human breast carcinoma cells (seeded at a density of $2x10^3$ cells/100 µl; HTB-26; American Type Culture Collection). No striking or consistent potentiation of cell death is seen in p53 (*TP53*) mutated/deficient cell lines.

A549 cells are seeded at a density of $0.1x10^6$ cells/10 ml per 10cm plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are incubated with suboptimal concentrations of gemcitabine (e.g. 5 to 12.5 nM) either in combination with 20nM 40-O-(2-hydroxyethyl) rapamycin or with the vehicle-control DMSO for an additional 72 hours. Cell extracts corresponding to 50 µg total protein are resolved by 8% SDS-PAGE electrophoresis and immunoblot analysis is performed using rabbit polyclonal antibodies raised against Poly (ADP-Ribose) Polymerase (PARP) (Cell Signalling Technology catalogue #9542). In this assay, the presence of the mTOR inhibitor, e.g. 40-O-(2-hydroxyethyl) rapamycin, causes increased PARP cleavage (a marker of apoptosis) at suboptimal gemcitabine concentrations (as compared to gemcitabine or the mTOR inhibitor alone at the same concentrations). This confirms the above results that, in the p53 (*TP53*) wild-type A549 cells, the presence of the mTOR inhibitor results in higher levels of cell death at suboptimal gemcitabine concentrations.

Example 2

p53 (*TP53*) wild-type human lung adenocarcinoma A549 cells are seeded at a density of $5x10^3$ cells/100 µl per well in 96-well plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are incubated with suboptimal concentrations of cisplatin (e.g. 3 to 10 µg/ml) either in combination with 20 nM 40-O-(2-hydroxyethyl) rapamycin or with the vehicle-control DMSO for an additional 24 hours. The YO-PRO® assay is performed as above to determine cell death or cytotoxicity and, after cell lysis, the relative cell proliferation. In this assay, the mTOR inhibitor, e.g. 40-O-(2-hydroxyethyl) rapamycin, causes a statistically significant potentiation of the cell killing effect of suboptimal concentrations of cisplatin ($p<0.05$; ANOVA with Tukey test). Subsequent analysis using two-way ANOVA indicates that the interaction between RAD001 and cisplatin was highly significant ($p<0.001$). Similar results as disclosed above are obtained when using a p53 (*PT53*) wild-type cell line other than the human lung adenocarcinoma A549, e.g. human MCF7 breast carcinoma cells. In the latter case, incubation with compounds is for 30 hours.

This procedure is repeated however with the use of p53 (*TP53*) mutated/deficient tumor cell lines, e.g. PC3M (seeded at a density of $3x10^3$ cells/100 µl) or DU145 (seeded at a density of $5x10^3$ cells/100µl:HTB-81; American Type Culture Collection). The incubation with compounds in this case is 22 hours for DU145 or 30 hours for PC3M. No striking or consistent potentiation of cell death is seen in p53 (*TP53*) mutated/deficient cell lines.

A549 cells are seeded at a density of $0.1x10^6$ cells/10 ml per 10 cm plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are incubated with suboptimal concentrations of cisplatin (e.g. 0.5 to 4 µg/ml) either in combination with 20nM 40-O-(2-hydroxyethyl) rapamycin or with the vehicle-control DMSO for an additional 24 hours. Cell extracts corresponding to 50 µg total protein are resolved on 8% SDS-PAGE electrophoresis and immunoblot analysis is performed using rabbit polyclonal antibodies raised against Poly (ADP-Ribose) Polymerase (PARP) and p53. In this assay, the presence of the mTOR inhibitor, e.g. 40-O-(2-hydroxyethyl) rapamycin, causes increased PARP cleavage (a marker of apoptosis) at suboptimal cisplatin concentrations (as compared to cisplatin or the mTOR inhibitor alone at the same concentrations). This confirms the above results that, in the p53 (*TP53*) wild-type A549 cells, the presence of the mTOR inhibitor results in higher levels of cell death at suboptimal cisplatin concentrations.

The p53 (*TP53*) status predicts sensitivity of e.g. a tumor in a subject to a combination of an mTOR inhibitor with a cytotoxic agent. p53 status can be assessed using DNA, RNA or protein obtained from tumor tissue as disclosed in order to predict likely responsiveness to a combination of an mTOR inhibitor with a cytotoxic agent.

Example 3

p53 (*TP53*) wild-type A549 and MCF7 cells are seeded at a density of $0.3x10^6$ and $0.4x10^5$ cells/4 ml per 6 cm plates, respectively, and incubated for 24 hours at 37°C and 5% CO_2. Cells are incubated with suboptimal concentrations of cisplatin (e.g. 0.5 to 4 µg/ml) either in combination with 20nM 40-O-(2-hydroxyethyl) rapamycin or with the vehicle-control DMSO for an additional 24 hours and 30 hours, respectively. Cell extracts corresponding to 30 µg total protein are resolved on 15% SDS-PAGE electrophoresis and immunoblot analysis is performed using mouse monoclonal antibodies raised against p21 (Oncogene Research Products, Clone EA10, catalogue #OP64). In both cell lines, cisplatin alone induces increased p21 protein expression in a concentration-dependent manner. Strikingly, the presence of the mTOR inhibitor, e.g. 40-O-(2-hydroxyethyl) rapamycin, attenuates cisplatin-induced upregulation of p21 protein expression. In contrast, Bax protein expression, a p53-

regulated pro-apoptotic protein, is unaffected by either agent alone or in combination. In this assay cytotoxic-induced p21 protein expression is inhibited by the presence of the mTOR inhibitor. This provides an explanation for the enhanced cell killing/apoptotic response observed with cisplatin and mTOR inhibitor combinations.

Example 4

p53 (*TP53*) wild-type A549 cells are seeded at a density of $0.1x10^6$ cells/5 ml per 6 cm plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are left untransfected or transiently transfected with 100 nM siRNA targeting either human p53 (Accession number: NM000546; target sequence: 5'-GCA TCT TAT CCG AGT GGA A-3') or LacZ (Accession number: M55068; target sequence: 5'-GCG GCT GCC GGA ATT TAC CTT-3') control siRNA, using Oligofectamine (Invitrogen, Cat # 12252-011). After 30 hours incubation, cells are incubated with increasing concentrations of cisplatin (e.g. 0.5 to 6 µg/ml) for an additional 24 hours. Cell extracts corresponding to 30 µg (p21) and 50 µg (p53 and PARP) total protein are resolved on 15 % (p21) and 10% (p53 and PARP) SDS-PAGE electrophoresis, and immunoblot analysis is performed using mouse monoclonal and rabbit polyclonal antibodies raised against p21 and p53 / PARP, respectively. Cisplatin treatment of untransfected or LacZ siRNA control transfected cells induces p53 and p21 protein expression in a concentration-dependent manner, with evidence of PARP cleavage (a marker of apoptosis) at higher cisplatin concentrations (2 to 6 µg/ml). Strikingly, attenuation of cisplatin-induced p53 protein expression occurs in the p53 siRNA transfected cells, which correlates with a dramatic attenuation of p21 expression, PARP cleavage and a loss of cell viability. The same effects on p53 expression, p21 expression and PARP cleavage are also observed with two other siRNA's targeting human p53 (target sequences: 5'-GGA AGA CTC CAG TGG TAA T-3' and 5'-GAT ATT GAA CAA TGG TTC A-3'). These data directly confirm that the enhanced cell killing/apoptotic response observed with cisplatin and mTOR inhibitor combinations are elicited through p53-dependent mechanisms.

Example 5

p53 (*TP53*) wild-type A549 cells are seeded at a density of $0.1x10^6$ cells/5 ml per 6 cm plates and incubated for 24 hours at 37°C and 5% CO_2. Cells are left untransfected or transiently transfected with 100 nM siRNA targeting either p21 (Accession number: NM000389; target sequence: 5'-GTG GAC AGC GAG CAG CTG A-3') or LacZ (as above) control siRNA, using Oligofectamine (Invitrogen, Cat # 12252-011). After 30 hours incubation, cells are incubated with suboptimal concentrations of cisplatin (e.g. 1 to 2 µg/ml) for an additional 24 hours. Cell extracts corresponding to 30 µg (p21) and 50 µg (PARP) total protein are resolved on 15 and

10% SDS-PAGE electrophoresis, respectively, and immunoblot analysis is performed using mouse monoclonal and rabbit polyclonal antibodies raised against p21 and PARP, respectively. Cisplatin treatment of untransfected or LacZ siRNA control transfected cells induces p21 protein expression in a concentration-dependent manner, with little evidence of PARP cleavage (a marker of apoptosis). Strikingly, attenuation of cisplatin-induced p21 protein expression occurs in the p21 siRNA transfected cells, which correlates with a dramatic induction of PARP cleavage. These data directly confirm that attenuation of cytotoxic-induced p21 protein expression is responsible for the enhanced cell killing/apoptotic response observed with cisplatin and mTOR inhibitor combinations.

CLAIMS

1. Use of the determination of p53 (*TP53*) status in a subject having a proliferative disease as a biomarker for determining the sensitivity of said subject to a treatment with an mTOR inhibitor in combination with a cytotoxic agent.

2. Use according to claim 1, comprising the use of p53 (*TP53*) gene analysis and the level of expression/post-translational modification of p53.

3. A method for determining the sensitivity of a proliferative disease in a subject to a combined treatment with an mTOR inhibitor and a cytotoxic agent, comprising determining the status of p53 (*TP53*) gene and/or the level of expression/post-translational modification of p53 in a sample derived from the subject.

4. A method or use according to any preceding claim, wherein the proliferative disease comprises a cancer.

5. A method according to any of claims 3 to 4, comprising determining the genetic status of p53 (*TP53*) and/or the level of expression of p53.

6. A method according to any of claims 3 to 5, wherein the sample is derived from a tumor in the subject.

7. A method of selecting subjects suffering from a proliferative disease for a combined treatment with an mTOR inhibitor and a cytotoxic agent, comprising determining the sensitivity of the proliferative disease to the combined treatment in each subject by a method as described in any of claims 3 to 6, and selecting those subjects showing wild-type p53 (*TP53*) status for the combined treatment.

8. A method or use according to any preceding claim, wherein the mTOR inhibitor comprises rapamycin or a rapamycin derivative.

9. A method or use according to claim 8, wherein the rapamycin derivative comprises 40-O-(2-hydroxyethyl) rapamycin, 40-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin or 40-epi-(tetrazolyl)-rapamycin.

10. A method or use according to any preceding claim, wherein the cytotoxic agent is selected from an antineoplastic antimetabolite, a platin compound, an alkylating agent, a topoisomerase I or II inhibitor, a microtubule active agent and irradiation.

11. Use of p21 as a biomarker for determining the sensitivity or response of a proliferative disease in a subject to treatment with an mTOR inhibitor in combination with a cytotoxic agent.

12. Use according to claim 11, comprising determining the level of p21 expression.

13. A method for determining the sensitivity or response of a proliferative disease in a subject to a treatment with an mTOR inhibitor in combination with a cytotoxic agent, comprising determining in a sample derived from the subject the level of p21 expression after treatment with the cytotoxic agent alone and after a combined treatment of the cytotoxic agent with an mTOR inhibitor.

14. A method for enhancing the activity of a cytotoxic agent or for overcoming resistance to a cytotoxic agent in a subject treated with said cytotoxic agent, comprising

- determining the level of p21 expression in a sample derived from the subject,
- if p21 expression is upregulated after administration of a cytotoxic agent, administering to said subject a therapeutically effective amount of an mTOR inhibitor in combination with the cytotoxic agent,
- determining again the level of p21 expression in a new sample derived from the subject after the treatment with the combination of the mTOR inhibitor and the cytotoxic agent, and
- if p21 expression is downregulated, further treating the subject with the mTOR inhibitor either concomitantly or sequentially with said cytotoxic agent.

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(54) Title: COMBINATIONS OF THERAPEUTIC AGENTS FOR TREATING CANCER

(57) Abstract: A combination therapy for treating patients suffering from proliferative diseases or diseases associated with persistent angiogenesis is disclosed. The patient is treated with a camptothecin derivative and one or more chemotherapeutic agents selected from a microtubule active agent; an alkylating agent; an anti-neoplastic anti-metabolite; a platin compound; a topoisomerase II inhibitor; a VEGF inhibitor; a tyrosine kinase inhibitor; an EGFR kinase inhibitor; an mTOR kinase inhibitor; an insulin-like growth factor I inhibitor; a Raf kinase inhibitor; a monoclonal antibody; a proteasome inhibitor; a HDAC inhibitor; and ionizing radiation.

WO 2006/065780 A2

COMBINATIONS OF THERAPEUTIC AGENTS FOR TREATING CANCER

The invention relates to a method of preventing or treating proliferative diseases or diseases that are associated with or triggered by persistent angiogenesis in a mammal, particularly a human, with a combination of pharmaceutical agents which comprises:

(a) a camptothecin derivative; and

(b) one or more chemotherapeutic agents.

The invention further relates to pharmaceutical compositions comprising:

(a) a camptothecin derivative;

(b) one or more chemotherapeutic agents; and

(c) a pharmaceutically acceptable carrier.

The present invention further relates to a commercial package or product comprising:

(a) a pharmaceutical formulation of a camptothecin derivative; and

(b) a pharmaceutical formulation of one or more chemotherapeutic agents for simultaneous, concurrent, separate or sequential use.

The combination partners (a) and (b) can be administered together, one after the other or separately in one combined unit dosage form or in two separate unit dosage forms. The unit dosage form may also be a fixed combination.

Background of the Invention

Camptothecin and derivatives thereof are cytotoxic agents, which exhibit antitumor activity primarily by inhibiting topoisomerase I, a clinically validated drug target which is usually overexpressed in malignant cells. Camptothecin and its derivatives act by interfering with the unwinding of supercoiled DNA by the cellular enzyme topoisomerase I which triggers events leading to apoptosis and programmed death in malignant cells.

Summary of the Invention

It has now been found that surprisingly camptothecin derivatives are even more efficacious when used in combination with other chemotherapeutic agents. There are both synergistic and additive advantages, both for efficacy and safety. Therapeutic effects of

combinations of chemotherapeutic agents with a camptothecin derivative can result in lower safe dosages ranges of each component in the combination.

The invention relates to a method of preventing or treating proliferative diseases or diseases that are associated with or triggered by persistent angiogenesis in a mammal, particularly a human, with a combination of pharmaceutical agents which comprises:

(a) a camptothecin derivative; and

(b) one or more chemotherapeutic agents.

The invention further relates to pharmaceutical compositions comprising:

(a) a camptothecin derivative;

(b) one or more chemotherapeutic agents; and

(c) a pharmaceutically acceptable carrier.

The present invention further relates to a commercial package or product comprising:

(a) a pharmaceutical formulation of a camptothecin derivative; and

(b) a pharmaceutical formulation of one or more chemotherapeutic agents for simultaneous, concurrent, separate or sequential use.

The Chemotherapeutic Agents

The term "chemotherapeutic agents" is a broad one covering many chemotherapeutic agents having different mechanisms of action. Combinations of some of these with camptothecin derivatives can result in improvements in cancer therapy. Generally, chemotherapeutic agents are classified according to the mechanism of action. Many of the available agents are anti-metabolites of development pathways of various tumors, or react with the DNA of the tumor cells.

By the term "chemotherapeutic agent" is meant especially any chemotherapeutic agent other than a topoisomerase I inhibitor or a derivative thereof. It includes, but is not limited to one or more of the following:

i. a microtubule active agent;

ii. an alkylating agent;

iii. an anti-neoplastic anti-metabolite;

iv. a platin compound;

v. topoisomerase II inhibitor;

vi. a compound targeting/decreasing a protein or lipid kinase activity or a protein or lipid phosphatase activity;
vii.monoclonal antibodies;
viii. proteasome inhibitors;
ix. HDAC inhibitors; and
x. tumor cell damaging approaches, such as ionizing radiation.

The term "microtubule active agent", as used herein, relates to microtubule stabilizing, microtubule destabilizing agents and microtublin polymerization inhibitors including, but not limited to, taxanes, e.g., paciltaxel and docetaxel; vinca alkaloids, e.g., vinblastine, especially vinblastine sulfate; vincristine, especially vincristine sulfate and vinorelbine; discodermolides; cochicine and epothilonesand derivatives thereof, e.g., epothilone B or a derivative thereof. Paclitaxel is marketed as TAXOL; docetaxel as TAXOTERE; vinblastine sulfate as VINBLASTIN R.P; and vincristine sulfate as FARMISTIN. Also included are the generic forms of paclitaxel, as well as various dosage forms of paclitaxel. Generic forms of paclitaxel include, but are not limited to, betaxolol hydrochloride. Various dosage forms of paclitaxel include, but are not limited to albumin nanoparticle paclitaxel marketed as ABRAXANE; ONXOL, CYTOTAX. Discodermolide can be obtained, e.g., as disclosed in U.S. Patent No. 5,010,099. Also included are Epotholine derivatives which are disclosed in U.S. Patent No. 6,194,181, WO 98/10121, WO 98/25929, WO 98/08849, WO 99/43653, WO 98/22461 and WO 00/31247. Especially preferred are epotholine A and/or B.

The term "alkylating agent", as used herein, includes, but is not limited to, cyclophosphamide, ifosfamide, melphalan or nitrosourea (BCNU or Gliadel), or temozolamide (TEMODAR). Cyclophosphamide can be administered, e.g., in the form as it is marketed, e.g., under the trademark CYCLOSTIN; and ifosfamide as HOLOXAN.

The term "anti-neoplastic anti-metabolite" includes, but is not limited to, 5-fluorouracil (5-FU); capecitabine; gemcitabine; DNA de-methylating agents, such as 5-azacytidine and decitabine; methotrexate; edatrexate; and folic acid antagonists, such as, but not limited to, pemetrexed. Capecitabine can be administered, e.g., in the form as it is marketed, e.g., under the trademark XELODA; and gemcitabine as GEMZAR.

The term "platin compound", as used herein, includes, but is not limited to, carboplatin, cisplatin, cisplatinum, oxaliplatin, satraplatin and platinum agents, such as ZD0473. Carboplatin can be administered, e.g., in the form as it is marketed, e.g., CARBOPLAT; and oxaliplatin as ELOXATIN.

The term "topoisomerase II inhibitor", as used herein, includes, but is not limited to, the anthracyclines, such as doxorubicin, including liposomal formulation, e.g., CAELYX; daunorubicin, including liposomal formulation, e.g., DAUNOSOME; epirubicin; idarubicin and nemorubicin; the anthraquinones mitoxantrone and losoxantrone; and the podophillotoxines etoposide and teniposide. Etoposide is marketed as ETOPOPHOS; teniposide as VM 26-BRISTOL; doxorubicin as ADRIBLASTIN or ADRIAMYCIN; epirubicin as FARMORUBICIN; idarubicin as ZAVEDOS; and mitoxantrone as NOVANTRON.

The term "compounds targeting/decreasing a protein or lipid kinase activity; or a protein or lipid phosphatase activity; or further anti-angiogenic compounds", as used herein, includes, but is not limited to, protein tyrosine kinase and/or serine and/or theroine kinase inhibitors or lipid kinase inhibitors, e.g.,

i. compounds targeting, decreasing or inhibiting the activity of the vascular endothelial growth factor (VEGF) receptors, such as compounds which target, decrease or inhibit the activity of VEGF, especially compounds which inhibit the VEGF receptor, such as, but not limited to, 7*H*-pyrrolo[2,3-*d*]pyrimidine derivative; BAY 43-9006; isolcholine compounds disclosed in WO 00/09495, such as (4-*tert*-butyl-phenyl)-94-pyridin-4-ylmethyl-isoquinolin-1-yl)-amine;

ii. compounds targeting, decreasing or inhibiting the activity of the platelet-derived growth factor (PDGF) receptors, such as compounds which target, decrease or inhibit the activity of PDGF receptors, especially compounds which inhibit the PDGF receptor, e.g., a *N*-phenyl-2-pyrimidine-amine derivative, e.g., imatinib, SU101, SU6668 and GFB-111;

iii. compounds targeting, decreasing or inhibiting the activity of the fibroblast growth factor (FGF) receptors;

iv. compounds targeting, decreasing or inhibiting the activity of the insulin-like growth factor receptor 1 (IGF-1R), such as compounds which target, decrease or inhibit the activity of IGF-IR, especially compounds which inhibit the IGF-1R receptor. Compounds include, but are not limited to, the compounds disclosed in WO 02/092599 and derivatives thereof of 4-amino-5-phenyl-7-cyclobutyl-pyrrolo[2,3-*d*]pyrimidine derivatives;

v. compounds targeting, decreasing or inhibiting the activity of the Trk receptor tyrosine kinase family;

vi. compounds targeting, decreasing or inhibiting the activity of the Axl receptor tyrosine kinase family;

vii. compounds targeting, decreasing or inhibiting the activity of the c-Met receptor;

viii. compounds targeting, decreasing or inhibiting the activity of the Ret receptor tyrosine kinase;

ix. compounds targeting, decreasing or inhibiting the activity of the Kit/SCFR receptor tyrosine kinase;

x. compounds targeting, decreasing or inhibiting the activity of the C-kit receptor tyrosine kinases (part of the PDGFR family), such as compounds which target, decrease or inhibit the activity of the c-Kit receptor tyrosine kinase family, especially compounds which inhibit the c-Kit receptor, e.g., imatinib;

xi. compounds targeting, decreasing or inhibiting the activity of members of the c-Abl family and their gene-fusion products, e.g., BCR-Abl kinase, such as compounds which target decrease or inhibit the activity of c-Abl family members and their gene fusion products, e.g., a *N*-phenyl-2-pyrimidine-amine derivative, e.g., imatinib, PD180970, AG957, NSC 680410 or PD173955 from ParkeDavis; or BMS354825;

xii. compounds targeting, decreasing or inhibiting the activity of members of the protein kinase C (PKC) and Raf family of serine/threonine kinases, members of the MEK, SRC, JAK, FAK, PDK and Ras/MAPK family members, or PI(3) kinase family, or of the PI(3)-kinase-related kinase family, and/or members of the cyclin-dependent kinase family (CDK) and are especially those staurosporine derivatives disclosed in U.S. Patent No. 5,093,330, e.g., midostaurin; examples of further compounds include, e.g., UCN-01; safingol; BAY 43-9006; Bryostatin 1; Perifosine; Ilmofosine; RO 318220 and RO 320432; GO 6976; Isis 3521; LY333531/LY379196; isochinoline compounds, such as those disclosed in WO 00/09495; FTIs; PD184352 or QAN697, a P13K inhibitor;

xiii. compounds targeting, decreasing or inhibiting the activity of protein-tyrosine kinase, such as imatinib mesylate (GLEEVEC); tyrphostin or pyrymidylaminobenzamide and derivatives thereof. A tyrphostin is preferably a low molecular weight (Mr <1500) compound, or a pharmaceutically acceptable salt thereof, especially a compound selected from the benzylidenemalonitrile class or the *S*-arylbenzenemalonirile or bisubstrate quinoline class of compounds, more especially any compound selected from the group consisting of Tyrphostin A23/RG-50810, AG 99, Tyrphostin AG 213, Tyrphostin AG 1748, Tyrphostin AG 490, Tyrphostin B44, Tyrphostin B44 (+) enantiomer, Tyrphostin AG 555, AG 494, Tyrphostin AG 556; AG957; and adaphostin (4-{[(2,5-dihydroxyphenyl)methyl]amino}-benzoic acid adamantyl ester; NSC 680410, adaphostin);

xiv. compounds targeting, decreasing or inhibiting the activity of the epidermal growth factor family of receptor tyrosine kinases (EGFR, ErbB2, ErbB3, ErbB4 as homo- or heterodimers), such as compounds which target, decrease or inhibit the activity of the epidermal growth factor receptor family are especially compounds, proteins or antibodies which inhibit members of the EGF receptor tyrosine kinase family, e.g., EGF receptor, ErbB2, ErbB3 and ErbB4 or bind to EGF or EGF-related ligands, and are in particular those compounds, proteins or monoclonal antibodies generically and specifically disclosed in WO 97/02266, e.g., the compound of Example 39, or in EP 0 564 409, WO 99/03854, EP 0520722, EP 0 566 226, EP 0 787 722, EP 0 837 063, U.S. Patent No. 5,747,498, WO 98/10767, WO 97/30034, WO 97/49688, WO 97/38983 and, especially, WO 96/30347, e.g., compound known as CP 358774, WO 96/33980, e.g., compound ZD 1839; and WO 95/03283, e.g., compound ZM105180, e.g., trastuzumab (HERCEPTIN), cetuximab, Iressa, OSI-774, CI-1033, EKB-569, GW-2016, E1.1, E2.4, E2.5, E6.2, E6.4, E2.11, E6.3 or E7.6.3, and {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine, erlotinib and gefitinib. Erlotinib can be administered in the form as it is marketed, e.g., TARCEVA, and gefitinib as IRESSA, human monoclonal antibodies against the epidermal growth factor receptor including ABX-EGFR; and

xv. compounds which target, decrease or inhibit the activity/function of serine/theronine mTOR kinase are especially compounds, proteins or antibodies which target/inhibit members of the mTOR kinase family, e.g., RAD, RAD001, CCI-779, ABT578, SAR543, rapamycin and derivatives/analogs thereof, AP23573 and AP23841 from Ariad, everolimus (CERTICAN) and sirolimus. CERTICAN (everolimus, RAD) an investigational novel proliferation signal inhibitor that prevents proliferation of T-cells and vascular smooth muscle cells.

The term "monoclonal antibodies", as used herein, includes, but is not limited to bevacizumab, cetuximab, trastuzumab, Ibritumomab tiuxetan, and tositumomab and iodine I 131. Bevacizumab can be administered in the form as it is marketed, e.g., AVASTIN; cetuximab as ERBITUX; trastuzumab as HERCEPTIN; rituximab as MABTHERA; ibritumomab tiuxetan as ZEVULIN; and tositumomab and iodine I 131 as BEXXAR.

The term "proteasome inhibitors", as used herein, includes compounds which target, decrease or inhibit the activity of the proteosome. Compounds which target, decrease or inhibit the activity of the proteosome include, but are not limited to, PS-341; MLN 341, bortezomib or velcade.

The term "HDAC inhibitor", as used herein, relates to relates to compounds which inhibit the histone deacetylase and which possess anti-proliferative activity. This includes but is not limited to compounds disclosed in WO 02/22577, especially *N*-hydroxy-3-[4-[[(2-hydroxyethyl)[2-(1*H*-indol-3-yl)ethyl]-amino]methyl]phenyl]-2*E*-2-propenamide; and *N*-hydroxy-3-[4-[[[2-(2-methyl-1*H*-indol-3-yl)-ethyl]-amino]methyl]phenyl]-2*E*-2-propenamide; and pharmaceutically acceptable salts thereof. It further especially includes suberoylanilide hydroxamic acid (SAHA); [4-(2-amino-phenylcarbamoyl)-benzyl]-carbamic acid pyridine-3-ylmethyl ester and derivatives thereof; butyric acid, pyroxamide, trichostatin A, oxamflatin, apicidin, depsipeptide, depudecin and trapoxin.

"Tumor cell damaging approaches" refers to approaches, such as ionizing radiation. The term "ionizing radiation", referred to above and hereinafter, means ionizing radiation that occurs as either electromagnetic rays, such as X-rays and gamma rays; or particles, such as alpha, beta and gamma particles. Ionizing radiation is provided in, but not limited to, radiation therapy and is known in the art. See Hellman, Cancer, 4th Edition, Vol. 1, Devita et al., Eds., pp. 248-275 (1993).

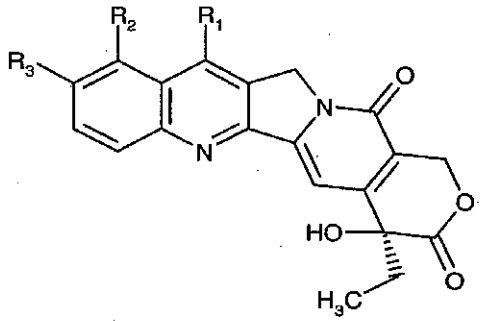
In each case where citations of patent applications or scientific publications are given, in particular with regard to the respective compound claims and the final products of the working examples therein, the subject matter of the final products, the pharmaceutical preparations and the claims is hereby incorporated into the present application by reference to these publications. Comprised are likewise the corresponding stereoisomers, as well as the corresponding crystal modifications, e.g., solvates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations disclosed herein can be prepared and administered as described in the cited documents, respectively.

The structure of the active agents identified by code numbers, generic or trade names may be taken from the actual edition of the standard compendium "The Merck Index" or from databases, e.g., Patents International, e.g., IMS World Publications, or the publications mentioned above and below. The corresponding content thereof is hereby incorporated by reference.

It will be understood that references to the components (a) and (b) are meant to also include the pharmaceutically acceptable salts of any of the active substances. If active substances comprised by components (a) and/or (b) have, e.g., at least one basic center, they can form acid addition salts. Corresponding acid addition salts can also be formed having, if desired, an additionally present basic center. Active substances having an acid group, e.g.,

COOH, can form salts with bases. The active substances comprised in components (a) and/or (b) or a pharmaceutically acceptable salts thereof may also be used in form of a hydrate or include other solvents used for crystallization. 7-*t*-Butoxyiminomethyl-camptothecin is the most preferred combination partner (a).

The camptothecin derivatives for use in the present invention include those disclosed in U.S. Patent No. 6,242,457, incorporated herein by reference, and have the following formula (I):



(I)

wherein

R_1 is a -C(R_5)=N-O(*n*)R_4 group,

wherein

R_4 is hydrogen or a C_1-C_8 linear or branched alkyl or C_1-C_8 linear or branched alkenyl group or C_3-C_{10} cycloalkyl, or C_3-C_{10} cycloalkylC_1-C_8 linear or branched alkyl group, or C_6-C_{14} aryl, or C_6-C_{14} aryl C_1-C_8 linear or branched alkyl group, or a heterocyclic or heterocyclo C_1-C_8 linear or branched alkyl group, said heterocyclic group containing at least a heteroatom selected from the group consisting of nitrogen atom, optionally substituted with a C_1-C_8 alkyl group, and/or oxygen and/or sulfur; said alkyl, alkenyl, cycloalkyl, cycloalkylalkyl, aryl, aryl-alkyl, heterocyclic or heterocyclo alkyl groups, being optionally substituted with one or more groups selected from the group consisting of: halogen, hydroxyl, keto, C_1-C_8 alkyl, C_1-C_8 alkoxy, phenyl, cyano, nitro, -NR_6R_7, wherein R_6 and R_7, the same or different between them, are hydrogen, C_1-C_8 linear or branched alkyl; the -COOH group or a pharmaceutically acceptable ester thereof, or the -$CONR_8R_9$ group, wherein R_8 and R_9, the same or different between them, are hydrogen, C_1-C_8 linear or branched alkyl; phenyl, or

R_4 is a C_6-C_{10} aroyl or C_6-C_{10} arylsulfonyl group, optionally substituted with one or more groups selected from the group consisting of: halogen, hydroxy, C_1-C_8 linear or branched alkyl, C_1-C_8 linear or branched alkoxy, phenyl, cyano, nitro, -$NR_{10}R_{11}$, wherein R_{10} and R_{11}, the same or different between them are hydrogen, C_1-C_8 linear or branched alkyl, or

R_4 is a polyaminoalkyl group;

n is the number 1;

R_5 is hydrogen, C_1-C_8 linear or branched alkyl, C_1-C_8 linear or branched alkenyl, C_3-C_{10} cycloalkyl, C_3-C_{10} cycloalkyl C_1-C_8 linear or branched alkyl, C_6-C_{14} aryl, C_6-C_{14} aryl C_1-C_8 linear or branched alkyl;

R_2 and R_3, the same or different between them are hydrogen, hydroxy, C_1-C_8 linear or branched alkoxy; and

their N_1-oxides, their single isomers, their possible enantiomers, diastereoisomers and relative admixtures, the pharmaceutically acceptable salts thereof and their active metabolites; with the proviso that when R_5, R_2 and R_3 are hydrogen, then R_4 is different from hydrogen.

Within the scope of the present invention, as examples of C_1-C_8 linear or branched alkyl group, methyl, ethyl, propyl, butyl, pentyl and acetyl are meant and their possible isomers, such as, e.g., isopropyl, isobutyl or *tert*-butyl.

Examples of C_1-C_8 linear or branched alkenyl group are methylene, ethylidene, vinyl, allyl, proparyl, butylenes, pentylene, wherein the carbon-carbon double bond, optionally in the presence of other carbon-carbon unsaturations, can be situation in the different possible positions of the alkyl chain, which can also be branched with the allowed isomery.

Examples of C_3-C_{10} cycloalkyl group are cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloctyl and polycyclic groups, such as, e.g., adamantyl.

Examples of C_3-C_{10} cycloalkyl C_1-C_8 linear or branched alkyl group are cyclopropylmethyl, 2-cyclopropylethyl, 1-cyclopropylethyl, 3-cyclopropylpropyl, 2-cyclopropylpropyl, 1-cyclopropylpropyl, cyclobutylmethyl, 2-cyclobutylethyl, 1-cyclobutylethyl, 3-cyclobutylpropyl, 2-cyclobutylpropyl, 1-cyclobutylpropyl, cyclohexylmethyl, 2-cyclohexylethyl, 1-cyclohenxylethyl, 3-cyclohexylpropyl, 2- cyclohexylpropyl, 1- cyclohexylpropyl, 5-cyclohexylpentyl, 3-cyclohexylpentyl, 3-methyl-2-cyclohexylbutyl, 1-adamantylethyl, 2-adamantylethyl and adamantylmethyl.

Examples of C_6-C_{14} aryl, or C_6-C_{14} aryl C_1-C_8 linear or branched alkyl group arephenyl, 1- or 2-naphthyl, anthryl, benzyl, 2-phenylethyl, 1-phenylethyl, 3-phenylpropyl, 2-anthrylpropyl, 1-anthrylpropyl, naphthylmethyl, 2-naphthylethyl, 1- naphthylethyl, 3-napthylpropyl, 2-napthylpropyl, 1-napthylpropyl, cyclohexylmethyl, 5-phenylpentyl, 3-phenylpentyl and 2-phenyl-3-methylbutyl.

Examples of heterocyclic- or heterocyclo C_1-C_8 linear or branched alkyl group are thienyl, quinolyl, pyridyl, *N*-methylpyperidinyl, 5-tetrazolyl, 2-(4,5-dihydroxazolyl)1,2,4-oxadiazolidin-3-yl-5-one, purine and pyrimidine bases, e.g., uracyl, optionally substituted as shown in the general definitions above mentioned.

Examples of C_6-C_{10} aroyl groups are benzoyl and naphthoyl.

Examples of C_6-C_{10} arylsulfonyl groups, optionally substituted with an alkyl group, are tosyl and benzenesulfonyl. As halogen, it is intended fluorine, chlorine, bromine and iodine.

Examples of substituted groups are pentafluorophenyl, 4-phenylbenzyl, 2,4-difluorobenzyl, 4-aminobutyl, 4-hydroxybutyl, dimethylaminoethyl and p-nitrobenzoyl, p-cyanobenzoyl.

Examples of polyaminoalkyl group is $-(CH_2)_m-NR_{12}-(CH_2)_p-NR_{13}-(CH_2)_q-NH_2$, wherein

m and p are an integer from 2-6;

q is an integer from 0-6, extremes included; and

R_{12} and R_{13} are a C_1-C_8 linear or branched alkyl group, e.g., *N*-(4-aminobutyl)2-aminoethyl, *N*-(3-aminopropyl)-4-aminobutyl and *N*-[*N*-(3-aminopropyl)-*N*-(4-aminobutyl)]-3-aminopropyl.

Examples of glycosyl groups are 6-*D*-galactosyl, 6-*D*-glucosyl, *D*-galactopyranosyl, the glycosyl group being optionally protected with a suitable ketal group, isopropylidene, for instance.

Examples of pharmaceutically acceptable salts are, in case of nitrogen atoms having basic character, the salts with pharmaceutically acceptable acids, both inorganic and organic, such as, e.g., hydrochloric acid, sulfuric acid and acetic acid; or in the case of acid group, such as carboxyl; the salts with pharmaceutically acceptable bases, both inorganic and organic, such as, e.g., alkaline and alkaline-earth hydroxides; ammonium hydroxide; amine; and also heterocyclic ones.

R_1 is preferably $-C(R_5)=N-O(_n)R_4$, wherein R_4 is preferably a C_1-C_8 linear or branched alkyl; and

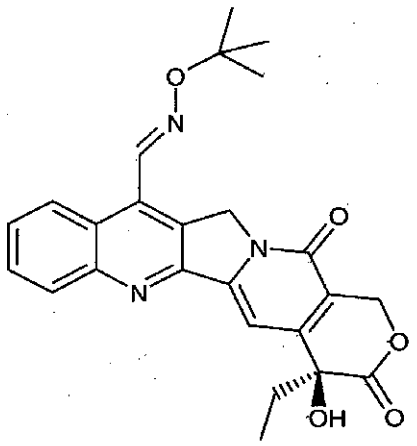
R_2 and R_3 are preferably hydrogen.

High preference is given to a compound selected from the group consisting of: 7-methyoxyiminomethylcamptothecin;

7-methoxyiminomethyl-10-hydroxycamptothecin;
7-(*tert*-butoxycarbonyl-2-propoxy)iminomethylcamptothecin;
7-ethoxyiminomethylcamptothecin;
7-isopropoxyiminomethylcamptothecin;
7-(2-methylbutoxy)iminomethylcamptothecin;
7-*t*-butoxyiminomethylcamptothecin;
7-*t*-butoxyiminomethyl-10-hydroxycamptothecin;
7-*t*-butoxyiminomethyl-10-methoxycamptothecin;
7-(4-hydroxybutoxy)iminomethylcamptothecin;
7-triphenylmethoxyiminomethylcamptothecin;
7-carboxymethoxyiminomethylcamptothecin;
7-(2-amino)ethoxyiminomethylcamptothecin;
7-(2-*N,N*-dimethylamino)ethoxyiminomethylcamptothecin;
7-allyloxyiminomethylcamptothecin;
7-cyclohexyloxyiminoethylcamptothecin;
7-cyclohexylmethoxyiminomethylcamptothecin;
7-cyclooctyloxyiminomethylcamptothecin;
7-cyclooctylmethoxyiminomethylcamptothecin;
7-benzyloxyiminomethylcamptothecin;
7-[(1-benzyloxyimino)-2-phenylethyl] camptothecin;
7-(1-benzyloxyimino)ethylcamptothecin;
7-phenoxyiminomethylcamptothecin;
7-(1-*t*-butoxyimino)ethylcamptothecin;
7-*p*-nitrobenzyloxyiminomethylcamptothecin;
7-*p*-methylbenzyloxyiminomethylcamptothecin;
7-pentafluorobenzyloxyiminomethylcamptothecin;
7-*p*-phenylbenzyloxyiminomethylcamptothecin;
7-[2-(2,4-difluorophenyl)ethoxy]iminomethylcamptothecin;
7-(4-*t*-butylbenzyloxy)iminomethylcamptothecin;
7-(1-adamantyloxy)iminomethylcamptothecin;
7-(1-adamantylmethoxy)iminomethylcamptothecin;
7-(2-naphthyloxy)iminomethylcamptothecin;
7-(9-anthrylmethoxy)iminomethylcamptothecin;
7-oxiranylmethoxyiminomethylcamptothecin;
7-(6-uracyl)methoxyiminomethylcamptothecin;
7-[2-(1-urcyl)ethoxy]iminomethylcamptothecin;

7-(4-pyridyl)methoxyiminomethylcamptothecin;
7-(2-thienyl)methoxyiminomethylcamptothecin;
7-[(*N*-methyl)-4-piperidinyl]methoxyiminomethylcamptothecin;
7-[2-(4-morpholininyl]ethoxy]iminomethylcamptothecin;
7-(benzoyloxyiminomethyl)camptothecin;
7-[(1-hydroxyimino)-2-phenylethyl)camptothecin;
7-*tert*-butyloxyiminomethylcamptothecin-*N*-oxide; and
7-methoxyiminomethylcamptothecin-*N*-oxide.

In a very preferred embodiment of the invention, the camptothecin derivative of formula (I) has the following structure:



Camptothecin derivatives of formula (I) and their preparation are disclosed in U.S. Patent No. 6,242,457, which is incorporated herein in its entirety.

The Combinations

Thus, in a first aspect, the present invention relates to a method for the prevention of treatment of proliferative diseases or diseases that are triggered by persistent angiogenesis in a mammal, preferably a human patient, which comprises treating the patient concurrently or sequentially with pharmaceutically effective amounts of a combination of:

(a) a camptothecin derivative, preferably of formula (I); and

(b) one or more chemotherapeutic agents.

In another aspect, the present invention relates to a pharmaceutical composition comprising a combination of:

(a) a camptothecin derivative, preferably of formula (I); and

(b) one or more chemotherapeutic agents.

In a yet further aspect, the present invention provides a pharmaceutical preparation comprising:

(a) a camptothecin derivative of formula (I); and

(b) one or more chemotherapeutic agents, together with a pharmaceutically acceptable carrier.

In preferred embodiment, the present invention provides a pharmaceutical preparation comprising:

(a) a camptothecin derivative of formula (I); and

(b) one or more chemotherapeutic agents selected from a microtubule active agent; an alkylating agent; an anti-neoplastic anti-metabolite; a platin compound; a topoisomerase II inhibitor; a VEGF inhibitor; a tyrosine kinase inhibitor; an EGFR kinase inhibitor; an mTOR kinase inhibitor; an insulin-like growth factor I inhibitor; a Raf kinase inhibitor; a monoclonal antibody; a proteasome inhibitor; a HDAC inhibitor; and ionizing radiation.

In another preferred embodiment, the present invention provides a pharmaceutical preparation comprising:

(a) a camptothecin derivative of formula (I); and

(b) one or more chemotherapeutic agents selected from paclitaxel; docetaxel; epothilone B; temozolamide; 5-FU; gemcitabine; oxaliplatin; cisplatinum; carboplatin; doxorubicin; {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine; everolimus; imatinib; erlotinib, bevacizumab, cetuximab, and velcade;

Any of the combination of components (a) and (b), the method of treating a warm-blooded animal comprising administering these two components, a pharmaceutical composition comprising these two components for simultaneous, separate or sequential use, the use of the combination for the delay of progression or the treatment of a proliferative disease or for the manufacture of a pharmaceutical preparation for these purposes or a commercial product comprising such a combination of components (a) and (b), all as mentioned or defined above, will be referred to subsequently also as COMBINATION OF THE INVENTION (so that this term refers to each of these embodiments which thus can replace this term where appropriate).

Simultaneous administration may, e.g., take place in the form of one fixed combination with two or more active ingredients, or by simultaneously administering two or more active ingredients that are formulated independently. Sequential use (administration) preferably

means administration of one (or more) components of a combination at one time point, other components at a different time point, that is, in a chronically staggered manner, preferably such that the combination shows more efficiency than the single compounds administered independently (especially showing synergism). Separate use (administration) preferably means administration of the components of the combination independently of each other at different time points.

Also combinations of two or more of sequential, separate and simultaneous administration are possible, preferably such that the combination component-drugs show a joint therapeutic effect that exceeds the effect found when the combination component-drugs are used independently at time intervals so large that no mutual effect on their therapeutic efficiency can be found, a synergistic effect being especially preferred.

The term "delay of progression", as used herein, means administration of the combination to patients being in a pre-stage or in an early phase, of the first or subsequent manifestations; or a relapse of the disease to be treated in which patients, e.g., a pre-form of the corresponding disease is diagnosed; or which patients are in a condition, e.g., during a medical treatment or a condition resulting from an accident, under which it is likely that a corresponding disease will develop.

"Jointly therapeutically active" or "joint therapeutic effect" means that the compounds may be given separately (in a chronically staggered manner, especially a sequence-specific manner) in such time intervals that they preferably, in the warm-blooded animal, especially human, to be treated, still show a (preferably synergistic) interaction (joint therapeutic effect).

"Pharmaceutically effective" preferably relates to an amount that is therapeutically or in a broader sense also prophylactically effective against the progression of a proliferative disease.

The term "a commercial package" or "a product", as used herein defines especially a "kit of parts" in the sense that the components (a), which is the camptothecin derivative and (b), which includes one or more chemotherapeutic agents, as defined above, can be dosed independently or by use of different fixed combinations with distinguished amounts of the components (a) and (b), i.e., simultaneously or at different time points. Moreover, these terms comprise a commercial package comprising (especially combining) as active ingredients components (a) and (b), together with instructions for simultaneous, sequential (chronically staggered, in time-specific sequence, preferentially) or (less preferably) separate use thereof in the delay of progression or treatment of a proliferative disease. The parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time

points and with equal or different time intervals for any part of the kit of parts. Very preferably, the time intervals are chosen such that the effect on the treated disease in the combined use of the parts is larger than the effect which would be obtained by use of only any one of the combination partners (a) and (b) as can be determined according to standard methods. The ratio of the total amounts of the combination partner (a) to the combination partner (b) to be administered in the combined preparation can be varied, e.g., in order to cope with the needs of a patient sub-population to be treated or the needs of the single patient which different needs can be due to the particular disease, age, sex, body weight, etc. of the patients. Preferably, there is at least one beneficial effect, e.g., a mutual enhancing of the effect of the combination partners (a) and (b), in particular, a more than additive effect, which hence could be achieved with lower doses of each of the combined drugs, respectively, than tolerable in the case of treatment with the individual drugs only without combination, producing additional advantageous effects, e.g., less side effects or a combined therapeutic effect in a non-effective dosage of one or both of the combination partners (components) (a) and (b), and very preferably a strong synergism of the combination partners (a) and (b).

Both in the case of the use of the combination of components (a) and (b) and of the commercial package, any combination of simultaneous, sequential and separate use is also possible, meaning that the components (a) and (b) may be administered at one time point simultaneously, followed by administration of only one component with lower host toxicity either chronically, e.g., more than 3-4 weeks of daily dosing, at a later time point and subsequently the other component or the combination of both components at a still later time point (in subsequent drug combination treatment courses for an optimal anti-tumor effect) or the like.

The COMBINATION OF THE INVENTION can also be applied in combination with other treatments, e.g., surgical intervention, hyperthermia and/or irradiation therapy.

The pharmaceutical compositions according to the present invention can be prepared by conventional means and are those suitable for enteral, such as oral or rectal, and parenteral administration to mammals including man, comprising a therapeutically effective amount of a camptothecin derivative and at least one chemotherapeutic agent alone or in combination with one or more pharmaceutically acceptable carriers, especially those suitable for enteral or parenteral application.

The pharmaceutical compositions comprise from about 0.00002% to about 100%, especially, e.g., in the case of infusion dilutions that are ready for use) of 0.0001-0.02%, or, e.g., in case of injection or infusion concentrates or especially parenteral formulations, from about

0.1% to about 95%, preferably from about 1% to about 90%, more preferably from about 20% to about 60%, active ingredient (weight by weight, in each case). Pharmaceutical compositions according to the invention may be, e.g., in unit dose form, such as in the form of ampoules, vials, dragées, tablets, infusion bags or capsules.

The effective dosage of each of the combination partners employed in a formulation of the present invention may vary depending on the particular compound or pharmaceutical compositions employed, the mode of administration, the condition being treated and the severity of the condition being treated. A physician, clinician or veterinarian of ordinary skill can readily determine the effective amount of each of the active ingredients necessary to prevent, treat or inhibit the progress of the condition.

In the instance where the chemotherapeutic agent is selected from the group consisting of doxorubicin, paclitaxel, docetaxel, epothilones and derivatives thereof, temozolamide, 5-FU; gemcitabine, oxaliplatin, carboplatin, 7*H*-pyrrol-[2,3-*d*]pyrimidine derivatives, isochinoline compounds, RAD001, GLEEVEC, erlotinib, bevacizumab, cetuximab, velcade, *N*-hydroxy-3-[4-[[(2-hydroxy-ethyl)[2-(1*H*-indol-3-yl)ethyl]-amino]methyl]phenyl]-2*E*-2-propenamide and 4-amino-5-phenyl-7-cyclobutyl-pyrrolo[2,3-*d*]pyrimidine derivatives pharmaceutically acceptable salts or solvates thereof; and pharmaceutically acceptable prodrug esters thereof; and the patient to be treated is a human, an appropriate dose of, e.g., 5-FU is administered at an appropriate dose in the range from 100-1500 mg daily, e.g., 200-1000 mg/day, such as 200, 400, 500, 600, 800, 900 or 1000 mg/day, administered in one or two doses daily. 5-FU may be administered to a human in a dosage range varying from about 50-1000 mg/m^2/day, e.g., 500 mg/m^2/day. Among the topoisomerase II inhibitors, DOXORUBICIN may be administered to a human in a dosage range varying from about 10-100 mg/m^2/day, e.g., 25 or 75 mg/m^2/day, e.g., as single dose. PACLITAXEL may be administered to a human in a dosage range varying from about 50-300 mg/m^{2}day. DOCETAXEL may be administered to a human in a dosage range varying from about 25-100 mg/m^2/day. CARBOPLATIN may be administered to a human in a dosage range varying from about 200-400 mg/m^2 about every four weeks. OXALIPLATIN may be administered to a human in a dosage range varying from about 50-85 mg/m^2 every two weeks.

Pharmaceutical preparations for the combination therapy for enteral or parenteral administration are, e.g., those in unit dosage forms, such as sugar-coated tablets, capsules or suppositories; and furthermore ampoules. If not indicated otherwise, these formulations are prepared by conventional means, e.g., by means of conventional mixing, granulating, sugar-coating, dissolving or lyophilizing processes. It will be appreciated that the unit content of a combination partner contained in an individual dose of each dosage form need not in itself

constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units. One of skill in the art has the ability to determine appropriate pharmaceutically effective amounts of the combination components.

Preferably, the compounds or the pharmaceutically acceptable salts thereof, are administered as an oral pharmaceutical formulation in the form of a tablet, capsule or syrup; or as parenteral injections if appropriate.

In preparing compositions for oral administration, any pharmaceutically acceptable media may be employed, such as water, glycols, oils, alcohols, flavoring agents, preservatives or coloring agents. Pharmaceutically acceptable carriers include starches, sugars, microcrystalline celluloses, diluents, granulating agents, lubricants, binders and disintegrating agents.

Solutions of the active ingredient, and also suspensions, and especially isotonic aqueous solutions or suspensions, are useful for parenteral administration of the active ingredient, it being possible, e.g., in the case of lyophilized compositions that comprise the active ingredient alone or together with a pharmaceutically acceptable carrier, e.g., mannitol, for such solutions or suspensions to be produced prior to use. The pharmaceutical compositions may be sterilized and/or may comprise excipients, e.g., preservatives, stabilizers, wetting and/or emulsifying agents, solubilizers, salts for regulating the osmotic pressure and/or buffers, and are prepared in a manner known *per se*, e.g., by means of conventional dissolving or lyophilizing processes. The solutions or suspensions may comprise viscosity-increasing substances, such as sodium carboxymethylcellulose, carboxymethylcellulose, dextran, polyvinylpyrrolidone or gelatin. Suspensions in oil comprise as the oil component the vegetable, synthetic or semi-synthetic oils customary for injection purposes.

The isotonic agent may be selected from any of those known in the art, e.g., mannitol, dextrose, glucose and sodium chloride. The infusion formulation may be diluted with the aqueous medium. The amount of aqueous medium employed as a diluent is chosen according to the desired concentration of active ingredient in the infusion solution. Infusion solutions may contain other excipients commonly employed in formulations to be administered intravenously, such as antioxidants.

The present invention further relates to "a combined preparation", which, as used herein, defines especially a "kit of parts" in the sense that the combination partners (a) and (b) as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners (a) and (b), i.e., simultaneously or at different

time points. The parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partner (a) to the combination partner (b) to be administered in the combined preparation can be varied, e.g., in order to cope with the needs of a patient sub-population to be treated or the needs of the single patient based on the severity of any side effects that the patient experiences.

The present invention especially relates to a combined preparation which comprises:

(a) one or more unit dosage forms of a camptothecin derivative; and

(b) one or more unit dosage forms of an chemotherapeutic agent.

The Diseases to be Treated

The compositions of the present invention are useful for treating proliferative diseases or diseases that are associated with or triggered by persistent angiogenesis.

A proliferative disease is mainly a tumor disease (or cancer) (and/or any metastases). The inventive compositions are particularly useful for treating a tumor which is a breast cancer; lung cancer, including non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC); gastrointestinal cancer, including esophageal, gastric, small bowel, large bowel, rectal and colon cancer; glioma, including glioblastoma; sarcoma, such as those involving bone, cartilage, soft tissue, muscle, blood and lymph vessels; ovarian cancer; myeloma; female cervical cancer; endometrial cancer; head and neck cancer; mesothelioma; renal cancer; uteran; bladder and urethral cancers; leukemia; prostate cancer; skin cancers; and melanoma. In particular, the inventive compositions are particularly useful for treating:

i. a breast tumor; a lung tumor, e.g., non-small cell lung tumor, including non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC); a gastrointestinal tumor, e.g., a colorectal tumor; or a genitourinary tumor, e.g., a prostate tumor; ovarian cancer; glioma, including glioblastoma;

ii. a proliferative disease that is refractory to the treatment with other chemotherapeutics; or

iii. a tumor that is refractory to treatment with other chemotherapeutics due to multidrug resistance.

In a broader sense of the invention, a proliferative disease may furthermore be a hyperproliferative condition, such as a leukemia, lymphoma or multiple myeloma.

The combination of the present invention can also be used to prevent or treat diseases that are triggered by persistent angiogenesis, such as Kaposi's sarcoma, leukemia or arthritis.

Where a tumor, a tumor disease, a carcinoma or a cancer are mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis.

The compositions are selectively toxic or more toxic to rapidly proliferating cells than to normal cells, particularly in human cancer cells, e.g., cancerous tumors, the compound has significant anti-proliferative effects and promotes differentiation, e.g., cell cycle arrest and apoptosis.

The invention is further defined by reference to the following examples describing in detail the compounds, compositions and combinations of the present invention, as well as their utility. It will be apparent to those skilled in the art, that many modifications, both to materials, and methods, may be practiced with out departing from the purpose and interest of this invention. The examples that follow are not intended to limit the scope of the invention as defined hereinabove or as claimed below.

EXAMPLE 1: Combination of 7-*t*-butoxyiminomethylcamptothecin and Oxaliplatin

IN VIVO EXPERIMENTAL PROCEDURES

Test system: Female CD1 nu/nu mice for tumor models were used.

Number of animals: 176 (88 for each tumor model)

A2780 tumor model: human ovarian carcinoma cells (2x106) are implanted s.c. in the right flank of female mice, 7-8 mice/group are treated 3 days after tumor injection with the following drugs.

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg, p.o. (qdx5/w, days 3-7)
3. Oxaliplatin 7 mg/10 ml/kg and 4.7 mg/10 ml/kg, i.p. (q4dx2, days 3, 7).
4. Combination groups: 7-t-butoxyiminomethylcamptothecin + oxaliplatin: 0.28+7, 0.28+4.7, 0.19+7, 0.19+4.7.

5. 7-t-butoxyiminomethylcamptothecin 0.19 mg/10 ml/kg, p.o. (qdx5/wx2w, days 3-7, 10-14).

6. Oxaliplatin 7 mg/10 ml/kg, i.p. (q7dx2, days 3, 10).

7. Combination groups: 7-t-butoxyiminomethylcamptothecin + oxaliplatin 0.19+7.

The drug is administered 1 h before 7-t-butoxyiminomethylcamptothecin.

DATA ANALYSIS

All raw data are recorded on appropriate forms bound in numbered registers, stored and processed by a computer system. The formula TV (mm3) = length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. LCK (log10 cell kill) is calculated using the following formula: (T-C)/3.32 x DT where T – C are the mean time (in days) required for treated (T) and control (C) tumors, respectively, to reach a determined volume, and DT is the doubling time of control tumors. CR is defined as disappearance of the tumor lasting at least 10 days after the end of treatments. The effect of the combination of 7-t-butoxyiminomethylcamptothecin and the different agents is evaluated according to the method of Romanelli et al. (1998). An R index of 1 (additive effect) or lower indicates the absence of synergism. Synergism is defined as any value of R greater than unity. R was calculated from expected and observed T/C% values.

A2780 ovarian carcinoma: 7-t-butoxyiminomethylcamptothecin at the MTD of 0.28 mg/kg shows a potent antitumor activity in terms of tumor volume inhibition (TVI = 100%), CR = 8/8 and LCK >2. The combination of 7-t-butoxyiminomethylcamptothecin with oxaliplatin on the same tumor gave activity comparable to 7-t-butoxyiminomethylcamptothecin as single agent but with some complete responders at 60 days.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with oxaliplatin [B] (i.p., q4dx2, +3) against A2780 ovarian carcinoma

Drug	Dose (mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	6	0/8	100	8/8	2.15

A	0.28	13	0/8	100	8/8	2.44
B	4.7	2	1/8	67	1/8	0.57
B	7	10	0/8	91	0/8	1.0
B+A	4.7+0.19	14	0/8	100	8/8	2.29
B+A	4.7+0.28	22	0/8	100	8/8	2.58
B+A	7+0.19	24	0/8	100	8/8	2.44
B+A	7+0.28	31	4/8	100	4/4	3.15

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. [5]LCK = log10 cell kill. DT = 2.1 days. On day +60 the following mice were without tumor lesions: 4.7+0.19 (1/8), 7+0.19 (1/8).

EXAMPLE 2: Combination of 7-*t*-butoxyiminomethylcamptothecin and Docetaxel

IN VIVO EXPERIMENTAL PROCEDURES

Test system: Female CD1 nu/nu mice for tumor models are used.

Number of animals: 176 (88 for each tumor model)

MCF-7 human breast carcinoma estrogen-dependent cells (5x106) are implanted in the right flank of female mice previously implanted with slow-release pellets of 17 ß-estradiol (0.72 mg/pellet). The pellets are placed in the inter scapular region one day before the tumor cells inoculation. 8 mice/group are treated 6 days after tumor injection with the following drugs:

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg p.o. (qdx5/w, days 3-7)
3. Docetaxel 20 mg/10 ml/kg. and 13.3 mg/10 ml/kg i.p. (q3-4dx2, days 3, 7).

4. Combination groups 7-t-butoxyiminomethylcamptothecin + docetaxel: 0.28+20, 0.28+13.3, 0.19+20, 0.19+13.3.

A2780/DDP human platinum ovarian carcinoma cells (2x106) are implanted in the right flank of female mice. 8 mice/group are treated 3 days after tumor injection with the following drugs.

1. Vehicle
2. 7-T-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg p.o. (qdx5/w, days 3-7)
3. Docetaxel 20 mg/10 ml/kg and 13.3 mg/10 ml/kg, i.p. (q4dx2, days 3, 7)
4. Combination groups 7-t-butoxyiminomethylcamptothecin + docetaxel: 0.28+20, 0.28+13.3, 0.19+20, 0.19+13.3.

NCI-H460 human non-small cell lung carcinoma cells (3x106) are implanted in the right flank of female mice. 8 mice/group are treated 3 days after tumor injection with the following drugs:

1. Vehicle
2. 7-t-butoxyiminomethylcamptotheciN 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg p.o. (qdx5/w, days 3-7)
3. Docetaxel 20 mg/10 ml/kg and 13.3 mg/10 ml/kg i.p. (q4dx2, days 3, 7)
4. Combination groups 7-t-butoxyiminomethylcamptothecin + docetaxel: 0.28+20, 0.28+13.3, 0.19+20, 0.19+13.3.

7 mice/group are treated 3 days after tumor injection with the following drugs:

1. 7-t-butoxyiminomethylcamptothecin 0.19 mg/10 ml/kg, p.o. (qdx5/wx2w, days 3-7, 10-14)
2. Docetaxel 20 mg/10 ml/kg, i.p. (q7dx2, days 3, 10)

DU145 human prostate carcinoma cells are inoculated (3x106) s.c. into the right flank of male mice. 8 mice/group are treated 14 days after tumor injection with the following drugs:

1. Vehicle

2. 7-t-butoxyiminomethylcamptothecin 0.25 mg/10 ml/kg and 0.19 mg/10 ml/kg, p.o. (qdx5/w, days 14-18)

3. Docetaxel 20 mg/10 ml/kg and 13.3 mg/10 ml/kg, i.p. (q4dx2, days 14-18)

4. Combination groups 7-t-butoxyiminomethylcamptothecin + docetaxel: 0.19+13.3, 0.25+13.3.

8 mice/group are also treated 14 days after tumor injection with the following drugs:

1. 7-t-butoxyiminomethylcamptothecin 0.17 mg/10 ml/kg, p.o. (qdx5/wx3w, days 14-18, 21-25, 28-32)

2. Docetaxel 20 mg/10 ml/kg, i.p., (q7dx3, days 14, 21, 28).

3. Combination groups: 7-t-butoxyiminomethylcamptothecin + docetaxel 0.17+20.

DATA ANALYSIS

All raw data are recorded on appropriate forms bound in numbered registers, stored and processed by a computer system. The formula TV (mm3) = length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. LCK (log10 cell kill) is calculated using the following formula: (T-C)/3.32 x DT where T – C are the mean time (in days) required for treated (T) and control (C) tumors, respectively, to reach a determined volume, and DT is the doubling time of control tumors. CR is defined as disappearance of the tumor lasting at least 10 days after the end of treatments. The effect of the combination of 7-t-butoxyiminomethylcamptothecin and the different agents is evaluated according to the method of Romanelli et al. (1998) Cancer Chemother. Pharmacol. 41, 385-390. An R index of 1 (additive effect) or lower indicates the absence of synergism. Synergism is defined as any value of R greater than unity. R was calculated from expected and observed T/C% values.

MCF-7 estrogen-dependent breast carcinoma: 7-t-butoxyiminomethylcamptothecin delivered p.o. according to the schedule qdx5 at 0.28 mg/kg (MTD) shows a potent antitumor activity (TVI = 70%) with 1 out of 8 mice without tumor lesion 32 days after the last treatment. On the same tumor model, docetaxel at the MTD of 20 mg/kg, i.p. q4dx2, shows a comparable antitumor activity to that of 7-t-butoxyiminomethylcamptothecin (TVI = 73%) and CR = 1/8. When 7-t-butoxyiminomethylcamptothecin is combined with docetaxel, both at their MTD, a strong toxicity is observed (7/8 mice died), whereas at their suboptimal doses (0.19+13.3 mg/kg) a synergistic

interaction (R = 9.5) was found in terms of increase in tumor volume inhibition and in number of complete responses.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +6) in combination with docetaxel [B] (i.p., q3-4dx2, +6) against MCF-7 human breast ca.

Drug	Dose (mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR
A	0.28	9	1/8	70	1/8
A	0.19	10	2/8	81	1/8
B	13.3	4	1/8	0	1/8
B	20	7	0/8	73	1/8
B+A	13.3+0.19	12	3/8	98	5/8
B+A	20+0.19	15	3/8	96	2/8
B+A	13.3+0.28	14	2/8	96	2/8
B+A	20+0.28	31	7/8	/	1/8

Treatment starts 6 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]LCK = log10 cell kill. [5]CR = complete response. DT = 18.9 days. R = 7.5 (0.28+13.3); R = 9.5 (0.19+13.3); R = 1.3 (0.19+20).

A2780/DDP platinum-resistant ovarian carcinoma: 7-t-butoxyiminomethylcamptothecin at the approximate maximum tolerated dose of 0.28 mg/kg (qdx5/w) is slightly efficacious (TVI = 46%). Docetaxel (20 mg/kg, i.p., q4dx2) at the MTD shows a comparable antitumor effect to that of 7-t-butoxyiminomethylcamptothecin. The effect of the combination of 7-t-butoxyiminomethylcamptothecin with docetaxel is additive on this tumor model.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with docetaxel [B] (i.p., q4dx2, +3) against A2780/DDP platinum-resistant ovarian carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	LCK[4]
A	0.19	1	0/8	35	0.18
A	0.28	7	0/8	46	0.23
B	13.3	8	0/8	7	0.09
B	20	17	0/8	43	0.18
B+A	13.3+0.19	12	0/8	43	0.62
B+A	20+0.19	14	0/8	23	0.18
B+A	13.3+0.28	13	0/8	54	0.62
B+A	20+0.28	19	0/8	65	0.71

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]LCK = log10 cell kill. DT = 1.7 days. R = 1.06 (13.3+0.19); 0.48 (20+0.19); 1.09 (13.3+0.28); 0.88 (20+0.28).

NCI-H460 NSCLC: 7-t-butoxyiminomethylcamptothecin (0.28 mg/kg, p.o., qdx5) reveals a stronger antitumor effect (TVI = 94%) compared with docetaxel (20 mg/kg, i.p. q4dx2) (TVI = 63% and 1 out of 8 mice with complete response 10 days after the last treatment). The interaction of 7-t-butoxyiminomethylcamptothecin with docetaxel is additive or synergistic depending on the dose regiment.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with docetaxel [B] (i.p., q4dx2, +3) against NCI-H460 non-small cell lung carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	8	0/8	79	1/8	1.14
A	0.28	16	1/8	94	0/8	1.45

B	13.3	2	0/8	4	0/8	0.21
B	20	7	0/8	63	1/8	0.72
B+A	13.3+0.19	8	0/8	87	0/8	1.87
B+A	20+0.19	9	0/8	94	0/8	1.87
B+A	13.3+0.28	16	0/8	95	1/8	2.3
B+A	20+0.28	18	0/8	95	0/8	1.87

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. [5]LCK = log10 cell kill. DT = 2.9 days. R = 1.5 (13.3+0.19); 1.3 (20+0.19); 1.15 (13.3+0.28); 0.44 (20+0.28).

DU145 prostate carcinoma: 7-t-butoxyiminomethylcamptothecin at the approximate maximum tolerated dose of 0.25 mg/kg (qdx5/w) shows an activity comparable to that found with the MTD of docetaxel delivered i.p. at 20 mg/kg, q4dx2 (TVI was 53%). The combination of the suboptimal doses of docetaxel with 7-t-butoxyiminomethylcamptothecin (13.3 and 0.19 mg/kg) produces a synergistic interaction. When 7-t-butoxyiminomethylcamptothecin is given for 3 weeks at the suboptimal dose of 0.17 mg/kg in combination with docetaxel at 20 mg/kg, i.p. (q7dx3), it produces a synergistic effect on tumor growth in terms of complete responses (3/8) and increase in LCK.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +14) in combination with docetaxel [B] (i.p., q4dx2, +14) against DU145 prostate carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	4	0/8	25	0/8	0.28
A	0.25	8	0/8	53	0/8	0.28
B	13.3	0	0/8	23	0/8	0.28

B	20	8	0/8	68	0/8	0.65
B+A	13.3+0.19	9	0/8	70	0/8	0.65
B+A	13.3+0.25	11	0/8	66	0/8	0.65

Treatment starts 14 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. [5]LCK = log10 cell kill. DT = 10.7 days. R = 1.92 (13.3+0.19); 1.06 (13.3+0.25).

EXAMPLE 3: Combination of 7-*t*-butoxyiminomethylcamptothecin and Paclitaxel

IN VIVO EXPERIMENTAL PROCEDURES

Test system: Female CD1 nu/nu mice for tumor models were used.

Number of animals: 176 (88 for each tumor model)

NCI-H460 human lung carcinoma from *in vitro* cell cultures are injected s.c. using 3x106 cells/100 µl/mouse into the right flank of CD1 nude mice. Mice are treated with 3 intravenously doses of paclitaxel at days 3, 10, 17 after tumor injection. 7-t-butoxyiminomethylcamptothecin is administered by oral route for 3 cycles (qdx5/w) starting 3 days after the tumor implantation. In other groups of mice 7-t-butoxyiminomethylcamptothecin is given p.o. in combination with paclitaxel by using the same schedule. Treatments in NCI-H460 tumor model are performed in the following groups of 8 mice each:

1. Vehicle
2. 50 mg/15 ml/kg, i.v. of paclitaxel (3, 10, 17)
3. 33.3 mg/15 ml/kg, i.v. of paclitaxel (3, 10, 17)
4. 25 mg/15 ml/kg, i.v. of paclitaxel (3, 10, 17)
5. 7-t-butoxyiminomethylcamptothecin 0.25 mg/10 ml/kg, p.o. (3-7), (10-14), (17-21)
6. 7-t-butoxyiminomethylcamptothecin 0.125 mg/10 ml/kg, p.o. (3-7), (10-14), (17-21)
7. 7-t-butoxyiminomethylcamptothecin 0.08 mg/10 ml/kg, p.o. (3-7), (10-14), (17-21)

8. Paclitaxel+7-t-butoxyiminomethylcamptothecin (33.3+0.125)

9. Paclitaxel+7-t-butoxyiminomethylcamptothecin (33.3+0.08)

10. Paclitaxel+7-t-butoxyiminomethylcamptothecin (25+0.125)

11. Paclitaxel+7-t-butoxyiminomethylcamptothecin (25+0.08)

To evaluate the antitumor activity of drugs on human xenografts, tumor volume is evaluated by measuring biweekly tumor diameters with a Vernier caliper. The formula TV (mm3) = [length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. When tumors of mice achieved a volume of about 2 g, the animals are sacrificed by cervical dislocation.

DATA ANALYSIS

All raw data are recorded on appropriate forms bound in numbered registers, stored and processed by a computer system. The formula TV (mm3) = length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. LCK (log10 cell kill) is calculated using the following formula: (T-C)/3.32 x DT where T – C are the mean time (in days) required for treated (T) and control (C) tumors, respectively, to reach a determined volume, and DT is the doubling time of control tumors. CR is defined as disappearance of the tumor lasting at least 10 days after the end of treatments. The effect of the combination of 7-t-butoxyiminomethylcamptothecin and the different agents is evaluated according to the method of Romanelli et al. (1998). An R index of 1 (additive effect) or lower indicates the absence of synergism. Synergism is defined as any value of R greater than unity. R is calculated from expected and observed T/C% values.

NCI-H460 tumor model: at the tolerated dose of paclitaxel of 50 mg/kg, i.v. administered according to the schedule q7dx3, starting 3 days after tumor inoculum, is able to induce a reduction of tumor growth (T/C%=38.6), with a low mean weight loss of 6%. The other two low doses of 33.3 mg/kg and 25 mg/kg, which are 2/3 of MTD and 1/2 of MTD, respectively, given i.v. according to the same schedule, are effective too. The T/C% evaluated are 36.8 and 55.2, respectively. The NCI-H460 tumor is very responsive to 7-t-butoxyiminomethylcamptothecin alone, since both the doses of 0.25 mg/kg and 0.125 mg/kg, administered for 5 days for 3 cycles, reduce the tumor volume of about 99% and 90%, respectively (T/C% were 1.5 and 9.9). These doses did not produce toxicity-related deaths (0 out of 8 mice) or reduction of body weight. A minor dose of 7-t-butoxyiminomethylcamptothecin (0.08 mg/kg), given according to

the same schedule, is effective (T/C%=59.5%). When suboptimal doses of each drug are combined (0.125 mg/kg of 7-t butoxyiminomethylcamptothecin and 33.3 or 25 mg/kg of paclitaxel), the combination groups achieve a tumor growth inhibition higher than with that achieved by the single-agent 7-t-butoxyiminomethylcamptothecin therapy (T/C% were 2.2 and 2.5%), with R index values of 1.6 and 2.2, respectively. These treatments did not induce toxicity in mice. Also the combination of a lower dose of 7-t-butoxyiminomethylcamptothecin (0.08 mg/kg) with the two suboptimal doses of paclitaxel (33.3 and 25 mg/kg, i.v.) produce R index values of 1.3 and 2.1.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with paclitaxel [B] (i.v., q7dx3, +3) against NCI-H460 NSCLC

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	T/C%[3]	R
A	0.25	0	0/8	1.5	
A	0.125	0	0/8	9.9	
A	0.08	0	0/8	59.5	
B	50	6	0/7	38.6	
B	33.3	2	0/8	36.8	
B	25	1	0/8	55.2	
B+A	33.3+0.125	3	1/8	2.2	1.6
B+A	25+0.125	6	0/7	2.5	2.2
B+A	33.3+0.08	2	0/8	20.9	1.3
B+A	25+0.08	3	0/7	15.7	2.1

[1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]TW in treated mice/TW in control mice x 100.

EXAMPLE 4: Combination of 7-*t*-butoxyiminomethylcamptothecin and Carboplatin

IN VIVO EXPERIMENTAL PROCEDURES

Test system: Female CD1 nu/nu mice for tumor models are used.

Number of animals: 176 (88 for each tumor model)

A2780/DDP human platinum ovarian carcinoma cells (2x106) are implanted in the right flank of female mice. 8 mice/group are treated 3 days after tumor injection with the following drugs.

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg p.o. (qdx5/w, days 3-7)
3. Carboplatin 50 mg/10 ml/kg and 33.3 mg/10 ml/kg, i.p. (q4dx2, days 3, 7)
4. Combination groups 7-t-butoxyiminomethylcamptothecin + carboplatin: 0.28+50, 0.28+33.3, 0.19+50, 0.19+33.3.

A2780 ovarian carcinoma cells (2x106) are implanted s.c. in the right flank of female mice. 7-8 mice/group are treated 3 days after tumor injection with the following drugs.

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg, p.o. (qdx5/w, days 3-7)
3. Carboplatin 50 mg/10 ml/kg and 33.3 mg/10 ml/kg, i.p. (q4dx2, days 3, 7)
4. Combination groups: 7-t-butoxyiminomethylcamptothecin + carboplatin: 0.28+50, 0.28+33.3, 0.19+50, 0.19+33.3..
5. 7-T-butoxyiminomethylcamptothecin 0.19 mg/10 ml/kg, p.o. (qdx5/wx2w, days 3-7, 10-14).
6. Carboplatin 50 mg/10 ml/kg, i.p. (q7dx2, days 3, 10).
7. Combination groups: 7-t-butoxyiminomethylcamptothecin + carboplatin 0.19+50.

NCI-H460 human non-small cell lung carcinoma cells (3x106) are implanted in the right flank of female mice. 8 mice/group are treated 3 days after tumor injection with the following drugs:

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.28 mg/10 ml/kg and 0.19 mg/10 ml/kg p.o. (qdx5/w, days 3-7)
3. Carboplatin 50 mg/10 ml/kg and 33.3 mg/10 ml/kg, i.p. (q4dx2, days 3, 7)
4. Combination groups: 7-t-butoxyiminomethylcamptothecin + carboplatin: 0.28+50, 0.28+33.3, 0.19+50, 0.19+33.3.

7 mice/group are also treated 3 days after tumor injection with the following drugs:

1. 7-t-butoxyiminomethylcamptothecin 0.19 mg/10 ml/kg, p.o. (qdx5/wx2w, days 3-7, 10-14)
2. Carboplatin 50 mg/10 ml/kg, i.p. (q7dx2, days 3, 10)

In all the combination groups, the drug were administered 1 h before 7-t butoxyiminomethylcamptothecin.

To evaluate the antitumor activity of drugs on human xenografts, tumor volume is evaluated by measuring biweekly tumor diameters with a Vernier caliper. The formula TV (mm3) = [length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. When tumors of mice achieve a volume of about 2 g, the animals are sacrificed by cervical dislocation.

DATA ANALYSIS

All raw data are recorded on appropriate forms bound in numbered registers, stored and processed by a computer system. The formula TV (mm3) = length (mm) x width (mm)2]/2 is used, where the width and the length are the shortest and the longest diameters of each tumor, respectively. LCK (log10 cell kill) is calculated using the following formula: (T-C)/3.32 x DT where T – C are the mean time (in days) required for treated (T) and control (C) tumors, respectively, to reach a determined volume, and DT is the doubling time of control tumors. CR is defined as disappearance of the tumor lasting at least 10 days after the end of treatments. The effect of the combination of 7-t-butoxyiminomethylcamptothecin and the different agents is evaluated according to the method of Romanelli et al. (1998). An R index of 1 (additive effect) or lower indicates the absence of synergism. Synergism is defined as any value of R greater than unity. R is calculated from expected and observed T/C% values.

A2780/DDP platinum-resistant ovarian carcinoma tumor model: 7-t-butoxyiminomethylcamptothecin at the approximate maximum tolerated dose of 0.28 mg/kg (qdx5/w) is slightly efficacious (TVI = 46%) and showed a comparable activity to that of carboplatin (50 mg/kg, i.p., q4dx2) (TVI = 34%). When 7-t-butoxyiminomethylcamptothecin is combined with carboplatin, an additive to synergistic interaction is found.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with carboplatin [B] (i.p., q4dx2, +3) against A2780/DDP platinum-resistant ovarian carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	LCK[4]
A	0.19	1	0/8	35	0.18
A	0.28	7	0/8	46	0.23
B	33	4	0/8	0	0
B	50	6	0/8	34	0.26
B+A	33+0.19	8	0/8	28	0.39
B+A	50+0.19	18	0/8	40	0.39
B+A	33+0.28	10	0/8	60	0.57
B+A	50+0.28	21	0/8	62	0.80

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]LCK = log10 cell kill. DT = 1.7 days. R = 0.9 (33+0.19); 0.71 (50+0.19); 1.35 (33+0.28); 0.94 (50+0.28).

A2780 ovarian carcinoma tumor model: 7-t-butoxyiminomethylcamptothecin at the MTD of 0.28 mg/kg shows a potent antitumor activity in terms of tumor volume inhibition (TVI = 100%), CR = 8/8 and LCK >2. When it is combined with carboplatin at the suboptimal dose of 33.3 mg/kg, i.p., q4dx2, an increase of LCK is observed, suggesting a major persistence of the effect in the inhibition of tumor growth after the end of the treatment. A similar result is obtained when 7-t-

butoxyiminomethylcamptothecin given for 2 weeks (0.19 mg/kg, qdx5/wx2w) is combined with carboplatin (50 mg/kg, q7dx2), since a higher LCK was reached.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with carboplatin [B] (i.p., q4dx2, +3) against A2780 ovarian carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	6	0/8	100	8/8	2.15
A	0.28	13	0/8	100	8/8	2.44
B	33.3	4	0/8	82	2/8	1.0
B	50	5	0/8	84	1/8	1.0
B+A	33.3+0.19	13	0/8	100	8/8	2.87
B+A	33.3+0.28	25	0/8	100	8/8	4.30
B+A	50+0.19	19	0/8	100	8/8	3.58
B+A	50+0.28	27	2/8	100	6/6	5.90

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. [5]LCK = log10 cell kill. DT = 2.1 days. On day +60 the following mice were without tumor lesions: 33.3+0.19 (1/8), 50+0.19 (2/8), 33.3+0.28 (5/8), 50+0.28 (3/6).

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/wx2w, +3) in combination with carboplatin [B] (i.p., q7dx2, +3) against A2780 ovarian carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	6	0/7	100	4/7	3.15

B	50	5	0/7	71	1/7	1.15
B+A	50+0.19	18	0/7	100	7/7	5.30

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR=complete response after the last treatment. [5]LCK=log10 cell kill. DT=2.1 days. On day +60 the following mice are without tumor lesions: 0.19 (4/7), 50 (1/7), 50+0.19 (7/7).

NCI-H460 NSCLC tumor model, 7-t-butoxyiminomethylcamptothecin (0.28 mg/kg, p.o., qdx5) reveals a strong antitumor effect (TVI = 94%) compared to carboplatin at 50 mg/kg, i.p. q4dx2, (MTD),with a moderate antitumor effect (TVI=59%). When 7-t-butoxyiminomethylcamptothecin (0.28 mg/kg) is combined with a suboptimal dose of carboplatin (33.3 mg/kg), a synergistic effect (R =3) is observed since TVI reached 100% and 3 out of 8 mice are without tumor lesion 10 days after tumor implantation. A therapeutic advantage is found with this type of combination since it exceeds the efficacy of the two drugs given alone. An additive effect is found with the combination of other doses. 7-t-butoxyiminomethylcamptothecin is also given according to the schedule qdx5/wx2w at 0.19 mg/kg in combination with carboplatin at 50 mg/kg, i.p., q7dx2 still producing a synergistic effect (R = 1.9)

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with carboplatin [B] (i.p., q4dx2, +3) against NCI-H460 non-small cell lung carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	8	0/8	79	1/8	1.14
A	0.28	16	1/8	94	0/8	1.45
B	33.3	4	0/8	49	0/8	0.73
B	50	8	0/8	59	0/8	0.83
B+A	33.3+0.19	12	0/8	91	0/8	1.87
B+A	50+0.19	21	0/8	95	0/8	1.87

B+A	33.3+0.28	26	0/8	100	3/8	2.6
B+A	50+ 0.28	25	0/8	97	1/8	2.1

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. [5]LCK = log10 cell kill. DT = 2.9 days. R = 1.2 (33.3+0.19); 1.7 (50+0.19); 3 (33.3+0.28); 0.82 (50+0.28).

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/wx2w, +3) in combination with carboplatin [B] (i.p., q7dx2, +3) against NCI-H460 non-small cell lung carcinoma

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	TVI%[3]	CR[4]	LCK[5]
A	0.19	7	0/7	94	0/7	2.3
B	50	3	0/7	38	0/7	0.73
B+A	50+0.19	18	0/7	98	0/7	2.9

Treatment starts 3 days after the tumor injection. [1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % in treated over control tumors. [4]CR = complete response after the last treatment. 5LCK = log10 cell kill. DT = 2.9 days. R = 1.86 (50+0.19)

EXAMPLE 5: Combination of 7-*t*-butoxyiminomethylcamptothecin and Doxorubicin

IN VIVO EXPERIMENTAL PROCEDURES

Test system: BALB/c *nu/nu* mice for tumor models are used.

MDA MB 435S human breast cancer cells (passage 4 from working stock VPStock 044) are obtained from ATCC (Rockville, MD, USA) and cultured in RPMI1640 cell culture medium, which is supplemented with 10% FCS and penicillinstreptomycin (50 IU/mL, 50 µg/mL final

concentration). The cells are harvested by trypsinisation, washed twice in HBSS and counted using Trypan Blue to distinguish viable cells. The cells are then resuspended in HBSS and adjusted to a final concentration of 1x107 cells/mL. For inoculation the injection site is liberally swabbed with alcohol and the needle introduced through the skin into the subcutaneous space just below the animal's right shoulder, where 100 µL of cells (1x106) are discharged. Acceptable tumor volumes are reached 30 days post inoculation.

7-t-butoxyiminomethylcamptothecin is administered orally (p.o.) 5 times per week, both on its own (i.e. as a mono-therapy) and in combination with doxorubicin. Doxorubicin (either on its own or in conjunction with Gimatecan) is administered 3 times per week intravenously, via the tail vein (i.v.).

1. Vehicle
2. 7-t-butoxyiminomethylcamptothecin 0.29 mg/kg, 0.17 mg/kg and, 0.09 mg/kg p.o. (qdx5/w)
3. Doxorubicin 50 mg/10 ml/kg and 4.5 mg/kg, 2.97 mg/kg, 1.49 mg/kg, i.v. ((qdx3/w))
4. Combination groups: 7-t-butoxyiminomethylcamptothecin + Doxorubicin: 0.17+2.97, 0.17+1.49, 0.17+0.45, 0.09+2.97, 0.09+1.49, 0.09+2.97, 0.03+2.97, 0.03+1.49, 0.03+0.45

DATA ANALYSIS

The paired t-test is used to determine differences in body weight changes from Day 0 to Day 13 for groups 14 and 15 and from Day 0 to Day 20 for all other groups (Table 4). All calculations are done using SigmaStat 3.0. The one way ANOVA procedure is used for statistical calculations of differences in the tumor volumes.

MDA MB 435S human breast cancer, 7-t-butoxyiminomethylcamptothecin in combination with doxorubicin shows additive to synergistic antitumor activity. Synergistic activity is most notable in the B 0.29+ A 0.07 group where the single agent non efficacious doses provide a combination activity of 48% T/C.

Antitumor activity of 7-*t*-butoxyiminomethylcamptothecin [A] (p.o., qdx5/w, +3) in combination with doxorubicin [B] (i.v., qdx3/w, +3) against MDA MB 435S human breast cancer

Drug	Dose(mg/kg)	BWL%[1]	Tox[2]	T/C%[3]
A	0.21	1	1/9	5
A	0.14	1	0/9	46
A	0.07	(+2.5)	9/9	93
B	2.9	9.9	9/9	45
B	1.91	8.4	8/9	51
B	0.96	(+2.5)	9/9	78
B+A	1.91+0.14	16	9/9	5
B+A	0.96+0.14	4.4	9/9	30
B+A	0.29+0.14	3.5	9/9	43
B+A	1.91+0.07	6	9/9	42
B+A	0.96+0.07	3.5	9/9	41
B+A	0.29+0.07	(+3.4)	9/9	48
B+A	1.91+0.03	2.6	8/9	68

[1]Body weight loss % induced by drug treatment. [2]Dead/treated mice. [3]Tumor volume inhibition % treated over control tumors.

EXAMPLE 6: Combination of 7-*t*-butoxyiminomethylcamptothecin and cis-Platinum

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

NCI-H460 non-small cell lung carcinoma (NSCLC) is obtained from the American Type Culture Collection (ATCC), A549 (NSCLC), HT-29 colon adenocarcinoma, A2780 and A2780/Dx,

A2780/DDP ovarian carinomas are from Istituto Nazionale Tumori, Milan, Italy. Cells are grown in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (GIBCO) and 50 µg/ml gentamycin sulfate (SIGMA). In order to test the effects of chemotherapeutic agents on cell growth, cells are seeded in 96-well tissue culture plates (Corning) at approximately 10% confluence and are allowed to attach and recover for at least 24 h. Varying concentrations of drugs alone or combined each other are then added to each well. The plates are incubated for 2 h and then washed before being incubated without drugs for additional 72 h. Other plates are treated with drugs sequentially (2 h with a drug followed by the other drug for 72 h). The number of surviving cells are then determined by staining with sulforhodamine B (SRB) as described by Skehan P et al. (1990) J. Natl. Cancer Inst. 82, 1107-1112.

DATA ANALYSIS

The interaction between 7-t-butoxyiminomethylcamptothecin and the different drugs is determined by using theanalysis of Drewinko et al. (1976) Cancer Biochem. Biophys. 1: 187-195. The analysis is performed as follows: (SFaxSFb/SFa+SFb)/100, where SFa is the survival fraction of 7-t-butoxyiminomethylcamptothecin and SFb is the survival fraction of the chemotherapeuticagent. Values indicated the following effects: a value > 1 synergism, <1 antagonism, =1 additive.

NCI-H460 non-small cell lung carcinoma: when cells are exposed simultaneously or sequential, the combination shows an additive cytotoxic effect (R index of 1).

A549 non-small cell lung carcinoma: when cells are simultaneously exposed to 7-t-butoxyiminomethylcamptothecin and cis-Platinum, or to a sequential treatment of cis-Platinum followed by 7-t-butoxyiminomethylcamptothecin show an additive cytotoxic effect, (R index of 1). When A549 cells are sequentially exposed to 7-t-butoxyiminomethylcamptothecin followed by cis-Platinum, a synergistic cytotoxic effect (R values of 1.2-1.3) is observed.

A2780 ovarian carcinoma: when cells were exposed simultaneously or sequential, the combination shows an additive cytotoxic effect (R index of 1).

A2780/DDP (Platinum resistant) ovarian carcinoma: when cells are exposed simultaneously or in the sequence cis-Platinum followed by 7-t-butoxyiminomethylcamptothecin, the combination shows an additive cytotoxic effect (R index of 1). When a2780/DDP cells are sequentially exposed to 7-t-butoxyiminomethylcamptothecin followed by cis-Platinum, a synergistic cytotoxic effect (R values of 1.2) is observed.

Schedules: (A) 7-*t*-butoxyiminomethylcamptothecin + cis-Platinum

(B) 7-*t*-butoxyiminomethylcamptothecin first then cis-Platinum

(C) cis-Platinum first then 7-*t*-butoxyiminomethylcamptothecin

Cell line	R value mean	Schedule	Comments
H460 NSCLC	1	ABC	
A549 NSCLC	1	AC	
A549 NSCLC	1.2-1.3	B	
A2780 ovarian	1	ABC	
A2780DDP ovarian	1	AC	Pt resistant cell line
A2780DDP ovarian	1.2	B	Pt resistant cell line

EXAMPLE 7: Combination of 7-*t*-butoxyiminomethylcamptothecin and Temozolamide

EXAMPLE 8: Combination of 7-*t*-butoxyiminomethylcamptothecin and Imatinib

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

A549 non-small cell lung carcinoma, A375 melanoma, 786-O renal cell adenocarcinoma SKOV3 ovary adenocarcinoma, 786-O renal cell adenocarcinoma, PANC-1 pancreas epithelioid carcinoma, U266B1 myeloma, SW620 colorectal adenocarcinoma, HeLa Cervical carcinoma and MIA PaCa-2 pancreatic carcinoma is obtained from the American Type Culture Collection (ATCC). The cell line of choice is diluted in appropriate media based on a cell count of 1,000 – 2,000 cells per well for adherent cell lines and 10,000 – 20,000 cells per well for suspension cell lines, cells are plated into 96 well plates using 100ul of the diluted cells per well. 4. The cells are grown overnight in an incubator at 37 deg C, 5%CO2 and 85% humidity prior to drug treatment. Compound dilutions are made from DMSO solutions for each compound. Typically these are centered on the EC50 and could be 6 or 9 dilutions which covered the full dose response of the

cell when exposed to the compound. There was a third series of dilutions made for the combination of the two compounds. For every dilution point in this series a fixed ratio of each compound is used. The cells are exposed simultaneously to the compounds for 72 hours and then the amount of proliferation is measured with Alamar Blue fluorescence (ex 535 em 590) for each well using an Envision (PerkinElmer) microplate reader.

DATA ANALYSIS

The interaction between 7-t-butoxyiminomethylcamptothecin and the different drugs is determined from the percent inhibition of proliferation defined as the ratio of the endpoint determination in each well divided by the control wells. The combination index (CI) is then determined for the 25, 50 and 75 % effect levels as described by Chou and Talay (Chou T-C, Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regulation 1984;22:27-55). CI of < 1 indicates synergistic cytotoxic effect, CI = 1 indicates additive cytotoxic effect and CI > 1 indicates an antagonistic cytotoxic effect

A549 non-small cell lung carcinoma: combination with Imatinib showed a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

SKOV3 ovary adenocarcinoma: combination with Imatinib showed a synergistic cytotoxic effect as indicated by CI values <1

786-O renal cell adenocarcinoma: combination with Imatinib showed a synergistic cytotoxic effect as indicated by CI values <1

MIA PaCa-2 pancreatic carcinoma: combination with Imatinib showed a synergistic or additive cytotoxic effect as indicated by CI values <1 or = 1 depending on the concentration of drug used.

A375 melanoma: combination with Imatinib showed a synergistic or additive cytotoxic effect as indicated by CI values <1 or = 1 depending on the concentration of drug used.

PANC-1 pancreas epithelioid carcinoma: combination with Imatinib showed an additive cytotoxic effect as indicated by CI values =1

7-t-butoxyiminomethylcamptothecin in combination with Imatinib			
Tumor cell line	Combination Index at cell fraction affected (cell kill)		
	25%	50%	75%

A549	0.32	0.67	1.95
SKOV3	0.88	0.86	0.88
786-O	0.78	0.92	0.62
MIA PaCa-2	0.62	0.76	1.03
A375	1.00	0.88	0.63
PANC-1	0.94	0.98	1.01

EXAMPLE 9: Combination of 7-*t*-butoxyiminomethylcamptothecin and Velcade

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

A549 non-small cell lung carcinoma, A375 melanoma, 786-O renal cell adenocarcinoma SKOV3 ovary adenocarcinoma, 786-O renal cell adenocarcinoma, PANC-1 pancreas epithelioid carcinoma, U266B1 myeloma, SW620 colorectal adenocarcinoma, HeLa Cervical carcinoma and MIA PaCa-2 pancreatic carcinoma is obtained from the American Type Culture Collection (ATCC). The cell line of choice is diluted in appropriate media based on a cell count of 1,000 – 2,000 cells per well for adherent cell lines and 10,000 – 20,000 cells per well for suspension cell lines, cells are plated into 96 well plates using 100ul of the diluted cells per well. 4. The cells are grown overnight in an incubator at 37 deg C, 5%CO2 and 85% humidity prior to drug treatment. Compound dilutions are made from DMSO solutions for each compound. Typically these are centered on the EC50 and could be 6 or 9 dilutions which cover the full dose response of the cell when exposed to the compound. There is a third series of dilutions made for the combination of the two compounds. For every dilution point in this series a fixed ratio of each compound is used. The cells are exposed simultaneously to the compounds for 72 hours and then the amount of proliferation is measured with Alamar Blue fluorescence (ex 535 em 590) for each well using an Envision (PerkinElmer) microplate reader.

DATA ANALYSIS

The interaction between 7-t-butoxyiminomethylcamptothecin and the different drugs is determined from the percent inhibition of proliferation defined as the ratio of the endpoint determination in each well divided by the control wells. The combination index (CI) is then determined for the 25, 50 and 75 % effect levels as described by Chou and Talay (Chou T-C, Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regulation 1984;22:27-55). CI of < 1 indicates synergistic cytotoxic effect, CI = 1 indicates additive cytotoxic effect and CI > 1 indicates an antagonistic cytotoxic effect

U266B1 myeloma: combination with Velcade shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

SKOV3 ovary adenocarcinoma: combination with Velcade shows a synergistic cytotoxic effect as indicated by CI values <1.

A375 melanoma: combination with Velcade shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

MIA PaCa-2 pancreatic carcinoma: combination with Velcade shows an additive cytotoxic effect as indicated by CI values around 1.

SW620 colorectal adenocarcinoma: combination with Velcade shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

7-*t*-butoxyiminomethylcamptothecin in combination with Velcade			
Tumor cell line	Combination Index at cell fraction affected (cell kill)		
	25%	50%	75%
U266B1	0.41	1.19	3.48
SKOV3	0.87	0.61	0.55
A375	1.23	0.85	0.66
MIA PaCa-2	1.16	0.97	1.15
SW620	1.39	0.75	0.88

EXAMPLE 10: Combination of 7-*t*-butoxyiminomethylcamptothecin and Epothilone B

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

Human non-small cell lung adenocarcinoma A549 (CCL 185) and ovarian carcinoma SK-OV-3 (ATCC HTB 77) cell lines are obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human metastatic prostate carcinoma PC-3M is obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). Cell culture media and supplements are from Animed/Bioconcept (Allschwil, Switzerland).

Cells are cultured with RPMI-1640 medium (complemented with 10% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM)) at 37 °C in an incubator with a 5 % v/v CO_2 and 80 % relative humidity atmosphere. Inhibition of monolayer cell proliferation by test compounds is assessed by methylene blue staining of fixed. Cells are seeded on day 0 at 1.5 x 10^3 cells/well into 96-well microtiter plates and incubated overnight. Drug interactions of

Epothilone B and the combination partner are assessed under conditions of simultaneous as well as sequential drug addition as follows. *Simultaneous drug addition*: Epothilone B and the combination partner are concomitantly added on day 1 and antiproliferative effects are assessed after incubation for 72 hrs on day 4. *Sequential drug addition, a)* "Epothilone B *before* combination partner": Epothilone B is added on day 1. After incubation for 24 hours, drug-containing medium is removed by aspiration on day 2 and replaced with medium containing the combination partner. Following additional incubation for 48 hrs, antiproliferative effects are assessed on day 4. *Sequential drug addition, b)* "Epothilone B *after* combination partner": The combination partner is added on day 1. After incubation for 24 hours, drug-containing medium is removed by aspiration on day 2 and replaced with medium containing Epothilone B. Following additional incubation for 48 hrs, antiproliferative effects are assessed on day 4. Epothilone B and the combination partner are tested at fixed ratios (multiples and fractions) of their respective single agent IC_{50}s on a given schedule, as determined in pilot experiments. Drugs are pre-mixed at the highest intended concentrations, followed by nine 1.5-fold serial dilutions in deep-well plates. When assessing single agent activities, which is performed in parallel as internal reference in each experiment, the combination partner is replaced by its respective vehicle. Each condition is present in duplicate. At the end of the incubation period, cells are fixed with 3.3 % v/v glutaraldehyde, washed with water and stained with 0.05 % w/v methylene blue. After washing, the dye is eluted with 3 % HCl and the optical density measured at 665 nm with a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Combination Index analysis of drug interaction effects

To determine the nature of the drug interaction (synergism, additivity or antagonism) with respect to *in vitro* cell growth inhibition, the combination index method based on the median dose effect principle (Chou TC and Talalay P Advanced Enzyme Regulation 1977;22:27-55) is used. This method takes into account the potency of each drug alone and each drug combination, as well as the shape of the dose-effect curves. Mathematical analysis (Chou TC, Motzer RJ, Tong Y and Bosl GJ Journal of the National Cancer Institute 1994; 86:1517-1524.) is performed using a commercial software (Calcusyn, Biosoft, UK). The Combination Index (CI) is calculated based on the following multiple drug effect equation: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2]$. $(D)_1$ and $(D)_2$ are the doses of drug 1 and drug 2 in combination that cause x% cell growth inhibition. $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 alone, respectively, that cause x% cell growth inhibition. CIs of <1 indicate greater than additive effects (synergism; the smaller the value, the greater the degree of synergy), CIs equal to 1 indicate additivity, and CIs >1 indicate antagonism. CI results are presented as mean +/- standard error of the mean (n = 3 independent experiments).

In A549 (CCL 185) non-small cell lung adenocarcinoma SK-OV-3 ovarian carcinoma and PC-3M metastatic prostate carcinoma the combination of 7-*t*-butoxyiminomethylcamptothecin and Epothilone B show a sequence dependence effect cytotoxic effect in cell culture. Simultaneous addition results in antagonism where as schedules gives additive to synergistic cytotoxic effect .

Antiproliferative Combination Index of 7-t-butoxyiminomethylcamptothecin and Epothilone B administered concomitantly or sequentially to A549 (lung) , PC-3M (prostate), and SK-OV-3 (ovarian) carcinoma cells in vitro.

Schedules: (A) 7-*t*-butoxyiminomethylcamptothecin + Epothilone B

(B) 7-*t*-butoxyiminomethylcamptothecin first then Epothilone B

(C) Epothilone B first then 7-*t*-butoxyiminomethylcamptothecin

		Combination Index (Mean ± SEM; n =3)		
Cell line	Fraction affected (cell kill)	Schedule A	Schedule B	Schedule C
A549	50 %	1.26 ± 0.12	0.89 ± 0.05	0.84 ± 0.09
	75 %	1.58 ± 0.18	0.94 ± 0.03	0.73 ± 0.04
	90 %	2.20 ± 0.74	1.07 ± 0.04	1.02 ± 0.22
PC-3M	50 %	1.48 ± 0.01	na[a]	0.57 ± 0.03
	75 %	1.80 ± 0.05	na[a]	0.86 ± 0.05
	90 %	2.37 ± 0.20	na[a]	1.37 ± 0.21
SK-OV-3	50 %	1.48 ± 0.03	1.35 ± 0.30	0.92 ± 0.08
	75 %	1.22 ± 0.09	0.98 ± 0.05	0.70 ± 0.11
	90 %	1.09 ± 0.18	1.10 ± 0.13	0.93 ± 0.11

Cell kill (fraction affected; corresponding to IC_{50}, IC_{75} and IC_{90}). The calculated combination index (CI) values are presented as mean ± standard error of the mean (n = 3 independent experiments). Per definition, CI = 1 indicates additivity. CI < 1.0 indicates synergy (the smaller the value, the stronger the degree of synergy), while CI > 1.0 indicates antagonism (the higher the value, the stronger the degree of antagonism). [a]not applicable, i.e due to narrow range of cellular effects within drug range tested (less than 50% fraction affected), calculated CI values display erroneously large error range and thus are not shown.

EXAMPLE 11: Combination of 7-*t*-butoxyiminomethylcamptothecin and Everolimus

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

A549 non-small cell lung carcinoma, A375 melanoma, 786-O renal cell adenocarcinoma SKOV3 ovary adenocarcinoma, 786-O renal cell adenocarcinoma, PANC-1 pancreas epithelioid carcinoma, U266B1 myeloma, SW620 colorectal adenocarcinoma, HeLa Cervical carcinoma and MIA PaCa-2 pancreatic carcinoma are obtained from the American Type Culture Collection (ATCC). The cell line of choice is diluted in appropriate media based on a cell count of 1,000 – 2,000 cells per well for adherent cell lines and 10,000 – 20,000 cells per well for suspension cell lines, cells are plated into 96 well plates using 100ul of the diluted cells per well. 4. The cells are grown overnight in an incubator at 37 deg C, 5%CO2 and 85% humidity prior to drug treatment. Compound dilutions are made from DMSO solutions for each compound. Typically these are centered on the EC50 and could be 6 or 9 dilutions which covered the full dose response of the cell when exposed to the compound. There was a third series of dilutions made for the combination of the two compounds. For every dilution point in this series a fixed ratio of each compound is used. The cells are exposed simultaneously to the compounds for 72 hours and then the amount of proliferation is measured with Alamar Blue fluorescence (ex 535 em 590) for each well using an Envision (PerkinElmer) microplate reader.

DATA ANALYSIS

The interaction between 7-t-butoxyiminomethylcamptothecin and the different drugs is determined from the percent inhibition of proliferation defined as the ratio of the endpoint determination in each well divided by the control wells. The combination index (CI) is then determined for the 25, 50 and 75 % effect levels as described by Chou and Talay (Chou T-C, Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regulation 1984;22:27-55). CI of < 1 indicates synergistic cytotoxic effect, CI = 1 indicates additive cytotoxic effect and CI > 1 indicates an antagonistic cytotoxic effect

A549 non-small cell lung carcinoma: combination with everolimus shows a synergistic cytotoxic effect as indicated by CI values <1.

SKOV3 ovary adenocarcinoma: combination with everolimus shows a spectrum of activity from synergistic to additive depending on the concentration of drug used.

PANC-1 pancreas epithelioid carcinoma: combination with everolimus shows a spectrum of activity from synergistic to additive depending on the concentration of drug used.

SW620 colorectal adenocarcinoma: combination with everolimus shows a spectrum of activity from synergistic to additive depending on the concentration of drug used.

7-*t*-butoxyiminomethylcamptothecin in combination with everolimus			
Tumor cell line	Combination Index at cell fraction affected (cell kill)		
	25%	50%	75%
A549	0.51	0.14	0.50
SKOV3	1.03	0.61	0.59
PANC-1	0.84	0.89	0.95
SW620	0.87	0.91	0.98

EXAMPLE 12: Combination of 7-*t*-butoxyiminomethylcamptothecin and {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}-((R)-1-phenyl-ethyl)-amine

IN VITRO EXPERIMENTAL PROCEDURES

Cell culture and cytotoxicity assay

A549 non-small cell lung carcinoma, A375 melanoma, 786-O renal cell adenocarcinoma SKOV3 ovary adenocarcinoma, 786-O renal cell adenocarcinoma, PANC-1 pancreas epithelioid carcinoma, U266B1 myeloma, SW620 colorectal adenocarcinoma, HeLa Cervical carcinoma and MIA PaCa-2 pancreatic carcinoma is obtained from the American Type Culture Collection (ATCC). The cell line of choice is diluted in appropriate media based on a cell count of 1,000 – 2,000 cells per well for adherent cell lines and 10,000 – 20,000 cells per well for suspension cell lines, cells are plated into 96 well plates using 100ul of the diluted cells per well. 4. The cells are grown overnight in an incubator at 37 deg C, 5%CO2 and 85% humidity prior to drug treatment. Compound dilutions are made from DMSO solutions for each compound. Typically these are centered on the EC50 and could be 6 or 9 dilutions which covered the full dose response of the cell when exposed to the compound. There is a third series of dilutions made for the combination of the two compounds. For every dilution point in this series a fixed ratio of each compound is used. The cells are exposed simultaneously to the compounds for 72 hours and then the amount of proliferation is measured with Alamar Blue fluorescence (ex 535 em 590) for each well using an Envision (PerkinElmer) microplate reader.

DATA ANALYSIS

The interaction between 7-t-butoxyiminomethylcamptothecin and the different drugs is determined from the percent inhibition of proliferation defined as the ratio of the endpoint determination in each well divided by the control wells. The combination index (CI) is then determined for the 25, 50 and 75 % effect levels as described by Chou and Talay (Chou T-C,

Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regulation 1984;22:27-55). CI of < 1 indicates synergistic cytotoxic effect, CI = 1 indicates additive cytotoxic effect and CI > 1 indicates an antagonistic cytotoxic effect.

A549 non-small cell lung carcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a synergistic cytotoxic effect as indicated by CI values <1.

SKOV3 ovary adenocarcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

PANC-1 pancreas epithelioid carcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

SW620 colorectal adenocarcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a spectrum of activity from synergistic to antagonistic depending on the concentration of drug used.

MIA PaCa-2 pancreatic carcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a synergistic cytotoxic effect as indicated by CI values <1.

A375 melanoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a synergistic cytotoxic effect as indicated by CI values <1.

HeLa Cervical carcinoma: combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine shows a spectrum of activity from synergistic to additive depending on the concentration of drug used.

7-*t*-butoxyiminomethylcamptothecin in combination with {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine			
Tumor cell line	Combination Index at cell fraction affected (cell kill)		
	25%	50%	75%
A549	0.13	0.26	0.54
SKOV3	1.30	0.93	0.66
PANC-1	0.10	0.45	2.01

SW620	1.58	0.91	0.65
MIA PaCa-2	0.93	0.84	0.76
A375	0.44	0.56	0.63
HeLa	1.08	0.50	0.50

What is claimed is:

1. A method for preventing or treating of a proliferative disease, which comprises administering pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) one or more chemotherapeutic agents selected from a microtubule active agent; an alkylating agent; an anti-neoplastic anti-metabolite; a platin compound; a topoisomerase II inhibitor; a VEGF inhibitor; a tyrosine kinase inhibitor; an EGFR kinase inhibitor; an mTOR kinase inhibitor; an insulin-like growth factor I inhibitor; a Raf kinase inhibitor; a monoclonal antibody; a proteasome inhibitor; a HDAC inhibitor; and ionizing radiation;

for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease.

2. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) one or more chemotherapeutic agents selected from paclitaxel; docetaxel; epothilone B; temozolamide; 5-FU; gemcitabine; oxaliplatin; cisplatinum; carboplatin; doxorubicin; {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine; everolimus; imatinib; erlotinib, bevacizumab, cetuximab, and velcade;

for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease.

3. A pharmaceutical composition comprising:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) one or more chemotherapeutic agents selected from a microtubule active agent; an alkylating agent; an anti-neoplastic anti-metabolite; a platin compound; a topoisomerase II inhibitor; a VEGF inhibitor; a tyrosine kinase inhibitor; an EGFR kinase inhibitor; an mTOR kinase inhibitor; an insulin-like growth factor I inhibitor; a Raf kinase inhibitor; a monoclonal antibody; a proteasome inhibitor; a HDAC inhibitor; and ionizing radiation.

4. A pharmaceutical composition comprising:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) one or more chemotherapeutic agents selected from paclitaxel; docetaxel; epothilone B; temozolamide; 5-FU; gemcitabine; oxaliplatin; cisplatinum; carboplatin; doxorubicin; {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine; everolimus; imatinib; erlotinib, bevacizumab, cetuximab, and velcade.

5. The method according to Claim 1, wherein the proliferative disease is selected from breast cancer, lung cancer, gastrointestinal cancer, including esophageal, gastric, small bowel, large bowel, colon and rectal cancer, glioma, sarcoma, ovarian cancer, myeloma, female cervical cancer, endometrial cancer, head and neck cancer, mesothelioma, renal cancer, ureter, bladder and urethral cancers, leukemia; prostate cancer, skin cancers and melanoma, a hyperproliferative condition, such as a leukemia, lymphoma or multiple myeloma.

6. The method according to Claim 2, wherein the proliferative disease is selected from colorectal cancer, ovarian cancer, breast cancer, prostate cancer, small cell lung cancer, non-small cell lung cancer, lung cancer, leukemia, and glioblastoma.

7. Use of 7-t-butoxyiminomethyl camptothecin for the preparation of a medicament, for use in combination of one or more chemotherapeutic agents selected from paclitaxel; docetaxel; epothilone B; temozolamide; 5-FU; gemcitabine; oxaliplatin; cisplatinum; carboplatin; doxorubicin; {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine; everolimus; imatinib; erlotinib, bevacizumab, cetuximab, and velcade for treatment of a proliferative disease.

8. The use of claim 7, wherein the proliferative disease is selected from colorectal cancer, ovarian cancer, breast cancer, prostate cancer, small cell lung cancer, non-small cell lung cancer, lung cancer, leukemia, and glioblastoma.

9. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) oxaliplatin; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is ovarian cancer.

10. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) docetaxel; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is selected from breast cancer, prostate cancer, non-small cell lung cancer and ovarian cancer.

11. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) paclitaxel; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is non-small cell lung cancer.

12. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) carboplatin; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is selected from ovarian cancer and non-small cell lung cancer.

13. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) doxorubicin; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is breast cancer.

14. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) cis-platinum; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferatihve disease is lung cancer, colon adenocarcinoma, and ovarian cancer.

15. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) temozolamide; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is glioblastoma.

16. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) imatinib; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is lung cancer, melanoma, renal cell adenocarcinoma, ovary adenocarcinoma, pancreas epithelioid carcinoma, myeloma, colorectal adenocarcinoma, cervical carcinoma, glioblastoma, and pancreatic carcinoma.

17. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) erlotinib; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is glioblastoma.

18. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) bevacizumab; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is glioblastoma.

19. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) velcade; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is lung cancer, melanoma, renal cell adenocarcinoma, ovary adenocarcinoma, pancreas epithelioid carcinoma, myeloma, colorectal adenocarcinoma, cervical carcinoma, leukemia, and pancreatic carcinoma.

20. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) epothilone B; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is selected from lung cancer, prostate cancer and ovarian cancer.

21. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) everolimus; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is selected from lung cancer, melanoma, renal cell adenocarcinoma, ovary adenocarcinoma, renal cell adenocarcinoma, pancreas epithelioid carcinoma, myeloma, colorectal adenocarcinoma, cervical carcinoma and pancreatic carcinoma.

22. A method for preventing or treating of a proliferative disease, which comprises administration of pharmaceutically effective amounts of a combination of:

(a) 7-*t*-butoxyiminomethylcamptothecin; and

(b) {6-[4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-((R)-1-phenyl-ethyl)-amine; for simultaneous, concurrent, separate or sequential use in for preventing or treating a proliferative disease wherein the proliferative disease is selected from lung cancer, melanoma, renal cell adenocarcinoma, ovary adenocarcinoma, renal cell adenocarcinoma, pancreas epithelioid carcinoma, myeloma, colorectal adenocarcinoma, Cervical carcinoma and pancreatic carcinoma.

AN - NLM21607321
DT - Journal Article
TI - Use of long-acting somatostetin analog, lanreotide, in **neuroendocrine tumors**.
AU - Canobbio L; Cannata D; Miglietta L; Pace M; Boccardo F
PUB - Oncology reports
- Greece
- Jan 1994
ORD - 1994-01-00
LNKD-pubmed:21607321
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VOL - 1
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LA - eng
AB - Somatostatin and its analogues have been reported highly effective in reducing the clinical syndrome in **endocrine tumours**. The effects of a long-acting analogue of somatostatin, lanreotide, were evaluated in 10 patients with **neuroendocrine tumours**. In patients with **carcinoid** tumour, a relief of flushing was achieved in 7 of 8 patients, of diarrhoea in 7 of 7 patients and of bronchoconstriction in 2 of 2 patients. In 5 of 8 patients there was a decrease of more than 50% of 5-hidroxyindolacetic acid excretion. The activity on tumour size was assessed in all the patients. No objective responses were observed. The tolerance to lanreotide was excellent. For the high symptomatic effect and mild toxicity lanreotide seems to be an appropriate treatment in symptomatic patients.

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(54) Title: COMBINATION OF MTOR INHIBITOR AND A TYROSINE KINASE INHIBITOR FOR THE TREATMENT OF NEOPLASMS

(57) Abstract: The invention features methods and compositions including an mTOR inhibitor and a tyrosine kinase inhibitor for reducing the proliferation of and enhancing the apoptosis of neoplastic cells. The addition of an MEK inhibitor to this combination further enhances the effectiveness of this therapeutic method.

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COMBINATION OF MTOR INHIBITOR AND A TYROSINE KINASE INHIBITOR FOR THE TREATMENT OF NEOPLASMS

Statement as to Federally Sponsored Research

This research has been sponsored by NIH Grants R01 DK50654 and P01 DK50693. The U.S. government has certain rights to the invention.

Background of the Invention

The present invention relates to pharmaceutical combinations and their use in the treatment of disorders associated with the proliferation of neoplasms.

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. Growth factor receptors ("GFRs") are an important part of the signal transduction pathway. GFRs are cell-surface proteins. When bound by a growth factor ligand, GFRs are converted to an active form which interacts with proteins on the inner surface of a cell membrane. As the result of this interaction, one of the key biochemical mechanisms of signal transduction is initiated; i.e., the reversible phosphorylation of various proteins within the cell. Protein kinases ("PKs") are enzymes that catalyze the phosphorylation of hydroxy groups on tyrosine, serine and threonine residues of proteins. This phosphorylation of intra-cellular proteins causes the formation of complexes with a variety of cytoplasmic signaling molecules that, in turn, effect numerous cellular responses such as cell division (proliferation), cell differentiation, cell growth, expression of metabolic effects to the extracellular microenvironment, among others (see Schlessinger and Ullrich, *Neuron* 9:303-391 (1992); Posada and Cooper, *Mol. Biol. Cell.* 3:583-392 (1992); and Hardie, *Symp. Soc. Exp. Biol.* 44:241-255 (1990)).

Growth factor receptors with tyrosine PK activity are known as receptor tyrosine kinases ("RTKs"). They comprise a large family of transmembrane receptors with diverse biological activity. At present, at least nineteen (19)

distinct subfamilies of RTKs have been identified, including the EGFR, epithelial growth factor receptor, RTKs (HER1-4); the insulin receptor RTKs (IR, IGF-1R, and IRR); the PDGFR, platelet derived growth factor receptor, RTKs (PDGFR-α, PDGFR-β, CSFIR, Flt-3, c-kit and c-fms); the VEGFR, vascular endothelial growth factor receptor, RTKs (VEGF R1/flt-1, VEGF R2/KDR/FLK-1, VEGF R3/flt-4); the Trk, tropomyosin receptor kinases (TrkA, TrkB, and TrkC); and the FGF, fibroblast growth factor, RTKs (FGFR1-4), among others.

In addition to the RTKs, there also exists a family of entirely intracellular PTKs or cellular tyrosine kinases ("CTKs"). At present, over 24 CTKs in 11 subfamilies (Src, Frk, Btk, Csk, Abl, Zap70, Fes, Fps, Fak, Jak, Pkc, and Ack) have been identified. The Src subfamily appear so far to be the largest group of CTKs and includes Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk, among others. For a more detailed discussion of CTKs see Bolen, *Oncogene* 8:2025-2031 (1993).

In addition to those listed above, there are tyrosine kinases that result from gene mutations. Examples include the BRC/ABL and TEL/ABL fusion genes, which encode for cytoplasmic proteins having constitutively active tyrosine kinase.

PTKs play an important role in the control of cellular processes including proliferation, differentiation, migration and survival. Enhanced PTK activity due to activating mutations or overexpression has been implicated in many human cancers. Malignant cell growth results from a breakdown in the mechanisms that control cell division and/or differentiation. It has been shown that the protein products of a number of proto-oncogenes are involved in the signal transduction pathways that regulate cell growth and differentiation. These protein products of proto-oncogenes include the extracellular growth factors, transmembrane growth factor PTK receptors (RTKs) and cellular PTKs (CTKs) discussed above. For example, EGFR is mutated and/or overexpressed in a variety of cancers, including brain, lung, squamous cell, bladder, gastric, breast, head and neck, oesophageal, gynecological and thyroid tumors.

Accordingly, it has been recognized that inhibitors of protein kinases are useful as selective inhibitors of the growth of mammalian cancer cells. Cancer therapy directed at specific and frequently occurring molecular alterations in signaling pathways of cancer cells has been validated through the clinical development and regulatory approval of agents such as Herceptin™, which targets HER-2 receptor, for the treatment of advanced breast cancer and Imatinib (Gleevec™), which targets the constitutively active tyrosine kinase BCR/ABL, for chronic myelogenous leukemia (CML) and Kit for gastrointestinal stromal tumors (GIST). See, for example, Shawver et al., *Cancer Cell* 1(2):117-123 (2002).

The macrolide fungicide rapamycin, a natural product with anti-tumor properties, is also capable of inhibiting signal transduction pathways that are necessary for the proliferation of cells. Rapamycin binds intracellularly to the immunophilin FK506 binding protein 12 (FKBP12), and the resultant complex inhibits the serine protein kinase activity of mammalian target of rapamycin (mTOR). The inhibition of mTOR, in turn, blocks signals to at least two separate downstream pathways which control the translation of specific mRNAs required for cell proliferation.

Although Imatinib represents a promising therapy for CML, it often does not provide a cure and, in some instances, the development of resistance complicates the therapy. A number of Imatinib resistant BCR/ABL positive cell lines have been described (see, for example, Mahon, F. X., et al., *Blood* 96(3):1070-1079 (2000)) and resistance to Imatinib has been demonstrated in a nude mouse model (Gambacorti-Passerini, C., et al., *J. Natl. Cancer inst.* 92(20):1641-1650 (2000)). In addition, CML progression is accompanied by secondary genetic alterations (Ahuja, H., et al., *J. Invest.* 78(6):2042-2047 (1991); and Honda, H., et al., *Blood* 95(4):1144-1150 (2000)); thus survival of late stage CML leukemia cells may no longer be dependent on BCR/ABL tyrosine kinase activity. Imatinib induced hematological responses have been less dramatic in blast crisis patients compared to what is observed in chronic phase patients (Druker, B. J., et al., *N. Engl. J Med.* 344(14):1038-1042 (2001)). Recently,

reactivation of BCR/ABL signaling either through mutation or amplification of BCR/ABL has been observed in patients that initially responded to Imatinib but then relapsed (Gorre, M. E., et al., *Science* 21:21 (2001); Barthe, C., et al., *Science* 293(5538):2163 (2001); and Hochhaus, A., et al., *Science* 293(5538):2163 (2001)). Finally, there have been no reports of patients undergoing Imatinib therapy becoming negative for the BCR/ABL translocation, suggesting the current therapy is not curative.

Additional therapies are needed to effectively eradicate cancers which are treated with signal transduction inhibitors that target tyrosine kinases, such as BCR/ABL positive leukemia.

Summary of the Invention

We have discovered that the combination of an mTOR inhibitor and a tyrosine kinase inhibitor is more effective than mTOR inhibitor monotherapy or tyrosine kinase inhibitor monotherapy for reducing the proliferation of and enhancing the apoptosis of cancer cells. The addition of an MEK (mitogen-activated protein kinase or extracellular signal-regulated kinase kinase) inhibitor to this combination further enhances the effectiveness of this combination therapy.

The present invention provides a method of treating a neoplasm in an individual in need thereof including administering to the patient at least one mTOR inhibitor in combination or in parallel with at least one tyrosine kinase inhibitor in amounts effective to treat the neoplasm.

Examples of mTOR inhibitors include, without limitation, any of the rapamycin macrolides described herein. Desirably, the mTOR inhibitor is a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

Tyrosine kinase inhibitors of the invention include small molecule inhibitors, tyrosine kinase antibodies, antisense oligomers, and RNAi inhibitors. Examples of tyrosine kinase inhibitors include any of those described herein. Desirable small molecule inhibitors include Imatinib, SU101, ZD1839, OSI-774,

CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, and MLN518. Desirable tyrosine kinase antibodies include trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

In another aspect, the invention features a method of treating leukemia in a patient in need thereof including administering rapamycin to the patient in amounts effective to treat the leukemia.

Cancers to be treated using the methods of the invention include, without limitation, carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage (i.e. leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell-lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkett's lymphoma); a hematopoietic tumor of myeloid lineage (i.e. acute myelogenous leukemia, chronic myelogenous leukemia, multiple myelogenous leukemia, myelodysplastic syndrome and promyelocytic leukemia); a tumor of mesenchymal origin (i.e. fibrosarcoma and rhabdomyosarcoma); a tumor of the central or peripheral nervous system (i.e. astrocytoma, neuroblastoma, glioma and schwannomas); melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

Any of the methods described herein can be used to treat a neoplasm having cells characterized by abnormally high levels of tyrosine kinase activity.

The abnormally high tyrosine kinase activity can be epithelial growth factor receptor (EGFR) kinase activity. Abnormally high EGFR activity can be characteristic of non-small-cell lung cancers, breast cancers, ovarian cancers, bladder cancers, prostate cancers, salivary gland cancers, pancreatic cancers, endometrial cancers, colorectal cancers, kidney cancers, head and neck cancers, and glioblastoma multiforme. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to EGFR can be used for the treatment of a cancers having abnormally high EGFR kinase activity. These include, but are not limited to, SU101, ZD1839, OSI-774, CI-1033, PKI166, GW2016, EKB-509,

BKB-569, trastuzumab, C225, MDX-H210, 2C4, MDX-447, EMD 72000, RH3 and ABX-EGF. In one embodiment, cancers having abnormally high EGFR kinase activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578. Desirably, the tyrosine kinase inhibitor targeted to EGFR is selected from trastuzumab, C225, and ZD1839.

The abnormally high tyrosine kinase activity can be human epidermal growth factor receptor-2 (HER2/ERB2) activity. Abnormally high HER2 activity can be characteristic of breast cancer, ovarian cancer, bladder cancer, salivary gland cancer, endometrial cancer, pancreatic cancer, and non-small-cell lung cancer. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to HER2 can be used for the treatment of a cancers having abnormally high HER2 activity. These include, but are not limited to, CI-1033, GW2016, trastuzumab, MDX-H210, MDX-447, ABX-EGF, and 2C4. In one embodiment, cancers having abnormally high HER2 activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578. Desirably, the tyrosine kinase inhibitor targeted to HER2 is trastuzumab.

The abnormally high tyrosine kinase activity can be platelet derived growth factor receptor (PDGFR) kinase activity. Abnormally high PDGFR activity can be characteristic of gastrointestinal stromal tumor, small cell lung cancer, glioblastoma multiforme, and prostate cancer. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to PDGFR can be used for the treatment of a cancers having abnormally high PDGFR kinase activity. These include, but are not limited to Imatinib, SU101, MLN518, and PTK787. In one embodiment, cancers having abnormally high PDGFR kinase activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578. Desirably, the tyrosine kinase inhibitor targeted to PDGFR is Imatinib.

The abnormally high tyrosine kinase activity can be Flt-3 activity. Abnormally high Flt-3 activity can be characteristic of acute myeloid leukemia. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to Flt-3 can be used for the treatment of a cancers having abnormally high Flt-3 kinase activity. These include, but are not limited to MLN518, SU11248, and PKC412. In one embodiment, cancers having abnormally high Flt-3 kinase activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578. Desirably, the tyrosine kinase inhibitor targeted to Flt-3 is PKC412.

The abnormally high tyrosine kinase activity can be tropomyosin receptor kinase (Trk) activity. Abnormally high Trk activity can be characteristic of prostate cancer and pancreatic cancer. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to Trk can be used for the treatment of a cancers having abnormally high Trk kinase activity. These include, but are not limited to CEP701 or CEP705. In one embodiment, cancers having abnormally high Trk kinase activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

Abnormally high vascular endothelial growth factor receptor (VEGFR) kinase activity is not typically found in the cells of a neoplasm, but is often found in the endothelial cells which vascularize the neoplasm. Thus, vascular endothelial growth factor receptor (VEGFR) kinase activity is a useful target for most solid tumors. Using the methods of the present invention, a tyrosine kinase inhibitor targeted to VEGFR can be used for the treatment of solid tumors. These include, but are not limited to SU5416, SU6668, ZD4190, ZD6474, PTK787, IMC-1C11, and rhu-Mab VEGF. In one embodiment, cancers having abnormally high VEGFR kinase activity are treated by administering any one of the above-listed tyrosine kinase inhibitors together or in parallel with a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

The abnormally high tyrosine kinase activity can be constitutively active tyrosine kinase BCR/ABL. BCR/ABL kinase activity can be characteristic of chronic myelogenous leukemia (CML). Using the methods of the invention, CML patients are treated, for example, with a combination of a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578 and a tyrosine kinase inhibitor which is active against BCR/ABL. Desirably, the kinase inhibitor which is active against BCR/ABL is Imatinib.

In any of the above methods, an MEK inhibitor can be included to enhance the effectiveness of the combination therapy. Examples of MEK inhibitors include any of those described herein. In one embodiment, the MEK inhibitor is selected from the group consisting of PD184352, PD198306, PD98059, UO126, Ro092210, and L783277. For example, an mTOR inhibitor, Imatinib, and UO126, an MEK inhibitor, can be used to treat CML or GIST.

Any of the combinations described herein can be used to treat a neoplasm which is resistant to monotherapy using the tyrosine kinase inhibitor of the combination. For example, the combination of an mTOR inhibitor and Imatinib, or the combination of an mTOR inhibitor and Imatinib and MEK inhibitor, can be used to treat Imatinib-resistant neoplasms. In one embodiment, the combination of an mTOR inhibitor and Imatinib can be used to treat Imatinib-resistant CML and GIST. In another example, the combination of mTOR inhibitor and PCK412, or the combination of an mTOR inhibitor and PCK412 and MEK inhibitor, can be used to treat PCK412-resistant neoplasms. In one embodiment, the combination of an mTOR inhibitor and PCK412 can be used to treat PCK412-resistant acute myeloid leukemia.

For any of the above methods, the mTOR and the tyrosine kinase inhibitors, or the mTOR, tyrosine kinase, and MEK inhibitors, can be administered in parallel within 30 days of each other. Desirably, the mTOR and tyrosine kinase inhibitors are administered in parallel within five days of each other, 24 hours of each other, simultaneously, or they are administered together. For the three component therapeutic combinations including mTOR, tyrosine kinase, and MEK inhibitors, each component is, desirably, administered in

parallel within five days of another component, 24 hours of another component, or all three are administered simultaneously, or together.

For any of the combinations described herein, the invention also features a method of determining whether a neoplasm in a human patient responds to a combination including an mTOR inhibitor and tyrosine kinase inhibitor. This method includes the steps of (a) administering the combination to the human patient; and (b) monitoring the patient to determine whether the neoplasm responds to the combination. Optionally, the combination includes an MEK inhibitor. This method can be performed, for example, to determine whether the combination has enhanced efficacy in comparison to monotherapy using any one of the inhibitors in the combination. This method can also be used to determine which regimens are effective for treating the neoplasm (e.g., variables include the amount of each inhibitor in the combination, routes of administration for each inhibitor, and/or the intervals between administrations). This method can also be used to determine which types of neoplasms respond to the combination therapy.

Administration of the mTOR, tyrosine kinase, and MEK inhibitors can be achieved by a variety of routes, such as by parenteral routes (e.g., intravenous, intraarterial, intramuscular subcutaneous injection), topical, inhalation (e.g., intrabronchial, intranasal or oral inhalation or intranasal drops), oral, rectal, or other routes.

The present invention features a pharmaceutical pack including a rapamycin macrolide and a tyrosine kinase inhibitor. Desirably, the rapamycin macrolide and the tyrosine kinase inhibitor are formulated separately and in individual dosage amounts. The pharmaceutical pack may further include an MEK inhibitor.

The present invention also features a pharmaceutical composition including an effective amount of a rapamycin macrolide and a tyrosine kinase inhibitor, together with a pharmaceutically acceptable carrier or diluent. The pharmaceutical composition may further include an MEK inhibitor.

Compounds useful in the present invention include those described herein in any of their pharmaceutically acceptable forms, including isomers such as diastereomers and enantiomers, salts, and solvates thereof, as well as racemic mixtures of the compounds described herein.

By "treating" is meant to slow the spreading of the cancer, to slow the cancer's growth, to kill or arrest cancer cells that may have spread to other parts of the body from the original tumor, to relieve symptoms caused by the cancer, or to prevent cancer. The symptoms to be relieved using the combination therapies described herein include pain, and other types of discomfort.

The terms "administration" and "administering" refer to a method of giving a dosage of a pharmaceutical composition to a patient, where the method is, e.g., topical, oral, intravenous, intraperitoneal, or intramuscular. The preferred method of administration can vary depending on various factors, e.g., the components of the pharmaceutical composition, site of the potential or actual disease, and severity of disease.

By administration "in parallel" is meant that the mTOR inhibitor, tyrosine kinase inhibitor, and, optionally, the MEK inhibitor are formulated separately and administered separately.

By administered "together" is meant that the mTOR inhibitor, tyrosine kinase inhibitor, and, optionally, the MEK inhibitor are formulated together in a single pharmaceutical composition and administered together.

By "effective amount" is meant the amount of a compound required to treat a neoplasm. The effective amount of mTOR inhibitor, tyrosine kinase inhibitor, and, optionally, the MEK inhibitor used to practice the present invention for the treatment of a neoplasm varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician, will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

As used herein, "individual" or "patient" includes humans, cattle, pigs, sheep, horses, dogs, and cats, and also includes other vertebrates, most preferably, mammalian species.

By "rapamycin macrolide" is meant naturally occurring forms of rapamycin in addition to rapamycin analogs and derivatives which target and inhibit mTOR.

By "tyrosine kinase inhibitor" is meant a molecule that inhibits the function or the production of one or more tyrosine kinases. Tyrosine kinase inhibitors include small molecule inhibitors of tyrosine kinases, antibodies to tyrosine kinases, and antisense oligomers and RNAi inhibitors that reduce the expression of tyrosine kinases.

By "small molecule" inhibitor is meant a molecule of less than about 3,000 daltons having tyrosine kinase antagonist activity.

By "antisense" or "antisense oligomer" is meant any oligonucleotide or oligonucleoside that acts to inhibit the expression or function of a tyrosine kinase.

By "RNAi inhibitor" is meant any double stranded RNA that acts to inhibit the expression or function of a tyrosine kinase (for an example of RNAi technology, see Zamore et al., *Cell* 101:25-33 (2000)).

By "abnormally high levels of kinase activity" is meant an increase in tyrosine kinase activity associated with malignant cell growth. An increase in tyrosine kinase activity can result from overexpression or mutation of a kinase gene. For example, the BCR/ABL mutation encodes a cytoplasmic protein with aberrant constitutive tyrosine kinase activity, resulting in uncontrolled proliferation.

As used herein, "resistance" or "resistant" refers to a neoplasm having cells that express a resistant mutant form of the tyrosine kinase or cells that overexpress the tyrosine kinase targeted by the tyrosine kinase inhibitor used in the combination therapy described herein. Resistance includes other known mechanisms of resistance (e.g., efflux pump in resistant cells). The net effect of the resistance is that the use of the tyrosine kinase inhibitor as a monotherapy for the treatment of the resistant cells is less effective than when used to treat a non-resistant cells.

As used herein, a neoplasm "responds" to a combination of mTOR inhibitor, tyrosine kinase inhibitor, and, optionally, MEK inhibitor, if the spread of the neoplasm is slowed, if the growth of the neoplasm is slowed, if neoplasm cells spreading from the site of origin to other parts of the body are killed or arrested, or if the combination relieves symptoms caused by the neoplasm. The symptoms relieved when a neoplasm responds to the combination therapies described herein include pain, and other types of discomfort.

As used herein, "monitoring" a patient to determine whether the neoplasm responds to the combination therapy includes any established protocol for monitoring the progression of a neoplastic disorder. Monitoring can include, for example, the use of biopsies, use of surrogate markers (e.g., PSA levels in prostate cancer patients), and the use of imaging techniques (e.g., CT scans, bone scans, chest x-rays, MRI scans) to see if the neoplasm has grown or spread, among other protocols.

The invention provides methods of treating neoplasms associated with enhanced tyrosine kinase activity, allowing for improved cancer therapy while permitting lower doses of mTOR, tyrosine kinase, and, optionally, MEK inhibitors to be used. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

FIG. 1A is a graph illustrating the effects of rapamycin (R) on BCR-ABL-transformed primary B lymphoblasts.

FIG. 1B is a graph illustrating the effects of rapamycin (R) and Imatinib (I) on BCR-ABL-transformed primary B lymphoblasts.

FIG. 2A is a graph illustrating the effects of Imatinib (I) and rapamycin (R) on BCR/ABL-evoked myeloid colony outgrowth.

FIG. 2B is a graph illustrating the effects of Imatinib (I) and rapamycin (R) on K562 cells derived from a blast crisis CML patient.

FIG. 3A is a graph illustrating the inhibitory effects of Imatinib (I) and rapamycin (R) in Ba/F3 cells expressing wild type BCR/ABL.

FIG. 3B is a graph illustrating the inhibitory effects of Imatinib (I) and rapamycin (R) in Ba/F3 cells expressing Imatinib-resistant BCR/ABL.

FIG. 4A is a picture of an immunoblotting assay illustrating the inhibitory effects of Imatinib (I) and rapamycin (R) in Ba/F-BCR/ABL WT and Imatinib-resistant Ba/F-BCR/ABL T315I cells on the activation of p70 S6K, Erk1/2 kinases.

FIG. 4B is a picture of an immunoblotting assay illustrating the inhibitory effects of Imatinib (I) and rapamycin (R) in Imatinib-resistant Ba/F-BCR/ABL T315I cells on the phosphorylation of 4E-BP1.

FIG. 5A is a graph illustrating the effects of PKC412 (P) and rapamycin (R) on the proliferation of PKC412-sensitive Ba/F-FLT3-ITD cells.

FIG. 5B is a graph illustrating the effects of PKC412 (P) and rapamycin (R) on the proliferation of PKC412-resistant Ba/F-FLT3-ITD F691I cells.

FIG. 5C is a picture of an immunoblotting assay illustrating the inhibitory effects of PKC412 (P) and rapamycin (R) in PKC412-sensitive Ba/F-FLT3-ITD cells on the activation of p70 S6K kinases.

FIG. 5D is a picture of an immunoblotting assay illustrating the inhibitory effects of PKC412 (P) and rapamycin (R) in PKC412-sensitive Ba/F-FLT3-ITD cells on the activation of 4E-BP1 kinases.

FIG. 6A is a graph illustrating the effects of Imatinib (I) or rapamycin (R) or UO126 (UO) alone or in various combinations, as indicated, on the proliferation of bone marrow cells expressing BCR/ABL.

FIG. 6B is a graph illustrating the effects of Imatinib (I) or rapamycin (R) or UO126 (UO) alone or in various combinations, as indicated, on the proliferation of Imatinib-resistant Ba/F-BCR/ABL T315I cells.

FIG. 6C is a graph illustrating the effects of PKC412 (P) or rapamycin (R) or UO126 (UO) alone or in various combinations, as indicated, on the proliferation of PKC412-resistant Ba/F-FLT3-ITD F691I cells.

FIG. 7 is a graph illustrating the effect of Herceptin and rapamycin (RAPA) on proliferation of MCF-7 cells.

FIG. 8 is a graph illustrating the effect of Herceptin and rapamycin (RAPA) on proliferation of SKBR3 cells.

FIG. 9 is a graph illustrating the effects of Imatinib and rapamycin (RAPA) on CWR-22 cells.

FIG. 10 is a graph illustrating the effects of Imatinib and rapamycin (RAPA) on LnCap cells.

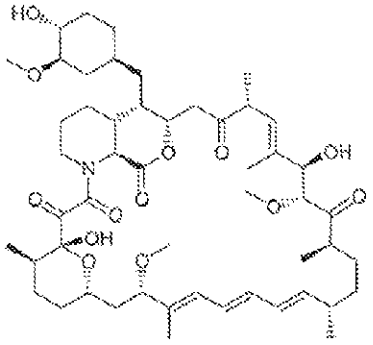
FIG. 11 is a graph illustrating the effect of Imatinib (I) and rapamycin (R) on survival rates in CML tumor bearing mice.

Detailed Description of the Invention

We have discovered that the combination of an mTOR and tyrosine kinase inhibitors, or mTOR, tyrosine kinase, and MEK inhibitors, is more effective than rapamycin macrolide monotherapy or tyrosine kinase inhibitor monotherapy for reducing the proliferation of and increasing the apoptosis of cancer cells.

Rapamycin Macrolides

Rapamycin (Sirolimus) is an immunosuppressive lactam macrolide that is produced by *Streptomyces hygroscopicus*, and which has the structure depicted in Formula I. See, for example, McAlpine, J. B., et al., *J. Antibiotics* 44: 688 (1991); Schreiber, S. L., et al., *J. Am. Chem. Soc.* 113: 7433 (1991); and U.S. Patent No. 3,929,992, incorporated herein by reference.



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Wherever the present application refers to "rapamycin macrolide", in addition to naturally occurring forms of rapamycin, the invention further includes rapamycin analogs and derivatives. Many such analogs and derivatives are known in the art. Examples include those compounds described in U.S. Patent Nos. 6,329,386; 6,200,985; 6,117,863; 6,015,815; 6,015,809; 6,004,973; 5,985,890; 5,955,457; 5,922,730; 5,912,253; 5,780,462; 5,665,772; 5,637,590; 5,567,709; 5,563,145; 5,559,122; 5,559,120; 5,559,119; 5,559,112; 5,550,133; 5,541,192; 5,541,191; 5,532,355; 5,530,121; 5,530,007; 5,525,610; 5,521,194; 5,519,031; 5,516,780; 5,508,399; 5,508,290; 5,508,286; 5,508,285; 5,504,291; 5,504,204; 5,491,231; 5,489,680; 5,489,595; 5,488,054; 5,486,524; 5,486,523; 5,486,522; 5,484,791; 5,484,790; 5,480,989; 5,480,988; 5,463,048; 5,446,048; 5,434,260; 5,411,967; 5,391,730; 5,389,639; 5,385,910; 5,385,909; 5,385,908; 5,378,836; 5,378,696; 5,373,014; 5,362,718; 5,358,944; 5,346,893; 5,344,833; 5,302,584; 5,262,424; 5,262,423; 5,260,300; 5,260,299; 5,233,036; 5,221,740; 5,221,670; 5,202,332; 5,194,447; 5,177,203; 5,169,851; 5,164,399; 5,162,333; 5,151,413; 5,138,051; 5,130,307; 5,120,842; 5,120,727; 5,120,726; 5,120,725; 5,118,678; 5,118,677; 5,100,883; 5,023,264; 5,023,263; and 5,023,262; all of which are incorporated herein by reference.

Desirable rapamycin macrolides for use in the present methods include rapamycin, CCI-779, Everolimus (also known as RAD001), and ABT-578. CCI-779 is an ester of rapamycin (42-ester with 3-hydroxy-2-hydroxymethyl-2-methylpropionic acid), disclosed in U.S. Patent No. 5,362,718. Everolimus is an alkylated rapamycin (40-O-(2-hydroxyethyl)-rapamycin, disclosed in U.S. Patent No. 5,665,772.

Tyrosine Kinase Inhibitors

Any tyrosine kinase inhibitor can be used in the methods of the present invention. These include small molecule inhibitors, antibodies to tyrosine kinase, antisense oligomers, and RNAi inhibitors.

The tyrosine kinase inhibitor used in the methods of the invention will be selected based upon the type of cancer being treated. Specifically, the inhibitor is

selected based upon which tyrosine kinases exhibit abnormally high levels of activity characteristic of the cancer to be treated. For example, the BCR/ABL fusion protein occurs in 95% of CML and 10-15% of acute lymphoblastic leukemia patients. Accordingly, using methods of the invention, CML patients can be treated, for example, with a combination of rapamycin and a tyrosine kinase inhibitor which is active against BCR/ABL.

Tyrosine kinase inhibitors and their respective targets are provided in Table 1 (small molecule inhibitors) and Table 2 (tyrosine kinase antibodies).

Table 1. Selected Small Molecule Tyrosine Kinase Inhibitors

Drug	Company	Target(s)
Imatinib (Gleevec™)	Novartis	PDGFR (c-kit), abl,
SU101 (Leflunomide)	Pharmacia	PDGFR, EGFR
ZD1839 (Iressa™)	AstraZeneca	EGFR (HER1)
OSI-774 (Tarceva™)	Oncogene Science	EGFR (HER1)
CI-1033	Pfizer	EGFR (HER1 and HER2)
SU5416	Pharmacia	VEGFR, PDGFR
SU6668	Pharmacia	VEGFR, PDGFR, FGFR
ZD4190	AstraZeneca	VEGFR (KDR/Flt-1)
ZD6474	AstraZeneca	VEGFR (KDR/Flt-1)
PTK787	Novartis	VEGFR (KDR/Flt-1), PDGFR, c-kit
PKI166	Novartis	EGFR (HER1)
GW2016	GlaxoSmithKline	EGFR (HER1 and HER2)
EKB-509	Wyeth	EGFR (HER1)
EKB-569	Wyeth	EGFR (HER1)
CEP-701	Cephalon	Trk
CEP-751	Cephalon	Trk
MLN518	Millenium	Flt-3, PDGFR (c-kit)
SU11248	Pharmacia	Flt-3
PKC412	Novartis	Flt-3

Table 2. Selected Antibody Tyrosine Kinase Inhibitors

Drug	Company	Target(s)
trastuzumab (Herceptin™)	Genentech	EGFR (HER2)
C225 (Erbitux™)	ImClone	EGFR
rhu-Mab VEGF (Avastin™)	Genentech	VEGFR
MDX-210	Medarex	EGFR (HER2)
2C4	Genentech	EGFR (HER2)
MDX-447	Medarex	EGFR
ABX-EGF	Abgenix	EGFR
EMD 72000	Merck	EGFR
RH3	York Medical	EGFR
IMC-1C11	ImClone	VEGFR2

MEK Inhibitors

Any MEK inhibitor can be used in the methods of the present invention. MEK inhibitors can be identified using known MEK inhibition assays. For example, the assays described in U.S. Patent No. 5,525,625 or in WO 02/06213 A1, can be used to identify MEK inhibitors. Examples of MEK inhibitors include those compounds described in U.S. Patent Nos. 6,545,030, 6,506,798, 6,492,363, 6,469,004, 6,455,582, 6,440,966, 6,310,060, 6,214,851, and 5,525,625, and U.S. Publication Nos. US 2003/0092748 A1, US 2003/0078428 A1, US 2003/0045521 A1, US 2003/0004193 A1, and US 2002/0022647 A1.

The MEK inhibitor used in the methods of the invention will be combined with a rapamycin macrolide and a tyrosine kinase inhibitor selected based upon the type of cancer being treated. Specifically, the inhibitor is selected based upon which tyrosine kinases exhibit abnormally high levels of activity characteristic of the cancer to be treated. For example, the BCR/ABL fusion protein occurs in 95% of CML and 10-15% of acute lymphoblastic leukemia patients. Accordingly, using methods of the invention, CML patients can be treated, for example, with a combination of rapamycin macrolide, MEK inhibitor, and a tyrosine kinase inhibitor which is active against BCR/ABL.

Several MEK inhibitors which can be used to practice the methods described herein are provided in Table 3.

Table 3. Selected MEK Inhibitors

Drug	Company
PD184352/CI-1044	Pfizer
PD198306	Pfizer
PD98059	Pfizer
UO126	Promega
Ro092210	Roche
L783277	Merck

Therapy

The methods of the present invention can be used for the treatment of a variety of cancers. In particular, the present methods can be used for the treatment of CML. CML progresses through distinct clinical stages. The earliest stage, termed the chronic phase, is characterized by the expansion of terminally differentiated neutrophils. Over several years the disease progresses to an acute phase termed blast crisis, characterized by maturation arrest with excessive numbers of undifferentiated myeloid or lymphoid progenitor cells. The BCR-ABL oncogene is expressed at all stages, but blast crisis is characterized by multiple additional genetic and molecular changes. Once the patient enters the blast crisis phase of the disease there are few curative options available.

The present methods can be used to treat both the acute and chronic stages of CML. This is demonstrated in FIGS. 1A, 1B, 2A, and 2B; and Table 4. The combination of rapamycin and Imatinib is much more effective in preventing proliferation and colony formation than either drug used alone.

In the methods of the present invention, the dosage and frequency of administration of the mTOR, tyrosine kinase, and, optionally, MEK inhibitors can be controlled independently. For example, one compound may be administered orally three times per day, while the second compound may be administered intravenously once per day. The compounds may also be formulated together such that one administration delivers two, or even all three, of the compounds.

The exemplary dosage of mTOR, tyrosine kinase, and MEK inhibitor to be administered will depend on such variables as the type and extent of the disorder,

the overall health status of the patient, the therapeutic index of the selected rapamycin macrolide and tyrosine kinase inhibitor, and their route of administration. Standard clinical trials maybe used to optimize the dose and dosing frequency for any particular combination of the invention.

Pharmaceutical Compositions

The invention features methods of treating cancer by administering an mTOR inhibitor, a tyrosine kinase inhibitor, and, optionally, an MEK inhibitor together or in parallel with each other. These may be formulated together or separately and administered to patients with a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Administration may be topical, parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration.

Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" (20th ed., ed. A.R. Gennaro AR., 2000, Lippincott Williams & Wilkins). Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the mTOR inhibitor and/or tyrosine kinase inhibitor and/or MEK inhibitor. Nanoparticulate formulations (e.g., biodegradable nanoparticles, solid lipid nanoparticles, liposomes) may be used to control the biodistribution of the rapamycin macrolide and/or tyrosine kinase inhibitor. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example,

lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel. The concentration of the mTOR inhibitor, tyrosine kinase inhibitor, and, optionally, MEK inhibitor in the formulation will vary depending upon a number of factors, including the dosage of the drug to be administered, and the route of administration.

The mTOR inhibitor and/or tyrosine kinase inhibitor and/or MEK inhibitor may be optionally administered as a pharmaceutically acceptable salt, such as a non-toxic acid addition salts or metal complexes that are commonly used in the pharmaceutical industry. Examples of acid addition salts include organic acids such as acetic, lactic, pamoic, maleic, citric, malic, ascorbic, succinic, benzoic, palmitic, suberic, salicylic, tartaric, methanesulfonic, toluenesulfonic, or trifluoroacetic acids or the like; polymeric acids such as tannic acid, carboxymethyl cellulose, or the like; and inorganic acid such as hydrochloric acid, hydrobromic acid, sulfuric acid phosphoric acid, or the like. Metal complexes include zinc, iron, and the like.

Administration of any of the mTOR inhibitor, tyrosine kinase inhibitor, or MEK inhibitor in controlled release formulations is useful where the inhibitor has (i) a narrow therapeutic index (e.g., the difference between the plasma concentration leading to harmful side effects or toxic reactions and the plasma concentration leading to a therapeutic effect is small; generally, the therapeutic index, TI, is defined as the ratio of median lethal dose (LD_{50}) to median effective dose (ED_{50})); (ii) a narrow absorption window in the gastro-intestinal tract; or (iii) a short biological half-life, so that frequent dosing during a day is required in order to sustain the plasma level at a therapeutic level.

Many strategies can be pursued to obtain controlled release in which the rate of release outweighs the rate of metabolism of the rapamycin macrolide and/or tyrosine kinase inhibitor. For example, controlled release can be obtained by the appropriate selection of formulation parameters and ingredients, including, e.g., appropriate controlled release compositions and coatings. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions,

emulsions, microcapsules, microspheres, nanoparticles, patches, and liposomes.

Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents or fillers (e.g., sucrose and sorbitol), lubricating agents, glidants, and antiadhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc).

Formulations for oral use may also be provided as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the methods and compounds claimed herein are performed, made, and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention.

Experimental Procedures

Cell lines and cell culture

Ba/F3 cell lines expressing p210 BCR/ABL wild type (Ba/F-BCR/ABL WT), p210 BCR/ABL T315I (Ba/F-BCR/ABL T315I), FLT3-ITD (Ba/F-FLT3-ITD), FLT3-ITD F691I (Ba/F-FLT3-ITD F691I) were grown in RPMI 1640 with 10% (v/v) fetal calf serum (FCS) plus antibiotics (Penicillin/Streptomycin). BCR/ABL-transformed B-lymphoblasts were generated as described previously (Sattler et al., *Cancer Cell* 1:479 (2002)) and maintained in RPMI plus 20% FCS, antibiotics and 50 μM 2-mercaptoethanol (2-ME). K562 cells (ATCC number CCL-243) were cultured in RPMI supplemented with 10% FCS and antibiotics (Penicillin/Streptomycin).

Reagents

Imatinib (Novartis Pharmaceuticals, Basel, Switzerland), rapamycin (Sigma Chemical Co., St. Louis, MO), PKC412 (Novartis), and UO126

(Calbiochem, La Jolla, CA) solutions were prepared in sterile DMSO and stored at -20 C.

Proliferation, cell cycle and apoptosis assays

BCR/ABL-transformed primary B-lymphoblasts (1×10^4 cells/well) were cultured in 96-well plates in RPMI medium containing 20% FCS for 24 hours. Ba/F-BCR/ABL WT, Ba/F-BCR/ABL T315I, Ba/F-FLT3-ITD, Ba/F-FLT3-ITD F691I and K562 cells (3.5×10^3 cells/well) were cultured in 96-well plates in RPMI containing 10% FCS for 48 or 60 hours. Cells were exposed to varying concentrations and combinations of drugs, as indicated. Four to six hours before harvesting, [^{3}H]-thymidine (1 μCi/well) was added and [^{3}H]-thymidine incorporation was determined using a Cell Harvester (Skatron, Sterling, VA).

For cell cycle and apoptosis assays, live cells from randomly growing BCR/ABL-transformed B-lymphoblasts were isolated using Histopaque (Sigma). After washing twice, the cells were resuspended in regular growth medium (RPMI containing 20% FCS and 50 μM 2-mercaptoethanol), exposed to inhibitors at the indicated concentrations for 24 hours, harvested and fixed in 70% ethanol for 3 hours at –20 C. Fixed cells were stained with propidium iodide, and cell cycle parameters were analyzed by using FACScan and Modfit LT software.

Bone marrow transduction and colony formation assays

A high-titer, helper virus-free retroviral stock of MSCV p210 (BCR/ABL)-IRES-GFP was prepared by transient transfection of 293T cells using the *kat* ecotropic packaging system (Million and Van Etten, *Blood* 96:664 (2000)). Bone marrow transduction and colony formation assays were performed as described previously (Sattler et al., *Cancer Cell* 1:479 (2002)). Briefly, bone marrow cells from mice were transduced with BCR/ABL-expressing retroviruses and then plated in triplicate in MethoCult M3234 medium (Stem Cell Technologies, Vancouver) at 1×10^5 cells/35 mm dish in the presence or absence of different

concentrations of drugs and maintained at 37 °C, 5% CO_2. Myeloid colonies were scored at day 10.

Immunoblotting

Cells were lysed in a buffer containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), NP40 (1% v/v), NaF (10 mM), 2 mM sodium orthovanadate and a cocktail of protease inhibitors, as described previously (Gu et al., *Mol. Cell. Biol.* 20:7109 (2000)). Cell lysates containing equivalent amounts of protein (50 g) were loaded and separated by SDS-PAGE. Immunoblotting was performed using phospho-specific antibodies reactive with Thr389 of p70 S6K, Ser65 of 4E-BP1 or Thr202/Tyr204 of p44/42 Erk (Cell Signaling Technology, Beverly, MA). Detection was by enhanced chemiluminescence (ECL). To control for equal loading, blots were reprobed with antibodies that detect total Erk2 or p70 S6K (Santa Cruz Biotechnology).

Example 1: Effects of rapamycin and/or Imatinib on BCR-ABL-transformed primary B lymphoblasts.

BCR/ABL-transformed primary B lymphoblast cells were seeded in triplicate in 96 well plates at 1×10^4 cells/well and exposed to various concentrations (0.25-10 nM) of rapamycin for 24 hours. As a control, cells were treated with DMSO vehicle for 24 hours. Cell proliferation was measured by [^{3}H]-thymidine incorporation, expressed as the percentage of control (DMSO-treated) incorporation. The results are provided in FIG. 1A.

BCR/ABL-transformed primary B lymphoblasts were exposed to the indicated concentrations provided in FIG. 1B of Imatinib (0.25-4 μM) alone or in combination with rapamycin (2 nM) for 24 hours, followed by measurement of [^{3}H]-thymidine incorporation. The results shown are representative of three independent experiments

Rapamycin inhibited the proliferation of these cells at doses significantly below typical serum levels (~5-15 nM) achieved in transplant patients (MacDonald et al., *Clin. Ther.* 22 Suppl. B:B101(2000)) (see FIG. 1A). As

expected, Imatinib also inhibited the proliferation of BCR/ABL-transformed primary B-lymphoblasts in a dose-dependent manner (FIG. 1B). Remarkably, combining rapamycin with Imatinib resulted in markedly decreased proliferation (FIG. 1B). Notably, the doses of each agent that, when used in combination, caused profound inhibition of cell proliferation were below the typical serum levels of the two drugs when used alone for anti-leukemic (Imatinib) or immunosuppressive (rapamycin) therapy (Druker et al., *N. Engl. J. Med.* 344:1038 (2002); MacDonald et al., *Clin. Ther.* 22 Suppl. B:B101(2000)).

Example 2: Effects of rapamycin and/or Imatinib on BCR/ABL-evoked myeloid colony outgrowth and on K562 cells derived from a blast crisis CML patient.

Bone marrow (BM) cells from wild type (WT) mice were transduced with BCR/ABL-expressing retroviruses and plated in methylcellulose, in triplicate using MethoCult M3234 medium, in the absence of cytokines. As expected, BCR/ABL promoted cytokine-independent myeloid colony outgrowth (Gishizky and Witte, *Science* 256:836 (1992)). Rapamycin (2-10 nM) or Imatinib (0.5 μM) alone inhibited myeloid colony formation by 50-60% (see FIG. 2A). However, combining these two agents resulted in greater than 90% decrease in BCR/ABL-induced myeloid colonies. This data shows that a combination of Imatinib and rapamycin may be more effective therapy for treatment of CML patients.

To ensure that the effects of the Imatinib /rapamycin combination were not restricted to murine cells, we carried out similar experiments on K562 cells, which are derived from a blast crisis CML patient (Lozzio and Lozzio, *Blood* 45:321 (1975)). Cells were exposed to the indicated concentrations (0.125 -1 μM), as indicated in FIG. 2B, of Imatinib alone or in combination with rapamycin (5 nM) for 60 hours. Cell proliferation was measured by [^{3}H]-thymidine incorporation, expressed as percentage of control (vehicle-treated) cells.

Even in this highly transformed cell line, rapamycin alone had some ability to inhibit [^{3}H]-thymidine incorporation up to ~25% inhibition at 5 nM (see FIG. 2B). Moreover, co-administration of rapamycin and Imatinib resulted in a dramatically increased inhibition. Together, these results suggest that

combination therapy with these two approved drugs may have broad efficacy against BCR/ABL-transformed cells, and may have activity in CML blast crisis.

Example 3: Rapamycin enhances the growth inhibitory effects of Imatinib in Ba/F3 cells expressing wild type and Imatinib-resistant BCR/ABL.

Imatinib resistance is an emerging clinical problem. Because rapamycin inhibits the proliferation of BCR/ABL-transformed lymphoid and myeloid cells, and rapamycin acts on a distinct downstream target of BCR/ABL, we tested whether rapamycin inhibited the proliferation of hematopoietic cells expressing Imatinib-resistant mutants of BCR/ABL. Ba/F-BCR/ABL WT (FIG. 3A) and Ba/F-BCR/ABL T315I (Imatinib-resistant) (FIG. 3B) cells were seeded in a 96-well plate at 3.5 X 10^3 cells/well in the presence of the indicated concentrations of Imatinib or rapamycin (5 nM) alone or in combination. Cell proliferation was measured after 48 hr of drug treatment. Values represent the means for triplicate determinations; *bars* ± SD (see FIGs. 3A and 3B).

As in the other cell systems (FIGS. 1, 2), rapamycin (5 nM) inhibited the proliferation of Ba/F-BCR/ABL WT cells (~ 60% after 48 hours of exposure). Likewise, Imatinib potently inhibited the proliferation of these cells, and combining the two agents resulted in enhanced inhibition (FIG. 3A).

Rapamycin alone showed comparable inhibition of proliferation of Ba/F3 cells expressing BCR/ABL WT and BCR/ABL T315I (FIGS. 3A,B), as would be predicted if mTOR were a critical downstream effector of BCR/ABL. Consistent with the Imatinib resistance of the T315I mutant, doses of Imatinib (0.5-1 μM) that inhibited proliferation of Ba/F-BCR/ABL WT cells by more than 50% caused little or no inhibition of Ba/F-BCR/ABL T315I cells (FIG. 3B). Remarkably, however, combining low dose rapamycin with Imatinib markedly enhanced the growth inhibitory effect of Imatinib on Ba/F-BCR/ABL T315I cells (FIG. 3B). These results suggest that combining rapamycin with Imatinib may be useful in treating Imatinib-resistant CML.

Example 4: Immunoblotting BCR/ABL-Expressing Cells with Phospho-Specific Antibodies.

Ba/F-BCR/ABL WT and Ba/F-BCR/ABL T315I cells were incubated with the indicated concentrations (see FIG. 4A) of Imatinib/Rapamycin for 18 hours. Cell lysates were prepared and equal amounts (50 μg) of protein were resolved by SDS-PAGE followed by immunoblot analysis with the indicated phospho-specific antibodies. To control for loading, the blots were stripped and reprobed with anti-Erk2 antibodies.

The effects of Imatinib and Rapamycin on 4E-BP1 phosphorylation were determined in Ba/F-BCR/ABL T315I cells by immunoblotting with phospho-4E-BP1 antibodies. Erk2 was used a loading control (see FIG. 4B).

Treatment of Ba/F-BCR/ABL WT cells with a low dose of Imatinib (0.5 μM), which causes ~70% inhibition of proliferation (FIG. 4A), resulted in partial inhibition of p70 S6K activation (FIG. 4B). Not surprisingly, the same dose failed to inhibit p70 S6K in Imatinib-resistant Ba/F-BCR/ABL T315I cells. At higher doses of Imatinib (4 μM), which completely inhibit Ba/F-BCR/ABL WT proliferation, p70 S6K activation was nearly completely inhibited as well, whereas in Ba/F-BCR/ABL T315I cells, which show only partial inhibition of proliferation at this dose, p70 S6K was inhibited by only ~50%. Thus, the partial inhibition of proliferation in Ba/F-BCR/ABL T315I cells treated with 4 μM Imatinib was paralleled by partial inhibition of p70 S6K activation. However, rapamycin at doses between 0.5-5 nM almost completely inhibited activation of p70 S6K in both Ba/F-BCR/ABL WT and Ba/F-BCR/ABL T315I cells (FIG 4A). Interestingly, 4E-BP1 phosphorylation displayed more resistance to treatment with either Imatinib or rapamycin than p70 S6K. Treatment of Ba/F-BCR/ABL T315I cells with Imatinib (0.5-4 μM) or rapamycin (5 nM) alone caused partial inhibition of 4E-BP1phosphorylation (FIG. 4B); at the same dose, rapamycin treatment totally inhibited p70 S6K phosphorylation. Combining Imatinib with rapamycin led to complete inhibition of 4E-BP1phosphorylation in Ba/F-BCR/ABL T315I cells (FIG. 4B). As expected, rapamycin treatment did not inhibit Erk activation in either of these cell lines. Although Imatinib potently

inhibited Erk activation in Ba/F-BCR/ABL WT cells, it failed to inhibit Erk activation in Imatinib-resistant cells (FIG. 4A). Indeed, Erk activation actually increased in Ba/F-BCR/ABL T315I cells at higher doses of Imatinib. The reason for the paradoxical effect of high doses of Imatinib in these cells is unclear, although similar results were obtained in studies of Imatinib-resistant K562 cells treated with Imatinib (Yu et al., *Cancer Res.* 62:188 (2002)).

Taken together, our results indicate that sub-therapeutic doses of Imatinib (e.g., low dose treatment of Ba/F-BCR/ABL WT cells or high dose treatment of Imatinib-resistant cells), leave the p70 S6K arm of the mTOR pathway partially active and thus susceptible to further inhibition by rapamycin.

Example 5: Effects of PKC412 and rapamycin on proliferation of Ba/F-FLT3-ITD cells.

PKC412-sensitive Ba/F-FLT3-ITD (FIG. 5A) and PKC412-resistant Ba/F-FLT3-ITD F691I (FIG. 5B) cells were exposed to the indicated concentrations of PKC412 for 48 hours in the presence or absence of 2 nM rapamycin, after which cell proliferation was measured by [^{3}H]-thymidine incorporation. Values represent the means for triplicate determinations; *bars* ± SD (see FIGS. 5A and 5B).

As expected, PKC412 inhibited the proliferation of Ba/F-FLT3-ITD cells in a dose-dependent manner. Moreover, whereas PKC412 (5 nM) or rapamycin (2 nM) alone caused approximately 55-60% inhibition, combining these drugs led to more than 94% inhibition of proliferation of Ba/F-FLT3-ITD cells (FIG. 5A). We also tested the effects of the rapamycin/PKC412 combination on PKC412-resistant Ba/F-FLT3-ITD cells. A PKC412-resistant cell line was derived by transduction of a PKC412-resistant FLT3-ITD F691I mutant into Ba/F3 cells. These cells also exhibit significantly higher expression of FLT3-ITD F691I protein compared to Ba/F-FLT3-ITD cells. As observed with rapamycin treatment of Imatinib-resistant BCR/ABL mutants, PKC412-resistant Ba/F-FLT3-ITD F691I cells remained sensitive to the growth inhibitory effects of rapamycin alone, but were resistant to doses of PKC412 as high as 20 nM (FIG. 5B).

However, rapamycin (2 nM) combined with PKC412 (5 nM) dramatically inhibited the proliferation of Ba/F-FLT3-ITD F691I cells (>93%, FIG. 5B).

PKC412-sensitive Ba/F-FLT3-ITD cells were treated with the indicated concentrations of rapamycin or PKC412 or both, after which cell lysates were subjected to immunoblotting with phospho-specific p70 S6 K (FIG. 5C) and 4E-BP1 (FIG. 5D) antibodies. The blots were reprobed for p70 S6 kinase and Erk2, respectively, to ensure equivalent loading.

Similar to the effects of the Imatinib/rapamycin combination on BCR/ABL-expressing cells, treatment of Ba/F-FLT3-ITD cells with the combination of PKC412 and rapamycin led to a greater decrease in phosphorylation of p70 S6K. Likewise, 4E-BP1 phosphorylation was >10 fold more resistant to rapamycin than p70 S6K in these cells (FIGS. 5C and 5D), and could only be substantially inhibited by the two-drug combination.

Example 6: Addition of an MEK inhibitor (UO126) increases the inhibitory effects of Rapamycin/Imatinib and Rapamycin/PKC412 combinations.

Bone marrow cells from mice were transduced with BCR/ABL-expressing retroviruses. Imatinib (0.5 μM), rapamycin (2 nM) or UO126 (2 μM) alone or in various combinations, as indicated, were added to the transduced bone marrow cells prior to plating for myeloid colony assays. The number of colonies from triplicate platings was determined after 10 days (see FIG. 6A).

Low doses of rapamycin (2 nM) or Imatinib (0.5 μM) alone inhibited BCR/ABL-evoked myeloid colony formation by ~50-60%, whereas low dose UO126 (2 μM) was only slightly (16%) inhibitory (FIG. 6A). UO126 plus rapamycin had an additive effect, whereas UO126 plus Imatinib or rapamycin plus Imatinib synergistically inhibited myeloid colony outgrowth (FIG. 6A). Remarkably, however, a combination of low doses of all three agents caused profound inhibition (96%) in this assay (FIG. 6A).

Imatinib-resistant Ba/F-BCR/ABL T315I cells were exposed to the indicated concentrations of Imatinib, rapamycin or UO126 alone or in various combinations for 48 hours, followed by measurement of cell proliferation by [^{3}H]-thymidine incorporation (see FIG. 6B).

UO126 (at 5 μM) or Imatinib (at 0.5 μM) alone exhibited only modest inhibitory effects on the proliferation of Ba/F-BCR/ABL T315I cells, although rapamycin (5 nM) alone caused about 70% inhibition of proliferation of these cells (FIG. 6B). UO126 plus Imatinib showed additive inhibitory effects, whereas UO126 plus rapamycin or Imatinib plus rapamycin greatly enhanced inhibition of Ba/F-BCR/ABL T315I cell proliferation (FIG. 6B). Again, however, the triple combination (rapamycin plus UO126 plus Imatinib) caused even more robust inhibition of Ba/F-BCR/ABL T315I cells.

PKC412-resistant Ba/F-FLT3-ITD F691I cells were incubated with the indicated concentrations of PKC412 or rapamycin or UO126 alone or in combination for 48 hours. Cell proliferation was measured by a [^{3}H]-thymidine incorporation assay (see FIG. 6C).

Whereas PKC412 at 10 nM had almost no effect, rapamycin (2 nM) or UO126 (5 μM) treatment inhibited PKC412-resistant Ba/F-FLT3-ITD cell proliferation by 68% or 40%, respectively. Moreover, the proliferation of PKC412-resistant Ba/F-FLT3-ITD F691I cells was profoundly inhibited when UO126 was added in combination with rapamycin (FIG. 6C). Rapamycin plus PKC412 also had synergistic inhibitory effects, whereas UO126 plus PKC412 exhibited additive effects on the proliferation of PKC412-resistant Ba/F-FLT3-ITD F691I cells. The three-drug combination (rapamycin plus PKC412 plus UO126) almost completely (>99%) inhibited the growth and survival of PKC412-resistant Ba/F-FLT3-ITD F691I cells.

Example 7: Effects of Imatinib, Rapamycin, and UO126 combinations on cell cycle distribution of BCR/ABL-transformed B-lymphoid cells.

3 million cells in media as described above and treated with the indicated drugs or left untreated for 24 hours, were fixed, stained with propidium iodide and subject to flow cytometry for cell cycle analysis. Results are provided in Table 4.

Decreased [^{3}H]-thymidine uptake could reflect a decreased rate of cell cycle progression and/or an increase in cell death. Treatment of BCR/ABL-transformed B lymphoblasts with low doses of Imatinib (1 μM) alone led to cell cycle arrest (Table 4); higher (therapeutic) doses were cytotoxic as expected (data not shown). Rapamycin (10 nM) alone caused G1 arrest, but no significant apoptosis. However, the combination of Imatinib (1 μM) and rapamycin (10 nM) evoked a significant increase in apoptosis, as indicated by sub-G1 DNA content (Table 4).

Whereas low doses of either agent alone caused G_1 arrest, cells exposed to Imatinib/ rapamycin exhibited substantial apoptosis (Table 4). This "gain of function" argues strongly that the drug combination has synergistic inhibitory effects.

Table 4. Effects of Imatinib, Rapamycin, andUO126 Combinations on Cell Cycle Distribution of BCR/ABL-transformed primary B-lymphoblast cells

Drugs Treated Cells	% of viable cells in phase: G_1	S	G_2/M	Total % Apoptotic
DMSO (control)	50.6	44.7	4.7	3.9
Imatinib (1 μM)	80.9	16	3.1	6.7
Rapamycin (10 nM)	79.9	18.3	1.8	8.6
Rapamycin (10nM) + Imatinib (1 μM)	83.4	16.6	0	16.5
UO126 (5 μM)	44.6	51.6	3.8	4.6
UO126 (5 μM) + Imatinib (1 μM)	83.2	15.2	1.6	6.6

Example 8: Effects of Herceptin™ and rapamycin on Neu-low and Neu-high cell lines.

MCF7 (Neu-low) and SKBR3 (Neu-high) were seeded at 30,000 cells per 6-well cluster (35mm plate). After 24 hours, cells were treated with herceptin (10 meg/ml) plus/minus the indicated concentrations of rapamycin. Media was changed every 2 days with fresh drugs. After 7 days, cell number was determined by Coulter Counter (see FIGS. 7 and 8).

This data shows that combining rapamycin with herceptin greatly enhances inhibitory effects on proliferation of breast cancer cells.

Example 9: Effects of rapamycin and/or Imatinib on PTEN-positive and PTEN-negative cell lines.

PTEN-positive human prostate cancer cells (CWR22) were seeded at 30,000 cells per 6-well cluster (35mm plate). After 24 hours, cells were treated with Imatinib (2 μg/ml) plus/minus the indicated concentrations of rapamycin. Media was changed every 2 days with fresh drugs. After 7 days, cell number was determined by hemocytometer (see FIG. 9).

PTEN-negative human prostate cancer cells (LnCaP) were seeded at 30,000 cells per 6-well cluster (35mm plate). After 24 hours, cells were treated with Imatinib (2 μg/ml) plus/minus the indicated concentrations of rapamycin. Media was changed every 2 days with fresh drugs. After 7 days, cell number was determined by hemocytometer (see FIG. 10).

Rapamycin alone has significant inhibitory effect on proliferation of both PTEN-positive and PTEN-negative prostate cancer cells. Combining rapamycin with Imatinib, which inhibits PDGFR, enhances the antiproliferative effect upon the growth of prostate cancer cells.

Example 11: Rapamycin/Imatinib combinations improve survival in mouse model CML.

BALB/C donor mice were primed with intraperitoneal injection of 5' fluorouracil (150 mg/kg). After 5 days, bone marrow cells were collected and transduced with MSCV p210 (*BCR/ABL*)-IRES-GFP viruses by two rounds of spinfection. After second round of transduction, cells were resuspended in Hanks balanced salt solution and injected (1 X 10^6 cells/0.5 ml) into the lateral tail vein of lethally irradiated (2 X 450 cGy) female recipient BALB/C mice. The trial design consisted of four groups (placebo/placebo; placebo/rapamycin; Imatinib/placebo; Imatinib/rapamycin), each group containing 9 mice. Rapamycin was administered at a dose of 7.0mg/kg/day and Imatinib at a dose of 70mg/kg/day. Imatinib and its placebo were administered by oral gavage, whereas rapamycin and its placebo were administered via intraperitoneal injection. All animals in the trial received two gavage treatments and one IP treatment per 24 hours. Treatment was started from day 9 after bone marrow transplantation (BMT) and continued until the mice died. The log rank statistics were used to attach a significance level to the difference in the survival curves.

At the time of death of the first double placebo animal (day 20), one mouse per group was sacrificed for full analysis. These mice were censored from the statistical analysis of survival. Peripheral blood was collected from the retroorbital cavity using a heparinized glass capillary. Blood smears were stained with Wright and Giemsa. Manual and automated (ADIVA 120 Hematology system, Bayer) total and differential blood cell counts were performed, as well as histopathologic exam of relevant organs (spleen, liver, heart, lungs, intestine, hindlimb bones, and kidneys). Preparation of single-cell suspensions from spleen and bone marrow for flow cytometry was performed as described previously (Schwaller et al, *Embo J* 17:5321 (1998), Kelly and Weisberg et al, *Cancer Cell* 1:433 (2002)).

This study validates the previous findings that imatinib treatment increases survival in the murine BCR/ABL disease model, even at a sub-optimal dose. As expected, animals treated with imatinib plus placebo showed better survival than those treated with double placebo (p=0.002). This trial also showed a protective effect of rapamycin alone against BCR/ABL disease. Mice treated with

rapamycin plus placebo survived longer than those treated with double placebo ($p=0.04$). Finally, animals treated with both imatinib and rapamycin showed a statistically significant improvement in survival when compared to those treated with either imatinib alone ($p=0.003$) or rapamycin alone, ($p=0.0003$).

Other Embodiments

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

Claims

1. A method of treating a neoplasm characterized by abnormally high levels of tyrosine kinase activity in a patient in need thereof, said method comprising administering to said patient at least one mTOR inhibitor together or in parallel with at least one tyrosine kinase inhibitor in amounts effective to treat said neoplasm.

2. The method of claim 1, wherein said mTOR inhibitor is a rapamycin macrolide.

3. The method of claim 1, wherein said rapamycin macrolide is rapamycin, CCI-779, Everolimus, or ABT-578.

4. The method of claim 1, wherein said said tyrosine kinase inhibitor is selected from the group consisting of a small molecule inhibitor, an antibody, an antisense oligomer, and an RNAi inhibitor.

5. The method of claim 4, wherein said small molecule inhibitor is selected from the group consisting of Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, and MLN518.

6. The method of claim 4, wherein said antibody is selected from the group consisting of trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

7. The method of claim 1, further comprising administering an MEK inhibitor.

8. The method of claim 7, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

9. The method of claims 1-8, wherein said neoplasm is selected from carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage; a hematopoietic tumor of myeloid lineage; a tumor of mesenchymal origin; a tumor of the central or peripheral nervous system; melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

10. The method of claim 9, wherein said hematopoietic tumor of lymphoid lineage is selected from leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell-lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkett's lymphoma.

11. The method of claim 9, wherein said hematopoietic tumor of myeloid lineage is selected from acute myelogenous leukemia, chronic myelogenous leukemia, multiple myelogenous leukemia, myelodysplastic syndrome and promyelocytic leukemia.

12. The method of claim 9, wherein said tumor of mesenchymal origin is fibrosarcoma or rhabdomyosarcoma.

13. The method of claim 9, wherein said tumor of the central or peripheral nervous system is selected from astrocytoma, neuroblastoma, glioma and schwannomas

14. The method of claims 2 or 3, wherein said tyrosine kinase activity is epidermal growth factor receptor activity; said neoplasm is selected from non-small-cell lung cancer, breast cancer, ovarian cancer, bladder cancer, prostate cancer, salivary gland cancer, pancreatic cancer, endometrial cancer, colorectal cancer, kidney cancer, head and neck cancer, and glioblastoma multiforme; and said tyrosine kinase inhibitor is selected from the group consisting of SU101, ZD1839, OSI-774, CI-1033, PKI166, GW2016, EKB-509, EKB-569, trastuzumab, C225, MDX-H210, 2C4, MDX-447, and ABX-EGF.

15. The method of claims 2 or 3, wherein said tyrosine kinase activity is human epidermal growth factor receptor-2 activity; said neoplasm is selected from the group consisting of breast cancer, ovarian cancer, bladder cancer, salivary gland cancer, endometrial cancer, pancreatic cancer, and non-small-cell lung cancer; and said tyrosine kinase inhibitor is selected from the group consisting of CI-1033, GW2016, trastuzumab, MDX-H210, MDX-447, ABX-EGF, EMD 72000, RH3, and 2C4.

16. The method of claim 15, wherein said tyrosine kinase inhibitor is trastuzumab.

17. The method of claims 2 or 3, wherein said tyrosine kinase activity is platelet derived growth factor receptor activity; said neoplasm is selected from the group consisting of gastrointestinal stromal tumor, small cell lung cancer, glioblastoma multiforme, and prostate cancer; and said tyrosine kinase inhibitor is selected from the group consisting of Imatinib, SU101, MLN518, and PTK787.

18. The method of claim 18, wherein said wherein said tyrosine kinase inhibitor is Imatinib.

19. The method of claims 2 or 3, wherein said tyrosine kinase activity is Flt-3 activity; said neoplasm is acute myeloid leukemia and said tyrosine kinase inhibitor is selected from MLN518, SU11248, and PKC412.

20. The method of claim 19, wherein said tyrosine kinase inhibitor is PKC412.

21. The method of claims 2 or 3, wherein said tyrosine kinase activity is tropomyosin receptor kinase activity; said neoplasm is prostate cancer or pancreatic cancer; and said tyrosine kinase inhibitor is Imatinib, CEP701 or CEP705.

22. The method of claims 2 or 3, wherein said tyrosine kinase activity is BCR/ABL activity; said neoplasm is chronic myelogenous leukemia or acute lymphoblastic leukemia; and said tyrosine kinase inhibitor is Imatinib.

23. The method of claims 2 or 3, wherein said tyrosine kinase is a vascular endothelial growth factor receptor kinase; said cancer is any solid tumor; and said tyrosine kinase inhibitor is selected from the group consisting of SU5416, SU6668, ZD4190, ZD6474, PTK787, IMC-1C11, and rhu-Mab VEGF.

24. The method of claims 1, 14, 15, 17, 18, 19, 21, 22, or 23, wherein said neoplasm is resistant to said tyrosine kinase inhibitor.

25. The method of claims 14, 15, 17, 18, 19, 21, 22, 23, or 24, further comprising administration of an MEK kinase inhibitor.

26. The method of claim 25, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

27. The method of claim 1, wherein said mTOR inhibitor and said tyrosine kinase inhibitor are administered in parallel within 30 days of each other.

28. The method of claim 27, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 5 days of each other.

29. The method of claim 28, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 24 hours of each other.

30. The method of claim 1, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered together.

31. The method of any of claims 27-30, further comprising administration of an MEK kinase inhibitor.

32. The method of claim 31, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

33. A method of treating leukemia in a patient in need thereof, said method comprising administering rapamycin to said patient in amounts effective to treat said leukemia.

34. A method of treating a neoplasm in a patient in need thereof, said method comprising administering to said patient at least one mTOR inhibitor together or in parallel with at least one tyrosine kinase inhibitor and at least one MEK inhibitor in amounts effective to treat said neoplasm.

35. The method of claim 34, wherein said neoplasm is selected from carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage; a hematopoietic tumor of myeloid lineage; a tumor of mesenchymal origin; a tumor of the central or peripheral nervous system; melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

36. The method of claims 34 or 35, wherein said mTOR inhibitor is selected from rapamycin, CCI-779, Everolimus, and ABT-578.

37. The method of claims 34 or 35, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzomab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

38. The method of claims 34 or 35, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

39. Use of an mTOR inhibitor and a tyrosine kinase inhibitor in the manufacture of a medicament for the treatment of a neoplasm in a patient in need thereof, wherein said neoplasm is characterized by abnormally high levels of tyrosine kinase activity.

40. The use according to claim 39, wherein said mTOR inhibitor is a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

41. The use according to claims 39 or 40, wherein said tyrosine kinase activity is epidermal growth factor receptor activity; said neoplasm is selected from non-small-cell lung cancer, breast cancer, ovarian cancer, bladder cancer, prostate cancer, salivary gland cancer, pancreatic cancer, endometrial cancer, colorectal cancer, kidney cancer, head and neck cancer, and glioblastoma multiforme; and said tyrosine kinase inhibitor is selected from the group consisting of SU101, ZD1839, OSI-774, CI-1033, PKI166, GW2016, EKB-509, EKB-569, trastuzumab, C225, MDX-H210, 2C4, MDX-447, and ABX-EGF.

42. The use according to claims 39 or 40, wherein said tyrosine kinase activity is human epidermal growth factor receptor-2 activity; said neoplasm is selected from the group consisting of breast cancer, ovarian cancer, bladder cancer, salivary gland cancer, endometrial cancer, pancreatic cancer, and non-small-cell lung cancer; and said tyrosine kinase inhibitor is selected from the group consisting of CI-1033, GW2016, trastuzumab, MDX-H210, MDX-447, ABX-EGF, EMD 72000, RH3, and 2C4.

43. The use according to claims 39 or 40, wherein said tyrosine kinase activity is platelet derived growth factor receptor activity; said neoplasm is selected from the group consisting of gastrointestinal stromal tumor, small cell lung cancer, glioblastoma multiforme, and prostate cancer; and said tyrosine kinase inhibitor is selected from the group consisting of Imatinib, SU101, MLN518, and PTK787.

44. The use according to claims 39 or 40, wherein said tyrosine kinase activity is Flt-3 activity; said neoplasm is acute myeloid leukemia and said tyrosine kinase inhibitor is selected from MLN518, SU11248, and PKC412.

45. The use according to claims 39 or 40, wherein said tyrosine kinase activity is tropomyosin receptor kinase activity; said neoplasm is prostate cancer or pancreatic cancer; and said tyrosine kinase inhibitor is CEP701 or CEP705.

46. The use according to claims 39 or 40, wherein said tyrosine kinase activity is BCR/ABL activity; said neoplasm is chronic myelogenous leukemia or acute lymphoblastic leukemia; and said tyrosine kinase inhibitor is Imatinib.

47. The use according to claims 39 or 40, wherein said tyrosine kinase is a vascular endothelial growth factor receptor kinase; said cancer is any solid tumor; and said tyrosine kinase inhibitor is selected from the group consisting of SU5416, SU6668, ZD4190, ZD6474, PTK787, IMC-1C11, and rhu-Mab VEGF.

48. Use of an mTOR inhibitor, tyrosine kinase inhibitor, and MEK inhibitor in the manufacture of a medicament for the treatment of a neoplasm.

49. The use according to claim 48, wherein said neoplasm is selected from carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage; a hematopoietic tumor of myeloid lineage; a tumor of mesenchymal origin; a tumor of the central or peripheral nervous system; melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

50. The use according to claims 48 or 49, wherein said mTOR inhibitor is selected from rapamycin, CCI-779, Everolimus, and ABT-578.

51. The use according to claims 48 or 49, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

52. The use according to claims 48 or 49, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

53. A pharmaceutical pack comprising an mTOR inhibitor and a tyrosine kinase inhibitor.

54. The pharmaceutical pack of claim 53, wherein said mTOR inhibitor and said tyrosine kinase inhibitor are formulated separately and in individual dosage amounts.

55. The pharmaceutical pack of claims 53 or 54, further comprising an MEK inhibitor.

56. The pharmaceutical pack of any of claims 53-55, wherein said mTOR inhibitor is a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

57. The pharmaceutical pack of any of claims 53-55, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

58. The pharmaceutical pack of any of claims 53-55, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

59. A pharmaceutical composition comprising an effective amount of a rapamycin macrolide and a tyrosine kinase inhibitor, together with a pharmaceutically acceptable carrier or diluent.

60. The pharmaceutical composition of claim 56, further comprising an MEK inhibitor.

61. The pharmaceutical composition of claims 59 or 60, wherein said mTOR inhibitor is a rapamycin macrolide selected from rapamycin, CCI-779, Everolimus, and ABT-578.

62. The pharmaceutical composition of claims 59 or 60, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

63. The pharmaceutical composition of claims 59 or 60, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

64. A method of determining whether a neoplasm in a human patient responds to a combination comprising an mTOR inhibitor and tyrosine kinase inhibitor, said method comprising the steps of:

a) administering said combination to said human patient; and

b) monitoring said patient to determine whether said neoplasm responds to said combination.

65. The method of claim 64, wherein said combination further comprises an MEK inhibitor.

66. The method of claims 64 or 65, wherein said neoplasm is selected from carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage; a hematopoietic tumor of myeloid lineage; a tumor of mesenchymal origin; a tumor of the central or peripheral nervous system; melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

67. The method of any of claims 64-66, wherein said mTOR inhibitor is selected from rapamycin, CCI-779, Everolimus, and ABT-578.

68. The method of any of claims 64-66, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

69. The method of any of claims 64-66, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

70. The method of claims 64 or 65, wherein said neoplasm is characterized by abnormally high levels of tyrosine kinase activity.

71. The method of claims 64 or 65, wherein said mTOR inhibitor and said tyrosine kinase inhibitor are administered in parallel within 30 days of each other.

72. The method of claim 71, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 5 days of each other.

73. The method of claim 72, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 24 hours of each other.

74. The method of claims 64 or 65, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered together.

75. The method of claim 65, wherein said MEK inhibitor is administered together with said tyrosine kinase inhibitor or said MEK inhibitor.

76. Use of an mTOR inhibitor and a tyrosine kinase inhibitor in the preparation of a medicament for determining whether a neoplasm in a human patient responds to a combination comprising an mTOR inhibitor and tyrosine kinase inhibitor, said method comprising the steps of:

a) administering said combination to said human patient; and

b) monitoring said patient to determine whether said neoplasm responds to said combination.

77. Use of an mTOR inhibitor, a tyrosine kinase inhibitor, and an MEK inhibitor in the preparation of a medicament for determining whether a neoplasm in a human patient responds to a combination comprising an mTOR inhibitor, tyrosine kinase inhibitor, and MEK inhibitor, said method comprising the steps of:

a) administering said combination to said human patient; and

b) monitoring said patient to determine whether said neoplasm responds to said combination.

78. Use according to claims 76 or 77, wherein said neoplasm is selected from carcinoma of the bladder, breast, colon, kidney, liver, lung, head and neck, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, or skin; a hematopoietic tumor of lymphoid lineage; a hematopoietic tumor of myeloid lineage; a tumor of mesenchymal origin; a tumor of the central or peripheral nervous system; melanoma; seminoma; teratocarcinoma; osteosarcoma; thyroid follicular cancer; and Kaposi's sarcoma.

79. Use according to any of claims 76-78, wherein said mTOR inhibitor is selected from rapamycin, CCI-779, Everolimus, and ABT-578.

80. Use according to any of claims 76-78, wherein said tyrosine kinase inhibitor is selected from Imatinib, SU101, ZD1839, OSI-774, CI-1033, SU5416, SU6668, ZD4190, ZD6474, PTK787, PKI166, GW2016, EKB-509, EKB-569, CEP-701, CEP-751, PKC412, SU11248, MLN518, trastuzumab, C225, rhu-Mab VEGF, MDX-H210, 2C4, MDX-447, IMC-1C11, EMD 72000, RH3, and ABX-EGF.

81. Use according to any of claims 76-78, wherein said MEK inhibitor is selected from PD184352, PD198306, PD98059, UO126, Ro092210, and L783277.

82. Use according to claims 76 or 77, wherein said neoplasm is characterized by abnormally high levels of tyrosine kinase activity.

83. Use according to claims 76 or 77, wherein said mTOR inhibitor and said tyrosine kinase inhibitor are administered in parallel within 30 days of each other.

84. Use according to claim 83, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 5 days of each other.

85. Use according to claim 84, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered in parallel within 24 hours of each other.

86. Use according to claims 76 or 77, wherein said rapamycin macrolide and said tyrosine kinase inhibitor are administered together.

87. Use according to claim 77, wherein said MEK inhibitor is administered together with said tyrosine kinase inhibitor or said MEK inhibitor.

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FIG. 1A

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
R (0.25 nM)
R (0.5 nM)
R (1 nM)
R (2.5 nM)
R (5 nM)
R (10 nM)

FIG. 1B

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
I (2 μM)
I (4 μM)
R (2 nM)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
I (2 μM)
I (4 μM)
+ R (2 nM)

FIG. 2A

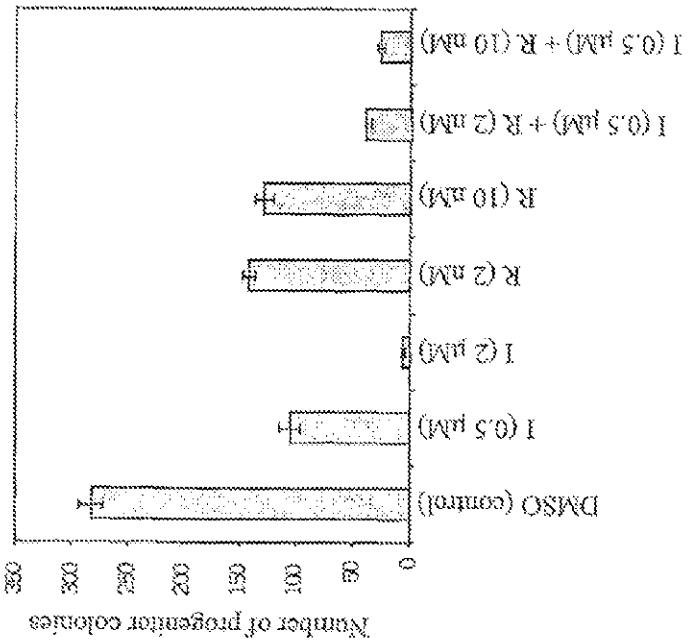

Number of progenitor colonies
350
300
250
200
150
100
50
0
DMSO (control)
I (0.5 μM)
I (2 μM)
R (2 nM)
R (10 nM)
I (0.5 μM) + R (2 nM)
I (0.5 μM) + R (10 nM)

FIG. 2B

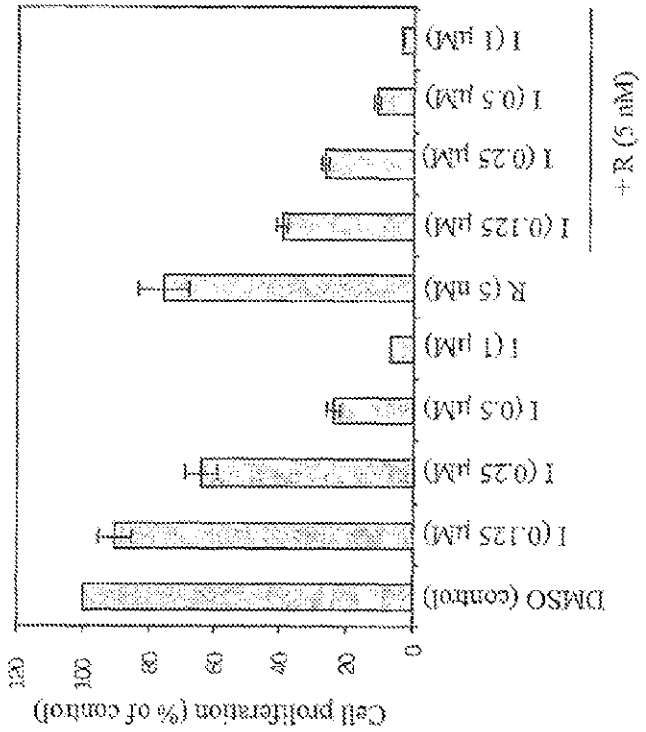

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
I (0.125 μM)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
R (5 nM)
I (0.125 μM)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
+ R (5 nM)

FIG. 3A

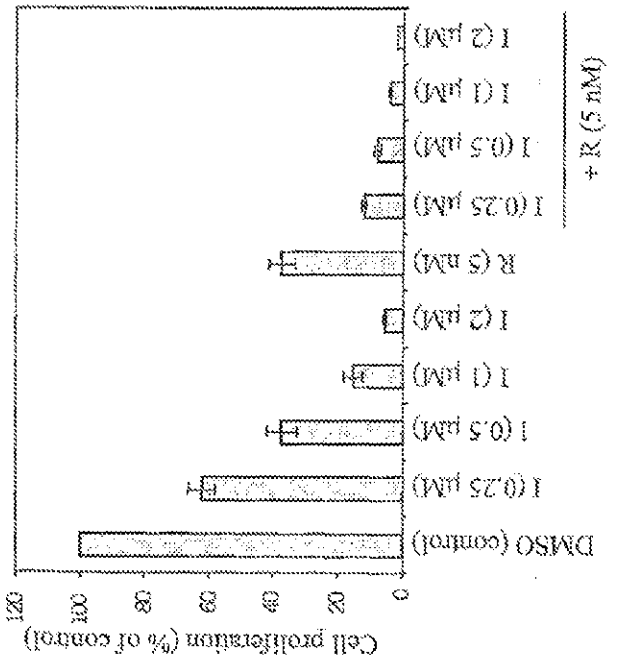

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
I (2 μM)
R (5 nM)
I (0.25 μM)
I (0.5 μM)
I (1 μM)
I (2 μM)
+ R (5 nM)

FIG. 3B

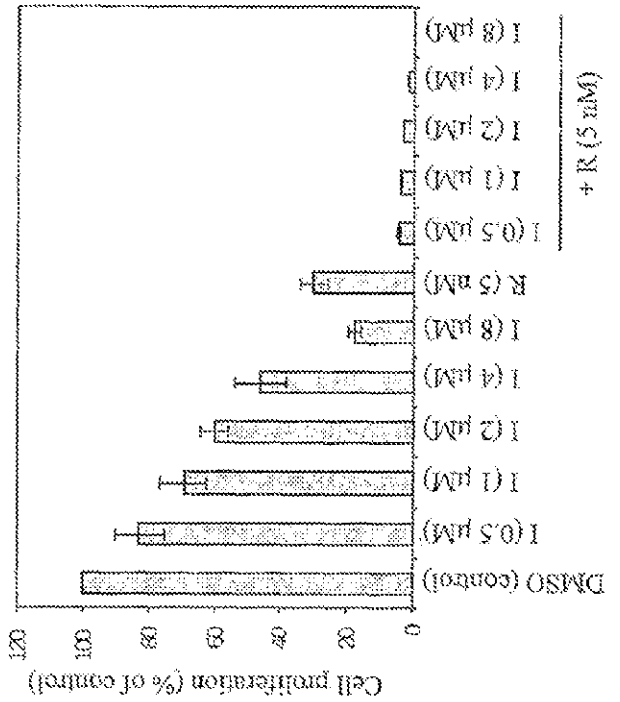

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
I (0.5 μM)
I (1 μM)
I (2 μM)
I (4 μM)
I (8 μM)
R (5 nM)
I (0.5 μM)
I (1 μM)
I (2 μM)
I (4 μM)
I (8 μM)
+ R (5 nM)

FIG. 4A

Ba/F-BCR/ABL WT
DMSO
I (4 μM)
I (0.5 μM)
R (5 nM)
I (0.5 μM) + R (5 nM)
Ba/F-BCR/ABL T315I
DMSO
I (4 μM)
I (0.5 μM)
R (5 nM)
I (0.5 μM) + R (5 nM)
Blots:
Phospho-p70 S6K
Phospho-Erk1/2
Erk2

FIG. 4B

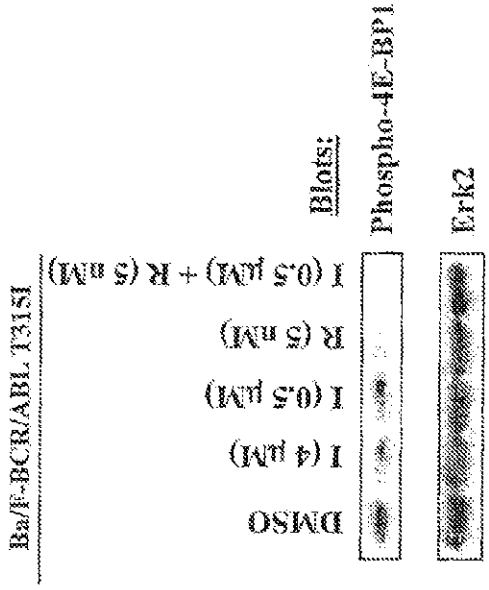

Ba/F-BCR/ABL T315I
DMSO
I (4 μM)
I (0.5 μM)
R (5 nM)
I (0.5 μM) + R (5 nM)
Blots:
Phospho-4E-BP1
Erk2

FIG. 5A

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
P (5 nM)
P (10 nM)
P (20 nM)
P (50 nM)
R (2 nM)
P (5 nM)
P (10 nM)
P (20 nM)
P (50 nM)
+ R (2 nM)

FIG. 5B

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
P (5 nM)
P (10 nM)
P (20 nM)
P (100 nM)
P (500 nM)
R (2 nM)
P (5 nM)
P (10 nM)
P (20 nM)
P (100 nM)
P (500 nM)
+ R (2 nM)

FIG. 5C

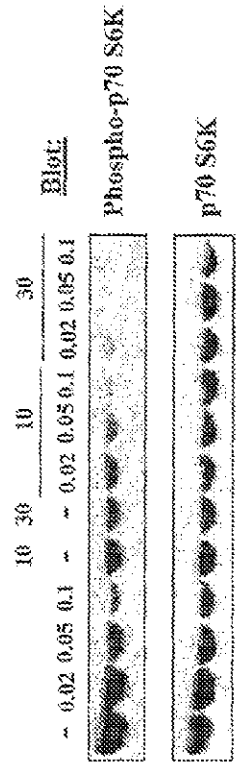

PKC412 (nM)
Rapamycin (nM)
10 30 10 30
- 0.02 0.05 0.1 - - 0.02 0.05 0.1 0.02 0.05 0.1
Blot:
Phospho-p70 S6K
p70 S6K

FIG. 5D

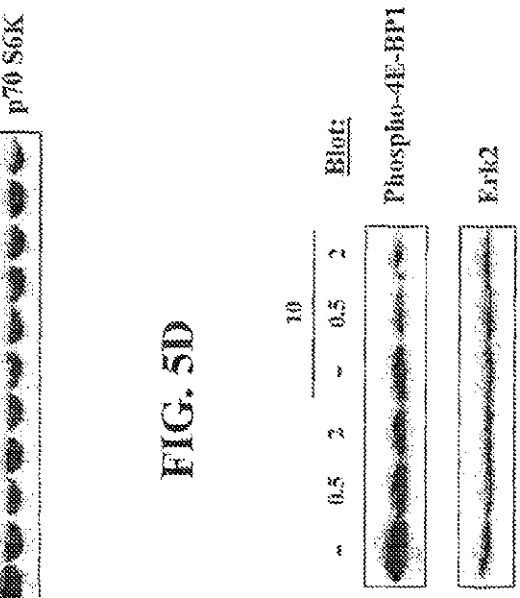

PKC412 (nM)
Rapamycin (nM)
10
- 0.5 2 - 0.5 2
Blot:
Phospho-4E-BP1
Erk2

FIG. 5C

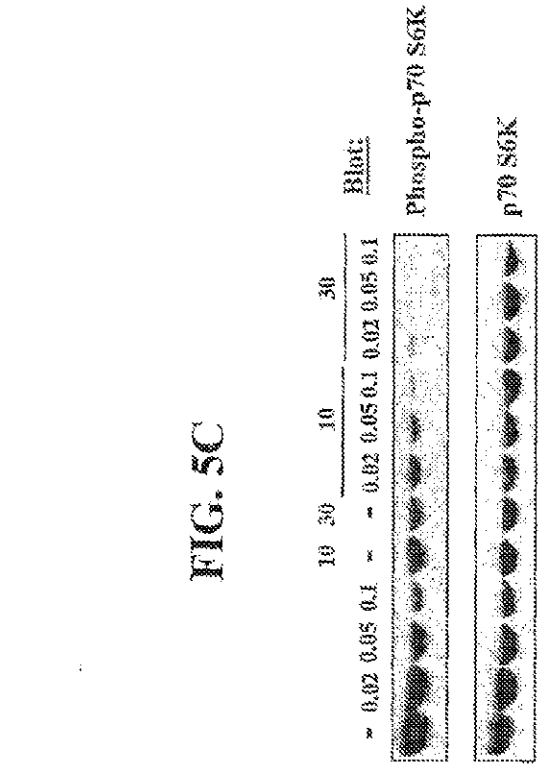

PKC412 (nM)
10 30 10 30
Rapamycin (nM)
- 0.02 0.05 0.1 - - 0.02 0.05 0.1 0.02 0.05 0.1
Blot:
Phospho-p70 S6K
p70 S6K

FIG. 5D

PKC412 (nM)
10
Rapamycin (nM)
- 0.5 2 - 0.5 2
Blot:
Phospho-4E-BP1
Erk2

FIG. 6B

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (control)
I (0.5 μM)
R (5 nM)
UO (5 μM)
I (0.5 μM) + R (5 nM)
I (0.5 μM) + UO (5 μM)
R (5 nM) + UO (5 μM)
R (5 nM) + UO (5 μM) + I (0.5 μM)

FIG. 6A

Number of progenitor colonies
350
300
250
200
150
100
50
0
DMSO (control)
I (0.5 μM)
R (2 nM)
UO (2 μM)
I (0.5 μM) + R (2 nM)
I (0.5 μM) + UO (2 μM)
R (2 nM) + UO (2 μM)
R (2 nM) + UO (2 μM) + I (0.5 μM)

FIG. 7

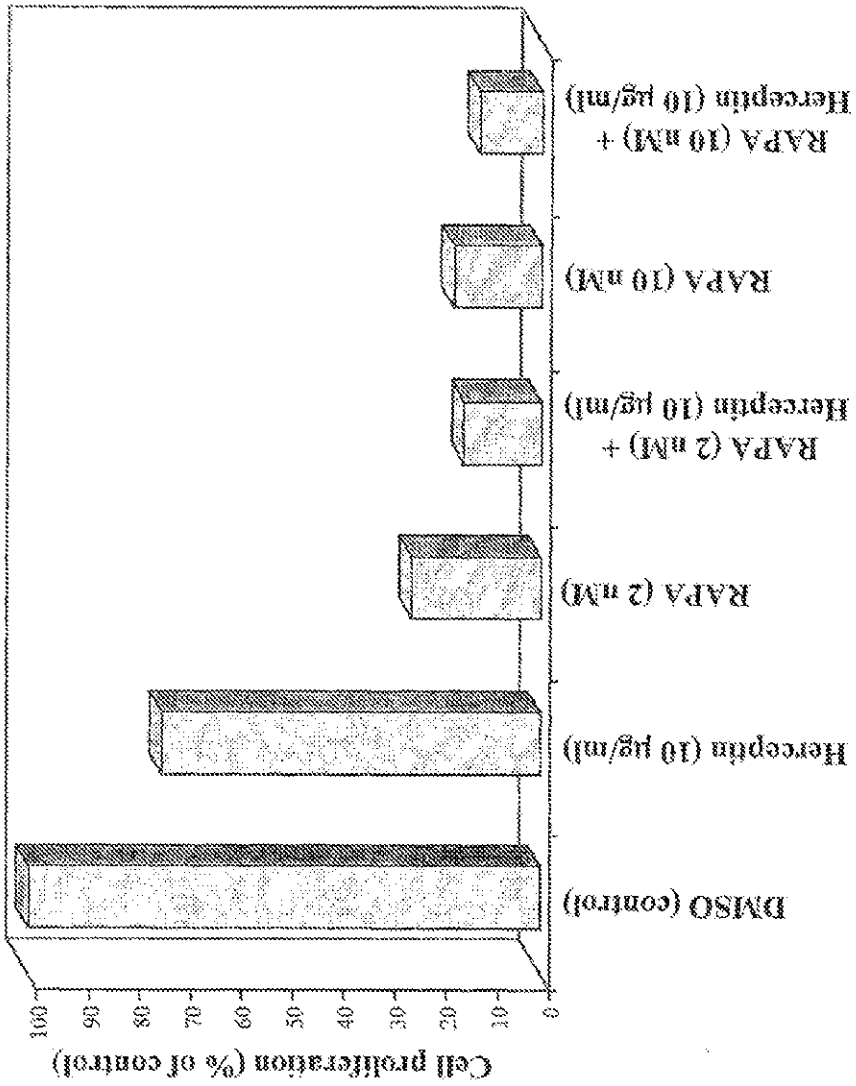

Cell proliferation (% of control)
100
90
80
70
60
50
40
30
20
10
0
DMSO (control)
Herceptin (10 μg/ml)
RAPA (2 nM)
RAPA (2 nM) + Herceptin (10 μg/ml)
RAPA (10 nM)
RAPA (10 nM) + Herceptin (10 μg/ml)

FIG. 8

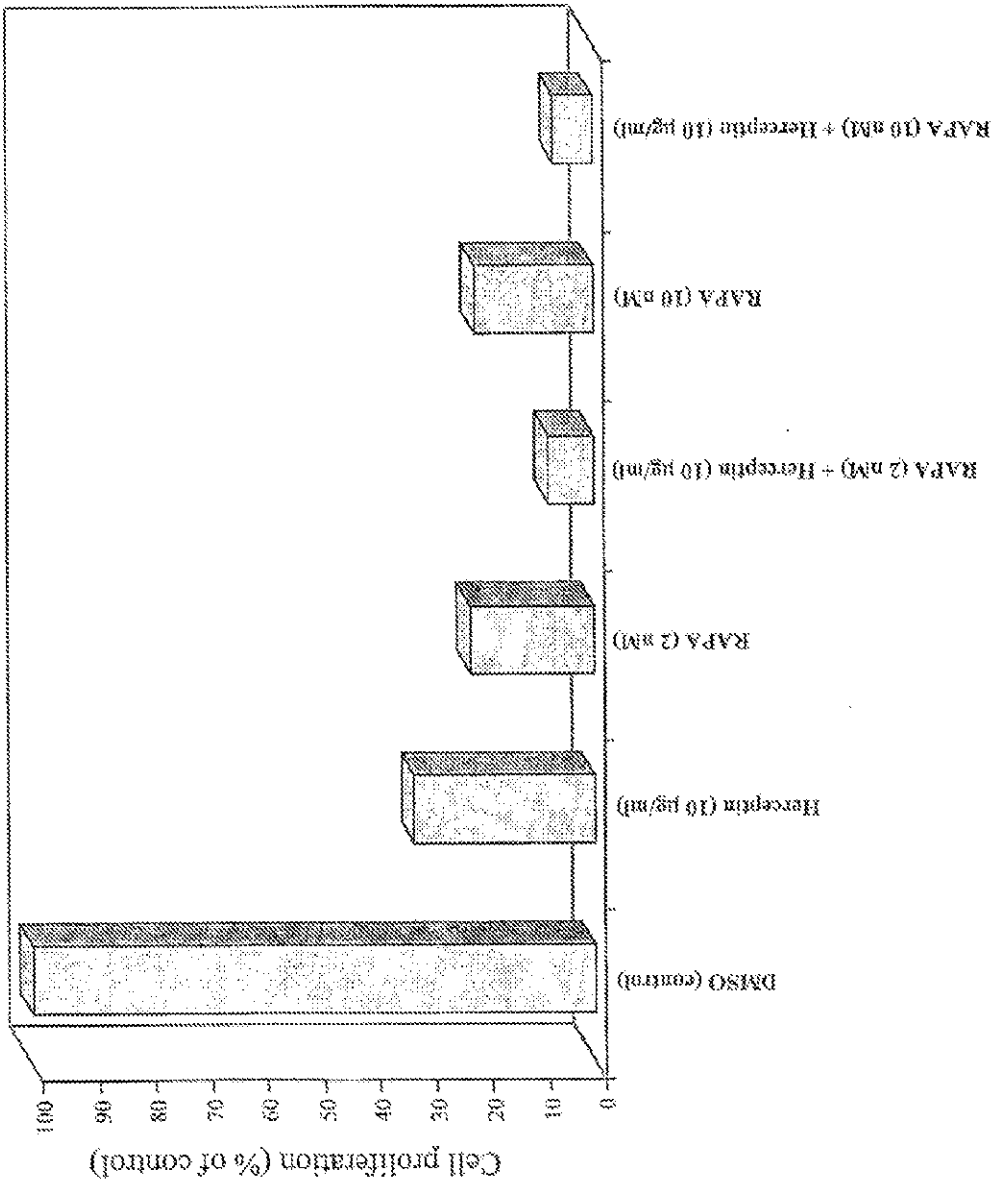

Cell proliferation (% of control)
0
10
20
30
40
50
60
70
80
90
100
DMSO (control)
Herceptin (10 μg/ml)
RAPA (2 nM)
RAPA (2 nM) + Herceptin (10 μg/ml)
RAPA (10 nM)
RAPA (10 nM) + Herceptin (10 μg/ml)

FIG. 9

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (Control)
Imatinib(2 μM)
Rapa (10 nM)
Rapa (2 nM)
Rapa (10 nM)
Rapa (2 nM)
+ Imatinib (2 μM)

FIG. 10

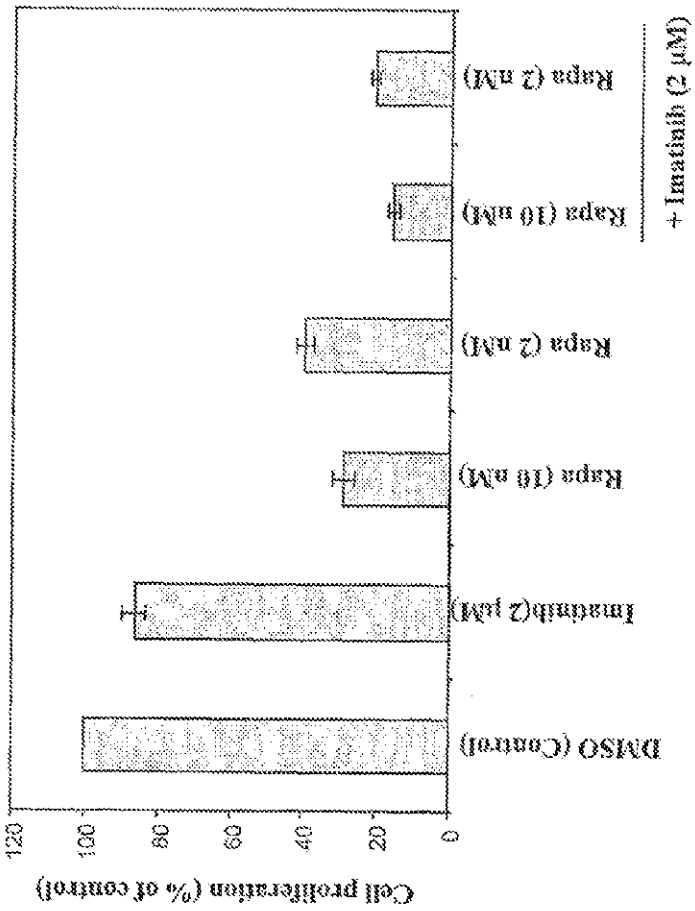
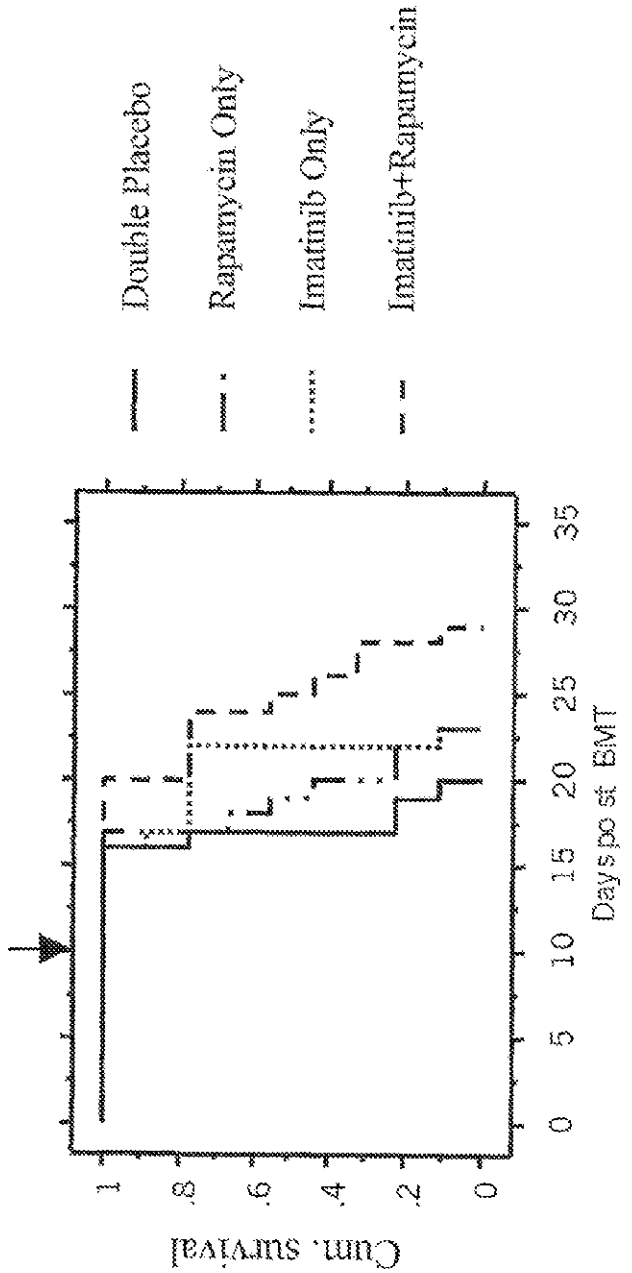

Cell proliferation (% of control)
120
100
80
60
40
20
0
DMSO (Control)
Imatinib(2 μM)
Rapa (10 nM)
Rapa (2 nM)
Rapa (10 nM)
Rapa (2 nM)
+ Imatinib (2 μM)

FIG. 11


Double Placebo
Rapamycin Only
Imatinib Only
Imatinib+Rapamycin
Cum. survival
1
.8
.6
.4
.2
0
0
5
10
15
20
25
30
35
Days post BMT

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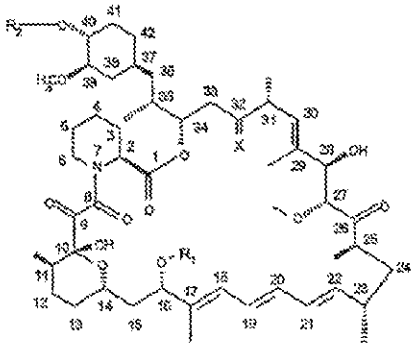
(54) Title: CANCER TREATMENT

(57) Abstract: Rapamycin derivatives have interesting effects in the treatment of solid tumours, optionally in combination with a chemotherapeutic agent.

Cancer Treatment

The present invention relates to a new use, in particular a new use for a compound group comprising rapamycin and derivatives thereof.

Rapamycin is a known macrolide antibiotic produced by Streptomyces hygroscopicus. Suitable derivatives of rapamycin include e.g. compounds of formula I



I

wherein

R_1 is CH_3 or C_{3-5}alkynyl,

R_2 is H or $-CH_2-CH_2-OH$, and

X is =O, (H,H) or (H,OH)

provided that R_2 is other than H when X is =O and R_1 is CH_3.

Compounds of formula I are disclosed e.g. in WO 94/09010, WO 95/16691 or WO 96/41807, which are incorporated herein by reference. They may be prepared as diclosed or by analogy to the procedures described in these references

Preferred compounds are 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32(S)-dihydro-rapamycin, 16-pent-2-ynyloxy-32(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin and, more preferably, 40-0-(2-hydroxyethyl)-rapamycin (referred thereafter as Compound A), disclosed as Example 8 in WO 94/09010.

Compounds of formula I have, on the basis of observed activity, e.g. binding to macrophilin-12 (also known as FK-506 binding protein or FKBP-12), e.g. as described in WO 94/09010, WO 95/16691 or WO 96/41807, been found to be useful e.g. as immunosuppressant, e.g. in the treatment of acute allograft rejection. It has now been found that Compounds of formula I have potent antiproliferative properties which make them useful

for cancer chemotherapy, particularly of solid tumors, especially of advanced solid tumors. There is still the need to expand the armamentarium of cancer treatment of solid tumors, especially in cases where treatment with anticancer compounds is not associated with disease regression or stabilization.

In accordance with the particular findings of the present invention, there is provided:

1.1 A method for treating solid tumors in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I.

1.2 A method for inhibiting growth of solid tumors in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I.

1.3 A method for inducing tumor regression, e.g. tumor mass reduction, in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I.

1.4 A method for treating solid tumor invasiveness or symptoms associated with such tumor growth in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I.

1.5 A method for preventing metastatic spread of tumours or for preventing or inhibiting growth of micrometastasis in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I.

By "solid tumors" are meant tumors and/or metastasis (whereever located) other than lymphatic cancer, e.g. brain and other central nervous system tumors (eg. tumors of the meninges, brain, spinal cord, cranial nerves and other parts of central nervous system, e.g. glioblastomas or medulla blastomas); head and/or neck cancer; breast tumors; circulatory system tumors (e.g. heart, mediastinum and pleura, and other intrathoracic organs, vascular tumors and tumor-associated vascular tissue); excretory system tumors (e.g. kidney, renal pelvis, ureter, bladder, other and unspecified urinary organs); gastrointestinal tract tumors (e.g. oesophagus, stomach, small intestine, colon, colorectal, rectosigmoid junction, rectum, anus and anal canal), tumors involving the liver and intrahepatic bile ducts, gall bladder, other and unspecified parts of biliary tract, pancreas, other and digestive organs); head and neck; oral cavity (lip, tongue, gum, floor of mouth, palate, and other parts of mouth, parotid gland, and other parts of the salivary glands, tonsil, oropharynx, nasopharynx, pyriform sinus, hypopharynx, and other sites in the lip, oral cavity and pharynx); reproductive system tumors (e.g. vulva, vagina, Cervix uteri, Corpus uteri, uterus, ovary, and other sites

associated with female genital organs, placenta, penis, prostate, testis, and other sites associated with male genital organs); respiratory tract tumors (e.g. nasal cavity and middle ear, accessory sinuses, larynx, trachea, bronchus and lung, e.g. small cell lung cancer or non-small cell lung cancer); skeletal system tumors (e.g. bone and articular cartilage of limbs, bone articular cartilage and other sites); skin tumors (e.g. malignant melanoma of the skin, non-melanoma skin cancer, basal cell carcinoma of skin, squamous cell carcinoma of skin, mesothelioma, Kaposi's sarcoma); and tumors involving other tissues including peripheral nerves and autonomic nervous system, connective and soft tissue, retroperitoneum and peritoneum, eye and adnexa, thyroid, adrenal gland and other endocrine glands and related structures, secondary and unspecified malignant neoplasm of lymph nodes, secondary malignant neoplasm of respiratory and digestive systems and secondary malignant neoplasm of other sites.

Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

In a series of further specific or alternative embodiments, the present invention also provides

1.6 A method for the treatment of a disease associated with deregulated angiogenesis in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I.

1.7 A method for inhibiting or controlling deregulated angiogenesis in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I.

1.8 A method for enhancing the activity of a chemotherapeutic agent or for overcoming resistance to a chemotherapeutic agent in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, either concomitantly or sequentially with said chemotherapeutic agent.

1.9 A method according to 1.8 wherein the chemotherapeutic agent is an inhibitor of signal transduction pathways directed either against host cells or processes involved in tumor formation and/or metastases formation or utilised by tumour cells for proliferation, survival, differentiation or development of drug resistance.

1.10 A method as indicated above, wherein rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I is administered intermittently.

CCI779 is a rapamycin derivative, i.e. 40- [3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin or a pharmaceutically acceptable salt thereof, and is disclosed e.g. in USP 5,362,718. ABT578 is a 40-substituted rapamycin derivative further comprising a diene reduction.

Examples of diseases associated with deregulated angiogenesis include without limitation e.g. neoplastic diseases, e.g. solid tumors. Angiogenesis is regarded as a prerequisite for those tumors which grow beyond a certain diameter, e.g. about 1-2 mm.

In a series of further specific or alternative embodiments, the present invention also provides:

2.1 A compound of formula I for use in any method as defined under 1.1 to 1.5 above.

2.2 Rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I for use in any method as defined under 1.6 to 1.10 above or 7 below.

3.1 A compound of formula I for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.1 to 1.5 above.

3.2 Rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I for use in the preparation of a pharmaceutical composition for use in any method as defined under 1.6 to 1.10 above or 7 below.

4.1 A pharmaceutical composition for use in any method as defined under 1.1 to 1.5 above comprising a compound of formula I together with one or more pharmaceutically acceptable diluents or carriers therefor.

4.2 A pharmaceutical composition for use in any method as defined under 1.6 to 1.10 above or 7 below comprising rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, e.g. Compound A, together with one or more pharmaceutically acceptable diluents or carriers therefor.

5.1 A pharmaceutical combination comprising a) a first agent which is rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, e.g. Compound A, and b) a co-agent which is a chemotherapeutic agent, e.g. as defined hereinafter.

5.2 A pharmaceutical combination comprising an amount of a) a first agent which is rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, e.g. Compound A, and b) a co-agent which is a chemotherapeutic agent selected from

the compounds defined under paragraph (iv) or (v) below, to produce a synergistic therapeutic effect.

6. A method as defined above comprising co-administration, e.g. concomitantly or in sequence, of a therapeutically effective amount of rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, e.g. Compound A, and a second drug substance, said second drug substance being a chemotherapeutic agent, e.g. as indicated hereinafter.

7. A method for treating post-transplant lymphoproliferative disorders or a lymphatic cancer, e.g. for treating tumor invasiveness or symptoms associated with such tumor growth in a subject in need thereof, comprising co-administering to said subject, e.g. concomitantly or in sequence, of rapamycin or a derivative thereof, e.g. CCI779, ABT578 or a compound of formula I, e.g. Compound A, and a second drug substance, said second drug substance being a chemotherapeutic agent, e.g. as indicated hereinafter.

By "lymphatic cancer" are meant e.g. tumors of blood and lymphatic system (e.g. Hodgkin's disease, Non-Hodgkin's lymphoma, Burkitt's lymphoma, AIDS-related lymphomas, malignant immunoproliferative diseases, multiple myeloma and malignant plasma cell neoplasms, lymphoid leukemia, myeloid leukemia, acute or chronic lymphocytic leukemia, monocytic leukemia, other leukemias of specified cell type, leukemia of unspecified cell type, other and unspecified malignant neoplasms of lymphoid, haematopoietic and related tissues, for example diffuse large cell lymphoma, T-cell lymphoma or cutaneous T-cell lymphoma).

By the term "chemotherapeutic agent" is meant especially any chemotherapeutic agent other than rapamycin or a derivative thereof. It includes but is not limited to,

i. an aromatase inhibitor,
ii. an antiestrogen, an anti-androgen (especially in the case of prostate cancer) or a gonadorelin agonist,
iii. a topoisomerase I inhibitor or a topoisomerase II inhibitor,
iv. a microtubule active agent, an alkylating agent, an antineoplastic antimetabolite or a platin compound,
v. a compound targeting/decreasing a protein or lipid kinase activity or a protein or lipid phosphatase activity, a further anti-angiogenic compound or a compound which induces cell differentiation processes,
vi. a bradykinin 1 receptor or an angiotensin II antagonist,

vii. a cyclooxygenase inhibitor, a bisphosphonate, a histone deacetylase inhibitor, a heparanase inhibitor (prevents heparan sulphate degradation), e.g. PI-88, a biological response modifier, preferably a lymphokine or interferons, e.g. interferon γ, an ubiquitination inhibitor, or an inhibitor which blocks anti-apoptotic pathways,

viii. an inhibitor of Ras oncogenic isoforms, e.g. H-Ras, K-Ras or N-Ras, or a farnesyl transferase inhibitor, e.g. L-744,832 or DK8G557,

ix. a telomerase inhibitor, e.g. telomestatin,

x. a protease inhibitor, a matrix metalloproteinase inhibitor, a methionine aminopeptidase inhibitor, e.g. bengamide or a derivative thereof, or a proteosome inhibitor, e.g. PS-341.

The term "aromatase inhibitor" as used herein relates to a compound which inhibits the estrogen production, i.e. the conversion of the substrates androstenedione and testosterone to estrone and estradiol, respectively. The term includes, but is not limited to steroids, especially atamestane, exemestane and formestane and, in particular, non-steroids, especially aminoglutethimide, rogletlhimide, pyridoglutethimide, trilostane, testolactone, ketokonazole, vorozole, fadrozole, anastrozole and letrozole. Exemestane can be administered, e.g., in the form as it is marketed, e.g. under the trademark AROMASIN™. Formestane can be administered, e.g., in the form as it is marketed, e.g. under the trademark LENTARON™. Fadrozole can be administered, e.g., in the form as it is marketed, e.g. under the trademark AFEMA™. Anastrozole can be administered, e.g., in the form as it is marketed, e.g. under the trademark ARIMIDEX™. Letrozole can be administered, e.g., in the form as it is marketed, e.g. under the trademark FEMARA™ or FEMAR™ Aminoglutethimide can be administered, e.g., in the form as it is marketed, e.g. under the trademark ORIMETEN™. A combination of the invention comprising a chemotherapeutic agent which is an aromatase inhibitor is particularly useful for the treatment of hormone receptor positive tumors, e.g. breast tumors.

The term "antiestrogen" as used herein relates to a compound which antagonizes the effect of estrogens at the estrogen receptor level. The term includes, but is not limited to tamoxifen, fulvestrant, raloxifene and raloxifene hydrochloride. Tamoxifen can be administered, e.g., in the form as it is marketed, e.g. under the trademark NOLVADEX™. Raloxifene hydrochloride can be administered, e.g., in the form as it is marketed, e.g. under the trademark EVISTA™. Fulvestrant can be formulated as disclosed in US 4,659,516 or it can be administered, e.g., in the form as it is marketed, e.g. under the trademark

FASLODEX™. A combination of the invention comprising a chemotherapeutic agent which is an antiestrogen is particularly useful for the treatment of estrogen receptor positive tumors, e.g. breast tumors.

The term "anti-androgen" as used herein relates to any substance which is capable of inhibiting the biological effects of androgenic hormones and includes, but is not limited to, bicalutamide (CASODEX™), which can be formulated, e.g. as disclosed in US 4,636,505.

The term "gonadorelin agonist" as used herein includes, but is not limited to abarelix, goserelin and goserelin acetate. Goserelin is disclosed in US 4,100,274 and can be administered, e.g., in the form as it is marketed, e.g. under the trademark ZOLADEX™. Abarelix can be formulated, eg. as disclosed in US 5,843,901.

The term "topoisomerase I inhibitor" as used herein includes, but is not limited to topotecan, irinotecan, 9-nitrocamptothecin and the macromolecular camptothecin conjugate PNU-166148 (compound A1 in WO99/17804). Irinotecan can be administered, e.g. in the form as it is marketed, e.g. under the trademark CAMPTOSAR™. Topotecan can be administered, e.g., in the form as it is marketed, e.g. under the trademark HYCAMTIN™.

The term "topoisomerase II inhibitor" as used herein includes, but is not limited to the anthracyclines such as doxorubicin (including liposomal formulation, e.g. CAELYX™), daunorubicin, epirubicin, idarubicin and nemorubicin, the anthraquinones mitoxantrone and losoxantrone, and the podophillotoxines etoposide and teniposide. Etoposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark ETOPOPHOS™. Teniposide can be administered, e.g. in the form as it is marketed, e.g. under the trademark VM 26-BRISTOL™ Doxorubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark ADRIBLASTIN™. Epirubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark FARMORUBICIN™. Idarubicin can be administered, e.g. in the form as it is marketed, e.g. under the trademark ZAVEDOS™. Mitoxantrone can be administered, e.g. in the form as it is marketed, e.g. under the trademark NOVANTRON™.

The term "microtubule active agent" relates to microtubule stabilizing and microtubule destabilizing agents including, but not limited to taxanes, e.g. paclitaxel and docetaxel, vinca alkaloids, e.g., vinblastine, especially vinblastine sulfate, vincristine especially vincristine sulfate, and vinorelbine, discodermolides and epothilones and derivatives thereof, e.g. epothilone B or a derivative thereof. Paclitaxel may be administered e.g. in the form as it is

marketed, e.g. TAXOL™. Docetaxel can be administered, e.g., in the form as it is marketed, e.g. under the trademark TAXOTERE™. Vinblastine sulfate can be administered, e.g., in the form as it is marketed, e.g. under the trademark VINBLASTIN R.P.™. Vincristine sulfate can be administered, e.g., in the form as it is marketed, e.g. under the trademark FARMISTIN™. Discodermolide can be obtained, e.g., as disclosed in US 5,010,099.

The term "alkylating agent" as used herein includes, but is not limited to cyclophosphamide, ifosfamide, melphalan or nitrosourea (BCNU or Gliadel™). Cyclophosphamide can be administered, e.g., in the form as it is marketed, e.g. under the trademark CYCLOSTIN™. Ifosfamide can be administered, e.g., in the form as it is marketed, e.g. under the trademark HOLOXAN™.

The term "antineoplastic antimetabolite" includes, but is not limited to 5-fluorouracil, capecitabine, gemcitabine, methotrexate and edatrexate. Capecitabine can be administered, e.g., in the form as it is marketed, e.g. under the trademark XELODA™. Gemcitabine can be administered, e.g., in the form as it is marketed, e.g. under the trademark GEMZAR™.

The term "platin compound" as used herein includes, but is not limited to carboplatin, cis-platin and oxaliplatin. Carboplatin can be administered, e.g., in the form as it is marketed, e.g. under the trademark CARBOPLAT™. Oxaliplatin can be administered, e.g., in the form as it is marketed, e.g. under the trademark ELOXATIN™.

The term "compounds targeting/decreasing a protein or lipid kinase activity or further anti-angiogenic compounds" as used herein includes, but is not limited to protein tyrosine kinase and/or serine and/or threonine kinase inhibitors or lipid kinase inhibitors, e.g. compounds targeting, decreasing or inhibiting the activity of the epidermal growth factor family of receptor tyrosine kinases (EGFR, ErbB2, ErbB3, ErbB4 as homo- or heterodimers), the vascular endothelial growth factor family of receptor tyrosine kinases (VEGFR), the platelet-derived growth factor-receptors (PDGFR), the fibroblast growth factor-receptors (FGFR), the insulin-like growth factor receptor 1 (IGF-1R), the Trk receptor tyrosine kinase family, the Axl receptor tyrosine kinase family, the Ret receptor tyrosine kinase, the Kit/SCFR receptor tyrosine kinase, members of the c-Abl family and their gene-fusion products (e.g. BCR-Abl), members of the protein kinase C (PKC) and Raf family of serine/threonine kinases, members of the MEK, SRC, JAK, FAK, PDK or PI(3) kinase family, or of the PI(3)-kinase-related kinase family, and/or members of the cyclin-dependent kinase family (CDK) and anti-angiogenic compounds having another mechanism for their activity, e.g. unrelated to protein or lipid kinase inhibition.

Compounds which target, decrease or inhibit the activity of VEGFR are especially compounds, proteins or antibodies which inhibit the VEGF receptor tyrosine kinase, inhibit a VEGF receptor or bind to VEGF, and are in particular those compounds, proteins or monoclonal antibodies generically and specifically disclosed in WO 98/35958, e.g. 1-(4-chloroanilino)-4-(4-pyridylmethyl)phthalazine or a pharmaceutically acceptable salt thereof, e.g. the succinate, or in WO 00/09495, WO 00/27820, WO 00/59509, WO 98/11223, WO 00/27819 and EP 0 769 947; those as described by M. Prewett et al in Cancer Research 59 (1999) 5209-5218, by F. Yuan et al in Proc. Natl. Acad. Sci. USA, vol. 93, pp. 14765-14770, Dec. 1996, by Z. Zhu et al in Cancer Res. 58, 1998, 3209-3214, and by J. Mordenti et al in Toxicologic Pathology, Vol. 27, no. 1, pp 14-21, 1999; in WO 00/37502 and WO 94/10202; Angiostatin™, described by M. S. O'Reilly et al, Cell 79, 1994, 315-328; Endostatin™, described by M. S. O'Reilly et al, Cell 88, 1997, 277-285; anthranilic acid amides; ZD4190; ZD6474; SU5416; SU6668; or anti-VEGF antibodies or anti-VEGF receptor antibodies,e.g. RhuMab.

By antibody is meant intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies formed from at least 2 intact antibodies, and antibodies fragments so long as they exhibit the desired biological activity.

Compounds which target, decrease or inhibit the activity of the epidermal growth factor receptor family are especially compounds, proteins or antibodies which inhibit members of the EGF receptor tyrosine kinase family, e.g. EGF receptor, ErbB2, ErbB3 and ErbB4 or bind to EGF or EGF related ligands, and are in particular those compounds, proteins or monoclonal antibodies generically and specifically disclosed in WO 97/02266, e.g. the compound of ex. 39, or in EP 0 564 409, WO 99/03854, EP 0520722, EP 0 566 226, EP 0 787 722, EP 0 837 063, US 5,747,498, WO 98/10767, WO 97/30034, WO 97/49688, WO 97/38983 and, especially, WO 96/30347 (e.g. compound known as CP 358774), WO 96/33980 (e.g. compound ZD 1839) and WO 95/03283 (e.g. compound ZM105180); e.g. trastuzumab (Herpetin®), cetuximab, Iressa, OSI-774, CI-1033, EKB-569, GW-2016, E1.1, E2.4, E2.5, E6.2, E6.4, E2.11, E6.3 or E7.6.3.

Compounds which target, decrease or inhibit the activity of PDGFR are especially compounds which inhibit the PDGF receptor, e.g. a N-phenyl-2-pyrimidine-amine derivative, e.g. imatinib.

Compounds which target, decrease or inhibit the activity of c-Abl family members and their gene fusion products, e.g. a N-phenyl-2-pyrimidine-amine derivative, e.g. imatinib; PD180970; AG957; or NSC 680410.

Compounds which target, decrease or inhibit the activity of protein kinase C, Raf, MEK, SRC, JAK, FAK and PDK family members, or PI(3) kinase or PI(3) kinase-related family members, and/or members of the cyclin-dependent kinase family (CDK) are especially those staurosporine derivatives disclosed in EP 0 296 110, e.g. midostaurin; examples of further compounds include e.g. UCN-01, safingol, BAY 43-9006, Bryostatin 1, Perifosine; ilmofosine; RO 318220 and RO 320432; GO 6976; Isis 3521; or LY333531/LY379196.

Further anti-angiogenic compounds are e.g. thalidomide (THALOMID) and TNP-470.

Compounds which target, decrease or inhibit the activity of a protein or lipid phosphatase are e.g. inhibitors of phosphatase 1, phosphatase 2A, PTEN or CDC25, e.g. okadaic acid or a derivative thereof.

Compounds which induce cell differentiation processes are e.g. retinoic acid, α-, γ- or δ-tocopherol or α-, γ- or δ-tocotrienol.

The term cyclooxygenase inhibitor as used herein includes, but is not limited to, e.g. celecoxib (Celebrex®), rofecoxib (Vioxx®), etoricoxib, valdecoxib or a 5-alkyl-2-arylaminophenylacetic acid, e.g. 5-methyl-2-(2'-chloro-6'-fluoroanilino)phenyl acetic acid.

The term "histone deacetylase inhibitor" as used herein includes, but is not limited to MS-27-275, SAHA, pyroxamide, FR-901228 or valproic acid.

The term "bisphosphonates" as used herein includes, but is not limited to, etridonic, clodronic, tiludronic, pamidronic, alendronic, ibandronic, risedronic and zoledronic acid. "Etridonic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark DIDRONEL™. "Clodronic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark BONEFOS™. "Tiludronic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark SKELID™. "Pamidronic acid" can be administered, e.g. in the form as it is marketed, e.g. under the trademark AREDIA™. "Alendronic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark FOSAMAX™. "Ibandronic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark BONDRANAT™. "Risedronic acid" can be administered, e.g., in the form as it is marketed, e.g. under the trademark ACTONEL™. "Zoledronic acid" can be administered, e.g. in the form as it is marketed, e.g. under the trademark ZOMETA™

The term "matrix metalloproteinase inhibitor" as used herein includes, but is not limited to collagen peptidomimetic and nonpeptidomimetic inhibitors, tetracycline derivatives, e.g. hydroxamate peptidomimetic inhibitor batimastat and its orally bioavailable analogue marimastat, prinomastat, BMS-279251, BAY 12-9566, TAA211 or AAJ996.

In each case where citations of patent applications or scientific publications are given, the subject-matter relating to the compounds is hereby incorporated into the present application by reference. Comprised are likewise the pharmaceutically acceptable salts thereof, the corresponding racemates, diastereoisomers, enantiomers, tautomers as well as the corresponding crystal modifications of above disclosed compounds where present, e.g. solvates, hydrates and polymorphs, which are disclosed therein. The compounds used as active ingredients in the combinations of the invention can be prepared and administered as described in the cited documents, respectively. Also within the scope of this invention is the combination of more than two separate active ingredients as set forth above, i.e. a pharmaceutical combination within the scope of this invention could include three active ingredients or more. Further both the first agent and the co-agent are not the identical ingredient.

Utility of the compounds of formula I in treating solid tumors as hereinabove specified, may be demonstrated in animal test methods as well as in clinic, for example in accordance with the methods hereinafter described.

A. *In Vitro*

A.1 Antiproliferative activity in combination with other agents

A cell line, e.g. the compound A resistant A549 line (IC_{50} in low nM range) versus the comparative Compound A resistant KB-31 and HCT116 lines (IC_{50} in the μM range), is added to 96-well plates (1,500 cells/well in 100 μl medium) and incubated for 24 hr. Subsequently, a two-fold dilution series of each compound (Compound of formula I or a known chemotherapeutic agent) is made in separate tubes (starting at 8 x the IC_{50} of each compound) either alone or in paired combinations, and the dilutions are added to the wells. The cells are then re-incubated for 3 days. Methylene blue staining is performed on day 4 and the amount of bound dye (proportional to the number of surviving cells that bind the dye) determined. IC_{50}s are subsequently determined using the Calcusyn program, which provides a measure of the interaction, namely the so-called non-exclusive combination index (CI), where: CI ~ 1 = the interaction is nearly additive; 0.85 – 0.9 = slight synergism; < 0.85 = synergy. In this assay, the compounds of formula I show interesting antiproliferative activity

in combination with another chemotherapeutic agent. For example the following CI values are obtained with a combination of Compound A and cisplatinum, paclitaxel, gemcitabine and doxorubicin, showing synergistic effects.

	CI			
Cell line	Cisplatinum	Paclitaxel	Gemcitabine	Doxorubicin
KB-31	0.74	0.9	0.79	0.7
A549	0.47	0.74	0.76	0.64
HCT116	0.47	0.3	0.9	0.52

Furthermore, in this assay, Compound A potentiates the loss of A549 cell viability and cell death when it is used in combination with gemcitabine.

A.2 Antiangiogenic activity

In vitro assay of the antiproliferative activity of rapamycin or a derivative thereof, e.g. Compound A, against human umbilical vein endothelial cells (HUVECs) demonstrates IC_{50} values of 120 ± 22 pM and 841 ± 396, and > 10 000 pM for VEGF- and bFGF- and FBS-stimulated proliferation, respectively. Additionally, no significant effects of Compound A on bFGF-stimulated normal human dermal fibroblast (NHDF) proliferation are observed over the same concentration range. These results indicate that Compound A inhibits the proliferation of HUVECs, being particularly potent against the VEGF-induced proliferation, VEGF being a key pro-angiogenic factor.

B. In Vivo

In the following assays, antitumor activity is expressed as T/C% (mean increase in tumor volumes of treated animals divided by the mean increase of tumor volumes of control animals multiplied by 100) and % regressions (tumor volume minus initial tumor volume divided by the initial tumor volume and multiplied by 100).

B.1 Activity in A549 human lung tumor xenografts

Fragments of A549 tumors (approx. 25 mg; derived from Cell line CCL 185, ATCC, Rockville MD, USA) are transplanted subcutaneously into the left flank of BALB/c nude mice. Treatment is started on day 7 or day 12 following tumor transplantation. The compound to be tested is administered p.o. once per day from day 7/12 to day 38/55, respectively. In this

assay, when administered at a daily dose ranging from 0.1 mg/kg to 2.5 mg/kg, the compounds of formula I exhibit dose-dependent inhibition of tumor growth; for example in one representative experiment Compound A when administered at a dose of 2.5 mg/kg results in persisting regressions (41 %); a dose of 0.5 mg/kg results in transient regressions (38 % on day 17), with a final T/C of 16 %, and a dose of 0.1 mg/kg slows tumor growth resulting in a final T/C of 43 % (T/C for control animals is 100%).

B.2 Activity in KB-31 human epidermoid tumor xenografts

Fragments of KB-31 tumors (approx. 25 mg; derived from the cell lines obtained from Roswell Park Memorial Institute Buffalo, NY, USA) are transplanted subcutaneously into the left flank of BALB/c nude mice. Treatment is started on day 7 or on day 10 following tumor transplantation. The compound to be tested is administered p.o. once per day from day 7/10 to day 25/35, respectively. Antitumor activity is expressed as T/C% as indicated above. In this assay, when administered at a daily dose ranging from 0.5 mg/kg to 2.5 mg/kg, the compounds of formula I inhibit tumor growth; for example in one representative experiment Compound A when administered at a dose of 2,5 mg/kg/day results in a final T/C cvalue of 25%(T/C for control animals is 100%).

B.3 Activity in CA20948 rat pancreatic tumors

Tumors are established in male Lewis rats by suboutaneous injection of CA20948 tumor cell suspension derived from donor rats into the left flank. Treatment is started on day 4 post inoculation. The compound to be tested is administered p.o. once per day (6 days a week) from day 4 to day 9-15 post inoculation. Antitumor activity is expressed as T/C% as indicated above. In this assay, when administered at a daily dose of 0.5 mg/kg to 2.5 mg/kg, the compounds of formula I inhibit tumor growth; for example in a representative experiment Compound A when administered p.o. at a daily dose of 2.5 mg/kg results in a final T/C value of 23 %. In the same experiment, intermittent administration of Compound A, 5mg/kg twice per week, results in a final T/C value of 32%. Compound A significantly and consistently decreases in these assays the rate of CA20948 pancreatic tumor growth when compared to vehicle controls (T/C for control animals is defined as 100%).

Compounds of formula I, e.g. Compound A, have been tested in further tumor models in accordance with the procedure as disclosed above. For example, a daily dosage of 2.5 mg/kg or 5 mg/kg Compound A produces final T/Cs of 18% and 9% when administered to the human NCI H-596 lung tumor model and the human MEXF 989 melanoma tumor model, respectively; 5 mg/kg produces final T/Cs of 20% (primary tumor) and 36% (cervical lymph

node metastases) when administered to the orthotopic mouse B16/BL6 melanoma tumor model and 24% when administered to the human AR42J pancreatic tumor model; 2.5 mg/kg produces a final T/C of 28% when administered to the multi-drug resistant (MDR) human KB-8511 epidermoid tumor model. Good antitumor responses are also obtained when compounds of formula I, e.g. Compound A, are administered intermittently, e.g. 2 subsequent days per week or twice a week, to mice transplanted with human AR42J pancreatic tumors.

B.4 Combination with doxorubicin

Mice transplanted with human KB-31 epidermoid tumors are treated for 21 days with doxorubicin at a dose of 5 mg/kg i.v. once per week, a compound of formula I, e.g. Compound A, at a dose of 2.5 mg/kg p.o once per day, or a combination of both. Thereafter compound of formula I treatment alone is continued in the combination group in order to determine if the compound of formula I can suppress the outgrowth of tumors that respond to conventional agents. Antitumor activity is expressed as T/C% or % regressions as indicated above. For example, the combination of Compound A and doxorubicin produces greater antitumor effect (74 % regressions) as compared to either agent alone (Compound A, T/C 32 %; doxorubicin 44 % regressions). No exacerbation of the body weight losses caused by doxorubicin occurs when Compound A treatment is added. Continuing Compound A treatment in the combination group, after ceasing doxorubicin, inhibits tumor outgrowth such that the tumor volumes of the doxorubicin monotherapy group are significantly larger than those of the combination group. Morever the combination appears to produce a greater cure rate (8/8 tumors) at 14 days post end of treatment than doxorubicin alone (3/8 tumors).

B.5 Combination with cisplatinum

Mice transplanted with human NCI H-596 lung tumors are treated for 21 days with cisplatinum at a dose of 2.5 mg/kg i.v. once per week, a compound of formula I, e.g. Compound A, at a dose of 2.5 mg/kg p.o. once per day, or a combination of both. Antitumor activity is expressed as T/C% or % regressions as indicated above. A combination of Compound A and cisplatinum produces a greater antitumor effect (5% regressions) as compared to either agent alone (Compound A, T/C 26%; cisplatinum, T/C 26%). The combination did not lead to worsened tolerability.

B.6 Antiangiogenic activity

B16/BL6 cells ($5 X10^4$) are injected intradermally into the ear of C57BL/6 mice. Seven

days later treatment with rapamycin or a derivative thereof e.g. Compound A, or vehicle is initiated. Primary tumor and cervical lymph nodes are collected after two weeks of daily treatment for measurement of vessel density. Endothelium of perfused vessels in the tumors is visualized using a nuclear staining dye (Hoechst 33342, 20 mg/kg) that is injected i.v. shortly before killing the mice. Tumors and metastases are snap frozen and sections examined under a light microscope equipped with an epifluorescent source. The fluorescence H33342-labelled endothelium cells is used to measure vessel number and size over the whole tumor section. Vessels are assigned to groups of 10 µm-size range. Distribution of vessel size is assessed using a histogram frequency analysis. At a dose of 5 mg/kg p.o., rapamycin or a derivative thereof reduces vessel density in both the primary tumor (e.g. T/C 50 % for Compound A) and the metastases (e.g.T/C 40 % for Compound A) as compared to controls. Rapamycin or a derivative thereof, e.g. Compound A, also changes vessel size distribution in the metastases.

B.7 Combination with an antiangiogenic agent

B16/BL6 cells ($5 X10^4$) are injected intradermally into the ear of C57BL/6 mice. Seven days later treatment with rapamycin or a derivative thereof, e.g. Compound A, a VEGF receptor tyrosine kinase inhibitor, e.g. 1-(4-chloroanilino)-4-(4-pyridylmethyl)phthalazine or a salt thereof, e.g. the succinate, or a combination of both is initiated and effects on the growth and weight of the primary tumor and cervical lymph node metastases are monitored, respectively. Daily administration of the antiangiogenic agent (100 mg/kg p.o.) or of rapamycin or a derivative thereof, e.g. Compound A, (1 mg/kg p.o.) alone, reduces the size of the primary tumor (final T/C: 65 % and 74 %, respectively), whereas the combination of these two agents is synergistic (T/C 12 %). Rapamycin or a derivative thereof, e.g. Compound A and the antiangiogenic agent treatment alone reduces cervical lymph node weights (related to regional metastases) (T/C: 75 % and 34 %, respectively), and the combination further reduces lymph node weights (T/C 13 %). The treatments significantly promote body weight gains as compared to controls. For the primary tumors, analysis of possible interaction shows synergy with Compound A and antiangiogenic agent as antiangiogenic agent /controls = 0.66; Compound A/controls = 0.77; Compound A and antiangiogenic agent /controls = 0.135. As Compound A and antiangiogenic agent /controls < Compound A/controls x antiangiogenic agent /controls (0.51), this is defined as synergy. For the metastases, analysis also shows synergy with Compound A and the antiangiogenic agent as antiangiogenic agent /controls = 0.337; Compound A/controls = 0.75; Compound A and antiangiogenic agent /controls = 0.122. As Compound A and antiangiogenic agent

/controls < Compound A/controls x antiangiogenic agent /controls (0.252), this is also defined as synergy (Clark, Breast Cancer Research Treatment 1997;46:255).

C. Clinical Trial

C.1 Investigation of clinical benefit of a compound of formula I, e.g. Compound A as monotherapy in solid tumours

Aim of the study: To identify the optimal dose of said compound, given orally once weekly, in a dose escalating study and the efficacy of the optimal dosage in solid tumours.

The study is divided into 2 parts:

Part 1:

Primary Aim: Identify the optimal dose of a compound of formula I, e.g. Compound A, given p.o. once weekly, assuming this should be the minimum dose associated with prolonged inhibition of mTOR and blood levels of said compound at least equivalent to those achieving an anti-tumor effect in in-vivo preclinical levels.

Secondary Aim: Assess safety of said compound when given alone to cancer patients and assess changes in tumor metabolic activity.

Design: Successive groups of 4 patients with advanced malignant solid tumors, refractory or resistant to standard therapies to receive a compound of formula I, e.g. Compound A, every 7 days different doses (group 1 to receive 5 mg; group 2 to receive 10 mg, group 3 to receive 20 mg) for 4 weeks. In week 4, establish the pharmacokinetic profile and the profile of mTOR inhibition as reflected by the inhibition of p70s6 kinase in peripheral lymphocytes. Carry out comparative 18-fluorodeoxyglucose (FDG) positron-emission tomography (FDG-PET) imaging (before 1st dose, after 3rd dose) to explore the change in tumor metabolism.

Patients main selection criteria: Adults with advanced-stage (III-V) solid tumors, resistant or refractory to standard therapies. At least one tumoral lesion should be measurable (>20 mm in one dimension).

Main variables for evaluation: Safety (adverse events), standard serum biochemistry and haematology, blood levels of the compound to be tested, lymphocyte p70-s6kinase activity, changes in tumor glucose uptake by FDG-PET.

Part 2:

Primary Aim: Explore the efficacy of a compound of formula I, e.g. Compound A, in patients with advanced solid tumors when given once a week at the optimal dosage, as identified in Part 1 as shown by tumor response.

Secondary Aim: Assess the safety of said compound at this dosage.

Design: 20 patients with progressing, advanced-stage solid tumors, resistant or refractory to standard therapies, to receive said compound at the dosage recommended as a result of Part 1. The general clinical state of the patient is investigated weekly by physical and laboratory examination. Changes in tumor burden are assessed every 2 months by radiological examination. Initially patients receive treatment for 2 months. Thereafter, they remain on treatment for as long as their disease does not progress and the drug is satisfactorily tolerated.

Main variables for evaluation: Safety (adverse events), standard serum biochemistry and haematology, tumor dimensions by computerised tomographic (CT) scan or magnetic resonance imaging (MRI).

C.2 Combined Treatment

Suitable clinical studies are, for example, open label non-randomized, dose escalation studies in patients with advanced solid tumors. Such studies prove in particular the synergism of the active ingredients of the combination of the invention. The beneficial effects on proliferative diseases can be determined directly through the results of these studies or by changes in the study design which are known as such to a person skilled in the art. Such studies are, in particular, suitable to compare the effects of a monotherapy using the active ingredients and a combination of the invention. Preferably, the dose of agent (a) is escalated until the Maximum Tolerated Dosage is reached, and the co-agent (b) is administered with a fixed dose. Alternatively, the agent (a) is administered in a fixed dose and the dose of co-agent (b) is escalated. Each patient receives doses of the agent (a) either daily or intermittent. The efficacy of the treatment can be determined in such studies, e.g., after 12, 18 or 24 weeks by radiologic evaluation of the tumors every 6 weeks.

Alternatively, a placebo-controlled, double blind study can be used in order to prove the benefits of the combination of the invention mentioned herein.

Daily dosages required in practicing the method of the present invention when a compound of formula I alone is used will vary depending upon, for example, the compound used, the host, the mode of administration and the severity of the condition to be treated. A preferred daily dosage range is about from 0.1 to 25 mg as a single dose or in divided doses. Suitable daily dosages for patients are on the order of from e.g. 0.1 to 25 mg p.o. Compound A may be administered by any conventional route, in particular enterally, e.g. orally, e.g. in the form of tablets, capsules, drink solutions, nasally, pulmonary (by inhalation) or parenterally, e.g. in the form of injectable solutions or suspensions. Suitable unit dosage forms for oral

administration comprise from ca. 0.05 to 12.5 mg, usually 0.25 to 10 mg Compound A, together with one or more pharmaceutically acceptable diluents or carriers therefor.

The combination of the invention can also be applied in combination with surgical intervention, mild prolonged whole body hyperthermia and/or irradiation therapy.

The administration of a pharmaceutical combination of the invention results not only in a beneficial effect, e.g. a synergistic therapeutic effect, e.g. with regard to slowing down, arresting or reversing the neoplasm formation or a longer duration of tumor response, but also in further surprising beneficial effects, e.g. less side-effects, an improved quality of life or a decreased mortality and morbidity, compared to a monotherapy applying only one of the pharmaceutically active ingredients used in the combination of the invention, in particular in the treatment of a tumor that is refractory to other chemotherapeutics known as anti-cancer agents. In particular, an increased up-take of the co-agent (b) in tumor tissue and tumor cells is observed, when applied in combination with the first agent (a).
A further benefit is that lower doses of the active ingredients of the combination of the invention can be used, for example, that the dosages need not only often be smaller but are also applied less frequently, or can be used in order to diminish the incidence of side-effects, while controlling the growth of neoplasm formation. This is in accordance with the desires and requirements of the patients to be treated.

According to one embodiment of the invention, a preferred pharmaceutical combination comprises
a) a compound of formula I, e.g. Compound A, and
b) as co-agent, one or more compounds as indicated in paragraphs (ii), (iii) or (iv) above, e.g. carboplatin, cisplatinum, paclitaxel, docetaxel, gemcitabine or doxorubicin.
A synergistic combination of a compound of formula I, e.g. Compound A, with carboplatin, cisplatinum, paclitaxel, docetaxel, gemcitabine or doxorubicin is particularly preferred.

A further preferred pharmaceutical combination is e.g. a combination comprising
a) rapamycin or a derivative thereof, e.g. CCI-779, ABT578 or Compound A, and
b) as co-agent, one or more compounds as indicated under paragraphs (i) and (v) to (x) above, preferably one or more compounds as specified in paragraph (v) above.
Preferred is e.g. a synergistic combination of rapamycin or a derivative thereof, e.g. CCI-779, ABT578 or Compound A, with a compound which target, decrease or inhibit the activity of VEGFR, EGFR family, PDGFR, c-ABl family members or protein kinase C, e.g. as disclosed above.

One specific embodiment of the invention relates to the use of a combination of the invention for the prevention, delay of progression or treatment of or for the preparation of a medicament for the prevention, delay of progression or treatment of breast cancer. Preferably, in such embodiment the combination comprises as co-agent b) an aromatase inhibitor, e.g. the aromatase inhibitor letrozole, an anti-estrogen, e.g. tamoxifen, a topoisomerase II inhibitor, e.g. doxorubicin, or a microtubule active agent, e.g. paclitaxel.

Another embodiment of the invention relates to the use of a combination of the invention for the prevention, delay of progression or treatment of or for the preparation of a medicament for the prevention, delay of progression or treatment of lung cancer. Preferably, in such embodiment the combination of the invention comprises as co-agent b) a platin compound, e.g. carboplatin, or a microtubule active agent, e.g. paclitaxel.

Another embodiment of the invention relates to the use of a combination of the invention for the prevention, delay of progression or treatment of or for the preparation of a medicament for the prevention, delay of progression or treatment of pancreatic cancer. Preferably, in such embodiment the combination of the invention comprises as co-agent b) an antineoplastic antimetabolite, e.g. gemcitabine.

Another embodiment of the invention relates to the use of a combination of the invention for the prevention, delay of progression or treatment of or for the preparation of a medicament for the prevention, delay of progression or treatment of glioblastomas. Preferably, in such embodiment the combination of the invention comprises as co-agent b) an alkylating agent, e.g. BCNU.

A further embodiment of the invention relates to the use of rapamycin or a derivative thereof in combination with a chemotherapeutic agent in the treatment of a lymphatic cancer, e.g. as disclosed above. The combination may additionally comprise as co-agent b) busulfan, cytarabine, 6-thioguanine, fludarabine, hydroxyurea, procarbazine, bleomycin or methotrexate. Topoisomerase II inhibitors e,g. daunorubicin or, particularly, compounds which target, decrease or inhibit the activity of PDGFR or of c-Abl family members and their gene fusion products, e.g. imatinib are preferred as co-agent (b).

The terms "co-administration" or "combined administration" or the like as utilized herein are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time.

It is one objective of this invention to provide a pharmaceutical composition comprising a quantity, which is jointly therapeutically effective against a proliferative malignant disease comprising a combination of the invention. In this composition, the first agent a) and co-agent (b) can be administered together, one after the other or separately in one combined unit dosage form or in two separate unit dosage forms. The unit dosage form may also be a fixed combination.

The pharmaceutical compositions for separate administration of the first agent a) and co-agent b) and for the administration in a fixed combination, i.e. a single galenical composition comprising at least two combination partners a) and b), according to the invention can be prepared in a manner known per se and are those suitable for enteral, such as oral or rectal, and parenteral administration to mammals (warm-blooded animals), including humans, comprising a therapeutically effective amount of at least one pharmacologically active combination partner alone, e.g. as indicated above, or in combination with one or more pharmaceutically acceptable carriers or diluents, especially suitable for enteral or parenteral application.

Suitable pharmaceutical compositions contain, for example, from about 0.1 % to about 99.9%, preferably from about 1 % to about 60 %, of the active ingredient(s). Pharmaceutical preparations for the combination therapy for enteral or parenteral administration are, for example, those in unit dosage forms, such as sugar-coated tablets, tablets, capsules or suppositories, or ampoules. If not indicated otherwise, these are prepared in a manner known per se, for example by means of conventional mixing, granulating, sugar-coating, dissolving or lyophilizing processes. It will be appreciated that the unit content of a combination partner contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units.

In particular, a therapeutically effective amount of each of the combination partner of the combination of the invention may be administered simultaneously or sequentially and in any order, and the components may be administered separately or as a fixed combination. For example, the method of delay of progression or treatment of a proliferative malignant disease according to the invention may comprise (i) administration of the first agent a) in free or pharmaceutically acceptable salt form and (ii) administration of a co-agent b) in free or pharmaceutically acceptable salt form, simultaneously or sequentially in any order, in jointly therapeutically effective amounts, preferably in synergistically effective amounts, e.g. in daily

or intermittently dosages corresponding to the amounts described herein. The individual combination partners of the combination of the invention may be administered separately at different times during the course of therapy or concurrently in divided or single combination forms. Furthermore, the term administering also encompasses the use of a pro-drug of a combination partner that convert *in vivo* to the combination partner as such. The instant invention is therefore to be understood as embracing all such regimens of simultaneous or alternating treatment and the term "administering" is to be interpreted accordingly.

The effective dosage of each of the combination partners employed in the combination of the invention may vary depending on the particular compound or pharmaceutical composition employed, the mode of administration, the condition being treated, the severity of the condition being treated. Thus, the dosage regimen of the combination of the invention is selected in accordance with a variety of factors including the route of administration and the renal and hepatic function of the patient. A physician, clinician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the single active ingredients required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentration of the active ingredients within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the active ingredients' availability to target sites.

Daily dosages for the first agent a) will, of course, vary depending on a variety of factors, for example the compound chosen, the particular condition to be treated and the desired effect. In general, however, satisfactory results are achieved on administration of rapamycin or a derivative thereof at daily dosage rates of the order of ca. 0.1 to 25 mg as a single dose or in divided doses. Rapamycin or a derivative thereof, e.g. a compound of formula I, may be administered by any conventional route, in particular enterally, e.g. orally, e.g. in the form of tablets, capsules, drink solutions or parenterally, e.g. in the form of injectable solutions or suspensions. Suitable unit dosage forms for oral administration comprise from ca. 0.05 to 10 mg active ingredient, e.g. Compound A, together with one or more pharmaceutically acceptable diluents or carriers therefor.

Fadrozole may be administered orally to a human in a dosage range varying from about 0.5 to about 10 mg/day, preferably from about 1 to about 2.5 mg/day. Exemestane may be administered orally to a human in a dosage range varying from about 5 to about 200 mg/day, preferably from about 10 to about 25 mg/day, or parenterally from about 50 to 500 mg/day, preferably from about 100 to about 250 mg/day. If the drug shall be administered in a

separate pharmaceutical composition, it can be administered in the form disclosed in GB 2,177,700. Formestane may be administered parenterally to a human in a dosage range varying from about 100 to 500 mg/day, preferably from about 250 to about 300 mg/day. Anastrozole may be administered orally to a human in a dosage range varying from about 0.25 to 20 mg/day, preferably from about 0.5 to about 2.5 mg/day. Aminogluthemide may be administered to a human in a dosage range varying from about 200 to 500 mg/day.

Tamoxifen citrate may be administered to a human in a dosage range varying from about 10 to 40 mg/day.

Vinblastine may be administered to a human in a dosage range varying from about 1.5 to 10 mg/m^{2}day. Vincristine sulfate may be administered parenterally to a human in a dosage range varying from about 0.025 to 0.05 mg/kg body weight - week. Vinorelbine may be administered to a human in a dosage range varying from about 10 to 50 mg/m^{2}day.

Etoposide phosphate may be administered to a human in a dosage range varying from about 25 to 115 mg/m^{2}day, e.g. 56.8 or 113.6 mg/m^{2}day.

Teniposide may be administered to a human in a dosage range varying from about 75 to 150 mg about every two weeks. Doxorubicin may be administered to a human in a dosage range varying from about 10 to 100 mg/m^{2}day, e.g. 25 or 50 mg/m^{2}day. Epirubicin may be administered to a human in a dosage range varying from about 10 to 200 mg/m^{2}day. Idarubicin may be administered to a human in a dosage range varying from about 0.5 to 50 mg/m^{2}day. Mitoxantrone may be administered to a human in a dosage range varying from about 2.5 to 25 mg/m^{2}day.

Paclitaxel may be administered to a human in a dosage range varying from about 50 to 300 mg/m^{2}day. Docetaxel may be administered to a human in a dosage range varying from about 25 to 100 mg/m^{2}day.

Cyclophosphamide may be administered to a human in a dosage range varying from about 50 to 1500 mg/m^{2}day. Melphalan may be administered to a human in a dosage range varying from about 0.5 to 10 mg/m^{2}day.

5-Fluorouracil may be administered to a human in a dosage range varying from about 50 to 1000 mg/m^{2}day, e.g. 500 mg/m^{2}day. Capecitabine may be administered to a human in a dosage range varying from about 10 to 1000 mg/m^{2}day. Gemcitabine hydrochloride may be administered to a human in a dosage range varying from about 1000 mg/m^2/week.

Methotrexate may be administered to a human in a dosage range varying from about 5 to 500 mg/m^{2}day.

Topotecan may be administered to a human in a dosage range varying from about 1 to 5 mg/m^{2}day. Irinotecan may be administered to a human in a dosage range varying from about 50 to 350 mg/m^{2}day.

Carboplatin may be administered to a human in a dosage range varying from about 200 to 400 mg/m^2 about every four weeks. Cisplatin may be administered to a human in a dosage range varying from about 25 to 75 mg/m^2 about every three weeks. Oxaliplatin may be administered to a human in a dosage range varying from about 50 to 85 mg/m^2 every two weeks.

Imatinib may be administered to a human in a dosage in the range of about 2.5 to 850 mg/day, more preferably 5 to 600 mg/day and most preferably 20 to 300 mg/day.

Alendronic acid may be administered to a human in a dosage range varying from about 5 to 10 mg/day. Clodronic acid may be administered to a human e.g. in a dosage range varying from about 750 to 1500 mg/day. Etridonic acid may be administered to a human in a dosage range varying from about 200 to 400 mg/day. Ibandronic acid may be administered to a human in a dosage range varying from about 1 to 4 mg every three to four weeks. Risedronic acid may be administered to a human in a dosage range varying from about 20 to 30 mg/day. Pamidronic acid may be administered to a human in a dosage range varying from about 15 to 90 mg every three to four weeks. Tiludronic acid may be administered to a human in a dosage range varying from about 200 to 400 mg/day.

Trastuzumab may be administered to a human in a dosage range varying from about 1 to 4 mg/m^2/week.

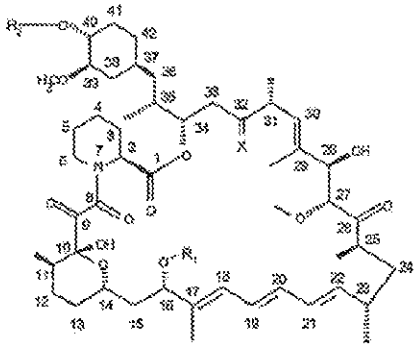
Bicalutamide may be administered to a human in a dosage range varying from about 25 to 50 mg/m^{2}day.

1-(4-chloroanilino)-4-(4-pyridylmethyl)phthalazine or salt thereof, e.g. succinate, may be administered to a human in a dosage range of about 50 to 1500, more preferably about 100 to 750, and most preferably 250 to 500, mg/day.

Rapamycin or derivatives thereof are well tolerated at dosages required for use in accordance with the present invention. For example, the NTEL for Compound A in a 4-week toxicity study is 0.5 mg/kg/day in rats and 1.5 mg/kg/day in monkeys.

CLAIMS

1. Use of a compound of formula I



I

wherein

R_1 is CH_3 or C_{3-5}alkynyl,

R_2 is H or $-CH_2-CH_2-OH$, and

X is =O, (H,H) or (H,OH)

provided that R_2 is other than H when X is =O and R_1 is CH_3,

in the preparation of a pharmaceutical composition for use in the treatment of solid tumors.

2. Use of a compound of formula I according to claim 1 in the preparation of a pharmaceutical composition for use in the treatment of solid tumor invasiveness or symptoms associated with such tumor growth.

3. Use of rapamycin or a rapamycin derivative in the preparation of a pharmaceutical composition for use to inhibit or control deregulated angiogenesis.

4. A pharmaceutical composition for use in the treatment of solid tumors comprising a compound of formula I as defined in claim 1, together with one or more pharmaceutically acceptable diluents or carriers therefor.

5. A pharmaceutical composition for use in the inhibition or controlling of deregulated angiogenesis comprising rapamycin or a rapamycin derivative, together with one or more pharmaceutically acceptable diluents or carriers therefor.

6. A pharmaceutical combination comprising a) a compound of formula I as defined in claim 1 and b) a co-agent which is a chemotherapeutic agent.

7. A pharmaceutical combination according to claim 6 wherein the co-agent is selected from

i. an aromatase inhibitor,

ii. an antiestrogen, an anti-androgen or a gonadorelin agonist,

iii. a topoisomerase I inhibitor or a topoisomerase II inhibitor,

iv. a microtubule active agent, an alkylating agent, an antineoplastic antimetabolite or a platin compound,

v. a compound targeting/decreasing a protein or lipid kinase activity or a protein or lipid phosphatase activity, a further anti-angiogenic compound or a compound which induces cell differentiation processes,

vi. a bradykinin 1 receptor or an angiotensin II antagonist,

vii. a cyclooxygenase inhibitor, a bisphosphonate, a histone deacetylase inhibitor, a heparanase inhibitor, a biological response modifier, an ubiquitination inhibitor, or an inhibitor which blocks anti-apoptotic pathways,

viii. an inhibitor of Ras oncogenic isoforms,

ix. a telomerase inhibitor, and

x. a protease inhibitor, a matrix metalloproteinase inhibitor, a methionine aminopeptidase inhibitor, or a proteosome inhibitor.

8. A pharmaceutical combination comprising a) rapamycin or a rapamycin derivative and b) a co-agent which is a chemotherapeutic agent selected from those listed under paragraphs (i.) and (v.) to (x.) as specified in claim 7.

9. A method for treating solid tumors in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula I as defined in claim 1, optionally concomitantly or sequentially with a chemotherapeutic agent.

10. A method for inhibiting or controlling deregulated angiogenesis in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of rapamycin or a rapamycin derivative, optionally concomitantly or sequentially with a chemotherapeutic agent.

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Stephen E. Johnson/Monika Van Houten
Attorney Docket Number:	34678-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt	
EFS ID:	11132332
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Monika Van Houten
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	07-OCT-2011
Filing Date:	19-MAY-2008
Time Stamp:	11:45:31
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	7886
Deposit Account	190134
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678-US-IDS.pdf	234309 3c620b1af6d464a04dbc9310764a0e8b6afcc9bc3	yes	4
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	2	
	Information Disclosure Statement (IDS) Form (SB08)		3	4	
Warnings:					
Information:					
2	Foreign Reference	WO05080593.pdf	1106155 67bf538dee9904b8c76126242ca16ff7ccffbfca1	no	21
Warnings:					
Information:					
3	Foreign Reference	WO06065780.pdf	2751417 28c39b6463d6b57b5cc3ffef38289af7742a1a1d	no	54
Warnings:					
Information:					
4	Non Patent Literature	Canobbio.pdf	245936 ecac32cfecaa31f3a6e0ecd17ae5f08495583ff	no	1
Warnings:					
Information:					
5	Foreign Reference	WO04004644.pdf	9132560 1ddcd537c6dfc8879931cd4d912082875b898d41	no	63
Warnings:					
Information:					
6	Foreign Reference	WO02066019.pdf	5246252 cf171aba79fc0f9714daf8d59a656829dd5b027	no	26
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	30272 bbd3c30661e69d9e25677947573 1b0b12e2f262f	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	18746901

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Case PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF Art Unit: 1627

Marks, Peter Wayne et al. Examiner: Jean-Louis, Samira J

INTERNATIONAL APPLICATION NO: PCT/EP06/068656

FILED: November 20, 2006

U.S. APPLICATION NO: 12/094173

35 USC §371 DATE: May 19, 2008

FOR: Neuroendocrine Tumor Treatment

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Sir:

This paper is being filed:

☒ supplemental to the Information Disclosure Statement filed May 19, 2008 and December 4, 2009.

☐ within three months of the date of entry of the national stage as set forth in 37 C.F.R. §1.491 of the international application. Therefore, no fees are required.

☐ before the mailing date of a first Office action on the merits, and so under 37 C.F.R. §1.97(b)(3) no fees are required.

If a fee is deemed to be required, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 19-0134 in the name of Novartis.

☐ This Information Disclosure Statement is being filed in accordance with 37 C.F.R. §1.97(c) or 37 C.F.R. §1.97(d).

☐ A letter for payment of fee set forth in 37 C.F.R. §1.17(p) is enclosed.

In accordance with 37 C.F.R. §1.56, applicants wish to call the Examiner's attention to the references cited on the attached form(s) PTO/SB/08A/B.

☒ The listed references were cited in an extended European search report dated September 8, 2011 and copies are enclosed herewith except for the US patents/applications.

The Examiner is requested to consider the foregoing information in relation to this application and indicate that each reference was considered by returning a copy of the initialed PTO/SB/08A/B form(s).

Respectfully submitted,

Stephen Johnson
for Applicant
Reg. No. 45,916

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Date: 10/7/11

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	34678-US-PCT	9572

1695 7590 10/13/2011

NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 101/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
JEAN-LOUIS, SAMIRA JM	
ART UNIT	PAPER NUMBER
1627	
MAIL DATE	DELIVERY MODE
10/13/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/094,173	MARKS ET AL.	
	Examiner	Art Unit	
	SAMIRA JEAN-LOUIS	1627	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 August 2011*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1-3,8 and 9* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-3 and 8-9* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Response to Arguments

This Office Action is in response to the amendment submitted on 08/02/11. Claims 1-3 and 8-9 are currently pending in the application, with claims 4-7 and 10-12 having being cancelled. Accordingly, claims 1-3 and 8-9 are being examined on the merits herein.

Receipt of the aforementioned amended claims is acknowledged and has been entered.

Applicant's argument with respect to the rejections of claims 10 and 12 under 35 U.S.C. 101 and 112, second paragraph has been fully considered. Given that applicant has cancelled claims 10 and 12, such arguments are now moot. Consequently, the rejections of claims 10 and 12 under 35 U.S.C. 101 and 112, second paragraph are hereby withdrawn.

Applicant's argument with respect to the claim objection of claim 9 has been fully considered. Given that applicant has amended the claim, such objection is now moot. Consequently, the objection of claim 9 is hereby withdrawn.

Applicant's argument with respect to the 102(b) rejection over claims 1-3 and 12 has been fully considered. Applicant argues that the claims as amended are no longer

anticipated by Terrence O'Reilly et al. Such arguments are not persuasive as applicant is arguing the amended claims. It is noted that the features upon which applicant relies (i.e., 40-O-(2-hydroxyethyl)-rapamycin in claim 1 and pancreatic neuroendocrine tumors in claim 2) are not recited in the rejected claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993). Nonetheless, the Examiner maintains that O'Reilly et al. still anticipates applicant's claims as O'Reilly teaches the use of RAD-001 (a.k.a. as 40-O-(2-hydroxyethyl)-rapamycin in the treatment of tumor growth including neuroendocrine tumors of the pancreas). As a result, such claims are still anticipated by the prior art. However, given that applicant has amended the claims and cancelled claim 12, the rejection of claims 1-3 and 12 over O'Reilly is being withdrawn and is modified below.

Applicant's argument with respect to the 103(a) rejection of claims 1-3, 8-9, and 12 over WeckBecker has been fully considered. Applicant argues that WeckBecker only refers to GEP tumors and does not define which tumors GEP refers to. Such arguments are not found persuasive as the examiner contends that applicant is arguing the newly amended claims. It is again noted to applicant that features upon which applicant now relies (i.e. treatment of pancreatic neuroendocrine tumors) are not recited in the rejected claims. Since the claims were directed to treatment of endocrine tumors and pituitary tumors are one type of endocrine tumors, the Examiner maintains that WeckBecker did indeed anticipate the claims. However, given that applicant has

amended the claims, the 102(b) rejection of claims 1-3, 8-9 and 12 over WeckBecker is hereby withdrawn.

For the foregoing reasons, the objection and rejections of record were indeed proper. However, in view of applicant's amendment, the following modified 102(b) and 103 (a) Final rejections are being made.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3 are rejected under 35 U.S.C. 102(b) as being anticipated by O'Reilly et al. (Proceedings of the American Association for Cancer Research Annual Meeting, 03/2002, Vol. 43, pg. 71, previously cited) as evidenced by Merck Manuals (Merck Manuals, Pancreatic endocrine tumors, 2009, pgs. 1-4, previously cited).

O'Reilly et al. teach the use of RAD001 (i.e. 40-O-(2-hydroxyethyl)-rapamycin; elected species; instant claim 1) as a bioavailable hydroxyethyl ether derivative of rapamycin that has demonstrated *in vitro* anti-proliferative activity against a panel of

human tumors (see pg. 71, #359). Importantly, O'Reilly et al. teach that RAD001 was found to be a potent inhibitor of tumor growth in 10 different cell lines and *in vivo* against pancreatic tumors (instant claims 1-2; see pg. 71, #359). Persistent tumor regressions were observed and O'Reilly et al. suggest that RAD001 may not only be effective against tumor cells, it may also affect angiogenesis (see pg. 71, #359). Additionally, O'Reilly et al. teach that doses ranging from 0.5-5.0 mg per day was administered and found to be potent in xenograft and cell line tumor models (see pg. 71, #359).

Merck Manual was provided to demonstrate that pancreatic cancer is characterized by endocrine tumors that tend to produce hormones that lead to aberrant functions and thus pancreatic tumors are classified as endocrine tumors (see pg. 1).

Accordingly, the teachings of O'Reilly et al. anticipate claims 1-3 and 12.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1-3 and 8-9 are rejected under 35 U.S.C. 103 (a) as being unpatentable over by Weckbecker (WO 97/47317, previously cited) as evidenced

by Novartis Data Sheet (Novartis, GEP NE tumors, Published online on 04/2005, pgs. 1-2).

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Weckbecker teaches a combination of a somatostatin analogue and a rapamycin for the prevention and treatment of cell hyperproliferation (see abstract and pg. 1, paragraph 1). Additionally, Weckbecker teaches that rapamycin or derivatives thereof are desired given that such compounds are immunosuppressive and known to inhibit cancer (see pg. 10, last paragraph and pg. 12, last paragraph). A preferred rapamycin compound is 40-O-(2-hydroxy)ethyl-rapamycin (i.e. elected species; instant claim 1; see pg. 12, paragraph 3). According to Weckbecker, such combination can be used for preventing or treating cell hyperproliferation including GEP tumors (i.e. Gastroentero-pancreatic neuroendocrine tumors: slow growing tumors of the pancreas and GI tract)

and pituitary adenomas (another type of endocrine tumor; see pg. 13 and pg. 14, paragraph 2).

Weckbecker does not specifically teach a method of treating pancreatic neuroendocrine tumors. Additionally, Weckbecker does not teach particular dosage administration of 40-O-(2-hydroxy)ethyl-rapamycin.

The Examiner however contends that because Weckbecker teaches administration of an effective amount of a rapamycin derivative (see pg. 13) and given that Weckbecker uses rapamycin in an amount of 5 mg, one of ordinary skill in the art would have found it obvious to administer such dosage amount for 40-O-(2-hydroxy)ethyl-rapamycin in the treatment of GEP and pituitary tumors.

Novartis Data Sheet is provided to demonstrate that GEP, a.k.a gastroenteropancreatic neuroendocrine tumors are slow growing tumors that occur in the pancreas and gastrointestinal tract and are thought to arise from neuroendocrine cells (see pg. 1, paragraphs 1-2). In fact Novartis Data Sheet teaches that pancreatic endocrine tumors are one subtype of such tumors and entail various subtypes including insulinomas, gastrinomas, VIPomas, PPomas, and glucgonomas (see pg. 1, last paragraph).

Thus, to one of ordinary skill in the art at the time of the invention would have found it obvious to treat pancreatic neuroendocrine tumors and other endocrine tumors

such as pituitary tumors since Weckbecker teaches that the combination of somatostatin analogue and a rapamycin derivative such as 40-O-(2-hydroxy)ethyl-rapamycin is effective in the treatment of pituitary tumors and GEP tumors and given that Novartis data Sheet teaches that GEP tumors encompass pancreatic neuroendocrine tumors. Given the teachings of Weckbecker and Novartis Data Sheet, one of ordinary skill would have been motivated to administer the somatostatin and rapamycin derivative of Weckbecker to treat neuroendocrine tumors with the reasonable expectation of providing a method that is effective in treating various endocrine tumors including pancreatic neuroendocrine tumors.

Conclusion

No claims are allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samira Jean-Louis whose telephone number is 571-270-3503. The examiner can normally be reached on 7:30-6 PM EST M-Th.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/S. J. L. /

Examiner, Art Unit 1627

10/06/2011

/SREENI PADMANABHAN/
Supervisory Patent Examiner, Art Unit 1627

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	11/26/2010	02/11/2011	10/05/2011						
	1	÷	✓	✓						
	2	÷	✓	✓						
	3	÷	✓	✓						
	4	÷	N	-						
	5	÷	N	-						
	6	÷	N	-						
	7	÷	N	-						
	8	÷	✓	✓						
	9	÷	✓	✓						
	10	÷	✓	-						
	11	÷	N	-						
	12	÷	✓	-						

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S42	4865	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 18:54
S43	29693	(endocrine tumor) or (neuroendocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic tumor) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (neuroendocrine carcinoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S44	0	S42 near3 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S45	0	S42 near30 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S46	0	S42 near300 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S47	345	S42 and S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S48	18514	somatostatin	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:27
S49	65	S47 and S48	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:27

10/ 6/ 2011 8:35:47 PM
C:\ Users\ sjeanlouis\ Documents\ EAST\ Workspaces\ 12094173-neuroendocrine tumor treatment.wsp

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

SEARCHED			
Class	Subclass	Date	Examiner
None			

SEARCH NOTES		
Search Notes	Date	Examiner
Palm Inventor Name Search	2/10/2011	SJL
STN-see enclosed search history	2/10/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	2/11/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub, Derwent)-see enclosed search notes	10/5/2011	SJL

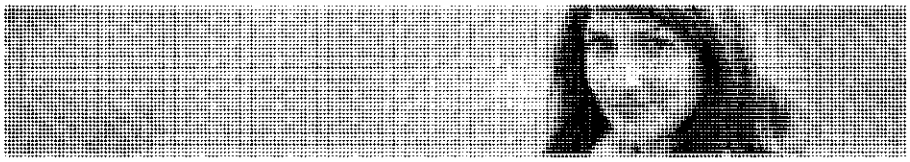
INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

NOVARTIS
oncology

Welcome to Novartis Oncology The Global Site (not intended for US residents)

Home Patients & Caregivers What is Cancer GEP NE Tumors

Home Patients and Caregivers Health Care Professionals Products Research About Us Media Care



Patients & Caregivers

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GEP NE Tumors

What is it? | Causes | Symptoms | Diagnosis | Treatment

GEP NE Tumors Article

Download GEP NE Tumors

What Are GEP NE Tumors?

Gastroenteropancreatic Neuroendocrine (GEP NE) tumors are rare, generally slow-growing tumors that occur in the pancreas and the gastrointestinal tract, which includes the stomach, small intestine and large intestine. GEP NE tumors include carcinoid tumors and pancreatic endocrine tumors (also called pancreatic islet cell tumors).

Normally, neuroendocrine cells in the pancreas and the gastrointestinal system produce hormones and other potent chemicals that help regulate various functions that keep the body in working order. GEP NE tumors are thought to arise from neuroendocrine cells. When they occur, the tumors sometimes have the ability to overproduce proteins and other substances, causing a variety of symptoms throughout the body.

Statistics

Carcinoid tumors are more common than pancreatic endocrine tumors. They are found in about 1 or 2 out of 100,000 people in the United States. They occur most frequently in the appendix, small intestine, and rectum. These tumors tend to grow slowly; in fact, many people are believed to have tiny carcinoids that never cause any health problems. Nevertheless, because it is not possible to distinguish such noncancerous (benign) carcinoids from potentially cancerous (malignant) ones, all discovered carcinoid tumors should be treated as having malignant potential.

Pancreatic endocrine tumors are found in about 4 out of every 1 million people. A number of different pancreatic endocrine tumors have been described. The most commonly occurring types include:

Insulinomas: These tumors are found most often in people between 30 and 60 years of age. They occur slightly more frequently in women. Patients with an insulinoma commonly come to medical attention because of symptoms of hypoglycemia.

Gastrinomas: These tumors are found most frequently in people between the ages of 45 and 50. They are somewhat more common in men than in women. Gastrinomas may be found either within the pancreas itself or in an area immediately adjacent to the pancreas. Patients with gastrinoma typically have symptoms of acid hypersecretion.

VIPomas: These tumors secrete a substance called vasoactive intestinal peptide that can cause profound diarrhea.

PPomas: These tumors are also called nonfunctioning pancreatic endocrine tumors. They do not secrete specific hormones but can be detected through their production of a protein called pancreatic polypeptide.

Glucagonomas: These tumors are less common than other pancreatic endocrine tumors. Patients with glucagonomas often have higher than normal blood sugars and are often diagnosed with diabetes. A rash is another common symptom of glucagonomas.

Risk Factors

Certain conditions that run in families can increase a person's risk of developing a GEP NE tumor. One genetic condition associated with pancreatic endocrine tumors is Multiple Endocrine Neoplasia type I (MEN1), a rare genetic inherited disorder linked to tumors in the pancreas and the parathyroid and pituitary glands. About 20% of gastrinomas and 7% to 8% of insulinomas are associated with MEN1. Genetic tests are available to detect mutations in the gene, MEN1, which are implicated in this disease.

Conditions that affect the production of stomach acid, such as Zollinger-Ellison syndrome, gastritis and pernicious anemia, can also increase the risk of developing gastric carcinoid tumors.

Screening

Unfortunately there is no general screening test to check for GEP NE tumors. However, the earlier a tumor is discovered, the better is a person's chance of survival. For this reason, people who notice symptoms of GEP NE tumors should discuss them with their doctor right away.

‹ previous section | next section ›

Notice of References Cited	Application/Control No. 12/094,173	Applicant(s)/Patent Under Reexamination MARKS ET AL.	
	Examiner SAMIRA JEAN-LOUIS	Art Unit 1627	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	O					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Novartis Data Sheet. Novartis, GEP NE tumors, published online on 04/2005, pgs. 1-2.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.: 9572

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

This Reply is submitted in response to the Final Office Action mailed October 13, 2011. Reconsideration of the present rejections and withdrawal of the present rejections are respectfully requested.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Electronic Acknowledgement Receipt

EFS ID:	11832885
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Andrea Jacquin
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	13-JAN-2012
Filing Date:	19-MAY-2008
Time Stamp:	14:33:16
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678-US-PCT_Amend_2012Jan13.pdf	1145934 6a5c607e22d02e306fbef1f2c24016790b016b81b	yes	6

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment After Final	1	1
	Claims	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	6
Warnings:			
Information:			
	Total Files Size (in bytes):	1145934	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Remarks/Arguments

Reconsideration of this application, as amended, is respectfully requested. Applicants acknowledge the withdrawal of Section 101, Section 102(b) and Section 112, 2nd paragraph rejections and claim objection by the Office in the Office Action dated October 13, 2011 at pages 2-4. Claims 1-3, 8 and 9 were pending in the present application. Claims 1 and 2 were amended. Accordingly, claims 1-3, 8 and 9 are currently pending. No new matter has been added by the present amendments. All amendments are made without prejudice or disclaimer. Applicants reserve the right to prosecute any cancelled or otherwise unclaimed subject matter in this or another application. Applicants believe these amendments place claims 1-3, 8 and 9 in condition for allowance. Consideration and entry of these amendments is respectfully requested.

Rejection under 35 U.S.C. § 102(b)

Claims 1-3 stand rejected under 35 U.S.C. § 102(b) as anticipated by a one paragraph, non-patent publication by Terrence O'Reilly, et al in the Proceedings of the American Association for Cancer Research, 43, 2002, 0359, page 71 (O'Reilly, et al. reference) as evidenced by a second reference , namely Merck Manuals (Merck Manuals, Pancreatic endrocrine tumors, 2009, pgs 1-4). Firstly Applicants respectfully submit that the Office has asserted an improper Section 102(b) rejection (MPEP § 2131, 2131.01). The Merck Manual relied on by the Office, "to show that the primary reference [*sic* O'Reilly et al, of record] has an "enabled disclosure" *In re Samour*, 571 F.2d 559, 197 USPQ 1 (CCPA 1978) and *In re Donohue*, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985)," was published four years later than Applicants' priority date for the claimed invention. As such, the Section 102(b) rejection is improper and Applicants respectfully request withdrawal of the rejection by the Office. As such, each and every element of Applicants' claimed invention is not disclosed for claims 1 and 2 by the primary O'Reilly, et al. reference as required by Section 102(b) (MPEP § 2131, emphasis added).

Applicants further traverse the rejection to the primary reference in view of amendments to claims 1 and 2. The O'Reilly, et al. reference (Abstract 359) does not disclose or suggest Applicants' composition for treating endrocine tumors, as claimed in claim 1 or for treating pancreatic neuroendrocine tumors, as claimed in claim 2. Applicants' amendments overcome the Section 102(b) rejection for claims 1 and 2 and corresponding dependent claim 3. Applicants respectfully request the Office to withdraw the Section 102(b) rejection.

Rejection under 35 U.S.C. § 103(a)

Claims 1-3, 8 and 9 stand rejected under 35 U.S.C. § 102(b) as being unpatentable over international patent publication WO 97/47317 (Weckbecker, et al. reference) as evidenced by

Novartis Data Sheet (Novartis, GEP NE tumors, published on line on 04/2005, pp 1-2). Applicants respectfully submit that the Section 103(a) rejection is improper for claims 1-3 and that the Office has not established a *prima facie* case of obviousness under Section 103(a) for the combination of references cited. The combination of references cited by the Office does not meet the requirement for a rejection of claims 1-3 under Section 103(a). The primary reference, the Weckbecker, et al. reference discloses a combination (emphasis added) of a compound of the somatostatin class and a rapamycin macrolide that is useful for the prevention or treatment of cell hyperproliferation. The Weckbecker, et al. reference does not disclose or suggest using Applicants claimed compound to treat cell hyperproliferation as a monotherapy anywhere in the contents of the reference. The Office has not considered the complete disclosure of the combination of references it has asserted against Applicants' claimed invention. The Novartis Data Sheet cited by the Office also does not evidence what the Office asserts with respect to the primary reference in the Section 103(a) rejection. The term "GEP tumors" are not found in the evidence reference to the primary reference, only GEP NE tumors are disclosed namely Gastroenteropancreatic Neuroendrocrine tumors. The Weckbecker, et al. reference only refers to a GEP tumor at one place in the specification. It does not define which tumors GEP refers to at page 14 or anywhere in the specification. The Office concedes that the Weckbecker, et al. reference does not specifically teach a method of treating pancreatic neuroendrocrine tumors or the dosage of administration using Applicants' claimed compound. Therefore the Office has failed to evidence two elements for Applicants' claimed invention according to claim 1 using the combination of the two references: (1) Applicants claimed method of treating endrocrine tumors comprising administering to a human subject in need thereof (2) a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. The Office has further failed to evidence two elements for Applicants' claimed invention according to claim 2 using the combination of the two references: (1) Applicants claimed method of treating pancreatic neuroendrocrine tumors comprising administering to a human subject in need thereof (2) a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. The Section 103(a) rejection of claims 1-3 is further improper from the hindsight analysis of the combined references asserted using Applicants' disclosure and claimed invention as the roadmap (MPEP § 2145). Applicants request the Office to withdraw the Section 103(a) rejection for claims 1-3. Applicants also point out that since claims 8 and 9 depend on claim 1, the rejection of claims 8 and 9 is improper as well, as dependent claims 8 and 9 are non-obvious with respect to the Section 103(a) rejection and request the Office to withdraw the rejection of claims 8 and 9.

With respect to the Section 103(a) rejection of claims 8 and 9 only, *arguendo*, Applicants respectfully disagree with the Section 103(a) rejection to claims 8 and 9 from the two references the Office has asserted. The primary reference, the Weckbecker, et al. reference discloses a combination of a compound of the somatostatin class and a rapamycin macrolide that is useful for the prevention or treatment of cell hyperproliferation. However, the Office has not

considered the complete disclosure of the combination of references it has asserted against Applicants' claimed invention. The Novartis Data Sheet cited by the Office also does not evidence what the Office asserts with respect to the primary reference in the Section 103(a) rejection. The term "GEP tumors" are not found in the evidence reference to the primary reference, only GEP NE tumors are disclosed namely Gastroenteropancreatic Neuroendrocrine tumors. The Weckbecker, et al. reference only refers to a GEP tumor at one place in the specification. It does not define which tumors GEP refers to at page 14 or anywhere in the specification. The Office concedes that the Weckbecker, et al. reference does not specifically teach a method of treating pancreatic neuroendrocrine tumors or the dosage of administration using Applicants' claimed compound. Therefore the Office has failed to evidence one element for Applicants' claimed invention according to claim 1 using the combination of the two references: (1) Applicants claimed method of treating endrocrine tumors comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. The Office has further failed to evidence one element for Applicants' claimed invention according to claim 2 using the combination of the two references: (1) Applicants claimed method of treating pancreatic neuroendrocrine tumors comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. One of skill in the art would not be able to have the reasonable expectation that Applicants' claimed methods would have been achieved based on the combined disclosure of the two references cited. The Section 103(a) rejection of claims 8 and 9 is improper from the hindsight analysis of the combined references asserted by the Office using Applicants' disclosure and claimed invention as the roadmap (MPEP § 2145). Claims 8 and 9 are in fact non-obvious over the Section 103 (a) rejection of record.

CONCLUSIONS

Consideration and entry of these amendments and remarks are respectfully requested. Claims 1-3, 8 and 9 are now in condition for allowance and Applicants respectfully request that a Notice of Allowance be issued as soon as possible. Should the Examiner have any questions, please contact the undersigned.

Respectfully submitted,

Stephen Johnson
for Applicant
Reg. No. 45,916

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-1422

Date: January 13, 2012

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
12/094,173	05/19/2008	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	01/13/2012									
	Total (37 CFR 1.16(i))	* 5	Minus	** 20	= 0	X $ =		OR	X $60=	0
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $250=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/DESHONNE T. MARTINO/

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. (Currently Amended) A method for treating endocrine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin.

2. (Currently Amended) A method for treating pancreatic neuroendrocine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin.

3. (Previously Presented) The method of claim 2, wherein the unit dose of 40-O-(2-hydroxyethyl)-rapamycin administered is from 0.1 mg to 15 mg.

4.-7. Cancelled

8. (Previously Presented) A method according to claim 1, comprising administering in addition a therapeutically effective amount of at least one second drug substance.

9. (Previously Presented) A method according to claim 8, wherein a second drug substance is somatostatin or a somatostatin analogue.

10.-12. Cancelled

DO NOT ENTER: /S.J.L./

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.: 9572

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

This Reply is submitted in response to the Final Office Action mailed October 13, 2011. Reconsideration of the present rejections and withdrawal of the present rejections are respectfully requested.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	34678-US-PCT	9572

1695 7590 01/23/2012

NOVARTIS
CORPORATE INTELLECTUAL PROPERTY
ONE HEALTH PLAZA 101/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
JEAN-LOUIS, SAMIRA JM	

ART UNIT	PAPER NUMBER
1627	

MAIL DATE	DELIVERY MODE
01/23/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Advisory Action Before the Filing of an Appeal Brief	**Application No.** 12/094,173	**Applicant(s)** MARKS ET AL.
	Examiner SAMIRA JEAN-LOUIS	**Art Unit** 1627

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 13 January 2012 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:
   a) ☒ The period for reply expires *3* months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a) ☒ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b) ☐ They raise the issue of new matter (see NOTE below);
   (c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): a) ☒ will not be entered, or b) ☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.
   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: _____.
   Claim(s) objected to: _____.
   Claim(s) rejected: *1-3,8 and 9*.
   Claim(s) withdrawn from consideration: _____.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☐ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: _____.
12. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
13. ☒ Other: IDS (10/07/2011).

/SAMIRA JEAN-LOUIS/
Primary Examiner, Art Unit 1627

The examiner acknowledges receipt of applicant's amendment and remarks filed on 01/13/2012. However, such amendment will not be entered as they raise new issues that would require further consideration and/or search. As a result, the amendment has not been entered.

Applicant's arugment with respect to the 102(b) rejection over O'Reilly in view of Merck has been fully considered. Applicant argues that the 102(b) rejection is improper as Merck Manual which was relied on by the Office to show that the primary reference is enabled, was published four years later than applicant's priority date for the claimed invention. Such arguments are however not found persuasive as the Examiner respectfully points out that Merk was provided to explain the meaning of a term used in the primary reference, i.e. what is meant by pancreatic tumors. The examiner further points out that extra references or other evidence can be used to show meaning of a term used in the primary reference and such references showing a universal fact need not antedate the filing date. See MPEP § 2124. As for applicant's argument that the O'Reilly reference does not disclose the instant claims as amended, such argument is again not found persuasive as applicant is arguing features not previously presented and yet to be examined. As a result, such arguments are moot. Consequently, the examiner maintains that the 102(b) rejection was indeed proper and is therefore maintained.

Applicant's arguments with respect to the 103(a) rejection over Weckbecker as evidenced by Novartis have been fully considered. Applicant argues that the Office has not established a prima facie case obviousness given that Weckbecker does not disclose or suggest using applicant's claimed compounds to treat cell hyperproliferation as a monotherapy. Additionally, applicant argues that the Novartis reference does not evidence what the Office asserts. Such arguments are however not found persuasive as the examiner contends that Weckbecker as evidenced by Novartis does indeed render obvious applicant's invention. Specifically, Weckbecker teaches a combination of somatostatin analogue with a rapamycin macrolide for the treatment and prevention of cell hyperproliferation (see abstract; pg. 1, lines 1-3, and pg. 13, lines 5-8). A preferred rapamycin is 40-O-(2-hydroxy)ethyl-rapamycin (see pg. 12, lines 6-7). Importantly, Weckbecker teaches that the aforementioned combination can be used for the prevention or treatment of malignant tumor growth including pituitatry adenoma (which is a type of endocrine tumor) and GEP tumors (see pg. 14, lines 4-6). Novartis, on the other hand, was provided to demonstrate that in the art GEP tumors are also known as gastroenteropancreatic neuroendocrine tumors (GEP NE) and are considered to be slow growing tumors that occur in the pancreas and the gastrointestinal tract (see Novartis Data Sheet , pg. 1). Additionally, Novartis Data Sheet reference teaches that GEP or GEP NE tumors include pancreatic endocrine tumors or pancreatic islet cell tumors. As a result, the examiner maintains that contrary to applicant's assertion, Weckbecker's teaching does indeed render obivous applicant's invention and the Novartis reference sheet was provided to explain the meaning of the term GEP tumors which encompasses pancreatic neuroendocrine tumors. Regardless, if GEF tumors were mentioned only once in the reference, the teachings of Weckbecker is clear in its purpose to treat cell hyperproliferation and malignant cancers and further suggests the use of the combination in treating GEP tumors or Gastroenteropancreatic tumors, i.e. tumors of the pancreas. It would have therefore been obvious to one skilled in the art to use such combination to treat tumors of the pancreas and endocrine tumors since Weckbecker teaches effective use of such combination in the treatment of such tumors. As for applicant's argument against claims 8 and 9, the examiner maintains that such claims are properly rejected given that Weckbecker as evidenced by Novartis has established a prima facie case of obviousness.

For the foregoing reasons, the examiner maintains that the rejections of record were indeed proper and are therfore maintained.

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO2005/080593 A2	09/01/2005	Novartis Pharma GmbH		☐
		WO2004/004644 A2	01/15/2004	Neel, Benjamin G		☐
		WO2006/065780	06-22-2006	Novartis AG		☐
		WO2002/066019	08-29-2002	Novartis AG		☐
						☐
						☐

Examiner Signature	/Samira Jean-louis/	**Date Considered**	01/17/2012

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw a line through citation if not in conformance and not considered. Include copy of this form with the next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kind Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L.

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Database Medline: Canobbio L. et al: "Use of long-acting somatostatin analog, lanreotide, in neuro-endocrine tumors." Oncology reports, vol. 1, no.1 Jan. 1994 p. 129- 131	☐
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Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw a line through citation if not in conformance and not considered. Include copy of this form with the next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L.

PTO/SB/30 (07-09)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	12/094173
Filing Date	May 19, 2008
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant must request non-entry of such amendment(s).
   - a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
     - ii. ☐ Other _____
   - b. ☒ Enclosed
     - i. ☒ Amendment/Reply
     - ii. ☐ Affidavit(s)/Declaration(s)
     - iii. ☐ Information Disclosure Statement (IDS)
     - iv. ☐ Other _____

2. Miscellaneous
   - a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   - a. ☒ The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 19-0134 in the name of Novartis.
     - i. ☒ RCE fee required under 37 CFR 1.17(e)
     - ii. ☒ Extension of time fee (37 CFR 1.136 and 1.17)
     - iii. ☐ Other _____
   - b. ☐ Check in the amount of $_____ enclosed
   - c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit Card information should not be included on this form. Provide credit card information and authorization on PTO-2038

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature		Date	February 2, 2012
Name (Print/Type)	Stephen Johnson	Registration No.	45,916

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature			
Name (Print/Type)		Date	

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.: 9572

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

This Amendment is submitted as part of a Request for Continued Examination pursuant to 37 C.F.R. § 1.114 in response to the Advisory Action mailed January 23, 2011. In addition, Applicants are filing a Notice of Appeal in response to the Advisory Action mailed January 23, 2011. Reconsideration of the present rejections and withdrawal of the present rejections are respectfully requested.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. (Currently Amended) A method for treating endocrine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin.

2. (Currently Amended) A method for treating pancreatic neuroendrocine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin.

3. (Previously Presented) The method of claim 2, wherein the unit dose of 40-O-(2-hydroxyethyl)-rapamycin administered is from 0.1 mg to 15 mg.

4.-12. Cancelled

Remarks/Arguments

Reconsideration of this application, as amended, is respectfully requested. Applicants acknowledge the withdrawal of Section 101, Section 102(b) and Section 112, 2nd paragraph rejections and claim objection by the Office in the Office Action dated October 13, 2011 at pages 2-4. Claims 1-3, 8 and 9 were pending in the present application. Claims 1 and 2 were amended. Claims 8 and 9 were cancelled without prejudice and applicants reserve the right to pursue the claims in a corresponding continuation application. Accordingly, claims 1-3 are currently pending. No new matter has been added by the present amendments. All amendments are made without prejudice or disclaimer. Applicants reserve the right to prosecute any cancelled or otherwise unclaimed subject matter in this or another application. Applicants believe these amendments place claims 1-3, 8 and 9 in condition for allowance. Consideration and entry of these amendments is respectfully requested.

Rejection under 35 U.S.C. § 102(b)

Claims 1-3 stand rejected under 35 U.S.C. § 102(b) as anticipated by a one paragraph, non-patent publication by Terrence O'Reilly, et al in the Proceedings of the American Association for Cancer Research, 43, 2002, 0359, page 71 (O'Reilly, et al. reference) as evidenced by a second reference , namely Merck Manuals (Merck Manuals, Pancreatic endrocrine tumors, 2009, pgs 1-4). Applicants traverse the rejection to the primary reference in view of amendments to claims 1 and 2. The O'Reilly, et al. reference (Abstract 359) does not disclose or suggest Applicants' composition for treating endrocine tumors in humans, as claimed in claim 1 or for treating pancreatic neuroendrocine tumors in humans, as claimed in claim 2. The O'Reilly Abstract is limited in its disclosure regarding Applicants' claimed invention. First, the O'Reilly Abstract does not disclose or suggest endrocrine tumors or pancreatic neuroendrocrine tumors. The Office further relies on an incorrect assertion that all pancreatic cancers and tumors are the same as all endrocrine cancers and tumors using the Merck Manual. Persons of skill in the art, e.g. clinical research MDs, oncologists, clearly distinguish pancreatic cancers/tumors and endocrine cancer/tumors. Applicants point to Appendices I, II which states categorically that pancreatic cancers and tumors are differentiated from endrocrine cancers and tumors (JHU, USC references respectively). The JHU reference recites at page 4, 1st paragraph that, Neoplasms (tumors) of the endocrine pancreas do occur, but are relatively rare, with an annual clinically recognized incidence approximating five cases per one million person years. By contrast, pancreatic cancer (and again by this we mean ductal andenocarcinoma) strikes about 9 or 10 per 100,000 people each year. Neoplasms (tumors) of the endocrine pancreas are known by two broad names; "islet cell tumor," and "well-differentiated pancreatic neuroendrocrine neoplasm" (Appendix I)." The USC reference (pages

1-2 pancreatic cancer, pages 1-3 endocrine tumors of abdomen, clearly and unambiguously differentiates pancreatic cancers/tumors from endocrine cancers/tumors (Appendix II) There is no such teaching or disclosure recuted in the O'Reilly Abstract, which only recites "...xenograft models of human tumors (including pancreatic , colon epidermoid, lung and melanoma." The O'Reilly Abstract does not disclose or suggest administering to a human subject a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin, as claimed. The reference is limited to disclosing and reciting in its explicit teaching of "xenograft models of human tumors (emphasis added)".

#359 *in vivo* activity of RAD001, an orally active rapamycin derivative, in experimental tumor models. Terence O'Reilly, Juliane Vaxelaire, Melanie Muller, Heinz-Herbert Flebig, Marc Hattenberger, and Heidi A. Lane. *Business Unit Oncology, Novartis Pharma AG, Basel, Switzerland, and Oncotest gmbH, Freiberg, Germany.*

RAD001 is a hydroxyethyl ether derivative of rapamycin that is orally bioavailable. RAD001 has demonstrated *in vitro* anti-proliferative activity against a panel of human tumor lines. For *in vivo* testing, tumor-bearing nude mice were administered RAD001 in a variety of doses and schedules. Tumors were established by transplantation of fragments generated from injection of cells, or by transplantation of fragments from stabilized tumors originating from surgically removed human tumors. When administered once daily p.o., at doses ranging from 0.5-5.0 mg/kg/day, RAD001 was a potent inhibitor of tumor growth in 10 different xenograft models of human tumors (including pancreatic, colon, epidermoid, lung and melanoma). In general, RAD001 was well tolerated and better tolerated in mouse xenograft models than standard cytotoxic agents (i.e. doxorubicin and 5-fluorouracil), while possessing similar antitumor activity. Only one instance of *in vivo* resistance has been observed (MAXF 401 mammary xenograft model), otherwise the activity of RAD001 was generally inhibition of tumor growth (persistent regressions in one tumor line, T/C values of 9 to 45 % in 8 tumor lines). Xenograft models sensitive to RAD001 treatment included tumors exhibiting comparative resistance *in vitro* (KB-31 and HCT116). Persistent tumor regressions (41-%) were observed in a line displaying sensitivity to RAD001 *in vitro* (A549). Pharmacokinetic analyses, following a 5 mg/kg administration, indicated rapid uptake into plasma (Cmax 2513 ng/ml; Tmax 1 h), but the time to Cmax was delayed in tumors (Cmax 102 ng/g; Tmax 2 h). Elimination from the tumor (t1/2, 16 hr) was apparently slower than for plasma (t1/2, 7.5 hr). RAD001 levels were above the IC50 of A549 cells for a 72 h period. Interestingly, tumor RAD001 levels, following a single 5 mg/kg administration, never exceeded the *in vitro* antiproliferative IC50 for either KB-31 or HCT116 cells; despite the sensitivity of these lines *in vivo*. From these observations, and given the extreme sensitivity of endothelial cells to RAD001, it is plausable that RAD001 may not only act on tumor cells but may also affect angiogenesis. Taken together, these data support the application of RAD001 as an antitumor agent.

Finally, Applicants disagree with the Office's assertion under MPEP 2124.

2124 Exception to the Rule That the Critical Reference Date Must Precede the Filing Date

IN SOME CIRCUMSTANCES A FACTUAL REFERENCE NEED NOT ANTEDATE THE FILING DATE

In certain circumstances, references cited to show a universal fact need not be available as prior art before applicant's filing date. *In re Wilson*, 311 F.2d 266, 135 USPQ 442 (CCPA 1962). Such facts include the characteristics and properties of a material or a scientific truism. Some specific examples in which later publications showing factual evidence can be cited include situations where the facts shown in the reference are evidence "that, as of an application's filing date, undue experimentation would have been required, *In re Corneil*, 347 F.2d 563, 568, 145 USPQ 702, 705 (CCPA 1965), or that a parameter absent from the claims was or was not critical, *In re Rainer*, 305 F.2d 505, 507 n.3, 134 USPQ 343, 345 n.3 (CCPA 1962), or that a statement in the specification was inaccurate, *In re Marzocchi,* 439 F.2d 220, 223 n.4, 169 USPQ 367, 370 n.4 (CCPA 1971), or that the invention was inoperative or lacked utility, *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA 1974), or that a claim was indefinite, *In re Glass*, 492 F.2d 1228,1232 n.6, 181 USPQ 31, 34 n.6 (CCPA 1974), or that characteristics of prior art products were known, *In re Wilson*, 311 F.2d 266, 135 USPQ 442 (CCPA 1962)." *In re Koller,* 613 F.2d 819, 823 n.5, 204 USPQ 702, 706 n.5 (CCPA 1980) (quoting *In re Hogan,* 559 F.2d 595, 605 n.17, 194 USPQ 527, 537 n.17 (CCPA 1977) (emphasis in original)). However, it is impermissible to use a later factual reference to determine whether the application is enabled or described as required under 35 U.S.C. 112, first paragraph. *In re Koller*, 613 F.2d 819, 823 n. 5, 204 USPQ 702, 706 n.5 (CCPA 1980). References which do not qualify as prior art because they postdate the claimed invention may be relied upon to show the level of ordinary skill in the art at or around the time the invention was made. *Ex parte Erlich*, 22 USPQ 1463 (Bd. Pat. App. & Inter. 1992).

Applicants respectfully disagree with the office's interpretation. However, in view of Applicants amendment and traverse, the point is moot. As such, each and every element of Applicants' claimed invention is not disclosed for claims 1 and 2 by the primary O'Reilly, et al. reference as required by Section 102(b) (MPEP § 2131, emphasis added). Applicants' amendments overcome the Section 102(b) rejection for claims 1 and 2 and corresponding dependent claim 3. Applicants respectfully request the Office to withdraw the Section 102(b) rejection for claims 1-3.

Rejection under 35 U.S.C. § 103(a)

Claims 1-3, 8 and 9 stand rejected under 35 U.S.C. § 102(b) as being unpatentable over international patent publication WO 97/47317 (Weckbecker, et al. reference) as evidenced by Novartis Data Sheet (Novartis, GEP NE tumors, published on line on 04/2005, pp 1-2). Applicants traverse the argument in view of the amended claims. Applicants respectfully maintain that the Section 103(a) rejection is improper for claims 1-3 and that the Office has not established a *prima facie* case of obviousness under Section 103(a) for the combination of references cited. The combination of references cited by the Office does not meet the requirement for a rejection of claims 1-3 under Section 103(a). The primary reference, the

Weckbecker, et al. reference discloses a combination (emphasis added) of a compound of the somatostatin class and a rapamycin macrolide that is useful for the prevention or treatment of cell hyperproliferation. The Weckbecker, et al. reference does not disclose or suggest using Applicants claimed compound to treat cell hyperproliferation as a monotherapy anywhere in the contents of the reference. The Weckbecker, et al. reference discloses at page 14 a list of indications of some types of neuroendrocrine tumors (e.g. GEP tumors, pituitary adenoma). However, the reference does not disclose or suggest any further data or statements in support for the disclosed combination to treat neuroendrocrine tumors (NET tumors, also cited in the reference the Office cites). The Examples in the reference recite an *in vitro* assay for breast cancer and an *in vivo* assay for azaserine induced exocrine pancreatic tumor. Applicants point out that an exocrine pancreatic tumor is a malignant neoplasm of the pancreas, which is not related to pancreatic neuroendrocrine tumors. The primary reference evidences a teaching away from Applicants' claimed method that was not considered by the office. The primary reference at best suggests or teaches combined therapeutic agents, not Applicants claimed monotherapy with everolimus (40-0-(2-hydroxyethyl)-rapamycin). The Office has not considered the complete disclosure of the combination of references it has asserted against Applicants' claimed invention. The Weckbecker, et al. reference only refers to a GEP tumor at one place in the specification. It does not define which tumors GEP refers to at page 14 or anywhere in the specification. The secondary reference (Novartis Data Sheet) does not clarify "GEP tumors" with respect to the Section 103(a) rejection. The term "GEP tumors" are not found in the evidence reference to the primary reference, only GEP NE tumors are disclosed namely Gastroenteropancreatic Neuroendrocrine tumors. The Office concedes that the Weckbecker, et al. reference does not specifically teach a method of treating pancreatic neuroendrocrine tumors or the dosage of administration using Applicants' claimed compound. Therefore the Office has failed to evidence two elements for Applicants' claimed invention according to claim 1 using the combination of the two references: (1) Applicants claimed method of treating endrocrine tumors comprising administering to a human subject in need thereof (2) a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. The Office has further failed to evidence two elements for Applicants' claimed invention according to claim 2 using the combination of the two references: (1) Applicants claimed method of treating pancreatic neuroendrocrine tumors comprising administering to a human subject in need thereof (2) a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin. The Section 103(a) rejection of claims 1-3 is further improper from the hindsight analysis of the combined references asserted using Applicants' disclosure and claimed invention as the roadmap (MPEP § 2145). Applicants request the Office to withdraw the Section 103(a) rejection for claims 1-3. Applicants also point out that since claims 8 and 9 depend on claim 1, the rejection of claims 8 and 9 is improper as well, as dependent claims 8 and 9 are non-obvious with respect to the Section 103(a) rejection and request the Office to withdraw the rejection of claims 8 and 9.

With respect to the Section 103(a) rejection of claims 8 and 9 only, Applicants traverse in view of cancelling claims 8 and 9 without prejudice, therby obviating the rejection.

CONCLUSIONS

Consideration and entry of these amendments and remarks are respectfully requested. Claims 1-3, 8 and 9 are now in condition for allowance and Applicants respectfully request that a Notice of Allowance be issued as soon as possible. Should the Examiner have any questions, please contact the undersigned.

Respectfully submitted,

Stephen Johnson
for Applicant
Reg. No. 45,916

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
(862) 778-1422

Date: February 6, 2012

CASE PAT034678-US-PCT

FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10	
Express Mail Label Number	Date of Deposit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

NOTICE OF APPEAL

Sir:

Applicants hereby appeal to the Board of Patent Appeals and Interferences from the Office Action dated October 13, 2011 finally rejecting claims 1-3 and 8-9.

☒ Please charge Deposit Account No. 19-0134 in the name of Novartis in the amount of $620 for payment of the appeal fee. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Account No. 19-0134 in the name of Novartis.

☐ The appeal fee was paid in a previous appeal herein. The examiner re-opened prosecution prior to any decision by the Board of Patent Appeals and Interferences. No fee is now due.

☒ Enclosed is a Petition for Extension of Time.

Respectfully submitted,

Stephen Johnson
for Applicant
Reg. No. 45,916

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Date: February 2, 2012

CASE PAT034678-US-PCT

FILING BY "EXPRESS MAIL" UNDER 37 CFR 1.10	
Express Mail Label Number	Date of Deposit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF — Art Unit: 1627

Marks, Peter Wayne et al. — Examiner: Jean-Louis, Samira J

INTERNATIONAL APPLICATION NO: PCT/EP06/068656

FILED: November 20, 2006

U.S. APPLICATION NO: 12/094173

35 USC §371 DATE: May 19, 2008

FOR: Neuroendocrine Tumor Treatment

MS: After Final
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

NOTICE OF APPEAL
PETITION FOR EXTENSION OF TIME

Sir:

The period for filing a Notice of Appeal has a shortened statutory time set to expire on January 13, 2012. A one-month extension is hereby requested pursuant to 37 CFR §1.136(a).

Please charge Deposit Account No. 19-0134 in the name of Novartis in the amount of $130 for payment of the extension fee. An additional copy of this paper is here enclosed. The Commissioner is hereby authorized to charge any additional fees under 37 CFR §1.17 which may be required, or credit any overpayment, to Account No. 19-0134 in the name of Novartis.

Respectfully submitted,

Stephen Johnson
for Applicant
Reg. No. 45,916

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Date: February 2, 2012

Electronic Acknowledgement Receipt	
EFS ID:	12008984
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Andrea Jacquin
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	06-FEB-2012
Filing Date:	19-MAY-2008
Time Stamp:	16:35:52
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1700
RAM confirmation Number	3433
Deposit Account	190134
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678-US_PCT_RCE_Amend_Noticeof Appeal_2012Feb6.pdf	2824832 430f23195c10dc6f4157d930ff347b75a119e894	yes	35

Multipart Description/PDF files in .zip description

Document Description	Start	End
Request for Continued Examination (RCE)	1	1
Amendment After Final	2	2
Claims	3	3
Applicant Arguments/Remarks Made in an Amendment	4	8
Notice of Appeal Filed	9	9
Extension of Time	10	10
Miscellaneous Incoming Letter	11	35

Warnings:

Information:

2	Fee Worksheet (SB06)	fee-info.pdf	33683 37ce1246568418 4c36c9ea5beb40f646adef bf63	no	2

Warnings:

Information:

Total Files Size (in bytes):	2858515

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/10S) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Stephen E. Johnson/Andrea Jacquin
Attorney Docket Number:	34678-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Notice of appeal	1401	1	620	620
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	150	150
Miscellaneous:				
Request for continued examination	1801	1	930	930
		Total in USD ($)		**1700**

APPENDIX 1

The Sol Goldman Pancreatic Cancer Research Center

- For Patients & Family
- Cancer Clinic
- Cyst Clinic
- NFPTR
- Medical Professionals
- Donate
- Blog

Expand All Collapse All

Johns Hopkins & You

- Our Pledge to You
- Choosing Johns Hopkins
  - Importance of Experienced Surgeon
  - Our Multidisciplinary Approach
  - Meet Our Team
  - Importance of Accurate Diagnosis
- Evaluating My Case
  - My tumor can be surgically removed
  - My tumor cannot be removed
  - I am not sure
  - Get a second opinion
- Becoming a Hopkins Patient
  - Making an Appointment
  - Preparing for an Appointment
  - Your Appointment
  - Getting Here
  - Housing Options
  - About Baltimore
- Pancreas Cancer Clinic
- Pancreas Cyst Clinic
- Participating in Research
  - National Familial Pancreas Tumor Registry
  - Gastrointestinal Cancer Rapid Medical Donation Program
  - Specific to Individuals of Ashkenazi Jewish Ancestry
  - Specific to African-American population
  - Pain & Quality of Life
  - Kimmel Cancer Center Clinical Trials Database
- Supporting Research
- Raising Awareness
- Our Blog

Patient Education

- Pancreas News
- Basics of Pancreatic Cancer
  - FAQs
  - The Pancreas
  - Types of Tumors
  - Causes
  - Hereditary Factors
  - Known Risk Factors
  - Symptoms and Side Effects
  - Screening
  - Questions
  - Ask a Nurse Practitioner
- Diagnosis
  - Diagnostic Tests
  - Metastasis
  - Staging
- Treatment and Care
  - Surgical Treatment
  - Laparoscopic Surgery
  - Vaccine
  - Radiation Therapy
  - Chemotherapy
  - Clinical Trials
  - Pain Management
  - Nutrition and Exercise
  - Holistic Care
  - Pathology
- Intraductal Papillary Mucinous Neoplasms
- Islet Cell Tumors & Endocrine Neoplasms of the Pancreas
- Research Presentations
- Bibliography
- Other Pancreas Sites

Support Services & Research

- Support
- Research
  - Genetics - Kern Lab
  - Early Detection- Goggins Lab
  - Sol Goldman Center
- Discussion Board

Patient Education / Treatment and Care

- Patient Treatment

- Our Research
- Supporting Islet Cell Tumor Research

Islet Cell Tumors of the Pancreas / Endocrine Neoplasms of the Pancreas

Sections on this page:

While the bulk of this web site is dedicated to the more common adenocarcinomas of the pancreas, the research team at Johns Hopkins also extensively studies islet cell tumors (also known as well-differentiated pancreatic endocrine neoplasms). In addition, all of the surgeons and all of the oncologists listed in this web site also treat patients with islet cell tumors / endocrine neoplasms of the pancreas. These tumors differ from pancreatic cancer (by that we mean adenocarcinoma of the pancreas) in three important ways. First, islet cell tumors / endocrine neoplasms grow much slower than does pancreatic cancer. As a result, patients with an islet cell tumor / endocrine neoplasm have a much better prognosis than do patients with pancreatic cancer. In fact, some patients with small islet cell tumors / pancreatic endocrine neoplasms are cured if their tumor is treated early enough. Second, some islet cell tumors / endocrine neoplasms produce large amounts of specific hormones, and the release of these hormones can result in dramatic clinical symptoms. For example, some islet cell tumors / endocrine neoplasms produce large quantities of insulin that is released into the bloodstream causing a dramatic lowering of blood sugar levels (called hypoglycemia). These tumors are called insulinomas. Third, some islet cell tumors / endocrine neoplasms arise in patients with a familial genetic syndrome. For example, Multiple Endocrine Neoplasia type 1 (MEN 1) is caused by an inherited mutation in the gene called menin, and patients with MEN 1 develop pituitary tumors, tumors of their parathyroid gland, and multiple islet cell tumors / endocrine neoplasms of their pancreas. An understanding of the familial risk is important for family counseling and for early detection efforts in affected individuals.

This section of our web site reviews the basics of islet cell tumors / pancreatic endocrine neoplasms with emphasis on the classification, diagnosis and treatment of these tumors. We also review the ongoing research accomplishments in this area by the Hopkins team. Although slightly technical, it is hoped that the information provided below will be useful for those physicians treating patients with endocrine tumors of the pancreas and for those patients suffering from endocrine tumors of the pancreas.

INTRODUCTION

The pancreas is really two organs in one. Most of the pancreas produces enzymes that help with the digestion of food. This portion of the pancreas is called the "exocrine" pancreas, and it is thought to give rise to the most common type of pancreatic cancer called ductal adenocarcinoma (also commonly simply referred to as pancreatic cancer). The pancreas also contains numerous islands of endocrine cells called the "islets of Langerhans." These islands contain small round cells (the endocrine cells) that produce the hormones that control blood sugar levels. These cells make insulin, glucagon and somatostatin. Insulin lowers blood sugar levels and glucagon generally raises blood sugar levels. Neoplasms (tumors) of the endocrine pancreas do occur, but are relatively rare, with an annual clinically recognized incidence approximating five cases per one million person-years. By contrast, pancreatic cancer (and again by this we mean ductal adenocarcinoma) strikes about 9 or 10 per 100,000 people each year. Neoplasms (tumors) of the endocrine pancreas are known by two broad names; "islet cell tumor," and "well-differentiated pancreatic endocrine neoplasm."

Islet cell tumors / pancreatic endocrine neoplasms are best divided into those that produce symptoms because they release hormones into the bloodstream ("functional") and those that form a mass but do not cause symptoms by releasing hormones into the blood stream ("nonfunctional). It used to be that the majority of islet cell tumors / pancreatic endocrine neoplasms that were discovered clinically were functional, indicating that they elaborate one or more hormonal products into the blood, leading to a recognizable clinical syndrome. As more and more people are getting CT scans for a wide variety of reasons, more and more islet cell tumors / pancreatic endocrine neoplasms are non-functional. These nonfunctional islet cell tumors / pancreatic endocrine neoplasms are seen as masses in the pancreas on imaging. By convention, functional islet cell tumors / endocrine neoplasms are named according to their predominant clinical syndrome and hormonal product. For example, insulinomas are islet cell tumors / pancreatic endocrine neoplasms that produce insulin and which thereby cause low blood sugar levels. Patients with islet cell tumors / pancreatic endocrine neoplasms of the pancreas with no recognizable clinical syndrome and normal serum hormone levels are considered to have nonfunctional pancreatic endocrine tumors.

CAUSES

Many of the risk factors that have been identified for pancreatic cancer (ductal adenocarcinomas of the pancreas) are not risk factors for islet cell tumors / pancreatic neuroendocrine neoplasms. For example, cigarette smoking doubles the risk of pancreatic cancer, but it does not appear to be a factor in the development of islet cell tumors / pancreatic endocrine neoplasms.

Several genetic (familial) syndromes predispose to pancreatic neuroendocrine neoplasms. These are important to recognize for three reasons. First, because these genetic syndromes are caused by genetic changes that can be inherited, other family members may be at risk. Second, these genetic syndromes predispose to (increase the risk of) more than one tumor type. Individuals with one of these syndromes therefore have an increased risk of developing pancreatic and extra-pancreatic neuroendocrine tumors. Finally, these syndromes are important because they provide insight into the

biology of pancreatic neuroendocrine neoplasms. Because the genes that cause these genetic syndromes are known, scientists can understand the cellular pathways which lead to the development of pancreatic neuroendocrine neoplasms. The hope is that a better understanding of these pathways will lead to better treatments.

Multiple Endocrine Neoplasia Type 1, abbreviated MEN-1, is a familial syndrome caused by inherited mutations in the MEN1 gene on chromosome 11. The MEN-1 gene codes for the menin protein, and patients with an inherited mutation in the MEN1 are predisposed to develop tumors of the pituitary (the small "master" gland at the base of the brain), the parathyroids (four small glands in neck which help control blood calcium levels), and the pancreas.

von Hippel-Lindau Syndrome, abbreviated VHL, is a familial syndrome caused by inherited mutations in the VHL gene on chromosome3. Patients with von Hippel-Lindau are predisposed to developing tumors in a number of organs including the the brain (hemagioblastoma), the eye (hemagioblastoma), the kidney (renal cell carcinoma), and the adrenals (pheochromocytoma). Pancreatic disease may be the first manifestation of VHL, and most patients with VHL eventually develop a pancreatic tumor. The pancreatic tumors in patients with VHL are interesting because they can have unique appearances. Some have a "clear" appearance under the microscope, and others are mixed tumors.

Tuberous Sclerosis Complex (TSC) is a third genetic syndrome which predisposes to pancreatic neuroendocrine neoplasms (http://www.tsalliance.org/index.aspx). Tuberous sclerosis complex is caused by inherited mutations in one of two genes- TSC1 or TSC2. The TSC1 gene is on chromosome 9 and it codes for the protein hamartin. The TSC2 gene is on chromosome 16 and it codes for the protein tuberin. Patients with tuberous sclerosis complex can suffer from developmental delay, mental retardation and even autism, and they are predisposed to develop a number of different tumors. They are predisposed to develop three different brain lesions- "tubers," subependymal giant cell astrocytomas and subependymal nodules. In fact, the name tuberous sclerosis comes from the brain lesions that these patients develop- Tuber in Latin means swelling and skleros in Greek means hard. They also can develop distinctive lesions of the skin (hypomelanotic macules and facial angiofibromas), kidney (angiomyolipomas), lungs (lymphangioleiomatosis), heart (rhabdomyomas), and eye tumors (hamartomas). Most of these are entirely benign growths, but they can cause symptoms depending on their location. Although less common than the other manifestations of tuberous sclerosis, several patients with tuberous sclerosis complex have been reported who developed pancreatic neuroendocrine neoplasms.

Neurofibromatosis type 1, also known as von Recklinghausen disease, is a familial syndrome caused by inherited mutations in the NF1 gene on chromosome 17 (http://ghr.nlm.nih.gov/condition/neurofibromatosis-type-1). The NF1 gene codes for the protein neurofibromin. Patients with neurofibromatosis develop dark patches of skin (café-au-lait spots), and benign (non-cancerous) and malignant (cancerous) tumors of the nervous system. The benign nerous system tumors include neurofibromas, and the malignant the "malignant peripheral nerve sheath tumor." They can also develop distinctive eye lesions called Lisch nodules, and a small percentage develop somatostatinomas of the pancreas/duodenum.

Clinical genetic testing is now available for these syndromes, but such testing is best done with the help of a trained genetic counselor. Individuals found to have one of these genetic syndromes may benefit from increased screening for early tumors, and, it is our hope, that a better understanding of the genetics of these syndromes will lead to novel gene-specific therapies in the future.

PATHOLOGY

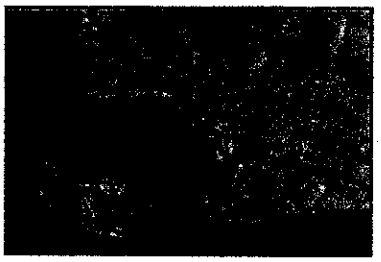

Figure 1 - click for larger

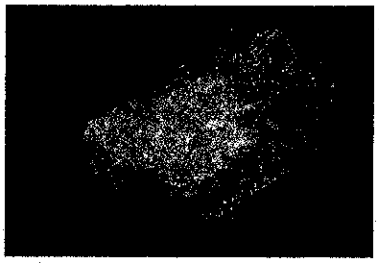

Figure 2 - click for larger

Figure 3 - click for larger

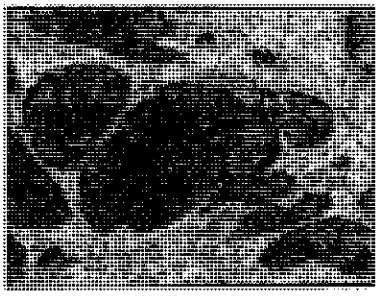

Figure 4 - click for larger

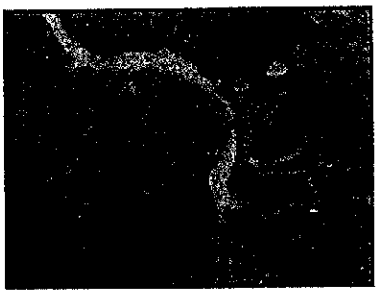

Figure 5 - click for larger

Islet cell tumors / pancreatic endocrine neoplasms of the pancreas have a distinct appearance. These tumors tend to form round well-demarcated masses **(figure 1)**. This appearance contrasts to the appearance of pancreatic cancer (ductal adenocarcinomas) which forms much more poorly-defined masses. As they enlarge islet cell tumors / pancreatic endocrine neoplasms can grow into blood vessels, out of the pancreas and even into adjacent organs such as the spleen **(figure 2)**. Islet cell tumors / endocrine neoplasms can also spread (metastasize) to lymph nodes and even to other organs such as the liver. Because they have the ability to spread, islet cell tumors / pancreatic endocrine neoplasms are generally considered "malignant" tumors.

All islet cell tumors / pancreatic endocrine neoplasms of the pancreas have a similar light microscopic appearance. These tumors are composed of round uniform cells with round uniform nuclei **(figure 3)**. Special stains, called immunohistochemical stains, can be used to demonstrate the production of endocrine hormones, such as insulin and glucagon, in tissue sections made from biopsies or resected tumors **(figure 4)**. Routine histologic examination does not predict the clinical behavior or the endocrine manifestations of these neoplasms. Prognosis is typically determined by the presence of local invasion, spread to regional lymph nodes, or the existence of hepatic or distant metastases.

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BLOOD TESTS

Elevated levels of the hormones produced by islet cell tumors / endocrine neoplasms can be detected in the blood by special tests that can be run on blood samples taken from a patient. These tests are measured using immunoassays, including radioimmunoassay. Tests for insulin, gastrin, VIP, glucagon, somatostatin, pancreatic polypeptide, prostaglandins and other hormonal markers are typically performed at selected large medical center or reference laboratories.

LOCALIZATION AND STAGING

At present, the initial imaging technique recommended for localization of an islet cell tumors / pancreatic endocrine neoplasms is a CT scan with intravenous and oral contrast (22-25). The accuracy of the CT scan in tumor localization is improved by the use of both oral and intravenous contrast. The CT scan is also used to assess for peripancreatic lymph node enlargement and for the presence of hepatic metastases. Islet cell tumors / pancreatic endocrine neoplasms typically produce solid masses on CT scanning and, importantly, these tumors usually "enhance" in the arterial phase **(figure 5)**. This latter feature is believed to be a manifestation of the rich blood supply that these tumors have, and it is a helpful diagnostic feature.

Should the CT scan fail to detect the primary tumor, the next step in radiographic assessment may be visceral angiography, focusing upon selective visualization of the arterial supply to the pancreas and peripancreatic regions (26).

Endoscopic ultrasonography can also be used to visualize islet cell tumors / pancreatic endocrine neoplasms and this technique has the advantage that a biopsy can be taken at the same time (27-29). Rosch and colleagues were able to correctly localize 32 of 39 tumors (82%) using endoscopic ultrasound, after a prior CT scan had failed to locate the tumor. In their experience, endoscopic ultrasonography was more sensitive than the combination of CT and visceral angiography. As further experience is gained with endoscopic ultrasound, it may offer distinct advantages in the evaluation of patients with islet cell tumors / pancreatic endocrine neoplasms.

Another technique that holds promise for the imaging of pancreatic endocrine tumors is somatostatin receptor imaging. These techniques rely upon the presence of somatostatin receptors on many islet cell tumors / pancreatic endocrine neoplasms (33), and have the potential for identifying both primary tumors as well as liver and other metastases. These scans are called "octreotide scans". Basically, a radioactive form of octreotide, a drug similar to somatostatin, is injected into the blood stream. It travels in the bloodstream and binds to (attaches to) islet cell tumors / pancreatic endocrine neoplasms that have the receptor for somatostatin on their surface. A radiation-measuring device can then detect the radioactive octreotide localized to the tumor.

In a minority of patients with islet cell tumors / pancreatic endocrine neoplasms, the primary tumor will not be localized following initial imaging studies such as CT, visceral angiography or endoscopic ultrasound. Most commonly this situation arises in patients with very small insulinoma or gastrinoma. In these cases, localization of the occult neoplasm may be assisted by the performance of selective transhepatic portal venous hormone sampling (37-41). This invasive technique involves the insertion of a catheter in the blood stream. It is designed to demonstrate a step-up in hormone concentration at the site where the tumor drains its hormonal product into the blood vessels leading into the liver. The results of portal venous hormone sampling are used to define a region of the pancreas (or duodenum in the case of gastrinoma) harboring the occult tumor.

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SURGICAL EXPLORATION

Surgical resection remains the best hope for a cure for patients with islet cell tumors / pancreatic endocrine neoplasms.

At the time of surgical exploration for pancreatic endocrine neoplasm, a complete evaluation of the pancreas and peripancreatic regions is performed. The liver is carefully assessed for evidence of metastatic disease. Potential extrapancreatic sites of tumor are evaluated in all cases, with particular attention being paid to the duodenum, the area of the spleen (splenic hilum), small bowel and its blood vessels (mesentery), the lymph nodes around the pancreas and the reproductive tract in women. One technique that provides additional information in the intraoperative setting is real time ultrasonography, which can assist in tumor identification (47, 48). In general surgeons can perform a Whipple resection for tumors of the head of the pancreas and a distal pancreatectomy for tumors of the tail of the pancreas. The goals of surgical therapy for pancreatic endocrine neoplasms include control of symptoms from hormone excess, safe resection of maximal tumor mass and preservation of maximal pancreatic parenchyma.

Because islet cell tumors / pancreatic endocrine neoplasms grow relatively slowly, some patients with metastases may still benefit from surgical resection. If for instance, a patient has an islet cell tumor / pancreatic endocrine neoplasm in the tail of the pancreas and an isolated metastasis in the liver, that patient may benefit from having both lesions removed. This contrasts greatly with pancreatic cancer (ductal adenocarcinoma). Patients with ductal adenocarcinoma that has spread to the liver are generally not surgical candidates.

If you have an islet cell tumor / pancreatic endocrine neoplasm and would like to be treated at Johns Hopkins, please contact Dr. Barish Edil, Dr. Rich Schulick or Dr. Christopher Wolfgang directly at 410-933-PANC (410-933-7262). They specialize in the surgical treatment of these tumors.

MEDICAL TREATMENT

Because these tumors are so slow growing they often do not respond to conventional chemotherapy (which usually targets fast growing cells). However, the U.S. Food and Drug Administration (FDA) recently approved the drugs Sutent (sunitinib) and Afinitor (Everolimus) for the treatment of advanced pancreatic endocrine tumors (also known as islet cell tumors or pancreatic neuroendocrine tumors [PanNETs]). Sutent is manufactured by Pfizer, and Afinitor by Novartis. It is exciting to see that the options available for patients with pancreatic neuoendocrine tumors is growing.

Afinitor was approved for the treatment of patients with progressive pancreatic neuroendocrine tumors that are not resectable surgically, that are locally advanced or metastatic. This approval by the FDA was based on a phase III clinical trial of Afinitor, in which the drug was shown to prolong progression free survival in patients with advanced PanNETs. This phase III trial was reported in the New England Journal of Medicine (N Engl J Med. 2011 Feb 10;364(6):514-23.). The research team at Johns Hopkins has played a role in understanding the likely mechanism by which Afinitor works. The team sequenced all of the known human genes in a series of pancreatic neuroendocrine tumors and found that one in six of these tumors harbors an activating mutation (DNA change) in the mTOR pathway. (Jiao, Shi, Edil, de Wilde, Klimstra, Maitra, Schulick, Tang, Wolfgang, Choti, Velculescu, Diaz, Jr., Vogelstein, Hruban & Papadopoulos, Science, 2011). This is the very pathway that Afinitor targets, and it is likely that Afinitor will be most effective in patients with a tumor with an mTOR pathway gene mutation.

Sunitinib belongs to the class of drugs called "tyrosine kinase inhibitors," and it has both effects against blood vessels in tumors (antiangiogenic) and effects against the tumor itself (antitumor properties). In a phase III clinical trial treatment with Sunitinib has been shown to significantly improve progression free survival in patients with metastatic pancreatic neuroendocrine tumors (PNETs) (Reviewed in: Cancer Metastasis Rev, 2011, Suppl 1:19-26).

In some instance metastases to the liver can be treated by destroying their blood supply using techniques such as hepatic artery embolisation and chemoembolisation. These approaches involve the selective cannulation of the blood vessels leading to the liver. The vessels feeding the metastases are identified and then destroyed (embolized). In some instances drugs can be used to block the symptoms causes by the release of hormones from the tumor. For example, proton pump inhibitors can be used to counteract the high levels of stomach acid produced in patients with gastrinomas. Finally, in selected patients response rates as high as 70% have been reported following the treatment of islet cell tumors / pancreatic endocrine neoplasms with the drug streptozocin. If you have an islet cell tumor / pancreatic endocrine neoplasm, and would like to be treated by a medical oncologist at Johns Hopkins please contact Dr. Ross Donehower, Dr. Daniel Laheru, or Dr. Dung Li directly at 410 -933-PANC (410-933-7262). They specialize in the medical treatment of islet cell tumors.

PROGNOSIS

The most important prognostic factor is whether or not the tumor can be removed surgically. Other significant prognostic for patients with an islet cell tumor / pancreatic endocrine neoplasm include the size of the tumor, the presence or absence of blood vessel invasion, the presence or absence of metastases to lymph nodes or other organs, The 5-year survival rate ranges between 50 and 70% in most series.

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INSULINOMA

Insulinoma is the most common syndromic islet cell tumor / pancreatic endocrine neoplasm. The insulinoma syndrome is associated with the "Whipple's triad:" 1) symptoms of hypoglycemia (low blood sugar levels) during fasting, 2) documentation of hypoglycemia with blood glucose (sugar) less than 50 mg/dl and 3) relief of symptoms following administration of exogenous glucose (6). Symptoms include confusion, seizure, obtundation, personality change and coma, as well as palpitations, tremulousness, diaphoresis (sweating) and tachycardia (fast heart rate). In most cases, patients consume carbohydrate-rich meals and snacks to relieve or prevent these symptoms.

Insulinoma is most reliably diagnosed using the technique of a monitored fast. During a monitored fast, blood is sampled every four to six hours for glucose and insulin determinations, and at the time of symptom occurrence. Additional support for the diagnosis of insulinoma comes from the calculation of the insulin to glucose ratio (I:G ratio) at different time points during the monitored fast. Normal individuals will have I:G ratios less than 0.3, while patients with insulinoma typically demonstrate I:G ratios greater than 0.4 after a prolonged fast.

After the diagnosis of insulinoma is confirmed by biochemical analyses, the appropriate localization and staging studies as described above are performed. For insulinoma the standard imaging studies include abdominal CT, endoscopic ultrasound and visceral angiography. The treatment of insulinoma is surgical in nearly all cases. Insulinomas are found evenly distributed within the pancreas, with approximately one-third being located in the head and uncinate process of the pancreas, one-third in the body of the gland, and one-third in the tail of the gland (51). Ninety percent of patients will be found to have small solitary tumors amenable to surgical cure. Less than 10% of patients with insulinoma will be found to have some form of the multiple endocrine neoplasia-1 (MEN-1) syndrome (see below).

In approximately 10% of all cases insulinoma will be found to have spread (metastasized) to peripancreatic lymph nodes or to the liver, justifying a diagnosis of malignant insulinoma. Under these circumstances, cautious and safe resection of the primary tumor and accessible metastases should be considered (56-58). Such tumor debulking can be helpful in reducing problematic hypoglycemic symptoms which can threaten long-term survival. The average patient survives several years following diagnosis and treatment of malignant islet cell tumors, indicating that the natural history of these malignant tumors typically follows an indolent course (25, 59).

Chemotherapeutic agents with some efficacy against malignant insulinoma include streptozocin, dacarbazine (DTIC), doxorubicin and 5-fluorouracil (60-62). The highest response rates to chemotherapy have been observed using combination therapy.

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GASTRINOMA (ZOLLINGER-ELLISON SYNDROME)

Gastrinomas are islet cell tumors / pancreatic endocrine neoplasms that release large quantities of the hormone gastrin into the blood stream leading to stomach/duodenal ulcers. In 1955 Zollinger and Ellison described two patients with severe peptic ulcer disease and pancreatic endocrine tumors, postulating that an ulcerogenic agent originated from the pancreatic tumor (9, 63). At present it is estimated that one in 1,000 patients with primary duodenal ulcer disease and two in 100 patients with recurrent ulcer following ulcer surgery harbor a gastrinoma (64). Seventy-five percent of gastrinomas occur sporadically, whereas 25% are associated with the MEN-1 syndrome (see below). In the past, the majority of gastrinomas had already metastasized at the time of diagnosis. More recently, with increased awareness and earlier screening, the diagnosis of gastrinoma is being made earlier, leading to the discovery of a higher percentage of curable neoplasms (65, 66).

Peptic ulceration of the upper GI tract and abdominal pain are seen in up to 90% of patients. Fifty percent of patients have some degree of diarrhea, while about 10% of patients present with diarrhea as the solitary symptom. The diagnosis of gastrinoma should be suspected in several clinical settings, and the liberal use of serum gastrin measurement for screening is encouraged. Gastric acid analysis is an important test in the evaluation of patients with suspected gastrinoma, as it can differentiate between ulcerogenic (high gastric acid) causes of hypergastrinemia and nonulcerogenic (low gastric acid) causes of hypergastrinemia.

Following the biochemical confirmation of the diagnosis of gastrinoma, two steps are important in patient management. First, gastric acid hypersecretion is pharmacologically controlled. So called "proton pump inhibitors" (lansoprazole, pantoprazole esomeprasole, etc) in high doses can be used to control acid production in the stomach in patients with gastrinoma (70, 71). Second, after the initiation of proton pump therapy, all gastrinoma patients should undergo imaging studies in an effort to localize the primary tumor and to assess for metastatic disease. The modalities appropriate for localization and staging of gastrinoma patients have already been discussed and include dynamic abdominal CT scanning with intravenous and oral contrast, endoscopic ultrasonography, somatostatin receptor imaging, percutaneous transhepatic portal venous sampling for gastrin, and the selective arterial secretin stimulation test. The majority of patients should be offered surgical exploration with curative intent. At the time of exploration the entire abdomen is carefully assessed for tumors within the pancreas and outside of the pancreas (72). The majority of gastrinomas have been identified to the right of the superior mesenteric vessels within the head of the pancreas or the duodenum: the so-called "gastrinoma triangle" (51, 73).

Gastrinomas within the pancreas are usually surgically resected either by distal pancreatectomy or pancreaticoduodenectomy (54, 76). Gastrinomas may also arise within the duodenum and they often can be surgically resected with primary closure of the duodenal defect (78, 79). In a small percentage of patients gastrinoma may be found only in peripancreatic lymph nodes, with these lymph nodes harboring the primary tumor. Resection of these apparent lymph node primary gastrinomas has been associated with long-term eugastrinemia (normal gastrin levels) and biochemical cure in up to 50% of cases (80).

Patients with gastrinomas that cannot be removed surgically can be treated long-term omeprazole therapy to alleviate their symptoms.

The overall results in patients with gastrinoma have improved markedly since the initial description of the syndrome. Up to 35% of patients explored for gastrinoma with curative intent have been rendered eugastrinemic at follow-up, and considering only those patients explored and thought to be successfully resected, the cure rates approach 60 to 70%. These recent results represent a major

improvement in the management of gastrinoma patients over the past decades, and support the practice of initial pharmacologic control of gastric hypersecretion using omeprazole, followed by tumor localization and staging, in hopes of curative resection.

Most patients with incurable metastatic gastrinoma succumb to eventual tumor growth and dissemination. Multiple modalities have been utilized in an effort to treat patients with such metastatic gastrinoma. The overall objective response rate to chemotherapy appears to be less than 50%.

Hepatic transplantation, hepatic artery embolization, and interferon therapy have all been used in small numbers of patients with gastrinoma metastatic to the liver (89-91). None of these therapies appears to be associated with reproducible improvements in survival.

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VIPoma (VERNER-MORRISON SYNDROME)

VIPomas are islet cell tumors / pancreatic endocrine neoplasms that cause symptoms by releasing large amounts of the hormone VIP into the blood stream. Synonyms for this syndrome include the WDHA syndrome (watery diarrhea, hypokalemia (low potassium levels in the blood), and either achlorhydria or hypochlorhydria (low chloride)) and the pancreatic cholera syndrome. Patients characteristically present with intermittent severe diarrhea, typically of a watery nature, averaging 5 liters/day (that is an awful lot of diarrhea!).

Hypokalemia (low blood potassium) results from the fecal loss of large amounts of potassium (up to 400 meq/day), and low blood potassium levels may be associated with muscular weakness, lethargy, and nausea.

The diagnosis of VIPoma is typically made after excluding other more common causes of diarrhea. The active agent in the VIPoma syndrome is the hormone vasoactive intestinal polypeptide (VIP) (93), with a minority of patients having elevations of other candidate mediators such as peptide histidine-isoleucine (PHI) or prostaglandins (94).

After biochemical documentation of elevated VIP levels, tumor localization and staging begins with abdominal CT scan with intravenous and oral contrast.

The preparation for surgical exploration in patients with VIPoma must include correction of fluid and electrolyte losses involving vigorous intravenous fluid administration and appropriate electrolyte replacement. Therapy with octreotide can be an important adjunct in the preoperative setting, as octreotide leads to a reduction in circulating VIP levels with a resultant decrease in the volume of diarrhea.

Surgical excision of the VIPoma is appropriate in all patients with the Verner-Morrison syndrome. The majority of VIPomas have been located in the tail of the pancreas, where they are amenable to resection via distal pancreatectomy. Metastatic disease to lymph nodes and the liver have been reported in half of all cases. In the presence of metastatic disease, safe palliative surgical debulking of metastatic tumor is indicated (95).

In patients with recurrent or unresectable VIPoma, octreotide therapy is used to reduce circulating VIP levels and control diarrhea. Chemotherapy specific for VIPoma patients has not been studied prospectively, although small numbers of patients have appeared to show partial responses to streptozocin, combination chemotherapy or interferon.

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GLUCAGONOMA

Glucagonomas are islet cell tumors / pancreatic endocrine neoplasms that cause symptoms by releasing large quantities of the hormone glucagon into the blood stream. The most common findings in the glucagonoma syndrome include severe dermatitis (skin rash), mild diabetes, stomatitis (mouth sores), anemia (low red blood cell count), and weight loss (96). The dermatitis is manifested by a characteristic skin rash termed "necrolytic migratory erythema." This rash exhibits cyclic migrations with erythematous (red) patches that spread with central healing points of resolution.

The diagnosis of glucagonoma may be suggested by the clinical presentation and biopsy of the skin lesions, but is secured by the documentation of elevated levels of fasting serum glucagon.

Patients with biochemical documentation of hyperglucagonemia in the appropriate clinical setting should undergo radiographic localization and staging with contrast-enhanced abdominal CT scan. Because these tumors are usually large and solitary, the CT scan localizes the tumor in the majority of patients.

Most glucagonomas have been located in the body and tail of the pancreas. These tumors are typically large and bulky, and surgical resection has required distal pancreatectomy. Metastases have been found in the majority of patients, and safe surgical debulking of these metastatic lesions should be considered.

Glucagonoma patients with incurable or recurrent disease appear to have low response rates to standard chemotherapeutic agents such as streptozocin and dacarbazine (97). Octreotide can be successful in reducing elevated glucagon levels, and in controlling the hyperglycemia and dermatitis associated with incurable glucagonoma (98, 99).

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SOMATOSTATINOMA

Somatostainomas are islet cell tumors / pancreatic endocrine neoplasms that cause symptoms by releasing large quantities of the hormone somatostatin into the blood stream. The somatostatinoma syndrome is the least common of the five functional pancreatic endocrine neoplasia syndromes (listed above), with an estimated annual incidence of less than one in forty million people. The clinical features of the somatostatinoma syndrome are nonspecific and include steatorrhea (oily stools), diabetes, hypochlorhydria (low blood chloride levels), and cholelithiasis (stones in the gallbladder). A fasting blood somatostatin level can be used to confirm the diagnosis of a somatostatinoma.

The majority of somatostatinomas have been located in the head of the pancreas. The most useful test for localization and staging has been the abdominal CT scan, which has been used to identify and stage these typically large tumors.

At surgery resection for cure has been uncommon, because of the presence of metastatic disease in most cases. Safe resection of the primary tumor and careful debulking of liver metastases appear indicated. At the time of exploration, cholecystectomy (removal of the gallbladder) is indicated even in the absence of documented gallstones, because of the concern about the development of gallstones with persistently elevated somatostatin levels.

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NONFUNCTIONAL ISLET CELL TUMORS / PANCREATIC ENDOCRINE NEOPLASMS

A growing number of patients with islet cell tumors / pancreatic endocrine neoplasms do not have a clinical syndrome caused by hormone production from the tumor. These patients are considered to have nonfunctional islet cell tumors / pancreatic endocrine neoplasms.

These nonfunctional islet cell tumors / pancreatic endocrine neoplasms present with clinical manifestations such as abdominal pain, weight loss and jaundice (105, 106), resulting from space-occupying lesions in the pancreas. In some patients the islet cell tumors / pancreatic endocrine neoplasms is detected by chance when the patient has a CT scan for another indication.

Nonfunctional islet cell tumors / pancreatic endocrine neoplasms are most commonly located in the head, neck or unicinate process of the pancreas (107). The majority of these behave in a malignant fashion. However, in contrast to the poor prognosis associated with ductal adenocarcinoma of the pancreas (commonly known as pancreatic cancer), these nonfunctional islet cell tumors / pancreatic endocrine neoplasms tend to grow in a more indolent fashion and are associated with a longer survival.

Localization and staging studies are performed in similar fashion to patients with the more common diagnosis of ductal adenocarcinoma of the exocrine pancreas. The abdominal CT scan is used for evaluation of the primary tumor and to assess for hepatic metastases.

At surgery most of these nonfunctional neoplasms are larger than 2 cm, and are not safely excised by local techniques. Tumors in the head, neck or uncinate process of the pancreas typically require pancreaticoduodenectomy (the Whipple resection) for safe resection, while tumors arising in the body or tail of the pancreas are treated by distal pancreatectomy.

The overall 5-year survival rate in all patients with resected nonfunctional pancreatic neoplasms approaches 50% (108). In patients with unresectable disease, partial responses to combination chemotherapy have been reported. The highest response rate of 69% was seen in patients receiving streptozocin plus doxorubicin.

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APPENDIX II

Center for Pancreatic and Biliary Diseases

University of Southern California, Department of Surgery

PANCREATIC CANCER

pancreas cancer home page/ USC treatment protocols/
pancreas cancer: adenocarcinoma/ cystic tumors/ islet tumors/
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The pancreas is a complex organ with many different types of cells in it. Each of these cell types may give rise to different types of tumors. The correct diagnosis of the tumor type is important since the prognosis for survival is dependant on the tumor type and surgical removal of some tumors in the pancreas can be associated with a normal life span. Often the type of tumor that is present in the pancreas can be diagnosed from specialized studies such as radioisotope studies and CT scans.

There are many different types of tumors that can develop in the pancreas. A pancreatic specialist can evaluate your tumor and determine which type of tumor is present in your pancreas. Approximately 85% patients have very aggressive type of tumor called ***adenocarcinoma of the pancreas***. In about 15% of patients ***other tumors in the pancreas*** are found that are less aggressive types of tumors which are often curable. An evaluation in a center that is experienced in the treatment of pancreatic cancer is important for determining appropriate treatment for pancreatic tumors.

What are the steps in the work up and treatment of the tumor in the pancreas

The following questions are sequentially addressed when a patient is seen at USC with a pancreas mass

- Where is the tumor in the pancreas
- What is the likely type of the tumor (adenocarcinoma or a less aggressive tumor type)
- Is the tumor surgically removable
- Is laparoscopic approach possible for your tumor type
- Is chemotherapy and/or radiation therapy indicated

Adenocarcinoma of the pancreas

The most common type of cancer of the pancreas is an adenocarcinoma which is a tumor that arises from the cells that line the duct of the pancreas. 85% of all cancerous tumors of the pancreas are adenocarcinomas. Approximately 30,000 new cases of pancreatic adenocarcinoma are diagnosed each year and approximately 28,000 patients die from pancreatic cancer each year. Only about 20 to 40% of patients with adenocarcinoma of the pancreas have a tumor that is confined to the pancreas at the time of diagnosis. The 5-year survival for patients who undergo surgical resection of adenocarcinoma of the pancreas is about 20 to 40%.

Surgery is a treatment of choice for patients who have adenocarcinoma of the pancreas that is surgically removable. Careful selection of patients for surgery is important, since surgical removal is associated with the best outcome diagnosing testing to identify patients suitable for surgery is extremely important. Appropriate diagnostic testing will also avoid unnecessary surgeries in patients whose tumors are too advanced for surgical removal.

The surgical procedure that is done depends on the location of the tumor in the pancreas. For tumors that occur in the head (which is the first part) of the pancreas, the Whipple operation is usually performed. For tumors that are located in the body and tail of the pancreas a distal pancreatectomy that removes the bottom half of the pancreas is recommended.The results of surgery have dramatically improved in the last two decades such that today the mortality (death) rate from surgery is less than 3 to 4% in tertiary care centers.

Most patients will require chemotherapy and radiation therapy after the surgery. Patients with unresectable tumors are often treated with chemotherapy and radiation therapy, and in some patients response to the treatment may allow subsequent surgical removal of the tumor.

Other tumors in the pancreas

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85% of tumors that are found in the pancreas are adenocarcinoma of the pancreas. 15% of tumors that develop in the pancreas are not adenocarcinomas and these tumors often have a far better prognosis. Since many patients with these tumors are often cured after surgery, identification and aggressive treatment of these tumors is important.The tumor types that are found in this group include:

- cystic tumors or neoplasms including mucinous cystadenoma and serous cyst adenoma
- islet cell tumors also called neuroendocrine tumors
- papillary cystic neoplasms
- lymphoma of the pancreas
- acinar cell tumors of the pancreas
- metastatic tumors to the pancreas

The majority of these tumors are non-malignant or benign, however even malignant tumors have five year survival rates in the order of 40 to 80% depending on the tumor type. In view of the excellent outcome, aggressive surgical therapy is indicated for these tumors, and the part of the pancreas that is affected by the tumor is removed.

At USC our emphasis has been to preserve as much of the pancreas as possible when removing benign and precancerous tumors to minimize the consequences of removal of large amounts of the pancreas such as diabetes and malabsorption (inability to digest food).

Specialized pancreatic procedures that are performed only in few centers in the United States such as a pancreatic head resection where only the head of the pancreas is removed preserving the duodenum and the bile duct that would otherwise be removed in a Whipple operation, central pancreatectomy where only the central portion of the pancreas is removed for tumors in this location preserving the head and body and tail of the pancreas and laparoscopic procedures that emphasize minimal access surgical technique for more rapid recovery are offered to patients at USC with benign tumors of the pancreas.

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Center for Pancreatic and Biliary Diseases

University of Southern California, Department of Surgery

ENDOCRINE TUMORS OF THE ABDOMEN

adrenal tumors home page/ pancreatic islet cell tumors home page/
surgery for endocrine tumors of the abdomen

What is an endocrine gland?

An endocrine gland is a specialized structures that are found in different parts of the body that secrete hormones.

What is a hormone?

Hormones are chemical substances that are delivered directly into the blood from endocrine organs. Hormones can produce profound effects on many parts of the body. For example insulin is a hormone that controls blood sugar levels. Testosterone is a hormone that causes the development of male sexual characteristics.

What are endocrine tumors

Endocrine tumors are abnormal growths in endocrine glands. Since endocrine glands produce hormones, tumors of endocrine glands also produce hormones. These hormones are produced in excessive amounts by endocrine tumors and then released into the blood.

The excessive amounts of hormones in the blood produce markedly abnormal effects on the body. For example in normal individuals insulin is secreted from pancreatic islet cells in just the right amount to keep your blood sugar levels within normal limits. In patients with insulin producing islet cell tumors of the pancreas, excessive insulin is produced in the blood that cause large decreases in blood sugar levels so that patients suffer from severe effects of low blood sugar.

Are endocrine tumors benign (non-cancerous) or malignant (cancerous)?

Endocrine tumors can be benign or grow as cancers. Often this distinction between cancer and not cancer is very difficult even after removal of the tumors. In some patients removal of what was thought to be a benign growth may come back as a cancerous recurrence. All patients with endocrine tumors of the abdomen should be followed carefully to detect early recurrence of the cancer.

What are abdominal endocrine tumors?

There are many endocrine glands that found in the abdomen. The two most important are:

- Adrenal gland
- Islet cells in the pancreas

Tumors can arise from both these two endocrine glands. Up to half of all tumors produce excess amounts of hormones that produce symptoms in the affected patient.

What are adrenal tumors

The adrenal gland is really two organs in one. The outer part of the adrenal is called the cortex and the inner part of the adrenal medulla. The two parts of the adrenal perform different functions and have different embryological origins.

Tumors of the adrenal glands arise from the cortex or the medulla part of the adrenal gland. Adrenal tumors commonly present because of excess secretion of hormones by the tumor. The tumors that commonly occur in the adrenal gland are

- **Tumors of the adrenal cortex that produce excess secretion of steroid hormones:** a condition called Cushing's disease
- **Tumors of the adrenal cortex that produce excess secretion of aldosterone:** a condition called Conn's syndrome
- **Tumors of the adrenal medulla produce excessive amounts of**

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catecholamines: a condition called pheochromocytoma

Adrenal tumors can be benign (non-cancerous) or malignant (cancer). Often this separation is difficult to make and long term close follow up is necessary after removal to detect recurrences early in patients who have adrenal cancer.

What is the treatment for adrenal tumors?

Adrenal tumors should be removed by surgery. Some of the procedures offered at USC for adrenal tumors are:

- **Laparoscopic adrenalectomy:** This is the treatment of choice for tumors less than 10cm.
- **Open adrenalectomy:** This is recommended only in patients where there is suspicion of cancer
- **Laparoscopic removal of both adrenal glands:** Recommended for patients with disease in both adrenal glands causing Cushing's disease or pheochromocytoma.

What are islet cell tumors of the pancreas?

Islet cell tumors of the pancreas are rare tumors. *These tumors are often also called pancreatic neuroendocrine tumors.*

Islet cell tumors of the pancreas are different from adenocarcinoma of the pancreas. These tumors tend to be slow growing tumors that are treatable even after they have metastasized. Islet cell tumors can produce dramatic symptoms since up to half of these tumors may secrete hormones that produce side effects due to excessive secretion of the hormones.

What are the different types of islet cell tumors?

The following types of neuroendocrine tumors are recognized:

- ***Non-functioning islet cell tumors.*** Patients with non-functioning tumors do not have any symptoms from excess secretion of pancreatic hormones since the tumor does not secrete any hormones into the blood.
- ***Functional islet cell tumors.*** These tumors produce dramatic symptoms because of excess secretion of various different hormones from the tumor in the pancreas.

The following types of functional islet cell tumors are recognized

- **Insulinoma:** a tumor that produces excessive amounts of insulin.
- **Gastrinoma:** a tumor that produces excessive amounts of gastrin
- **Glucagonoma:** an extremely rare tumor that produces excessive amounts of Glucagon.
- **VIPoma:** an extremely rare neuroendocrine tumor the produces excessive amounts of VIP.
- **Somatostatinoma:** an extremely rare tumor that produces excessive amounts of somatostatin.

Treatment for non-functioning islet cell tumors

Surgical removal of non-functioning islet cell tumors is often curative. These tumors typically tend to be large and therefore enucleation of the tumor is usually not possible.

Our approach is to remove these tumors preferentially laparoscopically. An open procedure is usually offered to the patient if there is metastases, for very large tumors (greater than 10 centimeters), if there is invasion of the major blood vessels around the pancreas by the tumor, and by patient preference. For all other patients, the laparoscopic procedure is offered as the treatment of choice.

We offer the following laparoscopic procedures for removal of non-functioning islet cell tumors of the pancreas:

- **Enucleation**
- **Distal Pancreatectomy**
- **Spleen Preserving Distal Pancreatectomy**
- **Central Pancreatectomy**
- **Whipple Operation**

Functioning islet cell tumors

Functional islet cell tumors often present in a dramatic fashion due to secretion of

excessive amounts of hormones such as insulin, gastrin, or glucagon. These tumors tend to present at a very early stage when the tumor is tiny and is often not readily detectable.

Localization of these tumors in the pancreas is an important consideration since the tumors may be very small, only a few millimeters in size, when the patient presents with major symptoms

Treatment of functioning islet cell tumors

The different types of functioning islet cell tumors require special consideration for surgery. Surgery is tailored to the type of the tumor. For further details click on the following links:

- **Insulinoma**
- **Gastrinoma**
- **Other functioning neuroendocrine tumors**

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PATENT APPLICATION FEE DETERMINATION RECORD

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APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
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TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
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APPLICATION AS AMENDED – PART II

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	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 3	Minus	** 20	= 0	X $ =		OR	X $60=	0
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AMENDMENT

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(54) Title: RAPAMYCIN COMPOUNDS IN THE TREATMENT OF NEUROFIBROMATOSIS TYPE 1

(57) Abstract: The present invention is directed to methods for treating neurofibromatosis type 1 (NF1) using rapamycin, derivatives of rapamycin or prodrugs of rapamycin. The invention covers the treatment both of non-malignant fibromas, as well as a variety of tumors associated with cells that have mutations in the NF1 gene.

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Rapamycin Compounds in the Treatment of Neurofibromatosis Type 1

Cross Reference to Related Applications

The present application claims the benefit of, and priority to, United States provisional application 60/639,776 filed on December 29, 2004.

Field of the Invention

The present invention is directed to a method of treating neurofibromatosis type 1 (NF1) by administering rapamycin, a rapamycin derivative, or a rapamycin prodrug. For the most part, the NF1 patients will be treated for non-malignant neurofibromas. However, treatments may extend to malignant peripheral nerve sheath tumors, optic pathway gliomas, myeloproliferative disorders, and pheochromocytomas.

Background of the Invention

Neurofibromatosis typel (NF1) (also known as von Recklinghausen NF or Peripheral NF), is a genetic disorder characterized by the growth of tumors along various types of nerves. Although the tumors are usually benign "neurofibromas," they are often disfiguring, painful and, depending on location and size, can be debilitating. In addition, NF1 patients have a predisposition for the formation of myeloid malignancies, gliomas and pheochromocytomas. NF1 affects approximately 1 in every 3500 individuals world-wide (Stumpf, *et al.*, *Arch. Neurol. 45*:575-578 (1988)) and, apart from palliative surgery, there is currently no effective treatment available.

NF1 is caused by mutations in the "NF1" gene (U.S. 5,605,799), many of which have been specifically identified (DeLuca, *et al.*, *Hum. Mutat. 23*:629 (2004); Zou, *et al.*, *Oncogene 23*:330-339 (2004); Orgine, *et al.*, *Hum. Mutat. 22*:179-180 (2003); Baralle, *et al.*, *Am. J. Med. Genet. 119A*:1-8 (2003); Kluwe, *et al.*, *Am. J. Med. Genet. 40*:368-371 (2003); DeRaedt, *et al.*, *Am. J. Hum. Genet. 22*:1288-1292 (2003); DeLuca, *et al.*, *Hum. Mutat. 21*:171-172 (2003); and Kluwe, *et al.*, *Hum. Mutat. 19*:309 (2002)). This gene has been fully sequenced and identified as producing the tumor suppressor neurofibromin (U.S. 5,227,292; U.S. 6,238,861; Cawthon, *et al.*, *Cell 62*:193-201 (1990); Vickochil, *et al.*, *Cell 62*:187-192 (1990)). About half of the mutations are inherited from a parent and about half occur spontaneously in patients with no family history of the disease.

Rapamycin is a drug that has been marketed as an immunosuppressive agent to prevent organ or bone marrow rejection in transplant patients (Kessler, *et al.*, *Helv. Chim. Acta 76*:117 (1993)). It is available from Wyeth-Ayerst Laboratories as a 1 mg/ml solution and in tablets of 1, 2 and 5 mg. Unlike other immunosuppressive agents, rapamycin does not appear to promote the development of malignancies (Guba, *et al.*, *Nature Med. 8*:128-135 (1992)). In fact, studies suggest that it alters transcription or translation of multiple genes and that this leads to an inhibition of cellular growth. At present, rapamycin or its derivatives are being tested as treatment for several different cancers, including cancer of the prostate (van der Poel, *et al.*, *Urol. Res. 30*:380-386 (2003); pancreas (Stephan, *et al.*, *Clin. Cancer Res. 10*:6993-7000 (2000); kidney and (Luan, *et al.*, *Kidney Int. 63*:917-926 (2003); lung (Boffa, *et al.*, *Clin. Cancer Res. 10*:293-300 (2004)).

Summary of the Invention

The molecular target of rapamycin has been identified and is known as mTOR (mammalian Target of Rapamycin). mTOR has been shown to be critically involved in cell growth and proliferation. The present invention is based upon the discovery that cells isolated from benign and malignant tumors from NF1 patients (which therefore harbor a mutated NF1 gene), exhibit aberrant "activation" of the mTOR protein. Furthermore, rapamycin dramatically suppressed the proliferation and tumorigenic properties of these cells. Thus, the invention is based upon the concept that rapamycin is effective in treating the tumors and, particularly, the non-malignant neurofibromas, associated with NF1.

Thus, in its first aspect, the invention is directed to a method of treating a patient for neurofibromatosis type 1 by administering a therapeutically effective amount of a rapamycin compound. The term "therapeutically effective" indicates that a sufficient amount of drug is given to accomplish, at least in part, the therapeutic objective. In the case or the present claims, a sufficient amount of a rapamycin compound should be given to shrink the size of tumors or retard their formation or growth.

The term "rapamycin compound" refers to rapamycin, derivatives of rapamycin that have been described in the art which maintain the antitumor effect of rapamycin in NF1 patients, and prodrugs of rapamycin or rapamycin derivatives. The most preferred

rapamycin compounds are rapamycin itself and the derivatives RAD001 (Novartis), AP23573 (Ariad) and CCI-779 (Wyeth).

The method described extends to the treatment of malignant tumors which have been identified as having cells exhibiting a mutation in the NF1 gene resulting in reduced neurofibromin activity. For example, tumor cells may be removed from a patient and examined for an NF1 mutation. If such a mutation is present, then treatment with a rapamycin compound would be initiated. Examples of tumors or conditions that can be treated include malignant peripheral nerve sheath tumors, malignant gliomas, astrocytomas and pheochromocytomas. In preferred embodiments, the rapamycin compound is rapamycin itself, RAD001, AP23573 or CCI-779 and is administered to patients at a dose of 0.1 - 500 mg per day, preferably at 1-50 mg per day and more preferably at 1-10 mg per day.

In all treatment methods, the rapamycin compound may be administered topically, typically in the form of a cream, lotion or ointment or given orally. Alternatively, it may be injected directly into a tumor or administered by an alternative route.

Detailed Description of the Invention

A. Rapamycin Compounds

Rapamycin is an immunosuppressive lactam macrolide which may either be purchased commercially (*e.g.*, from A.G. Scientific, San Diego, CA) or synthesized using procedures that have been described in the art (Nicolaou, *et al.*, *J. Am. Chem. Soc. 115*:4419-4420 (1993); Schreiber, *J. Am. Chem. Soc. 115*:7906-7907 (1993); Danisheffky, *J. Am. Chem. Soc. 115*:9345-9346 (1993)).

Rapamycin derivatives suitable for use in the invention are described in numerous references, including: WO 94/02136 (16-O-substituted derivatives); U.S. 5,258,389 (40-O-substituted derivatives); WO 94/9010 (O-aryl and O-alkyl derivatives); WO 92/05179 (carboxylic acid esters); U.S. 5,118,677 and 5,118,678 (amide esters); U.S. 5,118,678 (carbamates); U.S. 5,100,883 (fluorinated esters); U.S. 5,151,413 (acetals); U.S. 5,120,842 (silyl esters); WO 93,11130 (methylene derivatives); WO 94/02136 (methoxy derivatives); WO 94/02385 and WO 95/14023 (alkenyl derivatives); U.S. 5,256,790 (32-O-dihydro or substituted derivatives); EP 96/02441; U.S. 2004/023562 (carbohydrate derivatives);

U.S. 4,316,885 (mono and diacylated derivatives); U.S. 5,120, 725 (bicylic derivatives); U.S. 5,120,727 (rapamycin dimers); EP 467606 (27-oximes of rapamycin); U.S. 5,023,262 (42-oxo analogs); U.S. 5,177,203 (arylsulfonates and sulfamates); U.S. 5,177,203. In addition, various rapamycin prodrugs have been described in U.S. 4,650,803; 5,672,605; 5,583,189; 5,527,906; 5,457,111; 5,995,100; and 6,146,658. Of particular interest for use in treatment methods are derivatives described in patents owned by Novartis (US 5,665,772; 5,912,253; 5,985,890; 5,912,253; 6,200,985; 6,384,046; and 6,440,990), Ariad (WO 96/41865); and Wyeth (US 5,362,718; 6,399,625; 6,399,627; 6,432,973; 6,440,991; 6,677,357; and 6,680,718). The teachings of all of these patents and published applications are hereby incorporated by reference in their entirety.

It will be understood that the drugs described above may be administered to patients in any pharmaceutically acceptable form, for example, as a pharmaceutically acceptable salt. The most critical factor with respect to the compounds is their ability to inhibit TOR (target of rapamycin).

B. Drug Formulations

The present invention is compatible with the delivery of drugs by any means known in the art, including peroral, internal, pulmonary, rectal, nasal, vaginal, lingual, transdermal, intravenous, intraarterial, intramuscular, intraperitoneal, intra-tumoral, intracutaneous and subcutaneous routes. The most preferred routes are orally (especially using dosage forms such as tablets, capsules or solutions), topically, transdermally or by intra-tumoral injection. The amount of rapamycin compound present in a composition should, in general, be in the range of about 0.01 to about 30% w/w and preferably in an amount of 1 to 20% w/w.

Guidance in preparing formulations may be obtained from compositions of rapamycin that are commercially available and from descriptions in the art. Compositions may contain any of the standard inert components that are found in drug tablets, capsules, etc., including polymers; polyethylene glycol; cyclodextrins; saccharides; surfactants; disintegrants; antioxidants; stabilizers; flavoring agents; coloring agents, etc. Specific guidance for the preparation of a dosage form may be found in Remington's Pharmaceutical Sciences (1980) A. Oslo ed.

C. Treatment Methods

Patients diagnosed as having neurofibromatosis type I may be treated by administering one or more of the rapamycin derivatives and prodrugs described above. In general, patients will receive between about 1 mg per day and 10 mg per day. Naturally, these dosages can be adjusted by the attending physician based upon clinical conditions.

Tumors that may occur in patients not having neurofibromatosis may, nevertheless, be treated using rapamycin compounds, provided that they are dependent, at least in part, upon a loss of activity of the NF1 gene. Thus, to determine whether a given tumor will respond to rapamycin, one may remove cells from the tumor, *e.g.*, at biopsy, and assay them to determine whether there are mutations in the NF1 gene, or whether there is an abnormally low level of the tumor suppressor neurofibromin. To test for the presence of mutations, standard techniques such as amplification of regions of the NF1 gene by PCR using primers based upon the known sequence of NF1 or blotting techniques may be employed. Tests for the presence of neurofibromin may take the form of ELISAs, radioimmunoassays or immunoblots.

Treatment involving the use of rapamycin compounds may be combined with other treatment methods to improve overall effectiveness. Once initiated, treatment should continue until tumors completely disappear or, alternatively, until tumor growth has been arrested.

Examples

I. Materials and Methods

Cell Culture and immunoblots

Litter-mate matched MEFs or NIH3T3 cells were plated in serum-free media at a density of $1.0x10^6$ cells/10-cm plate. After 18 hours, 200 nM insulin or 6 μM LPA was added. Where indicated, cells were pre-treated with DMSO, 20 nM rapamycin (Calbiochem), 200 nM Wortmannin (Sigma-Aldrich), or indicated concentrations of U0126 (Calbiochem) for 30-min. For amino acid withdrawal studies, cells were washed and media was replaced with D-PBS for the indicated times. Following stimulation, cells were lysed in 1% SDS boiling lysis buffer. Neurofibroma-derived patient matched $NF1^{+/-}$ and $NF1^{-/-}$

Schwann cells were isolated and cultured as previously described (Rosenbaum, *et al.*, *J. Neurosci. Res. 61*:524-532 (2000); Serra, *et al.*, *Hum. Mol. Genet. 9*:3055-3064 (2000)). Schwann cells were seeded at a concentration of $2.5x10^5$ cells/6-cm plate in DMEM containing 0.1% serum without forskolin, insulin or heregulin and lysed as described above. Identical results were obtained in Schwann cell experiments that were acutely performed in the presence or absence of forskolin. Human MPNST cell lines were generated from NF1 patients as previously described (Frahm, *et al.*, *Neurobiol. Dis. 16*:85-91 (2004)). First and second-hit mutations in the *NF1* gene in tumor cells were verified by sequence analysis. Clarified lysates were normalized for protein levels and analyzed by Western blotting with the following antibodies: Phospho-p70S6K(T-389), Phospho-Tuberin (S-939), Phospho-Tuberin (T-1462), Phospho-Akt(S-473), Phospho-p44/42 Erk (T-202/Y-204) (Cell Signaling Technologies), Tuberin (C-20) (Santa Cruz Biotechnology), Protein Kinase Bα/AKT1, Actin, β-Tubulin (Sigma-Aldrich) and avian p90RSK1.

Retroviruses

Primary MEFs or NIH3T3 cells were infected with retrovirus expressing empty vector, human NF1 GRD (MSCV-GRD-pac), a dominant negative p90RSK (K112R), a dominant-negative p53 (pBabe-hygro-p53DD), or pLPC-E1A12S in the presence of 7.5-μg/ml polybrene. Cells were briefly selected in 2-μg/ml puromycin and/or 100-μg/ml hygromycin.

Ras activation analysis

NIH3T3 cells were plated in DMEM containing 3% serum at a density of $1.0x10^6$ cells/10-cm plate. After 18 hours, cell lysates were normalized and Ras-GTP was detected using a Ras-activation assay per manufacturer's protocol (Upstate Biotechnology).

Immunoprecipitations

Primary MEFs were harvested in Triton X-100 IP buffer (10 mM Tris pH 7.5, 50 mM NaCl, 50 mM NaF, 30 mM $Na_4O_7P_2$, 1.0% Triton X-100, Complete® protease inhibitor cocktail (Roche), 1 μM Microcystin-LR (Calbiochem), 100 nM Calyculin-A (Calbiochem)) and lysed on ice for 20-min. For MEFs, clarified lysates were normalized for protein concentration and incubated with anti-Tuberin (C-20) (1:100 dilution, Santa Cruz

Biotechnology) where indicated. Immunoprecipitated proteins were resolved along with total cell extracts (10% of immunoprecipitation volume) via SDS-PAGE and immunoblots were performed with the indicated antibodies.

MPNST Proliferation studies

MPNSTs were seeded at $6.5x10^4$ cells/6-cm well in normal growth medium containing rapamycin (0.01, 0.1, 1.0, 10, 100 nM) or equal volume of vector (DMSO). After 7 days, cells were trypsinized and live cells counted on triplicate plates using trypan blue exclusion.

Soft agar assays

Nf1$^{-/-}$ and *Nf1*$^{+/+}$ MEFs were infected with pLPC-E1A12S-puro and pBabe-p53DD-Hygro. $1.0x10^4$ selected MEFs were suspended in a DMEM containing 0.05% agar containing either rapamycin (0.1, 1.0, 10, 20 or 50 nM) or equal volume of vector (DMSO). Cells were seeded on a 0.34% agar base. Four to six weeks after the initial seed, colony growth was assayed by photographing and counting ten random fields of view per sample, in triplicate plates or wells. Colony size was assessed by using ImageJ software (v1.32J, NIH).

II. Results

The mTOR pathway is aberrantly activated in Nf1-deficient primary cells

In higher eukaryotes mTOR is regulated by both growth factor and nutrient availability (Fingar, *et al.*, *Oncogene 23*:3151-3171 (2004)). Growth factors have been shown to activate mTOR via a PI3 kinase dependent mechanism, whereas nutrients (amino acids) affect this pathway further downstream, at the level of tuberin and/or mTOR itself. Because neurofibromin negatively regulates the Ras/PI3 kinase pathway, we examined whether the mTOR pathway was deregulated in primary *Nf1*-deficient cells. The most commonly utilized *in vivo* readout of mTOR activation is the phosphorylation of a well-characterized substrate, S6 kinase (S6K1), at T-389. Phosphorylation at this site is dependent on mTOR and is required for maximal S6K1 activation. As expected, serum starved wild-type mouse embryonic fibroblasts (MEFs) exhibited little activation of AKT or S6K1. However, AKT was aberrantly activated in serum deprived *Nf1*-null MEFs. Notably, S6K1 was also hyper-phosphorylated in these cells in the absence of any growth

factors. The aberrant activation of S6K1 was inhibited by rapamycin, demonstrating its dependence on mTOR.

We also examined whether amino acid deprivation would inhibit mTOR activation in *Nf1*-deficient cells. Consistent with the observation that nutrients integrate into the mTOR pathway downstream of PI3 kinase activation, amino acid withdrawal resulted in the appropriate termination of the mTOR signal in both wild-type and *Nf1*-deficient cells. Thus, neurofibromin-deficiency specifically results in the deregulation of the mTOR pathway in response to growth factor deprivation.

mTOR activation is dependent on Ras

Because neurofibromin is a RasGAP we investigated whether the aberrant activation of the mTOR pathway in *Nf1*-deficient cells was dependent on the inappropriate activation of Ras. Notably, we and others have previously shown that Ras-GTP levels are deregulated in *Nf1*-deficient cells (Kim, *et al.*, *Oncogene 11*:325-335 (1995); Bollag, *et al.*, *Nat. Genet. 12*:144-148 (1996); Cichowski, *et al.*, *Genes & Development 17*:449-454 (2003)). We therefore sought to determine whether expression of only the catalytic, GAP-related domain (GRD) of neurofibromin would be sufficient to suppress the observed defect in S6K1 activation. Importantly, expression of this domain resulted in a dramatic decrease in Ras-GTP levels and suppressed the activation of Ras effector pathways as has been reported by Hiatt and colleagues (Hiatt, *et al.*, *J. Biol. Chem. 276*:7240-7245 (2000)). Moreover, in *Nf1*-deficient cells it restored the level of phospho-S6K1 to levels equivalent to those observed in wild-type cells under the same conditions, indicating that the hyper-activation of this pathway in *Nf1*-deficient cells is dependent on the absence of its RasGAP activity, rather than an additional uncharacterized function of neurofibromin.

Growth factor-induced mTOR activation is known to be dependent on PI3 kinase activity. However, growth factor receptors activate PI3 kinase via both Ras-dependent and independent mechanisms. Currently, it is unclear whether Ras is required for maximal mTOR activation in the context of normal growth factor signaling. Because expression of the GRD of neurofibromin dramatically inhibited RasGTP levels in wild-type cells, this reagent was used to determine whether Ras activation was required for maximal activation of the mTOR pathway in response to growth factors. Importantly, the GRD significantly

attenuated AKT activation and blunted S6K1 activation in response to LPA and insulin. Interestingly, in both cases the GRD appeared to have an effect on AKT and S6K1 at later time points, rather than immediately after growth factor treatment. This may suggest that Ras-independent mechanisms of PI3 kinase activation are important for the initial activation of this pathway, while Ras is required for a more sustained activation. This model is consistent with the fact that Ras-independent activation of PI3 kinase occurs more proximal to receptor activation than Ras-dependent activation of its effectors, in both receptor tyrosine kinase and G protein-coupled receptor signaling. Regardless, these data indicate that Ras activation is required for maximal activation of the mTOR pathway in response to growth factors.

Hyper-activation of the mTOR pathway in Nf1–deficient cells is dependent on both PI3K and MEK activation

To determine whether the inappropriate activation of mTOR in *Nf1*-deficient cells was solely dependent on the Ras/PI3 kinase effector pathway, we examined S6K1 phosphorylation in *Nf1*-deficient cells in the presence of the PI3 kinase inhibitor wortmannin, or the MEK inhibitor, U0126. As anticipated, wortmannin dramatically reduced S6K1 phosphorylation in serum-deprived *Nf1*-deficient cells. Surprisingly however, the MEK inhibitor suppressed S6K1 phosphorylation in these cells to a similar extent. These results suggest that the aberrant activation of mTOR in *Nf1*-deficient cells is dependent on both the Ras/PI3 kinase and Ras/Raf/MEK effector pathways.

To investigate how PI3 kinase and MEK might both contribute to the inappropriate activation of mTOR observed in *Nf1*-deficient cells, we examined the potential involvement of the *TSC2* gene product, tuberin, in these cells. The primary target of AKT in this pathway is thought to be tuberin, which is phosphorylated at two distinct sites, T-1462 and S-939, by this kinase. AKT phosphorylation of tuberin has been shown to inactivate the TSC1/TSC2 complex, resulting in the subsequent activation of Rheb and mTOR, through an unknown mechanism. Importantly, in *Nf1*-deficient serum starved cells tuberin was constitutively phosphorylated at T-1462 and S-939, in contrast to wild-type cells where phosphorylation was minimal under these conditions. Phosphorylation at these sites was suppressed in the presence of wortmannin; however, the MEK inhibitor had no effect,

suggesting that the loss of neurofibromin affects tuberin phosphorylation at these sites exclusively via the PI3K/AKT pathway.

Interestingly, a third regulatory phosphorylation site on tuberin has recently been reported. Blenis and colleagues have shown that PMA induces tuberin phosphorylation at S-1798 via the activation of the MEK/ERK/RSK pathway. This site was shown to be phosphorylated by an activated RSK1 *in vivo* and *in vitro*, and phosphorylation was blocked by a dominant negative RSK1 construct *in vivo*, suggesting that RSK1 can function as a tuberin kinase. Importantly, an alanine substitution at this site (S-1798) significantly reduced S6K1 activation in response to PMA and insulin. Furthermore, this mutation cooperated with T-1462A and S-939A mutations to inhibit tuberin function, suggesting that all three sites may be critical under certain conditions. Presumably, the loss of neurofibromin might also effect tuberin phosphorylation at this site via its effects on the Ras/Raf/MEK pathway. To test this hypothesis we examined tuberin phosphorylation at S-1798 in *Nf1*-deficient cells. This phosphorylation site is recognized by a phospho-specific antibody that recognizes a R*X*R*XX*pS/T consensus sequence. Blenis and colleagues have shown that this is the primary site activated by the MEK/ERK/RSK pathway and the phosphorylation site recognized by this antibody is blocked by dominant negative RSK1. We found that in *Nf1*-deficient serum starved MEFs, immunoprecipitated tuberin is hyper-phosphorylated at this basophilic site. Importantly, phosphorylation at this site is suppressed by U0126 and is unaffected by wortmannin.

Because RSK1 is thought to mediate phosphorylation at this site, *Nf1*-deficient cells were infected with a retrovirus that expresses a dominant negative RSK1 protein. Importantly, expression of the DN-RSK1 suppressed the aberrant phosphorylation at this site in *Nf1*-deficient cells. Moreover, it reduced S6K1 phosphorylation to levels observed in wild-type cells. Taken altogether these results suggest that the loss of neurofibromin results in the inactivation of tuberin via both the Ras/PI3K/AKT and Ras/Raf/MEK/ERK/RSK pathways.

NF1-deficient human tumor cells exhibit an activated mTOR pathway and are hypersensitive to rapamycin

To determine if *NF1*-deficient tumors from NF1 patients similarly exhibit an aberrant activation of the mTOR pathway, we examined this pathway in *NF1*+/- and *NF1*-/- Schwann cells derived from human neurofibromas. Neurofibromas are known to be extremely heterogenous lesions. Based on genetic studies of human tumors as well as mouse modeling data, it is believed that neurofibromas develop in NF1 patients as a result of "second hit" mutations in Schwann cells or Schwann cell precursors, which then act as a seed population to recruit other cells (*NF1*+/- Schwann cells, fibroblasts, mast cells, perineurial cells) into a developing lesion. Methods for isolating Schwann cells and then further separating *NF1* +/- Schwann cells from *NF1* -/- Schwann cells have been well described. We obtained matched *NF1*+/- and *NF1*-/- Schwann cells from one cutaneous and one plexiform neurofibroma, derived from two different patients. In both cases S6K1, AKT, and tuberin phosphorylation was significantly higher in the *NF1*- null cells as compared to the matched *NF1* +/- cells. Furthermore, S6K1 phosphorylation was blocked by both wortmannin and U0126 in mutant cells, indicating that both the PI3K and MEK pathway contribute to activation of mTOR in these tumor cells as well.

It is commonly believed that tumors become dependent on the dysregulation of a specific signaling pathway and are therefore hypersensitive to its down-regulation. To determine if NF1-associated peripheral nerve sheath tumors have become dependent on hyper-activation of the mTOR pathway, we tested the effects of rapamycin on tumors from NF1 patients. For this experiment we utilized malignant tumors (MPNSTs), which arise from benign neurofibromas. We found that the proliferation of two independently derived MPNST cell lines from two NF1 patients was dramatically suppressed at low concentrations of rapamycin. The IC_{50} for these experiments was between 1-10 nM. Notably, these IC_{50} values are comparable to or lower than the IC_{50} values of rapamycin derivatives that were tested on *PTEN* – deficient tumors, in which activation of the mTOR pathway has been well characterized. In addition, in one of the two cell lines, higher concentrations of rapamycin induced cell death.

As an independent means of assessing the effects of rapamycin on the tumorigenic properties of NF1-deficient cells, we established a genetically engineered cell system.

Cellular transformation is known to require multiple genetic events affecting the Rb, p53 and Ras pathways (Hahn,, *Nat. Rev. Cancer 2*:331-341 (2002)). In MEFs, the combined expression of a dominant-negative p53 gene and the E1A oncogene, which binds and inactivates Rb family members, is not sufficient to promote growth in soft agar. However we found that in the absence of *Nf1*, cells expressing these genes did form colonies in soft agar. Importantly, rapamycin suppressed colony growth at low concentrations in this system as well. Taken together, these results suggest that *NF1*-deficient tumor cell lines are exquisitely sensitive to rapamycin and suggest the potential therapeutic utility of rapamycin or its derivatives in treating tumors in NF1 patients.

III. Discussion

The NF1 tumor suppressor was shown to function as a RasGAP shortly after the gene was cloned in 1990. Accordingly, elevated levels of Ras-GTP are observed in *NF1*-deficient tumors and cells. However, to date, the Ras-effector pathways that are responsible for disease pathogenesis have not been defined. We show here that the mTOR pathway is critically deregulated in *NF1*-deficient primary cells and human tumors. Importantly, tumor cells are highly sensitive to the mTOR inhibitor rapamycin, suggesting that it or its derivatives may be useful therapeutically. To date there is no effective treatment or cure for NF1. Neurofibromas, while benign, can be extremely problematic. In addition to a high tumor burden encumbering some patients, many cannot be surgically resected because of underlying nerve involvement. Furthermore, lesions that are surgically reduced typically re-grow. Therefore, the suggestion that deregulation of the mTOR pathway participates in NF1-related tumorigenesis may represent a therapeutic breakthrough for this disease. Notably, NF1 is quite prevalent: greater than 10 times more prevalent than most other phakomatotic disorders, and even more common than TSC, a disease for which the effects of rapamycin are being assessed in clinical trials.

While the activation of mTOR is known to be PI3 kinase-dependent, the extent to which Ras participates in this process is unknown. In fact, because growth factor receptors can activate PI3 kinase via a Ras-independent mechanism, Ras is generally excluded from discussions of the mTOR pathway entirely (Raught, *et al., Proc. Nat'l Acad. Sci. USA 98*:7037-7044 (2001); Hay, *et al., Genes Dev. 18*:1926-1945 (2004)). Notably, the over-expression of an activated Ras allele can activate S6K1. However it has been unclear

whether the activation of endogenous Ras might similarly feed into this signaling pathway, in a tumorigenic setting, or in the context of normal growth factor signaling. By using *Nf1*-deficient cells we were able to uniquely address this question. We found that the activation of endogenous Ras, via the loss of neurofibromin, results in pathogenic levels of mTOR activation. Furthermore, by over-expressing the catalytic domain of neurofibromin, we showed that in wild-type cells Ras activation is required for maximal mTOR activation in response to growth factors. While this may be a subtle point, the amplitude and duration of Ras effector pathways has been shown to be critical for specifying biological responses (Marshall, *Cell 80*:179-185 (1995)). Therefore, it is possible that threshold levels of mTOR activation are not achieved in the absence of a Ras signal, which may have important biological consequences in certain settings. Regardless, these data demonstrate that Ras does function to amplify this signal.

Interestingly, we found that both the PI3 kinase pathway and the MEK pathway are required for the observed hyper-activation of mTOR in *NF1*-deficient cells and tumors. Specifically, these signals converge to phosphorylate tuberin at distinct sites. Importantly, a dominant-negative RSK1 blocks tuberin phosphorylation at the MEK dependent site, and completely suppresses S6K1 activation in *Nf1*-deficient cells, suggesting that RSK1 is the kinase that mediates this critical event. These results are particularly interesting in the context of other recent findings. First, it has been reported that *tsc* regulates photoreceptor differentiation downstream or in parallel to Ras/MAPK in *Drosophila* (Bateman, *et al.*, *Cell 119*:87-96 (2004)). A second report has also implicated the ERK effector RSK1 in phosphorylating tuberin at S-1798, which participates in its inactivation downstream of PMA (Roux, *et al.*, *Proc. Nat'l Acad. Sci. USA 101*:13489-13494 (2004)). We have gone on to show that this phosphorylation event occurs in *NF1*-deficient cells, is mediated by Ras, MEK and RSK1, and that this signal is absolutely required for the aberrant activation of mTOR in these cells. It remains to be determined if this phosphorylation event is generally required for tuberin inactivation. Given that *NF1*-mutations result in the aberrant activation of the PI3K/AKT pathway, it would have been expected that this signal would be sufficient to induce mTOR activation, as is thought for *PTEN*-deficient cells and tumors. However, the potential involvement of the MEK pathway in *PTEN*-deficient cells has never formally been tested. In *PTEN*-deficient tumors, it is possible that a cooperating mutation in the Ras/Raf pathway, or a signal emanating from growth factors (that engages the Raf/MEK

pathway), might be required for maximal mTOR activation. Alternatively, known cross-talk between the PI3K and ERK pathways may also participate in this process and may occur in primary *PTEN*-deficient cells as well. Regardless, both pathways are required in *NF1*-deficient cells and human tumors.

Finally, rapamycin derivatives have been suggested as potential therapeutic agents for a variety of cancers (Luo, *et al.*, *Cancer Cell* 4:257-262 (2003); Sawyers, *Cancer Cell* 4:343-348 (2003)). The basis for this suggestion is that multiple genes are mutated in tumors, therefore inhibition of more than one pathway may be required for a therapeutic effect. On this basis neurofibromatosis type I may represent a uniquely treatable disease. Notably, benign symptoms, such as neurofibromas, can be quite severe. However, they are likely to occur from the mutation of a single gene: *NF1*. Therefore it is possible that rapamycin derivatives may represent a single hit therapy for these lesions, either by preventing tumor development and/or promoting their regression. The fact that a subset of these lesions are dermal, may also make them uniquely accessible to non-systemic modes of delivery, such as topical treatment. In any case, our data suggest that these agents may represent one of the first viable therapies for NF1.

What is Claimed is:

1. A method of treating a patient for neurofibromatosis type 1 (NF1) comprising: administering to said patient a therapeutically effective amount of a rapamycin compound.

2. The method of claim 1, wherein said rapamycin compound is rapamycin or a rapamycin derivative selected from the group consisting of: RAD001; AP23573 and CCI-779.

3. The method of claim 1, wherein said rapamycin compound is administered to said patient at a dosage of 0.1-50 mg per day.

4. The method of claim 3, wherein said rapamycin compound is administered to said patient at a dosage of 1-10 mg per day.

5. The method of claim 4, wherein said rapamycin compound is administered topically or by intra-tumor injection to said patient.

6. A method of treating a patient for a malignant or nonmalignant tumor, wherein said tumor comprises cells having one or more mutations in the NF1 gene, comprising administering to said patient a therapeutically effective amount of a rapamycin compound.

7. The method of claim 6, wherein said rapamycin compound is rapamycin or a rapamycin derivative selected from the group consisting of: RAD001; AP23573 and CCI-779.

8. The method of claim 6, wherein said rapamycin compound is administered to said patient at a dosage of 0.1-50 mg per day.

9. The method of claim 8, wherein said rapamycin compound is administered to said patient at a dosage of 1-10 mg per day.

10. The method of claim 8, wherein said rapamycin compound is administered topically or by intra-tumor injection to said patient.

11. A method of treating a patient for a malignant peripheral nerve sheath tumor, wherein said tumor comprises cells having one or more mutations in the NF1 gene, comprising administering to said patient a therapeutically effective amount of a rapamycin compound.

12. The method of claim 11, wherein said rapamycin compound is rapamycin itself or a rapamycin derivative selected from the group consisting of: RAD001; AP23573; and CCI-779.

13. The method of claim 11, wherein said rapamycin compound is administered to said patient at a dosage of 0.1-50 mg per day.

14. The method of claim 13, wherein said rapamycin compound is administered to said patient at a dosage of 1-10 mg per day.

15. The method of claim 13, wherein said rapamycin compound is administered topically or by intratumor injection to said patient.

16. A method of treating a patient for a glioma, astrocytoma, myeloproliferative disorder or pheochromocytoma, wherein said glioma, astrocytoma, myeloproliferative disorder or pheochromocytoma have one or more mutations in a NF1 gene that reduces the activity of neurofibromin.

17. The method of claim 16, wherein said rapamycin compound is rapamycin itself or a rapamycin derivative selected from the group consisting of RAD001; AP23573; and CCI-779.

18. The method of claim 16, wherein said rapamycin compound is administered to said patient at a dosage of 0.1-50 mg per day.

19. The method of claim 18, wherein said rapamycin compound is administered to said patient at a dosage of 0.5-10 mg per day.

20. The method of claim 18, wherein said rapamycin compound is administered topically or intra-tumorally to said patient.

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
		US-			
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 2006/071966	07-06-2006	Brigham & Women's Hospital		☐
						☐
						☐
						☐
						☐
						☐

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw a line through citation if not in conformance and not considered. Include copy of this form with the next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kind Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Electronic Acknowledgement Receipt

EFS ID:	12107223
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Andrea Jacquin
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	17-FEB-2012
Filing Date:	19-MAY-2008
Time Stamp:	17:01:12
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		34678-US-PCT_SuppIDS_2012Feb17.pdf	544647 6efdfc7cc1e15bc434f7ac95ba60c79af63f0fbc	yes	3

	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Transmittal Letter		1	2	
	Information Disclosure Statement (IDS) Form (SB08)		3	3	
Warnings:					
Information:					
2	Foreign Reference	WO2006071966.pdf	874645 972f66912d22f42816ce512cf1fb137bcf3fd52ca	no	18
Warnings:					
Information:					
		Total Files Size (in bytes):	1419292		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Sir:

This paper is being filed:

☒ supplemental to the Information Disclosure Statement filed May 19, 2008, December 4, 2009 and October 7, 2011.

☐ within three months of the date of entry of the national stage as set forth in 37 C.F.R. §1.491 of the international application. Therefore, no fees are required.

☐ before the mailing date of a first Office action on the merits, and so under 37 C.F.R. §1.97(b)(3) no fees are required.

If a fee is deemed to be required, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 19-0134 in the name of Novartis.

In accordance with 37 C.F.R. §1.56, applicants wish to call the Examiner's attention to the reference cited on the attached form PTO/SB/08A/B.

☐ The listed references were cited in the international stage search report and copies are enclosed herewith except for the US patents/applications.

☒ A copy of the reference is enclosed herewith.

☐ Some of the non-astersked references were cited in a search report in a corresponding application. Copies of these references and the search report are enclosed herewith.

The Examiner is requested to consider the foregoing information in relation to this application and indicate that each reference was considered by returning a copy of the initialed PTO/SB/08A/B form(s).

Respectfully submitted,

Stephen Johnston
for Applicant
Reg. No. 45,916

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
+1 862 7781422

Date: February 17, 2012

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2264405	RU	C2	2005-11-20	Navarro et al.	Abstract only	☐

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

	1		☐

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Examiner Signature		Date Considered	

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Stephen Johnson/	Date (YYYY-MM-DD)	2013-09-16
Name/Print	Stephen Johnson	Registration Number	45916

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	16862296
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Angel Matos
Filer Authorized By:	Stephen E. Johnson
Attorney Docket Number:	34678-US-PCT
Receipt Date:	16-SEP-2013
Filing Date:	19-MAY-2008
Time Stamp:	14:47:45
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	RU2264405-abstract.pdf	95067 /5bb33bb8c13a6e5e8f68824b2873ad6e33548caa	no	2

Warnings:	
Information:	

2	Information Disclosure Statement (IDS) Form (SB08)	PAT034678-US-PCT-IDS-2013-05.pdf	612090 d3282665f8002aab6dcca6e7d1df8174367912bb69	no	4

Warnings:

Information:

A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.

Total Files Size (in bytes):	707157

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

No documents available for this priority number.

Espacenet

Bibliographic data: RU2003136738 (A) — 2005-05-20

40-O-(2-HYDROXY)ETHYLRAPAMYCIN IN CRYSTALLINE NONSOLVATED FORM, PHARMACEUTICAL COMPOSITION COMPRISING SUCH MACROLIDE AS ACTIVE SUBSTANCE AND METHOD FOR ITS PREPARING

Inventor(s): NAVARRO FRANSUA, ; PETI SAMJUEHL', ; STOUN GAJ

Applicant(s):

Classification: **- international:** ***A61K31/436; A61P37/06; C07D498/18;*** (IPC1-7): C07D498/18

- cooperative:

Application number: RU20030136738 19991206

Priority number(s): RU20030136738 19991206

Also published as: RU2264405 (C2)

Abstract of RU2264405 (C2)

FIELD: organic chemistry, antibiotics, pharmacy. ^ SUBSTANCE: invention relates to a new crystalline nonsolvated form of 40-O-(2-hydroxy)-ethylrapamycin that shows crystalline lattice with the following parameters: a = 14.37AA; b = 11.24AA; c = 18.31 AA, and volume value is 2805AA. Also, invention relates to a method for preparing this crystalline form that involves crystallization of 40-O-(2-hydroxy)ethylrapamycin from a solvent with mixture with aliphatic hydrocarbon of the formula CnH2n+2 wherein n = 5, 6 or 7. Also, invention relates to a pharmaceutical composition based on thereof and its using in preparing medicinal agents used in treatment or prophylaxis of organ or tissue transplant rejection, autoimmune, inflammatory states, asthma, proliferative disorders, tumor or hyperproliferative vascular diseases.; invention provides preparing the novel crystalline nonsolvated form of 40-O-(2-hydroxy)ethylrapamycin possessing immunosuppressive properties. ^ EFFECT: improved preparing method, improved and valuable medicinal properties of compound and composition. ^ 7 cl, 2 ex

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne and Lebwohl, David

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.: 9572

INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, David Lebwohl, M.D., declare as follows:

1. I received a Doctor of Medicine degree from Yale University School of Medicine in 1981.

2. I have worked at Novartis Pharmaceuticals Corporation since October 2002, in the areas of Clinical Research, Oncology Clinical Development, and currently am a Senior Vice-President and Global Head for Oncology Clinical Development.

3. Prior to working at Novartis Pharmaceuticals Corporation, I worked as Group Director of Clinical Oncology at Bristol-Myers Squibb for 7 years and was a Clinical Assistant Physician at Memorial Sloan-Kettering Cancer Center for 8 years.

4. I am a co-inventor of the claimed subject matter in the pending patent application. Based on my education and experience of 36 years, I consider myself to be an expert in the field of Oncology clinical development, including clinical studies of treating pancreatic neuroendocrine cancer.

5. I have reviewed the Office Action issued from the USPTO in the above-named application and am familiar with the two references cited in the prior art and the two supplemental evidence documents. The following is a summary of my analysis of the claimed invention with respect to the prior art and the rejection of record as an inventor and as a person of skill in the art.

6. The claimed use of 40-O-(2-ethylhydroxy)-rapamycin for treating endocrine and pancreatic neuroendocrine tumors (claims 1-3) is neither anticipated or obvious in view of a one paragraph, non-patent publication by Terrence O'Reilly, et al in the Proceedings of the American Association for Cancer Research, 43, 2002, 0359, page 71 (O'Reilly, et al. Abstract), as cited in the USPTO Office Action of record. The xenograft pancreatic tumor model described in limited detail in the O'Reilly, et al. Abstract does not refer to tumor cells derived from pancreatic neuroendocrine cancer. Pancreatic cancer (pancreatic adenocarcinoma) is distinct from pancreatic neuroendocrine cancer in that they originate in different types of cells in the pancreas and present in patients with different symptoms and abnormal cell behavior (tumorogenesis). The Merck Manual cited describes and details only pancreatic neuroendocrine cancers, not pancreatic adenocarcinomas. As a person skilled in the art of Oncology clinical trials and different cancers of the pancreas, it is my analysis that the O'Reilly, et al. Abstract does not teach the claimed invention and is limited in the scope and teaching of its disclosure. The definitive clinical study (RADIANT-3, see Appendix I), which was the basis for and filed in support of the claimed invention and the subsequent FDA approval of the first line treatment for pancreatic neuroendocrine tumors (pNET), in 2011 was the first clinical study that confirmed that patients having advanced neuroendocrine tumors of pancreatic origin when treated with 40-O-(2-ethylhydroxy)-rapamycin more than doubled the time without tumor growth and reduced the risk of pNET progression in patients by 65% when compared with placebo. Therefore, the efficacy and effectiveness of 40-O-(2-ethylhydroxy)-rapamycin for treating pNET would not have been expected or predicted from the limited teaching of the O'Reilly, et al Abstract.

7. The claimed use of 40-O-(2-ethylhydroxy)-rapamycin for treating endocrine and pancreatic neuroendocrine tumors (claims 1-3) is not obvious over international patent publication WO 97/47317 (Weckbecker, et al. reference) as evidenced by Novartis Data Sheet (Novartis, GEP NE tumors, published on line on 04/2005, pp. 1-2). As a person skilled in the art of Oncology clinical trials and different cancers of the pancreas, it is my analysis that the Weckbecker, et al. reference does not teach the claimed invention and is limited in the scope and teaching of its disclosure. The Weckbecker, et al. reference discloses a combination of a compound of the somatostatin class and a rapamycin

macrolide that is useful for the prevention or treatment of cell hyperproliferation. The Weckbecker, et al. reference discloses at page 14 a list of indications of some types of gastrointestinal endocrine pituitary tumors (e.g. GEP tumors, pituitary adenoma) that are suggested as being treated by the specific combination. As a person of skill in the art, it would be difficult to predict if the disclosed combination of a specific mTor inhibitor and a somastatin would be a basis to support a clinical study of a 40-O-(2-hydroxyethyl)-rapamycin for treating pNET as a single agent based on its limited disclosure, since the reference does not disclose or suggest any further data or statements in support for the disclosed combination to treat GEP tumors. Moreover, the reference does not define which tumors GEP refers to at page 14 or anywhere in the specification. The Examples in the reference recite an *in vitro* assay for breast cancer and an *in vivo* assay for azaserine induced exocrine pancreatic tumor. Again, the point is that an exocrine pancreatic tumor is a malignant neoplasm of the pancreas, which is not related to pancreatic neuroendrocrine tumors. Most important to emphasize is that the Weckbecker, et al. reference does not disclose or suggest using 40-O-(2-hydroxyethyl) rapamycin to treat cell hyperproliferation as a monotherapy (40-O-(2-hydroxyethyl)-rapamycin for treating pNET) anywhere in the contents of the reference. It is not clear what The Novartis Data Sheet cited by the Office also evidences what the Office asserts with respect to the reference or rejection. The term "GEP tumors" are not found in the evidence reference to the primary reference, only GEP NE tumors are disclosed namely Gastroenteropancreatic Neuroendrocrine tumors. The definitive clinical study (RADIANT-3, see Appendix i), which was the basis for the claimed invention and the FDA approval of the first line treatment for pancreatic neuroendocrine tumors (pNET), in 2011 was the first clinical study that confirmed that patients having advanced neuroendocrine tumors of pancreatic origin when treated with 40-O-(2-ethylhydroxy)-rapamycin more than doubled the time without tumor growth and reduced the risk of pNET progression in patients by 65% when compared with placebo. Therefore, the efficacy and effectiveness of 40-O-(2-ethylhydroxy)-rapamycin for treating pNET would not have been expected or predicted from the limited teaching of the Weckbecker, et al. reference.

8. The claimed invention and the results of the clinical study that confirmed that patients having advanced neuroendocrine tumors of pancreatic origin when treated with 40-O-(2-ethylhydroxy)-rapamycin more than doubled the time without tumor growth and reduced the risk of pNET progression in patients by 65% when compared with placebo could not have been achieved from either of the prior art documents cited by the USPTO.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Dr. David Lebwohl, M.D.
Sr. Vice-President and Global Head
Oncology Clinical Development
Novartis Pharmaceuticals
Corporation

Date: 23 Sept 2013

APPENDIX 1

Novartis International AG
Novartis Global Communications
CH-4002 Basel
Switzerland
http://www.novartis.com

NOVARTIS

[redacted]

Novartis gains FDA approval for Afinitor® as first new treatment in nearly three decades for patients with advanced pancreatic NET

- *Data show Afinitor delays tumor growth and reduces risk of disease progression in patients with advanced neuroendocrine tumors (NET) of pancreatic origin*[1]
- *Afinitor represents a new approach to treat advanced pancreatic NET, an aggressive cancer for which there has been limited treatment options*[1,2,3]
- *Regulatory filings outside the US have been submitted for everolimus in advanced NET and are being reviewed by health authorities worldwide*

Basel, May 5, 2011 – Novartis announced today that the US Food and Drug Administration (FDA) approved Afinitor® (everolimus) tablets for the treatment of progressive neuroendocrine tumors of pancreatic origin (PNET) in patients with unresectable, locally advanced or metastatic disease[4]. This marks the first approval of a treatment for this patient population in the US in nearly 30 years[5].

The approval was based on Phase III data from the RADIANT-3 (RAD001 In Advanced Neuroendocrine Tumors) trial showing treatment with Afinitor more than doubled the time without tumor growth (median 4.6 to 11.0 months) and reduced the risk of cancer progression by 65% when compared with placebo in patients with advanced pancreatic NET (hazard ratio=0.35 [95% confidence interval (CI), 0.27 to 0.45]; p<0.001). A consistent improvement in progression-free survival was seen with Afinitor in all patient subgroups[1]. The FDA determined that the safety and effectiveness of Afinitor in the treatment of patients with carcinoid tumors have not been established[4].

"The FDA approval of Afinitor represents an important step forward for patients with advanced pancreatic NET," said James Yao, MD, Associate Professor of Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas. "Patients will now have access to a treatment that has been shown to significantly delay tumor growth and reduce the risk of disease progression."

Approximately 60% of pancreatic NET patients are diagnosed with advanced disease[2]. This means that the cancer has already spread to other parts of the body, and is considered aggressive and difficult to treat[3]. The five-year survival rate for these patients is 27%[6].

"With this approval, US physicians can now offer their patients with progressive pancreatic NET a new treatment helping to fulfill a critical unmet need," said Hervé Hoppenot, President, Novartis Oncology. "This is the third indication for Afinitor in the US in just over two years, providing further evidence that inhibiting mTOR plays an important role in treating multiple tumor types."

Afinitor targets mTOR, a protein that acts as an important regulator of tumor cell division, blood vessel growth and cell metabolism[7]. Preclinical and clinical data have established

the role of mTOR in the development and progression of several types of tumors, including advanced pancreatic NET[1,7].

Novartis has submitted marketing applications for everolimus for the treatment of patients with advanced NET of gastrointestinal, lung or pancreatic origin to the European Medicines Agency (EMA) and the Swiss Agency for Therapeutic Products (Swissmedic), and additional regulatory submissions are being reviewed by health authorities worldwide.

About neuroendocrine tumors of pancreatic origin (pancreatic NET)
Neuroendocrine tumors arise from cells that can produce and secrete a variety of hormones that regulate bodily functions[8]. These tumors can occur anywhere in the body; however, most are found in the pancreas (pancreatic NET), gastrointestinal tract or lungs (carcinoid tumors)[8,9]. Pancreatic NET, also known as islet cell tumors, is a rare type of cancer different from pancreatic exocrine cancer, which is generally referred to as pancreatic cancer[9,10]. There have been limited treatment options for patients with pancreatic NET[2].

About RADIANT-3
RADIANT-3 is a Phase III prospective, double-blind, randomized, parallel group, placebo-controlled, multicenter study. The trial examined the efficacy and safety of Afinitor plus best supportive care (BSC) versus placebo plus BSC in 410 patients with advanced, low- or intermediate-grade pancreatic NET. Patients who met the study entry criteria were randomized 1:1 to receive either Afinitor 10 mg once-daily (n=207) or daily placebo (n=203) orally, both in conjunction with BSC[1].

The primary endpoint of RADIANT-3 is progression-free survival. Secondary endpoints include safety, objective response rate (confirmed according to RECIST), duration of response and overall survival[1].

About Afinitor (everolimus)
Afinitor® (everolimus) tablets is approved in the US for the treatment of progressive neuroendocrine tumors of pancreatic origin in patients with unresectable, locally advanced or metastatic disease. The FDA determined that the safety and effectiveness of Afinitor in the treatment of patients with carcinoid tumors have not been established.

Afinitor is approved in the European Union (EU) for the treatment of patients with advanced renal cell carcinoma (RCC) whose disease has progressed on or after treatment with vascular endothelial growth factor (VEGF)-targeted therapy and also in the US for the treatment of patients with advanced RCC after failure of treatment with sunitinib or sorafenib.

Afinitor is also approved in the US to treat patients with subependymal giant cell astrocytoma (SEGA) associated with tuberous sclerosis who require therapeutic intervention but are not candidates for curative surgical resection. The effectiveness of Afinitor is based on an analysis of change in SEGA volume. Clinical benefit such as improvement in disease-related symptoms or increase in overall survival has not been shown. Novartis has submitted marketing applications for everolimus for this use to the European Medicines Agency (EMA) and the Swiss Agency for Therapeutic Products (Swissmedic), and additional regulatory submissions are under way worldwide.

In the EU, everolimus is available in different dosage strengths for the non-oncology patient population under the trade name Certican® for the prevention of organ rejection in heart and kidney transplant recipients. In the US, everolimus is available in different dosage strengths under the trade name Zortress® for the prophylaxis of organ rejection in adult patients at low-moderate immunologic risk receiving a kidney transplant.

Everolimus is exclusively licensed to Abbott and sublicensed to Boston Scientific for use in drug-eluting stents.

Not all indications are available in every country. As an investigational compound the safety and efficacy profile of everolimus has not yet been established in all countries in pancreatic or any other type of NET. Access to everolimus outside of the approved indications has been carefully controlled and monitored in clinical trials designed to better understand the potential benefits and risks of the compound. Because of the uncertainty of clinical trials, there is no guarantee that everolimus will become commercially available for pancreatic or any other type of NET, or additional indications anywhere else in the world.

Important Safety Information about Afinitor (everolimus) tablets
Afinitor can cause serious side effects including lung or breathing problems, infections, and renal failure which can lead to death. Mouth ulcers and mouth sores are common side effects. Afinitor can affect blood cell counts, kidney and liver function, blood sugar and cholesterol levels. Afinitor may cause fetal harm in pregnant women. Women taking Afinitor should not breast feed.

The most common adverse drug reactions (incidence ≥15%) are mouth ulcers, rash, diarrhea, fatigue, acneiform dermatitis, infections, weakness, nausea, peripheral swelling, decreased appetite, headache, pneumonitis, abnormal taste, nose bleeds, mucosal inflammation, weight decreased and vomiting. The most common grade 3-4 adverse drug reactions (incidence ≥2%) are mouth ulcers, fatigue, decreased white blood cell count, diarrhea, infections, pneumonitis and diabetes mellitus. Cases of hepatitis B reactivation and pulmonary embolism have been reported.

Disclaimer
The foregoing release contains forward-looking statements that can be identified by terminology such as "will," "risk," "under way," "potential," or similar expressions, or by express or implied discussions regarding potential submissions or approvals for new indications or labeling for Afinitor, or regarding the potential timing of any such submissions or approvals, or regarding potential future revenues from Afinitor. You should not place undue reliance on these statements. Such forward-looking statements reflect the current views of management regarding future events, and involve known and unknown risks, uncertainties and other factors that may cause actual results with Afinitor to be materially different from any future results, performance or achievements expressed or implied by such statements. There can be no guarantee that Afinitor will be submitted or approved for any additional indications or labeling in any market. Nor can there be any guarantee that Afinitor will achieve any particular levels of revenue in the future. In particular, management's expectations regarding Afinitor could be affected by, among other things, unexpected regulatory actions or delays or government regulation generally; unexpected clinical trial results, including unexpected new clinical data and unexpected additional analysis of existing clinical data; the company's ability to obtain or maintain patent or other proprietary intellectual property protection; government, industry and general public pricing pressures; competition in general; the impact that the foregoing factors could have on the values attributed to the Novartis Group's assets and liabilities as recorded in the Group's consolidated balance sheet, and other risks and factors referred to in Novartis AG's current Form 20-F on file with the US Securities and Exchange Commission. Should one or more of these risks or uncertainties materialize, or should underlying assumptions prove incorrect, actual results may vary materially from those anticipated, believed, estimated or expected. Novartis is providing the information in this press release as of this date and does not undertake any obligation to update any forward-looking statements contained in this press release as a result of new information, future events or otherwise.

About Novartis

Novartis provides healthcare solutions that address the evolving needs of patients and societies. Focused solely on healthcare, Novartis offers a diversified portfolio to best meet these needs: innovative medicines, eye care, cost-saving generic pharmaceuticals, consumer health products, preventive vaccines and diagnostic tools. Novartis is the only company with leading positions in these areas. In 2010, the Group's continuing operations achieved net sales of USD 50.6 billion, while approximately USD 9.1 billion (USD 8.1 billion excluding impairment and amortization charges) was invested in R&D throughout the Group. Headquartered in Basel, Switzerland, Novartis Group companies employ approximately 119,000 full-time-equivalent associates and operate in more than 140 countries around the world. For more information, please visit http://www.novartis.com.

Novartis is on Twitter. Sign up to follow @Novartis at http://twitter.com/novartis.

References

1. Yao, et al. Everolimus for Advanced Pancreatic Neuroendocrine Tumors. New Eng J Med 2011;364:514-23.
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10. American Cancer Society Detailed Guides. Pancreatic Cancer. Available at http://www.cancer.org/acs/groups/cid/documents/webcontent/003131-pdf.pdf. Accessed April 2011.

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Electronic Acknowledgement Receipt

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Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Stephen E. Johnson/Andrea Jacquin
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Application Type:	U.S. National Stage under 35 USC 371

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		_34678-US-PCT_132_Declaration_Appendix1_2013Sep24.pdf	2230411 b4/cacd9b0b9a20b6814 33ef812b5c533af4e/1a	yes	9

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	1
	Miscellaneous Incoming Letter	2	5
	Miscellaneous Incoming Letter	6	9
Warnings:			
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

Applicants now submit a Declaration under 37 C.F.R. §1.132 from inventor Dr. David Lebwohl. Also attached in support of the Rule 132 Declaration is Appendix 1. Applicants request reconsideration pursuant to the amendment and RCE filed February 2, 2012 of record.

The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Account No. 19-0134 in the name of Novartis.

Respectfully submitted,

/ Stephen Johnson /

Stephen Johnson
for Applicant
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Date: September 23, 2013

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT034678-US-PCT	9572

1695 7590 05/09/2014

NOVARTIS PHARMACEUTICAL CORPORATION
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EXAMINER	
JEAN-LOUIS, SAMIRA JM	
ART UNIT	PAPER NUMBER
1627	

NOTIFICATION DATE	DELIVERY MODE
05/09/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

phip.patents@novartis.com

Office Action Summary	Application No. 12/094,173	Applicant(s) MARKS ET AL.	
	Examiner SAMIRA JEAN-LOUIS	Art Unit 1627	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02/06/12 and 09/24/13*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-3* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-3* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *02/17/12 and 09/16/13*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Response to Arguments

This Office Action is in response to the amendment submitted on 02/06/12. Claims 1-3are currently pending in the application, with claims 4-12 having being cancelled. Accordingly, claims 1-3 are being examined on the merits herein.

Receipt of the aforementioned amended claims is acknowledged and has been entered.

Applicant's argument with respect to the 102(b) rejection over O'Reilly has been fully considered. Applicant argues that O'Reilly does not teach or suggest treatment of endocrine tumors or pancreatic neuroendocrine tumors. Additionally, the Affidavit provided by Dr. Lebwohl further states that the xenograft pancreatic tumor model depicted in the O'Reilly abstract does not refer to tumor cells derived from pancreatic neuroendocrine tumors as pancreatic tumors are distinct from pancreatic neuroendocrine cancer. Such arguments are found persuasive as the examiner contends that O'Reilly did not explicitly teach treatment of pancreatic neuroendocrine tumors or cancer. Given that every single feature being claimed needs to be recited in the disclosure, O'Reilly does not anticipate such claims. Consequently, the 102(b) of claims 1-3 over O'Reilly as evidenced by Merck Manuals is hereby withdrawn.

Applicant's argument with respect to the 103(a) rejection of claims 1-3 and 8-9 over Weckbecker in view of Novartis Data Sheet has been fully considered. Applicant and the Affidavit provided by Dr. Lebwohl argue that Weckbecker in view of Novartis does not establish a *prima facie* case of obviousness since Weckbecker teaches a combination therapy as opposed to the monotherapy discussed in the instant claims. Additionally, applicant argues that treatment of GEP tumors only appears once in the disclosure and that the data recited in Weckbecker only tested breast cancer and exocrine pancreatic tumor model. Moreover, applicant argues that Norvatis teaches GEP NET tumors whereas Weckbecker teaches GEP tumors. Such arguments are however not found persuasive as the examiner maintains that Weckbecker in view of Novartis does indeed render obvious as Weckbecker teaches the use of a somatostatin agent in combination with a rapamycin macrolide for treating malignant tumor growth (see pg. 14, paragraph 2). Additionally, Weckbecker teaches treatment of GEP tumors (i.e. neuroendocrine gastro-entero-pancreatic tumors) wherein applicant's elected species or RAD-001 is preferred. Moreover, Weckbecker teaches that the macrolide agent can be administered in an amount of 5 mg. As a result, the examiner maintains that Weckbecker does indeed render obvious applicant's invention and established a prima facie case of obviousness. As for applicant's argument that Weckbecker only recites treatment of GEP tumors once, the examiner contends that a recitation of the use of the compounds is all that is needed for rendering the invention obvious. Additionally, the examiner maintains that the term "comprising" is used in the instant claims and thus does not exclude addition of other ingredients. Since the instant claims

recite the term comprising which does not exclude addition of other claims, the examiner maintains that such claims do not recite the use of a monotherapy as claimed by applicant.

As for applicant's argument that Novartis teaches GEP NT tumors as opposed to GEP tumors, the examiner maintains that GEP tumors are gastro-entero-pancreatic tumors of neuro-endocrine origin. The fact that Weckbecker did not recite NT tumors does not negate the fact that such tumors are neuro-endocrine tumors. In fact, Arnold et al. teach that GEP tumors are rare neuroendocrine tumors and several terms have been used to describe the same pathological entity (see Arnold, pg. 195, left col.). Specifically, Arnold et al. teach that such tumors are called: carcinoid, neuroendocrine tumor, neuroendocrine carcinoma, APUDoma, islet tumors (in case of pancreatic origin) and gastro-entero-pancreatic (GEP) tumors (see Arnold, Pg. 195, left col.). As a result, the examiner maintains that the definition proffered by Norvatis does indeed describe GEP tumors as GEP tumors are neuroendocrine tumors.

As for applicant's argument that Weckbecker does not provide any data on the treatment of GEP tumors, the examiner maintains that there is no requirement to provide data on very single embodiment claimed in an invention. Moreover, the examiner reminds applicant that a patent is presumed to be operable for all that it teaches. Once such a reference is found, the burden is on applicant to provide facts rebutting the presumption of operability. *In re Sasse,* 629 F.2d 675, 207 USPQ 107 (CCPA 1980). See also MPEP 716.07. Thus, simply stating that such type of tumors were not tested is not qualified as a fact that obviates the presumption of operability.

Thus, it is incumbent upon applicant to demonstrate via side by side comparison that the combination taught by Weckbecker does not in fact treat the GEP tumors taught in the disclosure. As a result, the examiner maintains that Weckbecker in view of Norvatis did indeed render obvious applicant's invention. However, in light of applicant's cancellation of claims 8-9, the 103(a) rejection of claims 1-3 and 8-9 is hereby withdrawn.

For the foregoing reasons, the rejections of record are withdrawn. However, in view of applicant's amendment, the following modified 103 (a) Non-Final rejection is being made.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1-3 are rejected under 35 U.S.C. 103 (a) as being unpatentable over by Weckbecker (WO 97/47317, previously cited) as evidenced by Arnold et al. (Gastrointestinal and Liver Tumors" by Wolfgang Scheppack, 2004, Chapter 15, pgs. 195-233).

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Weckbecker teaches a combination of a somatostatin analogue and a rapamycin for the prevention and treatment of cell hyperproliferation (see abstract and pg. 1, paragraph 1). Additionally, Weckbecker teaches that rapamycin or derivatives thereof are desired given that such compounds are immunosuppressive and known to inhibit cancer (see pg. 10, last paragraph and pg. 12, last paragraph). A preferred rapamycin compound is 40-O-(2-hydroxy)ethyl-rapamycin (i.e. elected species; instant claim 1; see pg. 12, paragraph 3). According to Weckbecker, such combination can be used for preventing or treating cell hyperproliferation including GEP tumors (i.e. Gastroentero-pancreatic neuroendocrine tumors: slow growing tumors of the pancreas and GI tract) and pituitary adenomas (another type of endocrine tumor; see pg. 13 and pg. 14, paragraph 2).

Weckbecker does not specifically teach a method of treating pancreatic neuroendocrine tumors. Additionally, Weckbecker does not teach particular dosage administration of 40-O-(2-hydroxy)ethyl-rapamycin.

The Examiner however contends that because Weckbecker teaches administration of an effective amount of a rapamycin derivative (see pg. 13) and given that Weckbecker uses rapamycin in an amount of 5 mg, one of ordinary skill in the art would have found it obvious to administer such dosage amount for 40-O-(2-hydroxy)ethyl-rapamycin in the treatment of GEP and pituitary tumors.

Arnold et al. teach that GEP tumors are rare neuroendocrine tumors and several terms have been used to describe the same pathological entity (see Arnold, pg. 195, left col.). Specifically, Arnold et al. teach that such tumors are called: carcinoid, neuroendocrine tumor, neuroendocrine carcinoma, APUDoma, islet tumors (in case of pancreatic origin and gastro-entero-pancreatic (GEP) tumors (see Arnold, Pg. 195, left col.). Importantly, Arnold et al. teach that most endocrine tumors arise out of the pancreas (see pg. 197, right col.).

Thus, to one of ordinary skill in the art at the time of the invention would have found it obvious to treat pancreatic neuroendocrine tumors and other endocrine tumors such as pituitary tumors since Weckbecker teaches that the combination of somatostatin analogue and a rapamycin derivative such as 40-O-(2-hydroxy)ethyl-

rapamycin is effective in the treatment of pituitary tumors and GEP tumors and given that Arnold teaches that GEP tumors are endocrine tumors and encompass pancreatic neuroendocrine tumors. Given the teachings of Weckbecker and Arnold, one of ordinary skill would have been motivated to administer the somatostatin and rapamycin derivative of Weckbecker to treat neuroendocrine tumors with the reasonable expectation of providing a method that is effective in treating various endocrine tumors including pancreatic neuroendocrine tumors.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samira Jean-Louis whose telephone number is 571-270-3503. The examiner can normally be reached on 7:30-6 PM EST M-Th.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SAMIRA JEAN-LOUIS/

Primary Examiner, Art Unit 1627

05/04/2014

Notice of References Cited	Application/Control No. 12/094,173	Applicant(s)/Patent Under Reexamination MARKS ET AL.	
	Examiner SAMIRA JEAN-LOUIS	Art Unit 1627	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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FOREIGN PATENT DOCUMENTS

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Arnold et al. Chapter 15 of "Gastrointestinal and Liver Tumors" by Wolfgang Scheppack, 2004, Chapter 15, pgs. 195-233.
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	W	
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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EAST Search History

EAST Search History (Prior Art)

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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	**Art Unit**
	SAMIRA JEAN-LOUIS	1627

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
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	10	÷	✓	-	-					
	11	÷	N	-	-					
	12	÷	✓	-	-					

15 Neuroendocrine Gastro-Entero-Pancreatic (GEP) Tumors

R. ARNOLD, R. GÖKE, M. WIED, TH. BEHR

Summary

Neuroendocrine gastro-entero-pancreatic (GEP) tumors are rare but present with variable, sometimes dramatic clinical syndromes. The majority of these tumors is non-functioning and most functioning and non-functioning tumors are malignant. This chapter describes the various clinical entities, has a special focus on histopathology of these tumors as a reliable source for prognosis and summarizes current state and new trends in diagnosis and treatment of these tumors. The management of neuroendocrine GEP-tumors needs a multidisciplinary approach. Therefore, diagnostic and therapeutic aspects of this chapter recognize the important contributions of surgery, pathology, radiology, nuclear medicine and gastrointestinal endocrinology.

Definition

Several terms for endocrine tumors of the gastrointestinal tract are currently applied to describe the same pathological entity: "carcinoid", "neuroendocrine tumor", "neuroendocrine carcinoma", "APUDoma", "gastro-entero-pancreatic (GEP) tumor", "islet cell tumor" (in case of pancreatic origin). The term "carcinoid" was introduced by S. Oberndorfer in 1907 to distinguish carcinoids as less rapidly growing and well-differentiated epithelial tumors of the small intestine from the more aggressively growing adenocarcinoma of the gut and, thus, recognizing the decisive difference to carcinomas which is the very slow growth of most endocrine tumors and which is frequently associated with an uncompromized life quality. Strictly speaking is the term "carcinoid" reserved for endocrine tumors of the gastrointestinal tract (Table 2) and not for those of the pancreas (Table 1). Endocrine pancreatic tumors are assumed to arise from islets of Langerhans although their origin from the diffuse endocrine cell system scattered within the mucosa of the gastrointestinal tract and the pancreatic duct system cannot be excluded. At least in vertebrates, the islets of Langerhans arise within an independent islet organ which melts together with the exocrine pancreas during ontogenesis. The blurredness of the term "carcinoid" results from its histological features which are almost identical with those of endocrine pancreatic tumors. Therefore, even pathologists frequently use the term "carcinoid" to describe an endocrine pancreatic tumor. To avoid the dilemma, the term "carcinoid" should be used only for well-differentiated endocrine tumors of the gut and the term "malignant carcinoid" to designate the corresponding well differentiated endocrine carcinoma [1]. If a "carcinoid" is associated with a clinical syndrome as a Zollinger-Ellison syndrome in the case of a gastrin-producing endocrine tumor of the duodenum, the respective "carcinoid" should better be called gastrinoma.

The term "neuroendocrine" reflects the origin of the endocrine cells of the gastrointestinal tract fom the embryonic neural crest. They have been designated as "Helles Zellenorgan" by F. Feyrter. The acronym "APUDoma" (amine precursor uptake and decarboxylation) describes the potency of endocrine tumors to synthesize in addition to hormones biogenic amines as serotonin and other peptides characteristic to cells originating from the neural crest first described by A.E.G. Pearse.

Endocrine tumors of the gastrointestinal tract are epithelial tumors and differ histologically from neuronal tumors as neuroblastomas, pheochromocytomas and paragangliomas which also arise from the diffuse neuroendocrine system and are, therefore, of neural crest origin.

Amphicrine and mixed endocrine-exocrine tumors will not be discussed within this survey since their prognosis and biological features are determined by the exocrine cell department with a predominantly unfavourable prognosis.

Table 1
Classification and leading symptoms of the most frequent endocrine tumors of the gastrointestinal tract

Name (Syndrome)	Leading Symptoms	Responsible Hormone	Other Hormones in the Tumor	Malignancy (%)	Localization of primary	Extra-pancreas Localization
Insulinoma	Hypoglycaemia	Insulin	Glucagon, PP	5–10	Pancreas	very rare
Gastrinoma (Zollinger-Ellison syndrome)	Peptic Ulcers, Diarrhea, Reflux Disease	Gastrin	Insulin, PP, Glucagon, ACTH, Somatostatin, Chromogranin A	>90	Pancreas	Duodenum, Stomach, Mesenterium
Carcinoid syndrome	Flush, Diarrhoea, Bronchial Obstruction	Serotonin	Tachykinins, Prostaglandins, Chromogranin A	100	Ileum	Pancreas (rare)
VIPoma (Verner-Morrison syndrome), Pancreatic Cholera	Intractable Diarrhea, Hypokalemia	VIP, PHI	PP, Glucagon, Somatostatin, Chromogranin A	75	Pancreas	
Glucagonoma	Erythema necrolyticans migrans, Diabetes	Glucagon	PP, Insulin, Somatostatin, Chromogranin A	50	Pancreas	Rare
Somatostatinoma	Diabetes, Steatorrhea, Gallstones	Somatostatin	PP, Insulin, Calcitonin	50	Pancreas	Duodenum
GHRHoma	Acromegaly	GHRH	Somatostatin, Gastrin, Insulin, Chromogranin A	100	Pancreas	Lung
CRHoma, ACTHoma	Cushing's syndrome	CRH	Gastrin, PP, Chromogranin A	>90	Pancreas	Lung

ACTH Adreno-corticotrophic hormone; *CRH* Corticotropin releasing hormone; *GHRH* Growth hormone releasing hormone; *PHI* Peptide histidine isoleucine; *PP* Pancreatic peptide; *VIP* Vasoactive intestinal polypeptide.

Table 2
Characteristics of extra-pancreatic endocrine gastrointestinal tumors ("carcinoids")

Localization	% of all carcinoids	Peptides and hormones	Functional activity	Endocrine cell type	Malignancy
Esophagus	0.04	Chromogranin A	rarely	Grimelius positive, NSE positive	>50%
Stomach	2–3	Chromogranin A (histamine,gastrin), Ghrelin, VMAT-2	very rarely	ECL-cells, rarely EC-cells, rarely G-cells	highly variable
Duodenum and proximal jejunum	22	5-HT, gastrin, somatostatin, PP, calcitonin, ACTH	Zollinger-Ellison syndrome or functional inactive	EC-cells, G-cells, somatostatin-cells	50%
Distal jejunum and ileum	23–28	Chromogranin A, serotonin, substance P, tachykinins, others	mostly inactive; carcinoid syndrome in 5–7%	EC-cells	>50% in tumors larger then 1 cm
Appendix	19	Serotonin, GLP-1, GIP-2, PP/PYY	mostly inactive; carcinoid syndrome extremely rare	EC-cells, L-cells	risk factor size >2 cm and invasion of mesoappendix
Cecum	8	Serotonin	carcinoid syndrome in 5%	EC-cells	> 50%
Ascending Colon	8	GLP-1, GLP-2 PP/PYY		L-cells	
Rectosigmoid	20	Serotonin,	no	EC-cells	15% depends on tumor size and invasion
Rectum	20	GLP-1, GLP-2 PP/PYY		L-Cells	

Classification

Endocrine GEP tumors can be subdivided according to their origin into those originating from the foregut (esophagus, stomach, duodenum, proximal jejunum, pancreas), midgut (distal jejunum, ileum, appendix, cecum, right-sided colon) and hindgut (left-sided colon and rectum). This classification is based on the embryologic assignment of the different parts of the gut. Vary rarely endocrine tumors of the same histology can arise in the ovary, extrahepatic bile ducts, the liver, the kidney, testis, spleen, breast and larynx and other organs as the broncial system and thymus.

Clinically more relevant is a classification according to the functional activity of endocrine GEP tumors. Most benign and malignant endocrine GEP tumors are functionally inactive and patients commonly present with abdominal pain, weight loss, obstructive jaundice and intestinal obstruction depending on the localization and size of the tumor. Noteworthy, many tumors are asymptomatic even in the presence of metastases and are discovered incidentally during routine imaging procedures.

Survival of patients with GEP tumors is even in the metastatic state much more favourable than in patients with other malignancys and depends on the site of the primary tumor and the extent of metastatic spread. Of pancreatic endocrine tumors the best prognosis is associated with insulinomas which are in more that 95% of patients solitary and benign. In contrast, most of the other pancreatic entities are malignant (see Table 1).

As shown in Tables 1 and 2 the majority of functionally active endocrine tumors arise within the pancreas (see Table 1) whereas functionally active tumors within the gastrointestinal tract can cause the Zollinger-Ellison syndrome if originating from the duodenum or cause a Carcinoid syndrome due to a metastatic tumor of the ileum.

Endocrine GEP tumors may be benign or malignant. The majority of endocrine pancreatic tumors are malignant and present with metastases mostly to the liver (see Table 1). The malignancy rate of endocrine tumors within the gastrointestinal tract is highly variable and mostly depending on the size of the carcinoid.

Endocrine GEP tumors can arise sporadic or as part of the Multiple Endocrine Neoplasia (MEN) syndromes (Table 3). MEN-I syndrome is an autosomal dominantly inherited disorder characterized by the synchronous or metachronous occurrence of tumors in multiple endocrine organs, predominantly the pancreas, parathyroid, pituitary and duodenum. The genetic locus was ascribed to a segment of the long arm of chromosome 11, where the menin gene – a tumor suppressor gene – is located which is in MEN-I syndrome mutated [2, 3]. MEN-I syndrome is present in 20% of patients with gastrinoma

Table 3
MEN syndromes

Syndrome	Affected organ	Alterations
MEN-1 (Wermer's syndrome)	Parathyroid gland	Hyperplasia, multiple adenomas
	Pancreas	Islet cell tumors (insulinoma, gastrinoma, VIPoma, glucagonoma)
	Pituitary (anterior)	Adenoma (prolactin, ACTH, STH, GH, non-funtioning)
MEN-2A (Sipple's syndrome)	Thyroid gland	C-cell hyperplasia, medullary thyroid carcinoma
	Adrenal medulla	Phaeochromocytoma
	Parathyroid gland	Hyperplasia, multiple adenomas
MEN-2B	Thyroid gland	C-cell hyperplasia, medullary thyroid carcinoma
	Adrenal medulla	Phaeochromocytoma
	Mucosa	Neuromas
	Other abnormalities: Marfanoid habitus, Megacolon	

[4], 4% of patients with insulinoma [5] and 13–17% of patients with glucagonoma [6]. However, in MEN-I syndrome most endocrine pancreatic tumors are nonfunctional containing mostly pancreatic polypeptide or glucagon [5].

Epidemiology

Endocrine GEP tumors are rare events. The exact incidence and prevalence of these tumors is difficult to ascertain because many are asymptomatic. From autopsy studies an annual incidence of 8.4 gastrointestinal endocrine tumors (carcinoids) per 100.000 people has been calculated [7, 8] (Table 4). 90% of these tumors were incidental autopsy findings. For endocrine pancreatic tumors an annual incidence of 0,1–0,4 tumors per 100.000 has been reported. [8]. Table 4 summarizes the published annual incidence rates for the most common gastrointestinal (carcinoids) and pancreatic endocrine tumors. Endocrine tumors originating in the midgut encompass by far the majority of all endocrine tumors followed by the pancreatic endocrine tumors.

Almost all endocrine tumors originating within the hindgut are asymptomatic and do not create symptoms as a consequence of hormone overproduction. The reason for that is unknown since many of these tumors contain peptides and hormones which are also pro-

Table 4
Epidemiological data of endocrine GEP tumors

Localization	Incidence cases per 100.000 people per year	Remarks (% of all gastrointestinal carcinoids)	Mean age (years) [range]
Stomach	0.002–0.1	(11–14%)	50–60
		type I: 74%	63 [15–88]
		type II: 6%	50 [28–67]
		type III: 13%	55 [41–61]
		poorly differentiated: 6%	
Duodenum		(22%)	59 [30–90]
		Gastrin-producing: 62%	
		Somatostatin-producing: 21%	
		Gangliocytic pasaganglioma: 9%	
		Undefined tumors: 5,6%	
Proximal Jejunum		(1%)	
Distal Jejunum/Ileum	0.28–0.89	(28%)	60–70
[30–99]			
Appendix		(19%) more frequent in females	32–45
[6–80]			
Colon	0.07–0.21	(right-sided colon: 8%)	58
		(left-sided colon: 20%)	
Rectum	0.14–0.76		60
Pancreas			
all	0.01 – 0.3		
Insulinoma	0.1–0.2		47 [8–82]
Gastrinoma	0.05–0.15		[33–53]
VIPoma	0.005–0.02		
Glucagonoma	0.001–0.01		

duced in tumors responsible for the carcinoid syndrome. The same is true for most gastric carcinoids and carcinoids arising in the distal ileum. Even in metastatic tumors a hormone mediated symptomatology is mostly absent. Of the endocrine pancreatic tumors almost 50% are functionally inactive as well [8]. The incidence rates of the functionally active tumors with insulinoma as the most frequent tumor are listed in table 4.

According to an analysis of 8305 cases of carcinoid tumors identified by the "Surveillance, Epidemiology, and End Results" (SEER) program of the American National Cancer Institute (NCI) from 1973 to 1991 and by an earlier NCI program 5-year survival of patients was 50.4% [7]. The presence of regional and distant metastases reduced survival rate to 21,8%. If survival rates are calculated separately for tumors arising in the foregut, midgut and hindgut 5-year survival rates were 44.5%, 61% and 72% respectively [7]. Most favourable survival have appendiceal carcinoids with 85.9%. Surveillance rates for endocrine GEP tumors of specific localizations will be discussed in more detail later in this chapter.

Etiology

The etiology of endocrine GEP tumors is unknown. It is comprehensible to assume that they originate from cells or rather precursor cells of the diffuse neuroendocrine cell system. Endocrine tumor cells display certain cytochemical properties with endocrine cells scattered within the mucosa of the gastrointestinal tract and with the constituents of the islets of Langerhans as the expression of neuron-specific enolase, synaptophysin and chomogranin A and C [1, 16]. Chromogranins are acidic glycoproteins present in almost all endocrine and neuronal tissues. They are released into the circulation and can serve as tumor markers since they are found in more than 90% of patients with endocrine GEP tumors. Although endocrine pancreatic tumors are also called "islet cell tumors" it is unproven that pancreatic insulinomas, gastrinomas, VIPomas etc. originate from the islets of Langerhans. In favour of this assumption are findings in experimental settings which clearly demonstrate that insulinomas in rats can under defined conditions arise from islets. However, some endocrine pancreatic tumors produce hormones and peptides as gastrin or VIP which are not synthesized from islet cells after birth. Therefore, it is conceivable to assume that islet tumors originate from endocrine pancreatic multipotent precursor cells which are constituants of the pancreatic duct epithelium [13].

General Pathophysiology

The key event occurring in functionally active endocrine GEP tumor cells is the loss of capacity to store their hormonal product as insulin in insulinomas, gastrin in gastrinomas etc. within the tumor cell. Therefore, inappropriately released hormones and peptides not responding to the physiological feadback inhibition are responsible for the clinical manifestation of the disease. According to the concept of an impaired storage capacity of tumor cells, it has been shown, that insulinoma cells contain less insulin than normal β-cells, and the mean total insulin content of insulinomas was even lower than the mean insulin content of the whole pancreas of the respective patient [14]. Very similar is the gastrin content of the majority of gastrinomas lower compared to the gastrin content of the whole antral mucosa which contains more gastrin-producing cells than the tumor [15].

Histopathology

Most endocrine GEP tumors display a solid, trabecular or glandular arrangement of well-different (Fig. 1a–c) [1, 16]. However, not in every case these features permit recognition of the endocrine nature of the tumor. In these tumors special staining methods as silver methods or immunohistochemical staines for general endocrine markers as chromogranins (Fig. 1e), synaptophysin or neuron-specific enolase are needed for tumor identification [1, 16]. To characterize the tumor cell further with regard to their hormone/peptide production specific antibodies against polypeptide hormones are needed to identify a tumor cell as insulin-, gastrin-, glucagon- or other hormones producing cell (Fig 2a, b) [16]. Endocrine tumors with predominant insulin production can be classified as insulinoma, those with predominant glucagon- or gastrin production as glucagonoma or gastrinoma. This does not indicate that a tumor which histologically has been diagnosed as insulinoma or glu-

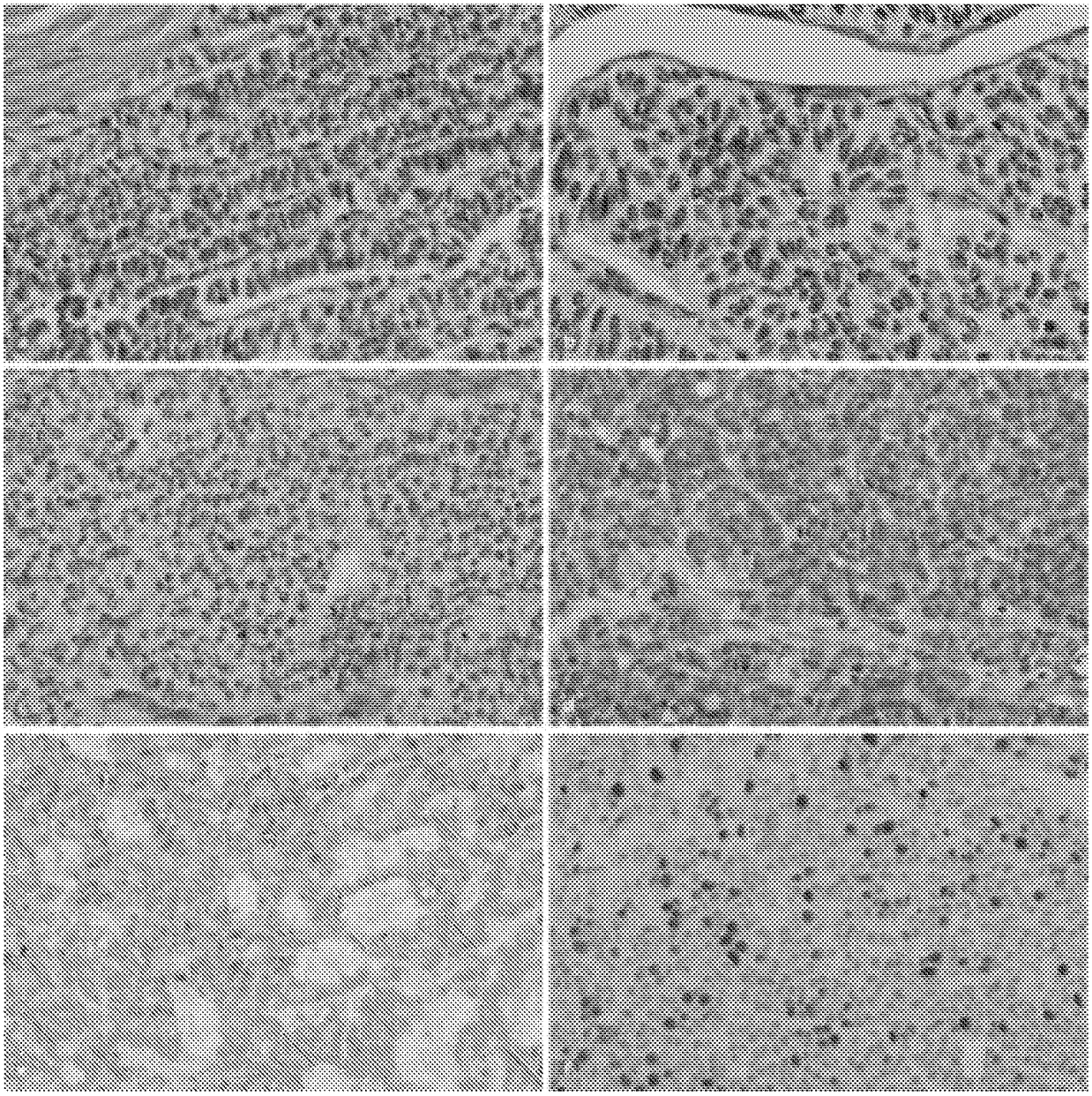


Figure 1a–f
Histopathological patterns in pancreatic endocrine tumors. a trabecular pattern; PAS staining; **b** glandular pattern; PAS staining; **c** solid pattern; PAS staining; **d** poorly differentiated neuroendocrine tumor; PAS staining; **e** staining with the endocrine marker chromogranin A; **f** staining with an antibody against the proliferation marker Ki-6

cagonoma acts as a functionally active endocrine tumor responsible for hypoglycemic attacks in the case of an insulinoma or giving rise to the typical symptoms of a glucagonoma syndrome. Functional activity or inactivity cannot be deducted from histology. Correspondingly and most characteristicly, many endocrine tumors as part of the MEN-I syndrome are functionally inactive [18].

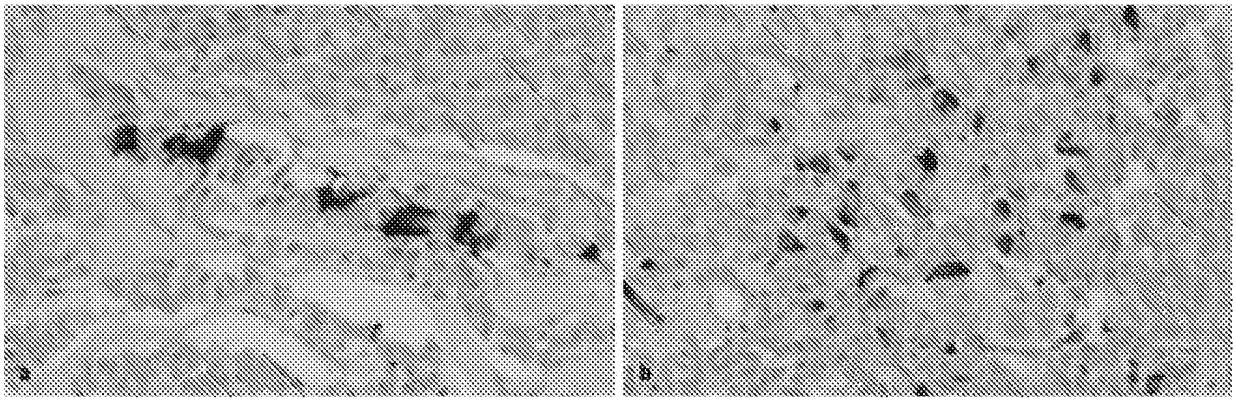


Figure 2a,b
Demonstration of several hormones present within the same endocrine Pancreatic tumor. **a** immunohistological staining for insulin; **b** immunohistological staining for gastrin

Most endocrine tumors are composed of more than one cell type. An endocrine pancreatic tumor with predominant insulin-producing cells can contain additional somatostatin- or glucagon- or pancreatic polypeptide-producing cells [15]. This feature is independent on the functional status of the tumor and can be observed in functionally active and inactive tumor [15]. It is unclear, why in the presence of multiple hormones within a single endocrine tumor only one or no clinical syndrome occurs. Nevertheless, in few patients, a second clinical syndrome can be present initially or develop later. This occurs preferably in patients with metastatic endocrine pancreatic tumors or in patients with MEN-I syndrome and multiple endocrine pancreatic tumors [4]. According to own observations which are in accordance with reports from the literature the combination of ectopic ACTH-producing and gastrin-producing pancreatic tumors giving rise to a combination of Cushing's syndrome and Zollinger-Ellison syndrome is frequent, although the condition itself with two functionally active tumors is a rare event.

Since most endocrine tumors are well-differentiated, their mitotic index visualized by the Ki67 labelling (Fig. 1f) index [9] is low which is in accordance with their slow growth behaviour. Therefore, it is difficult to predict the biological behaviour of well-differentiated tumors using classical histopathological malignancy criteria as cellular or structural atypia, necrosis, mitotic activity or microscopic invasion. A panel of international pathologists has, therefore, proposed to classify benign and malignant endocrine tumors into the categories listed in table 5 [1]. The basis for distinguishing a well-differentiated endocrine tumor from a well-differentiated endocrine carcinoma is the presence of metastases and/or evidence for local invasion. Benign or low risk endocrine tumors are distinguished from tumors with greater risk of malignancy on the basis of a combination or features such as tumor size, local extension, angioinvasion, cellular atypia, proliferative activity and the expression of hormones regularly found in the specific organ ("eutopic" hormone production) or the expression of "ectopic" hormonal products (as ACTH in an endocrine pancreatic tumor).

Poorly differentiated small cell carcinoma (Fig. 1d) is for experienced pathologists easy to distinguish from well-differentiated endocrine tumors on the basis of cellular atypia, the presence of markedly hyperchromatic nuclei, a high nuclear/cytoplasmic ratio, focal necrosis and high mitotic activity. To classify such an indifferentiated tumor as endocrine, tumors must react for

Table 5
General endocrine tumor categories

1	Well-differentiated endocrine tumor
2	Well-differentiated endocrine carcinoma
3	Poorly differentiated endocrine (small cell) carcinoma
4	Mixed exocrine-endocrine tumor
5	Tumor-like lesions

cytosolic neuroendocrine markers as synaptophysin and neuron-specific enolase [1]. However, these tumors are frequently negative for markers of endocrine granules as chromogranin and for specific hormonal products.

Additional histopathologic characteristics and tumor classifications will be discussed later when specific tumors are described in more detail.

Molecular Pathogenesis

Sporadic GEP Tumors

In sporadic pancreatic endocrine tumors (PETs) an allelic deletion of the tumor-suppressor gene MEN-I located on chromosome 11q13 has been found very frequently [3, 17]. However, the mutational frequency of MEN-I is different in functional and non-functional PETs: 30% of functional but only 8% of non-functional PETs showed mutations of the MEN-I gene [17, 18]. Furthermore, there are differences within the group of functional PETs: Alterations in MEN-I have been found in 54% (15/28) of gastrinomas, 50% (4/8) of VIPomas, 2/3 glucagonomas, 1/1 somatostatinoma but only in 7% (4/54) of insulinomas [17]. While such findings support the relevance of MEN-I for the pathogenesis of endocrine neoplasms, it is important to note that the incidence of MEN-I alteration is obviously tumor-type related and found more frequently in gastrinomas and non functional PETs than in insulinomas. Other frequent genetic abberations found in 25–50% of PETs analyzed are chromosomal deletions on 3p, 3q, 6q, 10q, 11q, 11p, 16p, 20q, 21q, 22q, Xq and Y. In up to 25% of PETs gains on chromosomes 5q, 7q, 7p, 9q, 12q, 17p and 20q were found.

The p53 tumor suppressor gene located on chromosome 17p13 encodes a nuclear protein which is involved in multiple cellular processes like cell cycle, DNA repair, replication, transcription, apoptosis and cell differentiation. p53 alterations are detectable in almost all cancers but are extremely rare in PETs. However, increased p53 protein concentrations were found in malignant insulinomas most likely due to inactivating mutations resulting in an increased stability or posttranslational events leading to overexpression [19].

The p16 (INK4a, MTS1) gene located on chromosome 9p21 encodes a protein that binds to cyclin-dependent kinase 4 inhibiting its interaction with cyclin. p16 alterations do not play a role in non-functional PETs and insulinomas. Since p16 was found abnormal in 42% of 8 gastrinomas analyzed [20] it might play a role in gastrinoma tumorigenesis. However, further studies are necessary to confirm this assumption.

DPC4/Smad4 is a tumor suppressor gene located on chromosome 18q21 encoding a protein which is involved in the TGF-β signaling pathway. Previous data suggested that Smad4 mutations seem to be common in non-functional PETs [21]. However, based on a more recent study it is unlikely that Smad4 plays a role in tumorigenesis of endocrine tumors.

Of the oncogenes c-myc, c-fos, K-ras and c-erbB-2 only K-ras was found to be overexpressed in PETs. However, only 10 of 90 PETs analyzed in the literature showed a ras mutation indicating that this is a rare event in these tumors. Most PETs with ras mutations were malignant insulinomas suggesting that alterations of ras might play a role in the pathogenesis of these tumors.

Recent data indicate that losses of sex chromosomes are common in PETs and are associated with presence of metastases, local invasion and poor survival.

Up to date the pathogenesis of neuroendocrine tumors of the gastrointestinal tract is not well characterized. Allelic loss of the MEN-I gene located on chromosome 11q13 was identified in type II ECL cell tumors and carcinoids of the jejunum and ileum. In type I ECL cell tumors abnormal RegIalpha gene was observed. In poorly differentiated neuroendocrine neoplasms allelic loss of p53 located on chromosome 17p13 were found in 4 of 9 cases suggesting a role for p53 in the development of these aggressive tumors.

Multiple Endocrine Neoplasia-Type 1

Multiple endocrine neoplasia-type 1 (MEN-I; Wermer's syndrome) is characterized by a combined occurrence of primary hyperparathyroidism, pancreatic endocrine tumors and pituitary adenomas [5]. The development of additional tumors in other endocrine or non-endocrine tissues indicates that the protein menin encoded by the MEN-I gene might have a function in a wide variety of tissues. Most MEN-I patients (90%) exhibit primary hy-

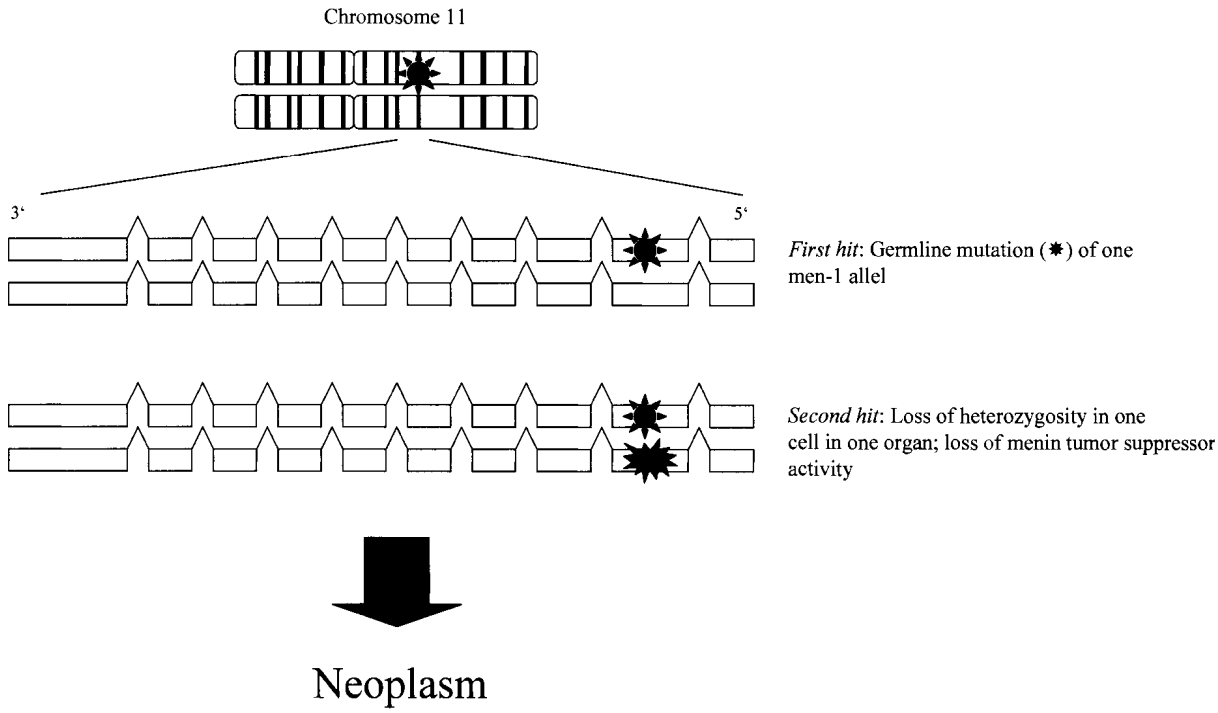


Figure 3

Tumorigenesis in MEN-1 according to the two hit model described by Knudson. Patients show a germline mutation of the MEN-1 gene. Later they acquire another mutation in the wild-type allele resulting in an loss of suppressor function of the gene

perparathyroidism. Pancreatic endocrine tumors occur in ~60% and are usually benign and non-functional [5]. The most common functional tumors are insulinomas and gastrinomas. The prevalence of pituitary adenomas is between 15–50%. A recent large study including 324 MEN-I patients showed pituitary adenomas in 42% of the cases which were larger in size and more aggressive than without MEN-I.

MEN-I is an autosomal dominant inherited syndrome and is related to mutations of the MEN-I gene located on chromosome 11q13 [2, 3]. The tumorigenesis of MEN-I is supposingly a process according to the two hit model by Knudson (Fig. 3). Patients inherit a mutated MEN-I gene and later aquire another mutation in the wild-type allel (loss of heterozygosity) in vulnerable endocrine tissue. This results in a loss of the tumor suppressor function of the MEN-I gene. The MEN-I gene contains 10 exons encoding the protein menin consiting of 610 amino acids [2]. Two transcripts have been identified which are most likely due alternative splicing. A 2.9 kb transcript was detected in all tissues while a 4.2 kb transcript was found in pancreas, stomach and thymus only [2]. Menin contains two nuclear localization sites and is predominantly a nuclear protein. However, during cell cycle menin was shown to shuttle from nucleus to cytoplasm.

In Ras-transformed NIH3T3 cells overexpression of menin resulted in decreased proliferation, suppression of clonogenicity in soft agar and inhibition of tumor growth in mice. Menin directly interacts with JunD, a transcriptional factor of the AP-1 complex, via three JunD interacting domains and inhibits JunD activation of transcription [22]. However, since JunD inhibits growth of Ras-transformed NIH3T3 cells the repressive effect of menin should result in enhanced growth. This indicates that the mechanism of action of menin is more complex than we know today and probably involves other genes and proteins. This assumption is supported by a recent observation that menin interacts with NF-κB proteins and inhibits NF-κB-mediated transactivation).

Up to date more than 400 mutations of the MEN-I gene have been identified. Most mutations were unique but some occurred twice or more in unrelated families (107, 110–112). Of 262 mutations observed in MEN-I patients between 1997 and 1999 approximately 22% are nonsense mutations, 48% frameshift deletions and insertions, 8% inframe deletions and insertions, 5% donor-splice site mutations and 17% missense mutations. The majority of mutations result in an inactivation of the MEN-I gene. There is no genotype-phenotype correlation in MEN-I. However, most patients with agressive phenotypes show truncating mutations.

Multiple Endocrine Neoplasia-Type 2

The term multiple endocrine neoplasia-type 2 (MEN-2) describes the combined occurrence of inherited forms of medullary thyroid carcinoma (MTC) with other malignomas. In MEN-2A (Sipple's syndrome) MTC is combined with pheochromocytoma and primary hyperparathyreoidism. MEN-2B (Gorlin's syndrome) is characterized by the occurrence of MTC, pheochromocytoma, neurinomas of the gastrointestinal tract and a marfanoid habitus [23].

Men-2 is caused by germline mutations of the RET gene located on chromosome 10q11–2 encoding a transmembrane tyrosine kinase receptor with cadherin-like and cystein-rich extracellular domains and a tyrosine kinase intarcellular domain [23]. RET genomic size is 60 kb and the gene contains 21 exons. GDNF (glia cell line-derived neurotrophic factor), neurturin, artemin and persephrin act as RET protein ligands inducing homodimerization through the cystein-rich region resulting in an activation of the tyrosine kinase domain and the Ras-MAP-kinase pathway. 95% of MEN-2A patients show mutations of the cystein-rich extracellular domain. The most common mutation affects codon 634 (Cys→Arg/Tyr/Gly). Missense mutations have also been identified in codons 609–611, 618 and 620. Approximately 98% of MEN-2B patients exhibit mutations in the intracellular tyrosine kinase domain (codon 918; Met→Thr). While mutations in the cystein-rich region result in the formation of constitutive active RET dimers, mutations in the intracellular tyrosine kinase domain lead to a switch to an abnormal signalling pathway.

Germline RET mutations were observed in approximately 100% of men-2 families. Therefore, genetic analysis of RET is advisable to identify young asymptomatic gene carriers and perform prophylactic thyroidectomy.

Other Inherited Syndromes Associated with GEP Tumors

In a recent report 12% of 158 patients with von Hippel-Lindau (VHL) syndrome had neuroendocrine tumors [24]. These patients showed no symptoms due to hormonal hypersecretion suggesting that the endocrine tumors were non-functioning. The VHL syndrome is caused by a germline mutation of the VHL gene which is located on chromosome 3p35–36 coding for a 213-aa protein. The VHL gene product is a component of an Skp1-Cdc53-F-box-like ubiquitin-ligase complex targeting the α-subunits of the hypoxia-inducible factor heterodimeric transcription factor for polyubiquitylation and proteasomal degradation. Somatostatinomas have been described in patients with von Recklinghausen's neurofibromatosis. Neurofibromatosis is caused by alterations of the NF1 gene located on chromosome 17q11.2 coding for neurofibromin which is a 2485-aa protein.

Growth Characteristics and Metastatic Spread and Secondary Non-Endocrine Malignancies

As recognized as early as in 1907 by S. Oberndorfer who introduced the term "carcinoid", endocrine GEP tumors grow slowly even in the metastatic state compared to adenocarcinomas of the gastrointestinal tract. However, the spontaneous tumor growth varies from one patient to another. Some tumors remain unchanged in size for months or even years without therapy, others grow slowly independent of any antiproliferative measures and still others exhibit exploding growth. The latter tumors are poorly differentiated and mostly small cell carcinomas. Even spontaneous tumor regression without any treatment has been reported in well-differentiated tumors. A schematic presentation, how malignant GEP tumors can grow is shown in fig. 4.

Unfortunately the use of proliferative markers and immunostaining of tumors for oncoproteins, tumor suppressor genes, and adhesion molecules gave contradic-

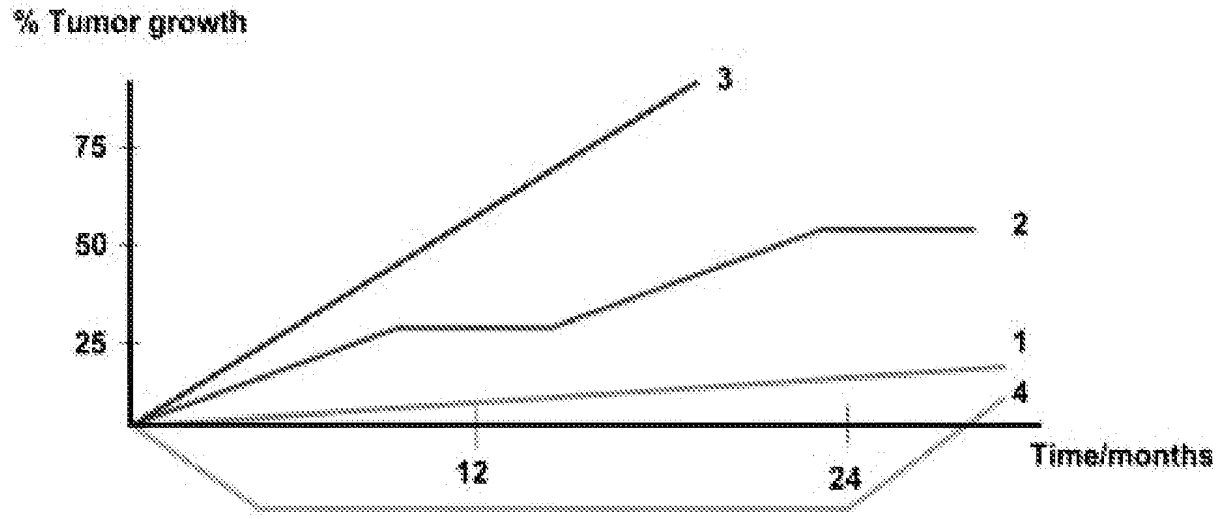


Figure 4
Schematic presentation how endocrine GEP tumors can grow. Some tumors grow so slowly that they do not meet the 25% increase according to the accepted NIH criteria of tumor progression even after 18 months (*1*). Tumor (*2*) displays an intermediate tumor growth. Tumor (*3*) grows very rapidly corresponding to its histology of a small cell neuroendocrine carcinoma. Tumor (*4*) decreased spontaneously in size, remained constant for 2 years and started to grow after 24 months of observation without any treatment

tory results. One study was performed in gastrointestinal carcinoid tumors and indicated that expression of p53, cyclin D1, Rb, bcl-2 and Ki-67 does not correlate with malignant behaviour, whereas p21 overexpression did. Others concluded from studies in bronchial carcinoid tumors and in gastrointestinal carcinoid tumors that high expression of ki-67, a tumor size >3 cm and a high mitotic index (in the case of ECL cell tumors of the stomach) is malignancy predictive. Possibly, these findings are dependent on the site of the primary tumor since in carcinoid tumors of the duodenum and ampulla of vater an aggressive behaviour of the tumor was not associated with higher proliferative indices as proliferating cell nuclear antigen (PCNA), Ki-67 and p21.

Malignant GEP tumors can spread in almost all organs: lymphnodes, peritoneal spread, liver, spleen, kidney, lung, skin, brain and bones. Table 6 is an example of the metastatic spread of patients with malignant gastrinoma and patients with carcinoid syndrome observed in the institution of the authors.

It is noteworthy that patients with malignant endocrine GEP tumors tend to synchronous and metachronous non-endocrine malignancies. In a 20-year retrospective study of 150 patients with gastrointestinal

Table 6
Metastatic spread in different endocrine GEP tumors (own data, values in percent)

Site	Non-functioning tumor	Carcinoid syndrome	Gastrinoma
Lymph nodes	64	76	54
Liver	61	88	57
Bones	13	17	11
Lung	9	14	7
CNS	2	0	4
Peritoneal	17	23	4
Other	28	24	4

carcinoids followed up for a median of 66 months 22% developed synchronous non-carcinoid tumors and 10% metachronous tumors. In another retrospective study on 69 patients with gastrointestinal carcinoid tumors 42% hat synchronous and 4% metachronous tumors. The most common site for the secondary primary malignancy was the gastrointestinal tract with carcinoma of the colon and rectum. Patients with colorectal carcinoids have, in addition, an increased risk for cancer in the colon, ano-rectum, small bowel, esophagus, stomach, lung, urinary tract and prostate.

Staging of Endocrine GEP Tumors

Staging of tumors is a useful and essential tool in oncology to define tumor size, invasion and infiltration into adjacent tissues and metastatic spread into regional lymph nodes, liver and other organs. This is important for further management using surgical, radiotherapeutic and chemotherapeutic approaches. For this, TNM classification has been elaborated for specific tumor entities as published by the AICC Cancer Staging Manual and UICC. No corresponding TNM classifications exist for endocrine GEP tumors and their malignant variants because management of endocrine tumors depends even in metastatic tumors primarily on growth behaviour (see Fig. 4) and functional activity. Insulinomas must be removed because they produce life-threatening symptoms independent on the size of the tumor which may be too small to be visualised by available imaging techniques. Metastatic insulinomas have to be partially resected if possible to reduce tumor burden and, thus, to facilitate control of hypoglycemic symptoms. In patients with very slowly growing metastatic GEP tumors the beneficial effect of many therapeutic measures is unsettled. The same applies for non-functioning small endocrine pancreatic tumors in patients with MEN-I syndrome for whom resection has not been shown to influence patient's outcome.

In an attempt to classify endocrine GEP tumors not having metastasized at diagnosis a panel of pathologists has recently proposed a classification of endocrine GEP tumors and assigned the tumors according to the categories summarised in table 5 [1]. The basis of distinguishing well differentiated endocrine carcinomas from other well differentiated endocrine tumors was usually the presence of metastases and/or evidence of local invasion. Benign or low risk endocrine tumors were distinguished from tumors with greater risk of malignancy on the basis of a combination of features such as tumor size and site, local extension, angioinvasion, cellular atypia and proliferative activity. Differentiation of poorly differentiated endocrine small cell carcinoma from well-differentiated endocrine tumors was performed by conventional histological characteristic featurs including high-grade cellular atypia, markedly hyperchromatic nuclei, focal necrosis and high proliferation.

In tables 7–11 these major principles have been transferred to endocrine tumors arising in different organs of the GI tract as the pancreas (Table 7), stomach (Table 8), the duodenum and upper jejunum (Table 9), the ileum, cecum, colon and rectum (Table 10) and the appendix (Table 11).

Clinical Entities: Symptoms and Laboratory Findings

Insulinoma

Epidemiology

Around 40% of endocrine pancreatic tumors are insulinomas. Based on a review of 224 insulinoma patients over a 60-year period the medium age was 47 (range 8–82) years and 41% of patients were male [25]. The yearly incidence is 0,5 to 4 per one million population and 5,8% had malignant insulinoma [25]. In 7,6% of patients the insulinoma was part of the MEN-I syndrome [4, 25]. Almost all insulinomas are situated in or close to the pancreas. 1–3% of insulinomas have been reported to arise in the duodenum, ileum and lung [26]. Insulinomas are evenly distributed within the pancreas [26]. Based on a study in 1067 cases most insulinomas are small: 5% smaller then 0.5 cm, 34% were 0.5 to 1 cm, 53% 1 to 5 cm and only 8% larger then 5 cm in size. With the exception of the rare malignant insulinomas prognosis of patients with a benign insulinoma and curative resection is excellent. For definition of "benign" insulinoma see Table 7.

Table 7
Clinicopathological staging of endocrine tumors of the pancreas. (Mod. according to [1])

1	Well-differentiated endocrine tumor
1.1	Benign behaviour: confined to the pancreas, nonangioinvasive, <2 cm in size*, ≤2 mitoses and ≤2% Ki67 positive cells/10HPF
1.2	Uncertain behaviour: confined to the pancreas ≥2 cm in size, >2 mitoses, >2% Ki67 cells/10 HPF, or angioinvasive
2	Well-differentiated endocrine carcinoma
2.1	Low grade malignant with gross local invasion and/or metastases
3	Poorly differentiated endocrine carcinoma – small cell carcinoma, high grade malignant

*<2 cm in size implies close to 100% probability of benign behaviour, >3 cm corresponds to 90% probability of malignancy. Functioning, associated with pertinent clinical syndrome of endocrine hyperfunction; nonfunctioning, not associated with pertinent clinical syndrome, irrespective of hormone detection in blood or tumor tissue.

Table 8
Clinicopathological staging of endocrine tumors of the stomach. (Mod. according to [1])

1	Well-differentiated endocrine tumor – carcinoid
1.1	ECL-cell carcinoid
1.1.1	ECL-cell carcinoid type I associated with type A gastritis
1.1.2	ECL-cell carcinoid type II associated with Zollinger-Ellison syndrome
1.1.3	Sporadic ECL-cell carcinoid
1.2	ECL-cell carcinoid
1.3	G-cell carcinoid
2	Small cell carcinoma – poorly differentiated endocrine tumor
3	Tumor like lesions: Hyperplasia, Dysplasia

Table 9
Clinicopathological staging of endocrine tumors of the duodenum and upper jejunum. (Mod. according to [1])

1	Well-differentiated endocrine tumor – carcinoid
1.1	Benign behaviour: nonfunctioning, confined to mucosa-submucosa, ≤1 cm in size, nonangioinvasive
1.1.1	Gastrin-producing tumour (proximal duodenum)
1.1.2	Serotonin-producing tumor
1.1.3	Gangliocytic paraganglioma, any size and extension (ampullary region)
1.2	Uncertain behaviour: confined to mucosa-submucosa >1 cm in size or angioinvasive
1.2.1	Gastrin-producing tumor, functioning (Zollinger-Ellison syndrome) or nonfunctioning, sporadic, or MEN-1-associated
1.2.2	Somatostatin-producing tumor (ampullary region) with or without Recklinghausen disease
1.2.3	Serotonin-producing tumor, nonfunctioning
2	Well-differentiated endocrine carcinoma – malignant carcinoid
2.1	Low grade malignant, extending beyond submucosa or with metastasis
2.2	Gastrin-producing carcinoma, functioning (Zollinger-Ellison syndrome) or nonfunctioning, sporadic, or MEN-1-associated
2.3	Somatostatin-producing carcinoma (ampullary region) with or without Recklinghausen disease
2.4	Serotonin-producing carcinoid, nonfunctioning or functioning (any size or extension) with carcinoid syndrome
2.5	Malignant gangliocytic paraganglioma
3	Poorly differentiated endocrine carcinoma – small cell carcinoma
4	High grade malignant (ampullary region)

Table 10
Clinicopathological staging of endocrine tumors of the ileum, cecum, colon and rectum. (Mod. according to [1])

1	Well-differentiated tumor – carcinoid
1.1	Benign behaviour: confined to mucosa-submucosa, nonangioinvasive, ≤1 (small int.) or ≤2 cm (large int.) in size
1.2	Uncertain behaviour: nonfunctioning, confined to mucosa-submucosa, >1 cm (small int.) or >2 cm (large int.) in size, or angioinvasive
2	Well-differentiated endocrine carcinoma – malignant carcinoid, low grade malignant, deeply invasive (muscularis propria or beyond), or with metastases
3	Poorly differentiated endocrine carcinoma – small cell carcinoma, high grade malignant
4	Mixed exocrine-endocrine carcinoma – moderate to high grade malignant

Table 11
Clinicopathological staging of endocrine tumors of the appendix. (Mod. according to [1])

1	Well-differentiated endocrine tumor – carcinoid, benign behaviour, nonfunctioning, confined to appendiceal wall, nonangioinvasive, ≤2 cm in size
1.1.1	Serotonin-producing tumor
1.1.2	Enteroglucagon-producing tumor – uncertain behaviour, nonfunctioning, confined to subserosa, >2 cm in size, or angioinvasive tumor
2	Well-differentiated endocrine carcinoma – malignant carcinoid
2.1	Low grade malignant, invading the mesoappendix or beyond, and/or with metastasis
2.2	Serotonin-producing carcinoid with or without carcinoid syndrome
3	Mixed exocrine-endocrine carcinoma
3.1	Low grade malignant – goblet-cell carcinoid

Pathophysiology

Insulinoma cells fail to respond adequately to low blood glucose levels. This is indicative of a defective negative feedback which maintains euglycemia in healthy subjects. In addition, the storage capacity of insulinoma cells is impaired, resulting in an inappropriate insulin release [14].

Symptoms

Insulinoma symptoms are the consequence of neurohypoglycemia and superimposed by symptoms which are the consequence of adrenergic counter regulation as sweating, tremulousness, palpitation (Table 12). As a rule, the frequently nonspecific symptoms are associated with fasting and occur more often after muscular exercise and during late night or early morning and when a meal is delayed. Whipple's triad is highly suggestive of an insulinoma and comprises of hypoglycemic symptoms, a parallel demonstration of low blood glucose levels less then 50 mg/dl (2.8 mmmol/l) and improvement of symptoms after administration of glucose [12, 25, 26]. There is often a long-lasting delay between onset of clinical signs and diagnosis, because symptoms listed in table 12 are unspecific and do not appear in a clear sequence. The mean duration of symptoms prior to diagnosis varies between 15 months and more then 3 years [12, 25]. Each patient displays his/her own pattern of symptoms which differ from patient to patient. Since regular food intake prevents the occurrence of symptoms, patients are used to eat regularly and often gain weight. If misinterpreted, severe and longer lasting hypoglycemia can progress to seizure and permanent brain damage. Some insulinoma patients are diagnosed only in psychiatric hospitals, having been admitted with misdiagnosed symptoms.

Differential Diagnosis

In addition to insulinoma hypoglycemia can have several causes which are summarised in table 13. Factitious hypoglycemia secondary to self-administration of insu-

Table 12
Clinical symptoms in patients with insulinoma

Symptoms of neurohypoglycemia	Symptoms of adrenergic counterregulatory response
Diplopia	Anxiety
Blurred vision	Sweating
Confusion	Tremulousness
Abnormal behaviour	Hunger
Weakness	Nausea
Amnesia	Fatique
Aphasia	Tremor
Transient motor defects	Palpitation
Dizziness	
Speech difficulty	
Headache	
Seizure	
Memory loss	
Lethargy	
Disorientation	
Mental change	
Convulsion	
Coma	
Obesity	

Table 13
Other causes of hypoglycemia

Insulin or insulin-like-mediated	Factitious hypoglycemia (153)
	Tumor associated hypoglycemia (151, 152)
	insulin autoantibodies (154)
	Transient hypoglycemia of infancy
	Nesidioblastosis
Postprandial (reactive) hypoglycemia	Previous gastric surgery (Billroth I and II gastrectomy)
	Idiopathic
Food stimulated	Ethanol
	Unripe kakee fruit (159)
Hormone deficiency	Addison's disease
	Growth hormone deficiency
	Hypothyroidism
Hepatic diseases	End-stage liver failure
	Glycogen storage disease
	Glycogen synthetase deficiencies
	Fructose-1,6-disphosphate deficiency
Metabolic diseases	Severe malnutrition
	Sepsis
Drugs	Sufonylureas (155)
	Quinine (156)
	Disopyramide (157)
	Haloperidol (158)

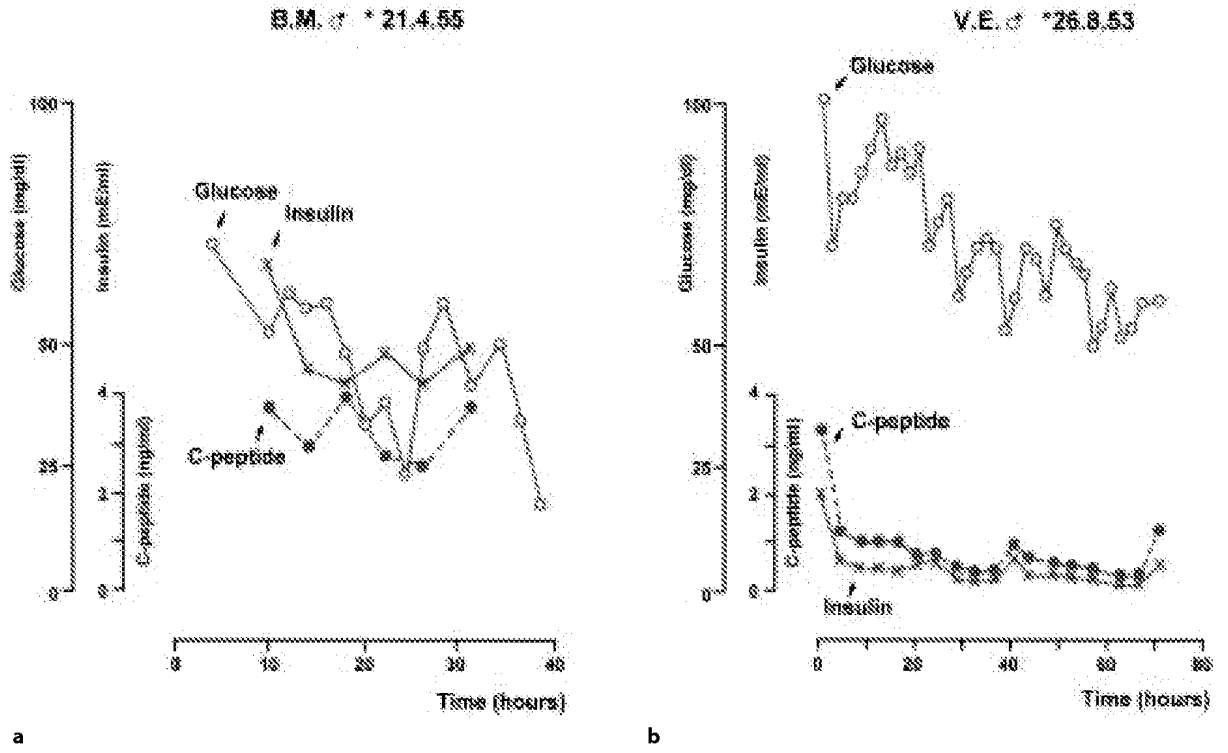


Fig. 5a,b
Blood glucose, insulin and C-peptide levels during a 40 h fast, suggesting an insulin producing tumor since insulin and C-peptide levels remained inadequately elevated despite low blood glucose levels (**a**). In **b** insulin and C-peptide levels dropped down to very low levels during a 72 hour fast

lin or oral intake of hypoglycemic agents should be considered if an insulinoma cannot be ascertained by diagnostic measures. In the author's institution the following conditions are mostly the cause of hypoglycemic episodes if an insulinoma could be excluded: postprandial hypoglycemia (late dumping syndrome) even in the absence of gastric resection (Billroth I and II resection) due to rapid gastric emptying; mesenchymal tumors, such as fibrosarcoma, liposarcoma, rhabdomyosarcoma, hemangiopericytoma, mesothelioma, leiomyosarcoma, IGF-II-producing tumors; endstage of liver cirrhosis.

Diagnosis

Biochemical testing. The gold standard for the diagnosis of an insulinoma is the "72 h fast" test. About 75% of insulinoma patients will develop symptoms and blood glucose levels of <40 mg/dl (2.2 mmol/l) within the first 24 h of the fast (Fig. 5), 90% within 48 h, and 100% within 72 h. The test should be performed using a standardised protocol as follows:

1. begin the test immediately after the last meal (breakfast); insert a capped intervenous cannula;
2. allow intake of calorie-free fluid ad lib (water);
3. encourage physical activity (walking);
4. analyze plasma glucose, insulin and C-peptide in the same specimen every 6 h until blood glucose drops to <60 mg/dl; then increase sampling to every 1–2 h;
5. terminate the test when the patient develops symptoms of hypoglycemia *and* glucose is <40 mg/dl. If the patient is asymptomatic, prolong the test until suggestive symptoms appear; sample always blood glucose, insulin and C-peptide at the end of the test;
6. when symptoms arise, give 10% glucose intravenously or orally until the patient is asymptomatic.

The pathophysiologic background of the prolonged fast test is that steadily decreasing blood glucose levels signal to the normal b-cell of the islets of Langerhans to turn down insulin and C-peptide levels which decrease to either not measurable or very low levels. In insulinoma patients insulin and C-peptide are inadequately suppressed despite even lower blood glucose levels then observed in healthy controls (see Fig. 5). Importantly, plasma insulin in insulinoma patients go rarely beyond levels normally found in the fasted and fed state of normal subjects; however, they are inappropriately high for the prevailing blood glucose concentration. Therefore, plasma insulin and C-peptide levels must always be assessed in relation to the corresponding blood glucose levels. Some authors have advised to calculate a ratio of insulin to glucose because borderline low levels of insulin have been reported in some patients with proven insulinoma in association with hypoglycemia. However, also these ratios do not reliably differentiate between insulinoma patients and healthy subjects since there is not in every subject a linear correlation of insulin and glucose levels. Normal subjects rarely exceed a ratio of plasma insulin (in μU/ml) to glucose (in mg/dl) of 0.3 which is based on the observation that insulin levels are in normals less than 6 μU/ml when blood glucose levels decrease to less than 40 mg/dl. For example: if plasma insulin measures 8 μU/ml and blood glucose is 40 μg/dl, than the ration is 8: 40=0.2. However, neither this ratio or an amended variation of such a ratio do reliably discriminate between insulinoma patients and healthy subjects because there is not in every patient a clear linearity between plasma insulin and glucose levels.

Some experts recommend estimation of proinsulin in insulinoma patients which is elevated in most insulinoma patients to more than 20% of the total plasma insulin levels. Indeed, some insulinomas produce and secrete predominately proinsulin.

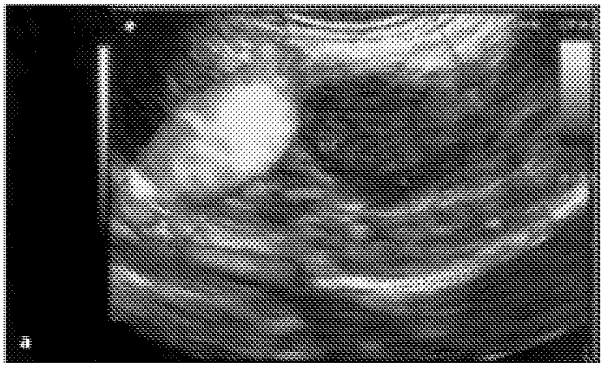
In previous literature, various stimulatory and suppressive tests have been recommended because they are believed to facilitate the diagnosis of an insulinoma as C-peptide suppression test, tolbutamide test, glucagon test, calcium infusion test, euglycaemic clamp procedure and others. These tests are neither specific nor sensitive and due to the possibly harmful side effects of prolonged hypoglycemia in case of the tolbutamide and calcium infusion not favoured in the more recent literature.

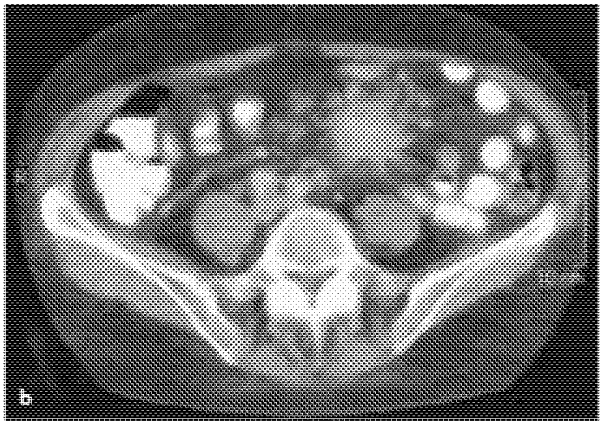
Figure 6a–f
Localization of endocrine GEP tumors by various imaging techniques. **a** Endoscopic ultrasound showing a small (11 mm) pancreatic insulinoma; **b** CT imaging of desmoplastic reaction in a patient with carcinoid syndrome due to an ileum carcinoid; **c** OctreoScan showing wide metastatic spread in a patient with a non-functioning endocrine pancreatic tumor; **d** MRT imaging of liver metastases in a patient with non-functioning endocrine pancreatic tumor; **e** MRT imaging of two brain metastases in a patient with malignant gastrinoma; **f** endoscopic demonstration of a rectal carcinoid. Notice the *yellow colour* ▸

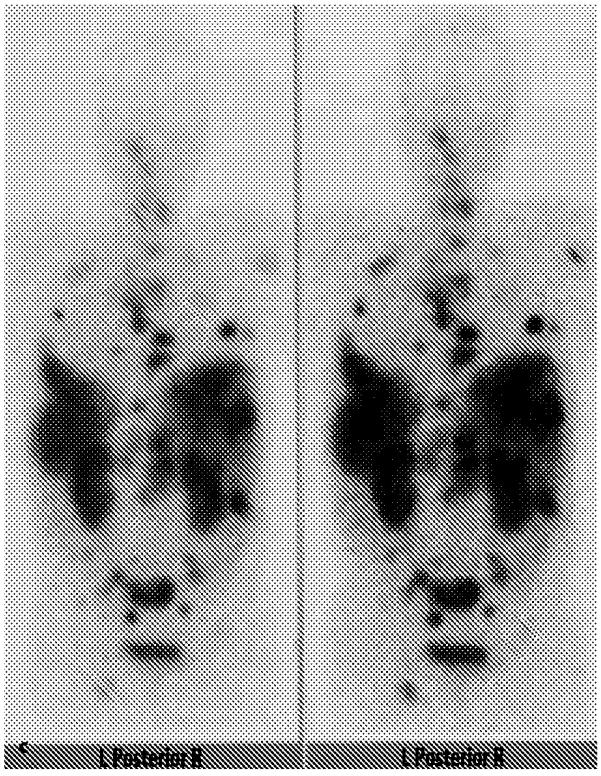
To exclude other causes of hypoglycemia as factitious hypoglycemia or postprandial hypoglycemia measurement of plasma sulfonylurea should be performed to exclude surreptitious use of these drugs and an oral glucose load with estimation of blood glucose and insulin levels in 30 minutes intervals for 3 h should be considered if reactive hypoglycemia is considered.

Localization. Imaging studies to localize an insulinoma should only be performed once the diagnosis of an insulinoma is highly suggestive by biochemical testing. Experienced surgeons claim that the most sensitive localization instrument is the finger of the skilled surgeon during intraoperative abdominal exploration and recommend no preoperative localization procedures. Indeed, almost all insulinomas are situated in the pancreas and only 1–3% found ectopically. On the other side, there is no localization procedure available with an 100% detection rate. At the institution of the authors, 50 patients with biochemically proven insulinoma had undergone operative exploration within the last 10 years and all insulinomas have been identified at the first operation (R. Rothmund, personal communication). These results have been confirmed by some but not all authors. The latter claim that 10–27% of insulinomas remained undetected and advocated, therefore, the need for preoperative localization procedures.

The most accurate and sensitive imaging procedure to localize and to stage (see table 7) an insulinoma is endoscopic ultrasound (EUS) which localizes an insulinoma in up to 85% and thus being superior to other imaging studies as CT, MRT, conventional ultrasound and arteriography (Fig. 6a). Of course, detection rate is mostly dependent on the experience of the investigator and expert investigators will detect tumors less than 0.4 cm in diameter. In such institutions EUS will be the



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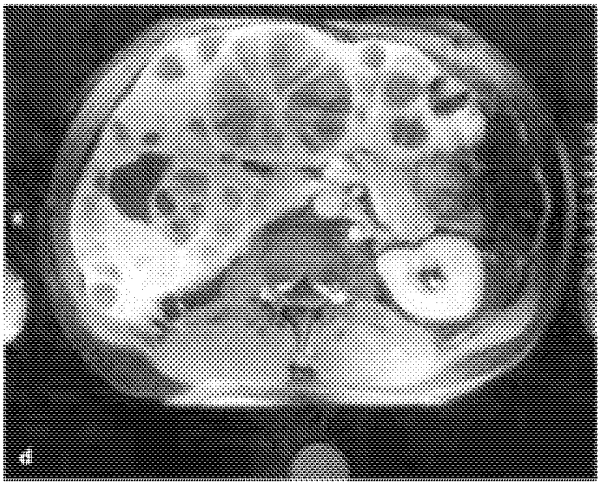
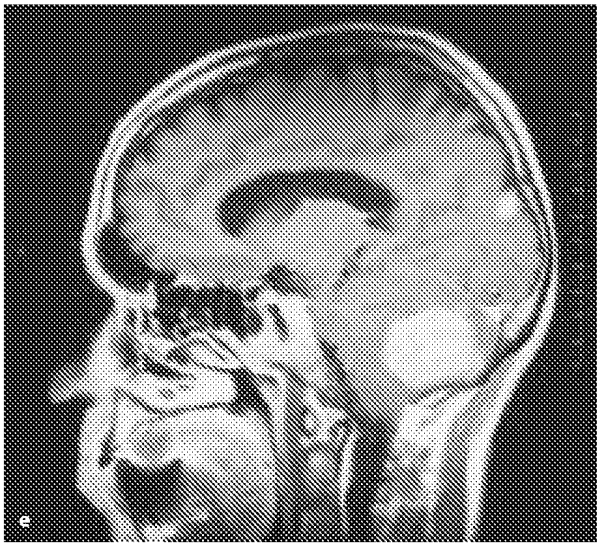




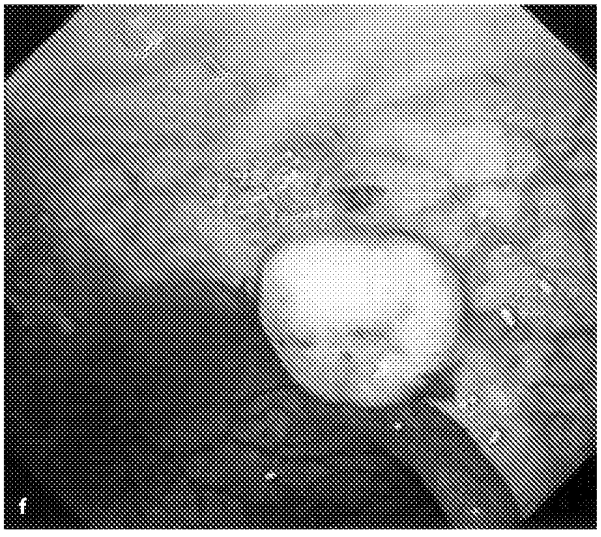


Table 14
Sensitivity of imaging studies in the detection of endocrine GEP tumors

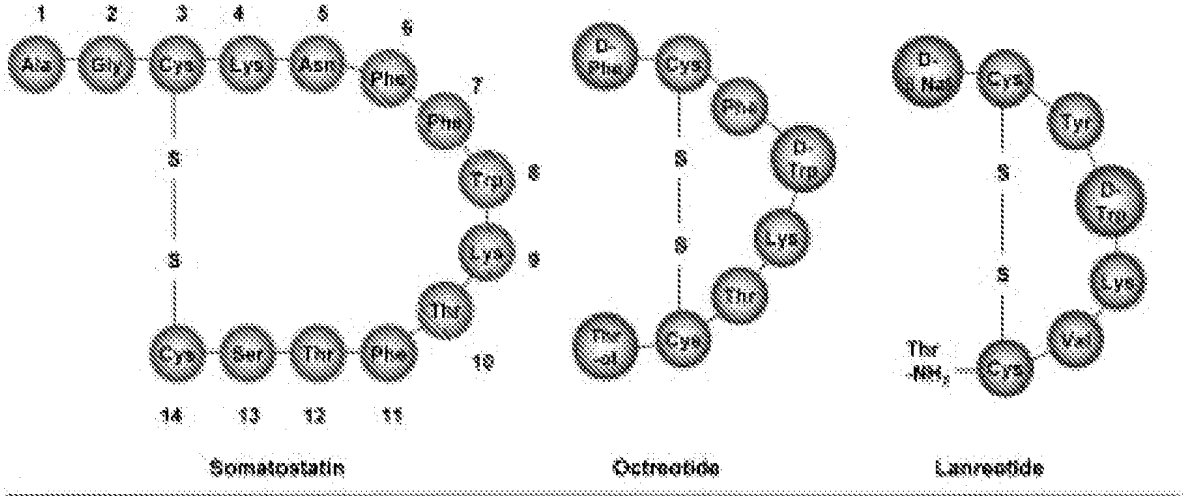
	SRS [%]	CT [%]	MRI [%]	EUS [%]
Gut tumors	72–96%	33%	n.d.	-
Pancreatic tumors	58–100%	25–38%	24–71%	58–86%
Insulinoma	12–50%	29%	13%	81–94%

primary and exclusive diagnostic modality for tumor imaging. Sensitivity of other imaging procedures are summarised in table 14. Angiography was for many years the preferential method in localising insulinomas due to the characteristic high blood supply of the tumor. In earlier reports successful tumor localization of up to 90% has been described whereas more recent data indicated a sensitivity of only 30–50%. The method is invasive, expensive and requires considerable experience in data interpretation. Therefore, both computed tomography (CT) with rapid-sequence spiral CT, with oral and intravenous contrast enhancement and magnetic resonance imaging (MRI) with the use of dynamic gadolinium enhancement and fat suppression have replaced angiography in many centers that prefer an exact preoperative tumor localization. Somatostatin receptor szintigraphy which recognizes high numbers of somatostatin receptors present on most endocrine GEP tumors detects, in contrast to other GEP tumors, only 50% of insulinomas due to the inconsistant frequency of somatostatin receptor subtype 2 on insulinomas. Therefore, negative somatostatin receptor scintigraphy does not exclude the presence of an insulinoma.

Treatment. The primary treatment option in patients with a biochemically proven insulinoma is surgery. Once the tumor is localized pre- and intraoperatively surgeons will decide whether the tumor can be enucleated or whether proximal or distal pancreatic resection is the perferred method. Total pancreatectomy or "blind" distal resection should be avoided if the insulinoma cannot be identified intraoperatively. In this case laparotomy should be terminated and tumor localization repeated. Surgery is also indicated in metastatic insulinoma since operative tumor debulking has been shown to provide long-lasting symptomatic improvement. Symptomatic antisecretory therapy is indicated in the pre-operative phase and in metastatic disease. To prevent hypoglycemic events regular intake of carbohydrates in required and a light carbohydrate meal in the late evening is important. If diet does not prevent hypoglycemia, oral administration of diazoxide and subcutaneous long-acting somatostatin analogues are the therapeutic priciples of choice to prevent hypoglycemia whereas β-blocking agents, glucocorticoids, calcium-channel blockers and phenytoin have been used earlier but with limited therapeutic effects.

Diazoxide is a non-diuretic benzothiadizine that inhibits the release of insulin from the secretory granules of normal β-cells and of insulinoma cells. Unfortunately, not all insulinoma patients respond to diazoxide but it should be tried with starting dosages of 25 μg b.i.d. and the dose can be escalated up to 200 μg t.i.d. Side effects including cardiac arrhythmia, cardiomyopathy, bone marrow depression, sodium retention and peripheral edema should be noticed and can force to discontinue therapy.

Also long-acting somatostatin analogues (Fig. 7) as octreotide and lanreotide suppress insulin secretion. They have been first introduced for the treatment is disabling acromegaly and later for functionally active endocrine GEP tumors to supress hormone secretion [27]. Octreotide, lanreotide and octreotide LAR are modifications of the naturally occuring somatostatin (Fig. 7). Lanreotide and octreotide-LAR bound to polylactid-glycolide microspheres permit sustained release allowing single subcutaneous injections of lancreotide every 2 weeks and of octreotide-LAR every 4 weeks. Somatostatin and its analogs act through a family of at least 5 receptors (sstr 1–5). Most encocrine GEP tumors express sstr 2, whereas the other 4 sstr are less frequently or not expressed (179). Unfortunately, long acting somatostatin analogs are effective in only 50% of insulinoma patients since sstr 2 is only expressed in 50% of insulimas. Therefore, the hypoglycemia preventing effect of somatostatin analogs is unpredictable. Importantly, somatostatin analogs can even aggravate hypoglycemic symptoms because they suppress also the counter regulatory hormone glucagon. Therefore, insulinoma patients must be monitored carefully if somatostatin analogs are considered to prevent hypglycemia. Treatment should be started with 50 μg short-acting octreotide b.i.d. and increased to 200 μg t.i.d. according to the patients re-



	Somatostatin	Octreotide	Octreotide LAR	Lanreotide	Lanreotide autogel
Dosing	3.5 µg/kg/h i.v.	50-200 µg s.c.	10-30 mg i.m.	30 mg s.c.	60 mg s.c.
Interval	continous	8h	28 days	14-28 days	28 days
Preferential receptors	sstr1-5	sstr2, (sstr5)	sstr2, (sstr5)	sstr2, (sstr5)	sstr2, (sstr5)

Figure 7
Structure and characteristics of native somatostatin and its long-acting analogues octreotide and lanreotide

sponse. If they respond adequately to the short lasting somatostatin formulations, the longer lasting octreotide-LAR and lanreotide-LAR which have to be administered only every 2 weeks (lanreotide-LAR) or every 28 days (octreotide LAR) can be offered.

In patients with malignant and metastatic insulinoma several therapeutic options have to be considered including palliative surgery, embolisation of liver metastases and chemotherapy with streptozotocin combinations (for further details see "General aspect of management" under "Medical treatment of symptoms")

Persistent Neonatal Hyperinsulinemic Hypoglycemia (PNHH). Persistent neonatal hyperinsulinemic hypoglycemia was earlier called "nesidioblastosis" and originally described by Laidlow in 1938 [28]. He called cells which originated from the pancreatic ductal epithelium nesidioblasts, their proliferation nesidioblastosis and the resulting tumor nesidioblastoma. Today, the term "nesidioblastosis" is substituted by the term "persistent neonatal hyperinsulinemic hypoglycemia (PNHH). This entity is characterized by the occurrence of hyperinsulinemic hypoglycemia in the absence of an endocrine pancreatic tumor. As in insulinomas hypoglycemia occurs in the fasting state and insulin secretion is not adequately suppressed. The disease affects newborn children within the first 6 months of life and only very rarely adults [28]. The term nesidioblastosis is misleading since it implies general islet cell hyperplasia which does not exist. The pancreas of the newborn enfant has physiologically much more and smaller islets of Langerhans compared to the situation in later life. The morphological abnormalities of the endocrine pancreas that underly PNHH are heterogeneous and encompass small endocrine tumors (insulinoma), unifocal or multifocal adenomatosis characterized by local and excessive proliferation of islet

cells, hyperplasia of islets of Langerhans and frequently even no recognizable pathomorphological abnormalities [28]. In the latter situation, a functional defect of the pancreatic β-cells is assumed to cause unrestrained insulin release. PNHH occurs in a sporadic and a familial autosomal recessive form [28]. In the familial variant of PNHH a genetic defect has been identified on the short arm of chromosome 11p14–15.1. The respective gene codes for the sulfonylurea receptor which is mutated in the familial form of PNHH. This mutation results in abnormal insulin secretion and altered sensitivity of the β-cell to glucose. The genetic defect responsible for the sporadic form of PNHH is also identified. A recent report suggests a dysfunction in the adenosine triphosphate-sensitive potassium channel present in the plasma membrane of pancreatic β-cells [29].

Clinically, the respective infants present with nonspecific symptoms resulting from neuroglucopenia. Medical management includes continuous glucose infusion via a central venous catheter, diazoxide and long-acting somatostatin analogs. Recently, it has been demonstrated that calcium channel blocking agents can be used with efficacy and safety to control hypoglycemia in PNHH. However, definitive cure requires in most patients subtotal pancreatectomy.

Adult onset PNHH is very rare and requires the same multimodal therapeutic approach as in the infantile form.

Gastrinoma

Epidemiology and Prognosis

Gastrinomas are as insulinomas very rare tumors. The yearly incidence is 0,5 to 3 per one million population [4, 8]. The mean age at diagnosis is 50 years. Unlike insulinomas, the majority of gastrinomas is malignant. Gastrinomas are in 30–50% part of the MEN-1 syndrome [8]. In a recent study with 151 patients with surgically removed non-metastasised gastrinoma, of whom 128 were part of MEN-1 syndrome, it has been shown that 34% of patients with sporadic gastrinoma but none of patients with MEN-1 syndrome were disease free after 10 years [30]. This demonstrates that in sporadic gastrinoma definitive cure can be achieved in a substantial proportion by surgery. In contrast, patients with gastrinoma as part of MEN-1 syndrome have either multiple gastrinoma or have metastatic disease at operation.

Whereas insulinomas are almost exclusively located in the pancreas and are not located in a special part of the pancreas, the vast majority of gastrinomas occur in the "gastrinoma triangle". This region is defined by the junction of the neck and body of the pancreas, the junction of the second and third part of the duodenum and the confluence of the cystic and common bile duct. 50% of gastrinomas are located in the duodenum. Very rarely, gastrinomas arise in the antrum, omentum, liver, lymph node or elsewhere [8]. In MEN-1 gastrinomas are more frequently located in the duodenum where they are mostly very small and multifocal. Sporadic gastrinomas, in contrast, occur more frequently in the pancreas. The malignant potential of sporadic gastrinomas and those arising in patients with MEN-1 syndrome is not uniform. Recent studies indicate that approximately one fourth of patients with sporadic gastrinomas persue an aggressive growth pattern, with a 10-year survival of 30%, whereas in the remaining 75% of patients gastrinomas display a less aggressive growth pattern with a 10 year survival of 95% [30]. Similarly, in patients with metastatic gastrinomas to the liver aggressive growth was demonstrated only in a minority of patients whereas the majority displayed indolent growth [30]. Tumor related deaths occur almost entirely in the aggressive growth group. According to a recent investigation in patients with gastrinoma and MEN-1 syndrome growth behaviour is also not uniform. 23% of patients with gastrinoma and MEN-1 syndrome developed liver metastases and 14% had an aggressively growing gastrinoma. Aggressive growth of the primary gastrinoma but not of liver metastases growing less aggressively was associated with decreased survival [30]. High serum gastrin levels, a primary tumor size of >3 cm and the presence of bone and liver metastases were associated with an aggressive gastrinoma growth [30].

Pathophysiology

The pathophysiological events occurring in patients with Zollinger-Ellison syndrome are summarized in Fig. 8. Hypergastrinemia as the result of unrestrained hormone release from the tumor displays two effects: stimulation of gastrid acid secretion from the parietal cell and stimulation of parietal and ECL-cells both located in the oxyntic mucosa of the proximal stomach. The consequences of gastric acid hypersecretion are summarized in Fig. 8. All patients with gastrinoma develop diffuse,

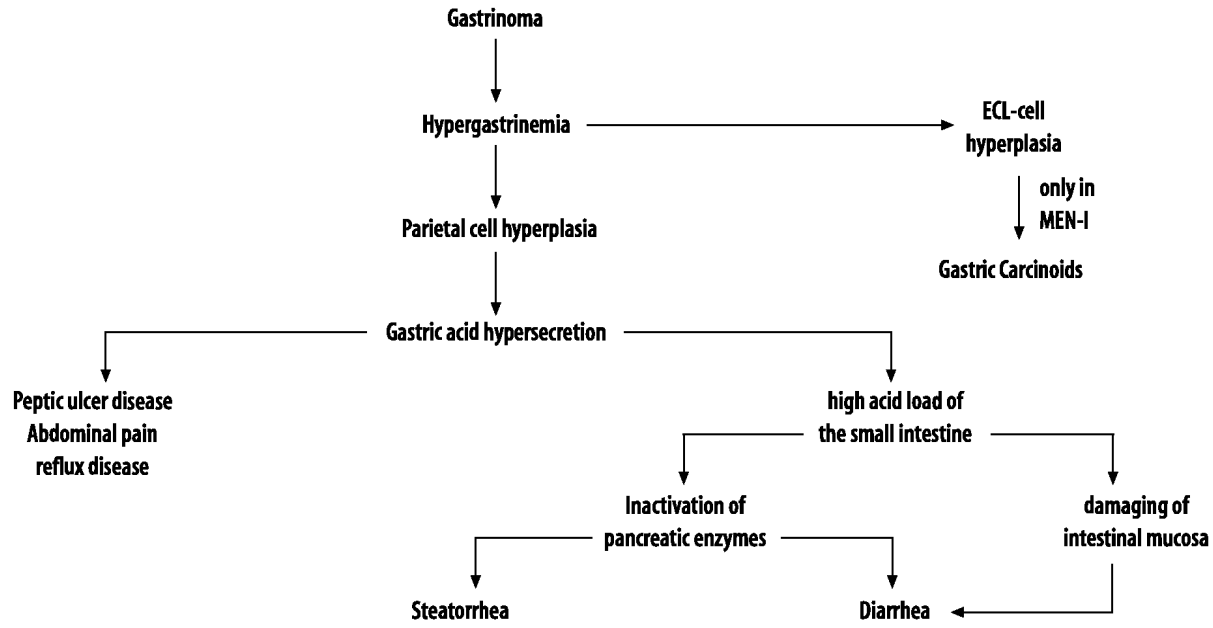


Figure 8
Pathophysiology of Zollinger-Ellison syndrome

linear and micronodular ECL-cell hyperplasia but gastric carcinoids arise almost exclusively in patients with MEN-1 syndrome. This indicates that a genetic trait must be present together with the trophic action of high serum gastrin levels.

Symptoms

Clinical presentation of patients with Zollinger-Ellison syndrome has changed considerably in the last two decades mainly due to the availability of potent antisecretory drugs. Before the discovery of H_2-blockers and proton pump inhibitors patients presented with severe relapsing peptic ulcer disease and its sequelae: life-threatening bleeding and perforation. Patients died from complications and not from the tumor itself. This has dramatically changed. Most patients with gastrinoma present today with severe and medically resistant reflux disease requiring higher than standard dosages of PPIs. More then 90% of gastrinoma patients suffer from epigastric discomfort and peptic ulcer disease. Most ulcers are situated in the duodenal bulb or the distal stomach, whereas ulcers located in atypical sites (distal to the duodenal bulb, jejunum) are the exception. 30% of gastrinoma patients have helicobacter pylori infection, but eradication has virtually no influence on peptic ulcer relapse rate. 5–10% of patients do not have peptic ulcer disease and present with secretory diarrhea (Fig. 8). Patients report on watery stools arising during late night and early morning with some improvement after intake of meals. Secretory diarrhea is present in more than 50% of gastrinoma patients. A history of nephrolithiasis and the presence of hypercalcemia are suspicious for hyperparathyroidism as part of MEN-1 syndrome. The association between Zollinger-Ellison syndrome and Cushing's syndrome due to ectopic ACTH production by the endocrine pancreas tumor is rare and patient's survival depends mainly on the control of hypercorticism (see later).

Differential Diagnosis

Differential diagnosis of Zollinger- Ellison syndrome encompass few states of relapsing ulcer disease, hypergastrinemia and gastric acid hypersecretion. Antral G-cell hyperfunction is are rare event and results, ac-

Table 15
Differential diagnosis of hypergastrinemia

With gastric acid hypersecretion	With hypo- or achlorhydria
Antral G-cell hyperfunction	Typ A gastritis
Massiv resection of small bowel	Renal insufficiency
"excluded antrum"	Prolonged acid-suppressive medication

cording to recent reports, from *H. pylori* infection. In these patients *H. pylori* inhibits antral somatostatin release much more powerfully then in other *H. pylori* infected individuals and leads to hypergastrinemia because antral gastrin producing G- and somatostatin-producing D-cells are situated in close vicinity. After food intake serum gastrin increases in patients with antral G-cell hyperfunction to much higher levels then in patients with normogastrinemic ulcer disease. Cure of infection prevents further peptic ulcer relapse and fasting and postprandial hypergastrinaemia normalize.

The "excluded antrum syndrome" is currently extremely rare and was more frequent in earlier decades when patients with peptic ulcer disease have been subjected to distal gastric resection (Billroth II). In this condition, a small part of the distal antrum adjacent to the duodenal bulb was inadvertently left on the blind loop. Hypergastrinemia results from the neutral environment of this part of antral mucosa and produces acid hypersecretion with the consequence of relapsing ulcer disease in the remaining stomach or around the gastro-jejunal anastomosis.

Diagnosis

Biochemical Testing. The triad: "excessive gastric acid hypersecretion, intractable peptic ulcer disease and the presence of a non-insulin-producing pancreatic endocrine tumor" was recognized as entity in 1955 by Zollinger and Ellison. Biochemically, diagnosis of a Zollinger-Ellison syndrome is based on the simultaneous presence of elevated serum gastrin levels and low intragastric pH. Elevated serum gastrin levels alone do not prove a gastrinoma since it can be found in several conditions mostly as consequence of reduced or absent gastric acid. Examples are the intake of PPIs, the presence of chronic atrophic gastritis (type A gastritis) and severe *H. pylori* associated chronic gastritis (Table 15). Therefore, diagnosis of Zollinger-Ellison syndrome is easily made if elevated serum gastrin levels combined with gastric acid hypersecretion exists. Since most patients with Zollinger-Ellison syndrome are under long-term PPIs, treatment should be stopped and shorter lasting H_2-blockers offered. Ten days after discontinuation of PPI-treatment basal acid secretion and serum gastrin should be studied after 12 hours withdrawal of H_2-blockers. A serum gastrin level of greater 500 pg/ml in the absence of conditions summarized in table 14 but in the presence of elevated basal acid output (BAO) are highly suggestive of Zollinger-Ellison syndrome. BAO is generally above 10 mEq/hr in an intact stomach and above 5 mEq/hr in patients after Billroth I and II resection. If serum gastrin levels are in the upper normal range or only moderately elevated Zollinger-Ellison syndrome can be confirmed by a secretin provocative test. After intravenous rapid injection of 2 U/kg secretin, serum gastrin rises "paradoxically" within 15 minutes by more then 50% or 200 pg/ml in patients with Zollinger-Ellison syndrome. Blood for gastrin measurement should be taken at times – 5 min and immediately before secretin and after 2, 5, 15 and 30 minutes post secretin. The mechanism of gastrin increase after secretin is not completely understood. There are no other conditions with gastrin increase after secretin. In contrast, hypergastrinemia due to chronic atrophic gastritis or "excluded antrum" declines after secretin (Fig. 9). The sensitivity of the secretin test is below 100% since in some gastrinoma patients no or only small increases of serum gastrin occur.

Localization. During the past decade significant advances in the localization of endocrine GEP tumors could be achieved. Imaging studies in patients with gastrinoma are focused on the detection of the primary (pancreas, duodenum, elsewhere) and to the presence of metastases. As other malignant endocrine tumors malignant gastrinomas tend to metastasize into lymph nodes, liver, bones and other sites as skin and brain. Somatostatin receptor szintigraphy using ^{111}indium-labelled octreotide (OctreoScan) has changed the imaging strategies of gastrinomas and other endocrine pancreatic tumors since somatostatin subtype 2 (sst_2) receptors have been demonstrated in approximately 90% of gastrinomas using in vitro autoradiography. It could be shown that OctreoScan has a sensitivity of 71–75% and a specificity of up to 82% (see Fig. 7c). According to a recent report in

which OctreoScan was compared with conventional imaging procedures in 80 gastrinoma patients, OctreoScan was the most sensitive modality for detection of primary and metastatic gastrinoma. However, size of the primary gastrinoma was an important factor and tumors smaller then 0.5 cm could not be visualized. According to this study 33% of primaries, especially small duodenal gastrinomas that could be detected later intraoperatively, were missed by OctreoScan [8]. Somatostatin receptor szintigraphy and MRI were the most sensitive imaging procedures to detect bone metastases and OctreoScan was the only method to distinguish small liver metastases from small hemangiomas. Therefore, OctreoScan is presently the imaging procedure of choice to localize the primary and to define the extent of metastatic spread. As in insulinomas endoscopic ultrasound is highly accurate in the localization of small pancreatic and duodenal gastrinomas whereas contrast medium enhanced CT and MRT have been shown to have sensitivities of approximately 80–85% [32].

The most important questions which have to be answered are:

- Is the biochemically and clinically highly suggestive gastrinoma sporadic or part of the MEN-1 syndrome?
- Is the gastrinoma malignant and has spread to lymph nodes, to the liver and elsewhere?
- What is the potential benefit of surgical intervention?

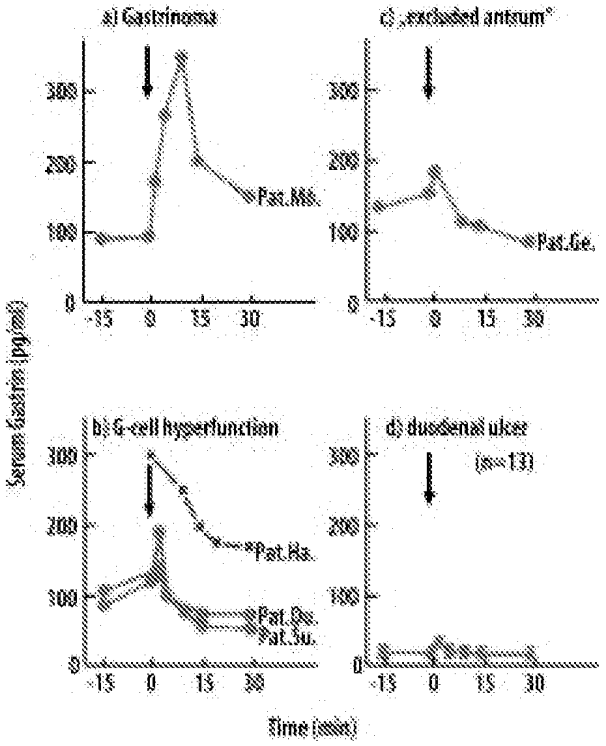


Figure 9a–d
Serum gastrin levels after secretin injection in patients with **a** Zollinger-Ellison syndrome; **b** antral G-cell hyperfunction; **c** patient with "excluded antrum"; **d** patients with regular duodenal ulcer disease

If the size of a gastrinoma is greater then 2 cm in case of a pancreatic tumor (see Table 7) and greater then 1 cm in case of a duodenal tumor (see Table 9) malignant behaviour is likely and the effect of surgical intervention with respect to cure the disease uncertain. In patients with MEN-1 syndrome frequently more then only 1 endocrine pancreatic tumor can be visualized. In this condition it is almost impossible to identify the tumor which is responsible for the Zollinger-Ellison syndrome.

For practical reasons one should always start with OctreoScan. If the primary as well as metastatic spread have been detected by OctreoScan the findings should be further confirmed by contrast-medium enhanced CT or MRT (see Fig. 7e). If a solitary gastrinoma, no primary and no metastases are found by OctreoScan and upper abdominal CT and/or MRT careful upper gastroduodenoscopy should be performed to confirm the presence of a duodenal primary. Endoscopic ultrasound should complete endoscopy. In case of a solitary primary and if no primary and no metastases exist laparotomy is indicated to localize and to remove the primary tumor within the "gastrinoma triangle".

Treatment. The sequelae of gastric acid hypersecretion as reflux disease, peptic ulceration and watery diarrhea can be effectively controlled by proton pump inhibitors (PPI). They have completely substituted surgical procedures as total gastrectomy and vagotomy. They are superior to all available histamin-H_2-blockers. For all currently available PPIs a comparable therapeutic efficacy and safety has been demonstrated. However, the exact dosage necessary for symptomatic control and for inhibition of gastric acid secretion has to be determined for every individual patient. The dosage can range from normal and 3–4 times elevated dosages. One should start with the regular PPI dose bid (2×20 mg Omeprazole; 2×30 mg Lanzoprazole; 2×40 mg Pantoprazole; 2×40 mg Rabeprazole etc.) and increase the dosage until patients are

symptom-free and BAO decreased to less then 5 mEq/hr in patients with intact stomach. BAO should be studied prior to the next scheduled dose. If BAO is around 0 mEq/hr PPI dosage should be reduced if desired.

There is no place for long-acting somatostatin analogs which have to be administered subcutaneously and which do not control acid secretion as reliably as PPIs.

In case of a sporadic non-metastasized gastrinoma, surgical removal should always be desired [31]. In patients with a gastrinoma as part of MEN-1 syndrome available data do not indicate whether removal of the primary has an survival advantage for the patient. In addition it is difficult to define which of several pancreatic tumors is the tumor responsible for the Zollinger-Ellison syndrome. Therefore, laparotomy in Zollinger-Ellison syndrome and MEN-1 syndrome is a controversial issue.

For management of metastatic disease see under "General aspect of management".

Glucagonoma

Definition, Epidemiology and Prognosis

Glucagonomas are endocrine pancreatic tumors that contain predominantly glucagon. However, only some of these tumors secrete excessive amounts of glucagon into the circulation and cause the glucagonoma syndrome. The latter tumors are malignant, sized 5 cm in diameter and even larger, frequently solitary but they can spread to the liver, lymphnodes, bone and other sites. Most glucagonomas, especially small tumors found in most patients with MEN-1 syndrome are non-functioning and not associated with a clinical syndrome. Their malignant potential is small.

All glucagonomas whether functionally inactive or active encompass less then 1% of all endocrine tumors of the GEP system. Whereas functionally inactive small and benign glucagonomas are found not only in patients with MEN-1 syndrome but even by chance at autopsy the glucagonoma syndrome is rare if compared with insulinoma and gastrinoma.

The prognosis of small functionally inactive glucagonomas is likely favourable whereas in the malignant variant as cause of the glucagonoma syndrome prognosis depends on the aggressiveness of tumor growth and the response to antiproliferative measures.

Pathophysiology

Elevated glucagon levels have metabolic consequences which explain some but not all clinical features in patients with glucagonoma syndrome. Weight loss results from the catabolic effects of glucagon and from a recently described anorectic substance found in animals with transplantable glucagonoma. Glucagon stimulates glycogenolysis and gluconeogenesis leading to impaired glucose tolerance and diabetes mellitus as found in most Patients with glucagonoma syndrome. Since exogenous administration of glucagon decreases erythropoiesis in animals anemia which is frequently observed in these patients can be attributed to glucagon hypersecretion. However, the role of hyperglucagonemia in the pathogenesis of thromboembolic complications including pulmonary embolism is poorly understood. The necrolytic migratory erythema (Fig. 10) is believed to be related to hypoaminoacidemia present in most but not all patients and not the consequence of hyperglucagonemia. Skin lesions disappear despite high glucagon levels if plasma amino acid levels normalize. They are similar to those observed in patients with zink deficiency and zink suplementation has been shown to improve skin lesions in some patients as well. According to the authors experience skin lesions can disappear after resection of the primary without normalization of hyperglycagonemia and hypoaminoacidemia due to metastases to the liver suggesting a still undetected substance released by the tumor as cause of the necrolytic migratory erythema.

Symptoms

Main symptoms in patients with glucagonoma syndrome are skin lesions, diabetes mellitus, weight loss and anorexia present in up to 90% of patients with glucagonoma syndrome. Less frequently normochromic and normocytic anemia, venous thrombosis and pulmonary embolism occur. Diarrhea and steathorhea are present in 14% of patients. Their etiology is unclear. Rare clinical features include, in addition, psychiatric disturbances including depression and complex neurologic symptoms with dementia and optic atrophy, possible related to protein hypercatabolism that can recover after treatment with long-acting somatostatin analogs.

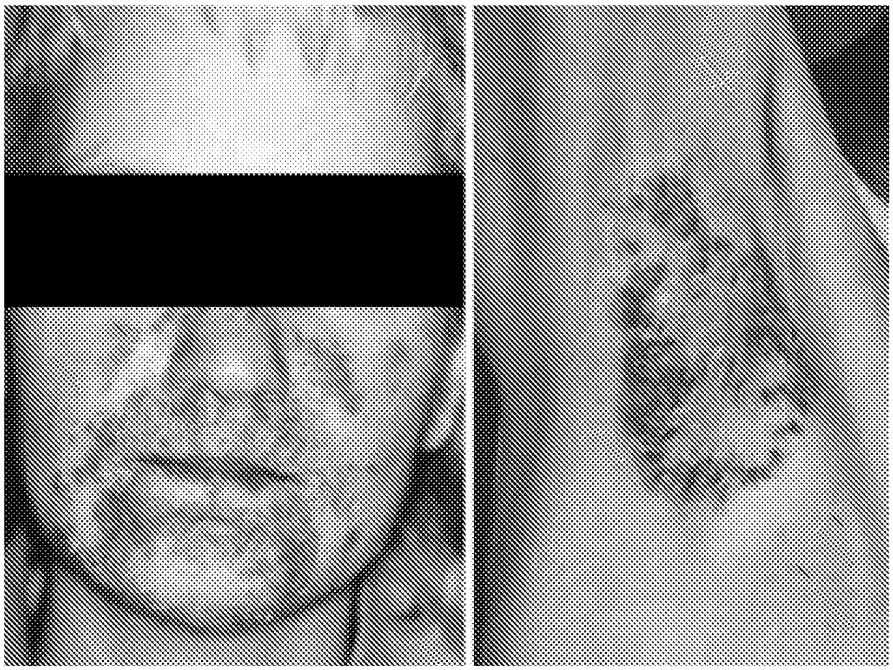


Figure 10
Necrolytic migratory erythema in two patients with glucagonoma syndrome

The necrolytic migratory erythema is one of the most impressive clinical symptoms in patients with glucagonoma syndrome. Skin lesions involve the lower abdomen, groin perineum, face, armpits (Fig. 10) and distal extremities. They start with an erythematous lesion which becomes papular. The erythema spreads peripherally and becomes raised with superficial central blistering. Lesions begin to heal in the center with a raised erythematous ring. Healing is followed by hyperpigmentation. The sequence of skin manifestations take 1–2 weeks while new lesions appear elsewhere. Skin lesions frequently precede the diagnosis of the syndrome for many years and are misdiagnosed as acrodermatitis enteropathica, psoriasis, contact dermatitis and other skin disorders.

Diagnosis

Diagnosis is based on the presence of a mostly large pancreatic tumor with and without metastases to the liver or elsewhere, clinical symptoms described in detail in "Symptoms" and the demonstration of high plasma glucagon levels. Total plasma aminoacids should be determined and support the diagnosis if they are markedly decreased. In most patients the pancreatic tumor can be visualized by conventional US. Fine-needle biopsy proves the endocrine nature of the tumor. Somatostatin receptor szintigraphy, CT and MRT imaging are helpful in evaluating tumor burden outside the pancreas [32].

Treatment

Surgical removal of the primary and of resectable liver metastases should always be considered because tumor debulking has been shown to have a favourable effect on clinical symptoms including skin lesions.

Medically, long-acting somatostatin analogs have been shown to be currently the medical principle of choice in the control of skin lesions, to improve symptoms as weight loss, anemia and diarrhea. Most studies recommend 200–600 μg per day or 10–20 mg octreotide-LAR every 28 days. Unfortunately, long-acting somatostatin analogs are not effective in up to 50% of patients. In these individuals debulking operation, embolization of liver metastases and chemotherapy with streptozotocin combinations or dacarbazine should be considered. For details see "General aspect of management".

Verner-Morrison Syndrome (VIPoma)

Epidemiology and Prognosis

An endocrine tumor mostly located within the pancreas and responsible for a syndrome characterized by extreme watery diarrhea, hypochlohydria and hypokalemia was first discribed by Priest and Alexander in 1957 and by Verner and Morrison in 1958. Alternative acronyms are "Verner-Morrison syndrome", "VIPoma" (due to the hormone vasoactive intestinal polypeptide responsible for the diarrhea), "WDHH (watery diarrhea, hypokalemia, hypochlorhydria) syndrome" and "Pancreatic Cholera". In adults VIPomas are mostly located in the pancreas and rarely outside the pancreas in the retroperitoneum, liver, small intestine and bronchial system. In children, a ganglioneuroma or an ganglioneuroblastoma can cause a VIPoma syndrome. Most VIPomas are malignant and have metastasized to the liver at diagnosis. VIPomas are as glucagonomas, somatostatinomas and endocrine pancreatic tumors with ectopic hormone production very rare. Prognosis of this malignant tumor was prior to the availability of long-acting somatostatin analogues unfavourable and patients died from the consequences of excessive watery diarrhea as dehydratation and pulmonary embolism. Presently, prognosis depends on the aggressiveness of tumor growth which differs between VIPomas and their responsiveness to chemotherapy.

Pathophysiology

Although VIPomas produce frequently several hormones as Peptide Histidine-Methionine-28 (PHM-27), secretin, pancreatic polypeptide and prostaglandines, vasoactive intestinal polypeptide (VIP) which is elevated in all patients with Verner-Morrison syndrome is the cause of watery diarrhea and electrolyte disturbances. VIP induces massive small intestinal net secretion of water and ions, especially potassium with an inconsistent effect on water absorption in the colon. If VIP is infused intravenously in healthy individuals in amounts reflecting circulating VIP-levels in patients with Verner-Morrison syndrome, subjects develop massive secretory diarrhea, hypokalemia and hypochloremic metabolic acidosis. VIP reduces water and sodium absorption from the colon and induces potassium secretion, thus explaining hypokalemia which may induce secondary hypoaldosteronism. In addition, VIP inhibits gastric acid secretion. Fluid secretion from the small intestine is attributed to activation of adenylate cyclase and cyclic adenosine monophosphate in intestinal cells. Some patients develop hypercalcemia which is attributed to the stimulation of bone osteolytic activity by VIP.

Symptoms

Patients with Verner-Morrison syndrome present with excessive watery diarrhea up to 5–10 L per day. Chronic diarrhea is a constant feature in 50% of patients but may be intermittent in others. Diarrhea decreases only slightly during fasting or exclusive parenteral nutrition proving its secretory nature. Untreated diarrhea leads to dehydration, weight loss, hypkalemia and hypomagnesemia resulting in paresthesia, muscle weakness and cardiac arrhythmia. Before the availability of long-acting somatostatin analogs patients died from the consequences of dehydration and electrolyte loss as cardiac arrhythmia and pulmonary embolism. 25% of patients report flushing episodes.

Differential Diagnosis

Secretory diarrhea of other origin as diarrhea in patients with Zollinger-Ellison syndrome, carcinoid syndrome, medullary carcinoma of the thyroid is mostly less pronounced compared to diarrhea in VIPoma patients. A stool volume of less then 700 ml per day excludes a VIPoma and is suggestive for other causes of secretory diarrhea. The most important differential diagnosis is laxative abuse and Münchhausen's syndrome. Repeatedly normal VIP-levels should induce search for laxatives in stool and urine.

Diagnosis

The combination of excessive watery diarrhea, hypokalemia and elevated plasma VIP-levels together with a pancreatic mass visualized by conventional US or CT/MRT imaging are highly suggestive for a VIPoma. The endocrine nature of the tumor can be ascertained by fine needle biopsy.

Treatment

Surgical intervention should always be considered if the primary is resectable and liver metastases could be removed with tenable risk for the patients. Medical therapy and patient's prognosis have been dramatically changed through the currently available long-acting somatostatin analogs. They substituted a number of drugs tried earlier to control diarrhea as loperamide, prednisone, clonidine, phenothiazines, indomethacin, lithium and others. Octreotide is in most patients effective and doses of 200–600 µg per day in two to three single injections are recommended. If the short lasting formulations are effective, long-acting depot formulations as octreotide-LAR 20 mg every 28 days can be offered. Unfortunately, some patients escape from treatment with long-acting somatostatin analogs after an initial longer lasting response. The mechanisms behind tachyphylaxis and/or desensitisation are not completely understood. Treatment should be terminated and started again after few weeks.

Somatostatinoma

Epidemilogy and Prognosis

Somatostatinoma is an endocrine tumor containing somatostatin as shown by immunihistology and arises either in the pancreas or in the duodenum where it is located close to the ampulla vateri. Most duodenal somatostatinomas are solitary and small (<1,5 cm) whereas pancreatic somatostatinomas tend to be larger. Malignancy rate in large tumors is 50–90% and metastases to liver, lymph nodes and bones have been described. According to table 9 duodenal somatostatinomas tend to metastasize if they are larger then 2 cm.

Approximately 90% of these tumors are functionally inactive and only 10% are cause of the somatostatinoma syndrome. Non-functional somatostatinomas located in the region of ampulla vateri are associated with von Recklingshausen' s disease. Small pancreatic somatostatinomas of the pancreas can be part of MEN-1 syndrome. Prognosis of somatostatinomas seems to be favourable in small, non-metastasized tumors. In larger tumors it depends on the aggressiveness of tumor growth.

Pathophysiology

Somatostatin inhibits the release of gastrointestinal hormones as CCK and gastrin, inhibits basal and simulated gastric acid secretion and stimulated pancreatic secretion and inhibits the absorption of food constituents from the intestine. Through inhibition of CCK release somatostatin inhibits gallbladder emptying. Inhibition of pancreatic secretion is believed to cause diarrhea and steatorrhea in the somatostatinoma syndrome. Since somatostatin inhibits insulin release impaired glucose tolerance is a frequent finding in patients with somatostatinoma syndrome.

Symptoms

Leading symptoms in patients with somatostatinoma syndrome are cholecystolithiasis, diabetes mellitus, diarrhea and steatorrhea, hypochlorhydria and as a consequence of a tumor situated close to the ampulla vateri obstructive jaundice. However, the reported symptoms are rarely present in all patients with somatostatinoma syndrome. Other symptoms are epigastric pain, weight loss and nausea.

Since symptoms in patients with somatostatinoma syndrome can arise although in other conditions as cholecystolithiasis, diabetes mellitus and diarrhea and steatorrhea some authors question the existence of this entity.

Treatment

Medical treatment should be directed to correct symptoms associated with cholecystolthiasis, diabetes mellitus and diarrhea. Few observations suggest that diarrhea and diabetes mellitus can improve by exogenous somatostatin. But this suggestion is clearly experimental. Surgery should considered to be reduce tumor burden.

Tumors with Ectopic Hormone Production

Epidemiology, Classification and Prognosis

Most benign and malignant endocrine GEP tumors produce more then one hormone within the same tumor. Insulinomas contain next to insulin frequently somato-

statin-producing and glucagon-producing cells, gastrinomas additional pancreatic-polypeptide-, insulin- and somatostatin-producing cells (see Fig. 2). As a rule the additional hormones are not released into the circulation and do not produce hormone-mediated symptoms. In patients with MEN-1 syndrome, however, two clinical syndromes from two independent pancreatic or one pancreatic and one duodenal endocrine tumor can arise, mostly the combination of a gastrin-producing tumor situated in the duodenal bulb with the clinical symptoms of Zollinger-Ellison syndrome and a pancreatic functionally active insulinoma. In the latter condition additional endocrine pancreatic tumors exist, mostly glucagonomas and somatostatinomas which are functionally inactive.

Apart from tumors producing multiple hormones regularly synthetized in the normal islets of Langerhans of adults (insulin, glucagon, pancreatic polypeptide, somatostatin) or during fetal life (gastrin) (eutopic hormone production) endocrine pancreatic tumors can produce hormones uncommon for the endocrine pancreas (ectopic hormone production) as growth hormone-releasing factor (GRF), ACTH or corticotropin-releasing factor (CRF) and parathyroid hormone-related peptide (PTH-RP) or an unknown hypercalcemic substence mimicking the action of PTH. These tumors are mostly malignant and very rare. In addition, few patients with neurotensin-producing tumors have been described. Tumor prognosis depends on the aggressiveness of tumor growth and the success of antiproliferative measures.

Clinical Symptoms, Pathophysiology and Diagnosis

GRFoma. The association of acromegaly with endocrine bronchial, intestinal and pancreatic tumors is rare and approximately 150 patients with this syndrome have been described. Pancreatic GRFomas are mostly large but multiple GRFomas have also been reported. They are part of MEN-1 syndrome. Different from pituitary adenoma GRFomas arise 3 times more frequent in females then in males. They can be associated with other endocrine syndromes as gastrinoma, insulinoma, Cushing's syndrome and pheochromocytoma. In 30–40% metastatic disease which spreads mostly to the liver is present.

Clinically, patients present with characteristic signs indistinguishable from pituitary derived acromegaly with elevated GH and IGF-1 levels. If GRFomas are associated with other endocrine functionally active tumors, symptoms of acromegaly could be blurred by hypoglycemia resulting from an insulinoma etc.

Treatment includes surgical resection of the primary and debulking procedures and, medically, the suppression GRF levels by long-acting somatostatin analogs.

ACTH-Producing Tumors. According to a recent report from the Mayo Clinic in 106 patients ectopic ACTH or CRF production results in 25% from bronchial carcinoids, 16% from malignant islet cell tumors, 16% from medullary thyroid carcinoma, 11% from small cell carcinoma of the lung, 7% from disseminated neuroendocrine tumors with unknown primary source, 5% from thymic carcinoids and 3% from pheochromocytoma. Whereas in MEN-1 syndrome Cushing's disease results from a pituitary adenoma ectopic ACTH-production in the case of an islet cell tumor has been found mostly in sporadic gastrinomas. In the latter condition the gastrinoma is metastatic and the prognosis poor.

In a recent prospective study Cushing's syndrome in patients with Zollinger-Ellison syndrome due to a solitary malignant gastrinoma was an independent predictor for poor survival. Patients with ectopic ACTH syndrome differ from those with pituitary adenoma because they present with muscle waisting and weight loss which is more frequently observed then the classic features of Cusing's syndrome.

Possibly, symptom differences in patients with ectopic and eutopic ACTH-production result from a different processing of pro-piomelanocortin with the release of high amounts of ACTH precursors and less intact ACTH in the circulation in patients with ectopic hormone production. However, symptoms may overlap those seen in pituitary dependent Cushing's disease. Ectopic ACTH-producing endocrine tumors are more resistant to chemotherapy and the severe hypercortisolism is responsible for a high rate of life-threatening complications.

Treatment is frequently difficult since only few tumors respond to long-acting somatostatin analogs. If ketoconazoles, animogluthetimide or mifepristone do not control hypercortisolism and curative or palliative resection of the primary tumor and its metastases is not possible, patients are likely to benefit from bilateral adrenalectomy. For additional antiproliferative measures see "general aspects of management" (below).

PTH-RP-Producing Tumors. Few endocrine pancreatic tumors present with hypercalcemia and secret parathyroid hormone-related peptide (PTH-RP) or not identified hypercalcemic substances mimicking the action PTH. According to a recent review of 19 patients common features were hypercalcemia, normal or low PTH levels associated with extremely vascular, large and usually malignant tumors (17 of 18) displaing positive stains for PTH-RP. Some patients respond favourably to long-acting somatostatin analogs and to streptozotocin combinations if tumor resection is not possible.

Nonfunctioning Endocrine Pancreatic Tumors

Epidemiology, Classification and Prognosis

Nonfunctioning endocrine pancreatic tumors can be subdivided into benign and small tumors as part of the MEN-1 syndrome and large, malignant tumors which mostly spread into lymph nodes, liver, bones, and elsewhere. The latter can or cannot secret hormonal products into the circulation. Pancreatic polypeptide or neurotensin are released by some tumors but do not produce a clinical syndrome. Other functionally inactive tumors do not secret any products with hormonal activity. However, they release chromogranin A, a constituent of the secretory machinery of endocrine cells. Exact incidence rates for malignant non-functioning tumors are missing but according to own experiences they are at least as frequent as all functionally active endocrine pancreatic tumor together. By histology and immunohistology functioning PETs cannot be distinguished from nonfunctioning tumors.

Clinically, tumors and their metastases are either found incidentally at routine abdominal check-up, by the patient itself who realizes the presence of an upper abdominal mass or by obstructive jaundice due to a pancreatic head tumor. Most patients report no or only little upper abdominal discomfort and few present with more severe abdominal pain and weight loss. Frequently, tumors are misdiagnosed as endocrine pancreatic carcinomas but the unrestricted life quality of patients and hypervascular lesions identified by imaging procedures lead the correct diagnosis. Other patients present during routine US investigations with cystic liver lesions misdiagnosed as benign liver cysts. Prognosis of patients with non-functioning malignant tumors depends on the aggressiveness of tumor growth which can vary considerably from exploding tumor growth to long intervals of stable disease even in the absence of any treatment.

Diagnosis

The correct diagnosis requires histology which can mostly be obtained by ultrasound or CT-guided fine-needle biopsy. If the endocrine nature of the primary tumor or of its metastases is ascertained, OctreoScan should be performed to identify tumor load and the presence of distant metastases as in bones. If the primary is not identified within the pancreas upper and lower endoscopy and careful CT/MRT examinations of the chest should be performed to localize the primary within the fore-, mid- and hindgut (Fig. 6d). Because tumors do not produce a hormone-mediated syndrome, expensive hormone analyses are not helpful and should be avoided. However, chromogranine A should be estimated which is elevated in most metastatic tumors and serves as tumor marker. Some tumors produce, in addition, pancreatic polypeptide which serves as tumor marker as well.

Treatment

Treatment should follow the principles of other endocrine pancreatic malignancies. If resectable, non-metastatic primaries should be removed either by Whipple's procedure or partial pancreatectomy. In metastatic diseases several antiproliferative measures are available which are summarized under "general aspect of management".

Endocrine Tumors of the Stomach and Gut

Gastric Carcinoids

Epidemiology, Classification. Endocrine tumors of the stomach are nonfunctioning. As shown in table 8 there are 4 types of tumors:

- ECL-cell carcinoids;
- EC-cell carcinoids;
- Gastrin-cell tumors;
- poorly differentiated or small cell endocrine carcinomas [9, 10].

With the exception of the latter tumor most endocrine tumors of the stomach are well differentiated. Gastric carcinoids have been reported to occur with an inci-

dence of 0,002–0,1 per 100.000 population per year and account for 11–41% of all gastrointestinal carcinoids from the esophagus to the rectum. The most frequent endocrine tumors of the stomach are gastric ECL-cell carcinoids representing 74% of gastric endocrine tumors and occuring more frequently in females. The mean age of detection is 63 years. Small cell undifferentiated carcinomas represent 6% of endocrine tumors of the stomach. EC-cell and Gastrin-cell carcinoids are extremely rare.

ECL-Cell Carcinoids. *Clinical and histopathological aspects and prognosis:* ECL-cell carcinoids can be subdivided into three entities:

- type I ECL-cell carcinoids are associated with type A-gastritis (autoimmune chronic atrophic gastritis) present in the oxyntic and fundic part of the gastric mucosa. Due to the loss of parietal cells type I ECL-cell carcinoids are always associated with achlorhydria and – as a consequence of achlorhydria – with massive hypergastrinemia of antral origin achieving levels comparable to that observed in Zollinger-Ellison syndrome. Carcinoids in type A-gastritis are usually small and multiple.
- type II ECL-cell carcinoids arise in patients with hypergastrinemia due to a Zollinger-Ellison syndrome, mostly as part of MEN-1 syndrome. In contrast to type I carcinoids oxyntic mucosa is hyperplastic due to the trophic action of gastrin but without any atrophic changes.
- type III (sporadic) ECL-cell carcinoids are neither associated with type- A atrophic gastritis although gastritis is present nor with hypergastrinemia. They arise in areas without ECL-cell hyperplasia which is a prerequisite for the formation of type-I and type-II ECL-cell carcinoids. Clinically, type III tumors present with relatively large (1–3 cm) tumors that are usually aggressive with local invasion and the formation of metastases to adjacent lymphnodes and the liver. They are mostly non-functioning but can be the source of gastric hemorrhage and obstruction. In rare cases they can be associated with an atypical carcinoid syndrome with red long-lasting flushing episodes but without diarrhea. These tumors secrete histamine and 5-hydroxytryptophane.

ECL-cell carcinoids arise from ECL-cells that are physiologically present in the oxyntic and fundic mucosa and contain histamine which stimulates gastric acid secretion via specific receptors on the parietal cells. They can proliferate by two mechanisms: both chronic atrophic gastritis and the trophic action of hypergastrinemia lead to ECL-cell hyperplasia which can further proliferate to type I ECL-cell carcinoids. Histologically ECL-cell hyperplasia consists of linear, diffuse, micronodular and adenomatoid ECL-cell hyperplasia (Fig. 11). The next step in tumor formation is dysplasia with enlarging and fusing micronodules, microinvasion and newly formed stroma. Nodules greater than 0.5 mm and invading into submucosa are called carcinoids. Smaller and multiple

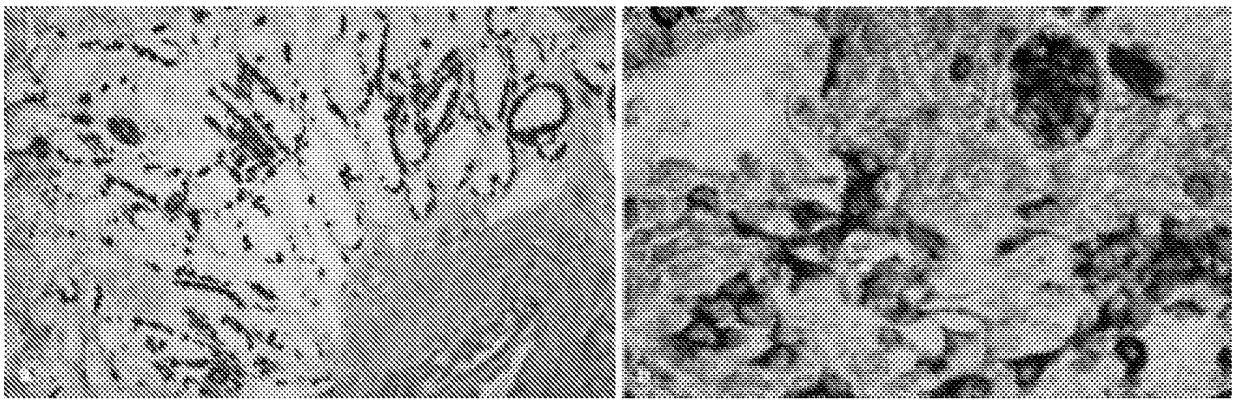


Figure 11
Histology of ECL-cell hyperplasia

nodules together with linear and diffuse ECL-cell hyperplasia a constituents of microcarcinoidosis. All these events are found in type A chronic atrophic gastritis and the ultimate step is the formation of type I carcinoids.

An identical spectrum of ECL-cell growth from ECL-cell hyperplasia to carcinoid formation is present in gastrinoma patients as part of MEN-1 syndrome. In contrast, sporadic gastrinoma patients develop ECL-cell hyperplasia but likely not ECL-cell carcinoids. This indicates the importance of a genetic trait (MEN-1) essential for the formation of type II carcinoids or the presence of severe atrophic gastritis as prerequisit for type I carcinoid formation through the trophic action of hypergastrinemia present in both.

Prognosis of type I and II ECL-cell carcinoids is excellent and favourable prognostic factors are: tumors confined to mucosa and submucosa, size <1 cm, low mitotic activity (Ki-67) and lack of angioinvasiveness. In contrast, angioinvasion, a size >1 cm, invasion of muscularis propria and beyond and high mitotic activity indicate a more aggressive behaviour. Metastatic spread into lymph nodes and to the liver is rare (5% rsp. 2.5% in type-I carcinoids) and slightly greater in type-II carcinoids.

Treatment: ECL-cell carcinoids (type I) in type-A chronic atrophic gastritis are mostly small and should be endoscopically excised. Yearly controls are recommended for further surveillance. If carcinoids are larger than 1 cm endoscopic US should estimate whether or not invasion of muscularis propria is present. In case of invasion local surgical excision is recommended. No agreement exists between experts whether or not antrectomy should be offered to remove the gastrin source as the trophic principle responsible for carcinoid formation.

Type II ECL-cell carcinoids should be handled as type I tumors but patients' prognosis depends mostly on the aggressiveness of underlying Zollinger-Ellison and MEN-I syndrome.

Type III ECL-cell tumors (sporadic carcinoids) should be removed according to the principles for non-endocrine gastric adenocarcinomas. No proven strategy exists if metastatic spread occurred. Tumors are mostly insensitive to available chemotherapeutic strategies.

EC-Cell Carcinoid. EC-cell carcinoids are very rare and always malignant. They present with rapid growth and are functionally inactive.

Gastrin-Cell Tumor. These very rare tumors are mostly well differentiated and small. They are found incidentally at endoscopy as small muscosal/submucosal nodules. They can and can't present with Zollinger-Ellison syndrome.

Small Cell Carcinoma (Poorly Differentiated Endocrine Neoplasm). These very rare tumors display exploding tumor growth similar to small cell carcinomas of the lung and of other origin.

Endocrine Tumors of the Duodenum and Proximal Jejunum

Epidemiology, Classification and Prognosis

Duodenal carcinoids account to 22% of all gastrointestinal carcinoids (see Table 9). In contrast, jejunal carcinoids are very rare. Most endocrine tumors of the duodenum are gastrinomas which in 7–21% are part of MEN-1 syndrome. Not all gastrinomas are cause for a Zollinger-Ellison syndrome and present as non-functional tumors. Gastrinomas can arise at multiple sites (15%) and are mostly small (<1 cm). Non-functioning gastrinomas have a more favourable prognosis as tumors associated with Zollinger-Ellison syndrome that have a higher incidence of metastases. However, compared to pancreatic gastrinomas duodenal gastrinomas have significantly less frequent metastases to the liver (5% versus 52%). Ten years survival of patients with duodenal gastrinoma is significantly better then those of pancreatic origin (59% versus 9%).

Somatostatinomas account for 21% of endocrine tumors in the upper small intestine and are located at, or very close to, the ampulla of vater. They are larger as gastrinomas (>2 cm) and can cause obstructive jaundice, pancreatitis and intestinal obstruction. Histologically, tumors contain characteristic concentrically laminated psammoma bodies. They are mostly malignant, with local invasion into surrounding tissues (sphincter of Oddi, head of the pancreas) and spread into paraduodenal lymph nodes and to the liver. Duodenal somatostatinomas are mostly non-functioning and genetically associated with neurofibromatosis von Recklinghausen.

Gangliocytic paragangliomas are very rare events characterized by an admixture of three cell types: spindle cells, epithelial cells and ganglionic cells. The spindle cells are neural in nature. They are usually benign but can be larger then 2 cm in size.

Treatment

Duodenal gastrinomas should be surgically removed either by local excision or Whipple's procedure. In cased of metastatic spread surgery should be avoided. The place of radical surgery in patients with duodenal and/or pancreatic endocrine tumors as part of MEN-1 syndrome is controversial even between experienced surgeons. Somatostatinomas and gangliocytic paragangliomas should be resected if possible. In case of metastatic spread, see "General aspect of management" under "Control of tumor growth in malignant and metastatic tumors".

Endocrine Tumors of the Distal Small Intestine (Jejunum, Ileum)

Epidemiology, Classification and Prognosis

The incidence of carcinoids arising in the distal small bowel has been reported to be 0,28–0,89 per 100.000 population and year. Most gastrointestinal carcinoids are localized in this area (see Table 10). Carcinoids of this site have been observed in equal proportion in males and females with and age peak in the 6th and 7th decade. Histologically, tumors are EC (enterochromaffine)- cell carcinoids containing serotonin. A minority of carcinoids consists of glucagon-like peptides- or pancreatic polypeptide- and peptide YY producing cells. EC-cell carcinoids can be multiple and are in 15% associated with other malignancies as gastrointestinal adenocarcinoma, breast cancer and others. The majority of tumors is located in the distal ileum close to the ileocecal valve. Only 5–7% of EC-cell carcinoids are functionally active and cause of the carcinoid syndrome. The primary is mostly situated in the ileum. Despite most EC-cell carcinoids are functionally inactive patients present with intermittent lower abdominal cramps due to incomplete intestinal obstruction and desmoplastic reaction of the mesenterium. Occasionally, small ileal carcinoids are incidentally detected during routine colonoscopy.

Prognosis of carcinoids arising in the distal jejunum and ileum is generally unfavourable if compared to that for duodenal, gastric (ECL-cell carcinoids) and rectal carcinoids since endocrine tumors arising in the distal small intestine frequently lead to metastases. 10 year survival is approximately 43% and more favourable if the primary tumor is removed and liver metastases absent. Therefore, patients with even small ileal carcinoids found incidentally during routine colonoscopy should be recommended right-sided hemicolectomy since metastatic spread into regional lymph nodes occurs early and is independent on the size of the primary.

Pathophysiology and Symptoms

Non-Functioning EC-Cell Tumors. Even tumors less than 1 cm, confined to the mucosa and submucosa discovered incidentally during colonoscopy and intubation of the terminal ileum are frequently malignant with metastatic spread to regional lymph nodes (see Table 10). They present very rarely with gastrointestinal bleeding.

Tumors measuring >1 cm in diameter are mostly malignant with metastases to the regional lymph nodes and later to the liver, bone and elsewhere. Patients frequently report on intermittent abdominal discomfort existing sometimes for years. Later, complaints worsen and diagnosis will be made if intermittent intestinal obstruction occurs. The latter is assigned to the role of growth factors secreted by the tumor cell as PDGF, TGFα, bFGF and others which stimulate neighburing fibroblasts which lead to new stroma formation and later to the typical desmoplastic reaction of the mesenterium. The consequence is angulation of the bowel with subsequent obstruction. Since also vasculature is affected by the stroma formation, ischemia develops in the involved segment and surgeons find the respective bowel dark-blue. Gastrointestinal bleeding is also in larger EC-cell tumors rare. Desmoplastic reaction can easily be detected by high-resolution MRT.

Functioning EC-Cell Tumors and Carcinoid Syndrome. EC-cell tumors responsible for the carcinoid syndrome do not different histologically from non-functioning tumors. However, carcinoid syndrome apperars only in patients with metastases to the liver. The symptoms are the consequence of unrestrained release of hormones and other mediators [11]. Flushing is present in up to 94% of patients and is characterized by red to purple discoloration of face, neck and upper chest. Duration of episodes is mostly short (few minutes) but can continue for hours. Frequently, patients themselves do not notice flushing and their attention will be drawn by others. Occasionally, flushing is associated with unpleasant sensations of lacrimation, warmth, facial and conjunctival edema. The hormonal mediator of flushing has not been identified with certainty. The elevation of blood serotonin during flush-

ing does not prove its causal relationship. In addition, serotonin antagonist as methysergide and odansetone have only little effect. A more likely mediator are tachykinins as substance P and related peptides as neurokinins.

Serotonin is synthesized and secreted from carcinoid tumor cells. It arises from the amino acid tryptophan which is hydroxylated to 5-hydroxytryptophan and decarboxilated to 5-hydroxytryptamin (serotonin). Through several enzymes as monoamine oxidase and aldehyde dehydrogenase sterotonin is converted to 5-hydroxyindolacetic acid (5-HIAA) and excreted into urine.

Diarrhea with watery bowel movements is a common symptom and present in up to 85% of patients. It is possibly related to serotonin production but other mediators as prostaglandins have been suggested to contribute to diarrhea as well. Diarrhea responds better to serotonin antagonists then flushing.

The most important consequence of long-standing carcinoid syndrome is carcinoid heart disease, present in 45–77% of patients. Heart disease threatens patients with carcinoid syndrome at least by the same extent then the tumor itself and patients can die from carcinoid heart disease despite tumor growth is well controlled. Carcinoid heart disease involves mostly tricuspidic valve, pulmonary valve and rarely mitral and aortic valve. The mediator responsible for carcinoid heart disease is unknown. Several studies suggest the concept that serotonin is involved by reporting higher serotinin levels in plasma and 5-HIAA levels in urine in patients with compared to those without heart involvement. However, anorectic factors as well are discussed to be involved. Typically, the affected valves are thickened, shortened and retracted by fibrous transformation leading to tricuspid and pulmonary valve insufficiency which can be easly visualized by two-dimensional echocardiography.

Additional symptoms in carcinoid syndrome encompass bronchoconstriction, Pellegra-like skin changes and abdominal pain. The latter can result from diffuse liver enlargement or desmoplastic mesenterial reaction (see Fig. 7b). Pellegra-like skin reactions are the consequence of niacin deficiency which is due to the formation of serotonin from 5-hydroxytryptophan.

Carcinoid crisis is a rare exacerbation in patients with carcinoid syndrome mostly arising during anesthesia or surgery if patients are not under continuous somatostatin treatment. Flushing, hyper- and hypotension, severe bronchospasm and cardiac arrhythmias are the main features and subsequent death is not uncommon.

Diagnosis

In non-functional tumors, incidental detection of an ileal carcinoid during colonoscopy and in case of intestinal obstruction surgery uncovers the responsible carcinoid, which, in the latter situation, has mostly metastasized into regional lymphnodes. Non-functioning liver metastases from an ileal carcinoid are frequently detected by US during routine check-up. Most sensitive measures to estimate total tumor burden are Octreo Scan, CT and MRT. Biochemically, chromogranin A serves as most reliable tumor marker. Diagnosis of carcinoid syndrome is based on the demonstration of high 5-HIAA levels in the urine and elevated plasma serotonin and chromogranin A levels in the presence of liver metastases from an neuroendocrine tumor. CT, MRT and OctreoScan define total tumor burden and mostly the site of the primary. All patients deserve careful cardiologic diagnosis with two-dimensional echocardiography to prove or disprove cardiac involvement.

Differential Diagnosis

Some patients with non-functional ileal carcinoids develop diarrhea after right-sided hemicolectomy. They are later misdiagnosed as having carcinoid syndrome and somatostatin treatment will be started to prevent diarrhea. However, somatostatin treatment can even worsen diarrhea which is not hormone mediated but the consequence of bile acid loss into colon or bacterial overgrowth.

Treatment

Surgery is the treatment of choice to remove the primary and its local metastases reponsible for the desmoplastic reaction and subsequent abdominal pain. Surgery should further be considered in case of few and large liver metastases. In addition, right-sided hemicolectomy should be offered to patients with small ileal carcinoids detected incidentally during routine colonoscopy. Symptom control in patients with carcinoid syndrome was difficult before the availability of long-acting somatostatin analogs which are currently the principle or first choice to control flushing and diarrhea. They are indispensable in the treatment and prevention of bronchial obstruction and carcinoid crisis. They have, therefore, to be administered perioperatively and during laparotomy.

Their effects on carcinoid heart disease and its progression have not yet been demonstrated. Octreotide should be started at doses of 2–3×50 µg, but higher doses up to 3×500 µg may be necessary in some patients. If effective, LAR formulations should be offered (Octreotide-LAR 20 mg every 28 days). Long-acting somatostatin analogs are safe. One of the most frequent side effects is the formation of gall stones since somatostatin inhibits the release of CCK and by this mechanism gallbladder contraction. In few patients, somatostatin analogs induce diarrhea which can be as severe as diarrhea restulting from carcinoid syndrome. In these patients and those with tachyphylaxis to long-acting somatostatin analogs diarrhea should be controlled by serotonin receptor blockade. 5-HT_1 (methysergide)-, 5-HT_{1+2} (cyproheptadine)-, 5-HT_2 (ketanserin)- and 5-HT_3 (ondansetron)-receptor antagonists have been recommended. But they do not completely stop diarrhea. To substitute niacin deficiency, patients should be offered niacin orally. Cardiac failure should be treated by conventional pharmacologic therapy.

Endocrine Tumors of the Appendix

Incidence rates of appendiceal carcinoids account for 0.075 new cases per 100.000 population and year. 19% of all gastrointestinal carcinoids have been reported to be localized in the appendix. Mean age of presentation is 32–43 years and females are more frequently affected.

Appendiceal carcinoids are mostly detected incidentally during appendectomy or described by pathologists in the resected appendix. They are mostly situated in the distal third of the appendix. By obstructing the lumen they can produce appendicitis. Clinicopathologic staging is summarized in Table 11. Most appendiceal carcinoids are well-differentiated tumors consisting of EC-cells but some contain glucagon-like peptides and PP/PYY-producing cells.

Most patients with appendiceal carcinoids have a favourable prognosis. Several well conducted studies demonstrate that carcinoids of less then 2 cm size confined to the appendiceal wall and not angioinvasive are completely cured by appendectomy. Invasion of the mesoappendix, a size >2 cm and angioinvasion carry an uncertain malignant potential and right sided hemicoletomy should, therefore, be performed as in patients with metastatic spread into regional lymph nodes. Also location of a carcinoid at the base of the appendix with involvement of the cecum has a more unfavourable prognosis requiring right sided hemicolectomy.

Goblet cell carcinoids are more aggressive tumors and should be treated by right sided hemicolectomy since these carcinoids frequently invade the wall of the appendix.

Endocrine Tumors of the Colon and Rectum

With an incidence of up to 0.21 cases per 100.000 population per year hindgut carcinoids (left sided colon, rectosigmoid) are less frequent then midgut carcinoids (see Fig. 7f) (jejunum, ileum, appendix). They account for 20% of all gastrointestinal carcinoids. Within the hindgut most carcinoids are situated in the rectum (54%). Average age for colonic carcinoids is 66 years and for rectal carcinoids 58 years. Histologically, colonic carcinoids consist of EC-cells or L-cells (glucagon-like peptides and PP/PYY-producing carcinoids) and rectal carcinoids mainly of L-cells. Most colonic carcinoids are found in the right colon with an average size of 4,9 cm. Rectal carcinoids represent as submucosal nodules or yellow polypoid lesions situated mostly 4 cm proximal the dentate line. Mostly, they are less then 1 cm in diameter and only in 13% greater then 2 cm (see Table 10). Carcinoids of the colon/rectum are generally non-functioning and present with abdominal pain due to bowel obstruction, bleeding or are detected incidentally during screening colonoscopy or in case of carcinoids >2 cm with liver metastases. Very few colonic carcinoids are associated with a carcinoid syndrome. Few hindgut carcinoids are poorly differentiated and aggressively growing. Their prognosis is poor. Carcinoids >2 cm have a higher malignancy rate with metastases mostly to the liver.

Established malignancy criteria of rectal carcinoids are a size >2 cm, invasion of the muscularis propria, DNA aneuploidy and the presence of 2 mitosis and more per 10 high power microscopic fields (magnification: ×400). The prognosis is in general more favourable then the prognosis of patients with carcinoids situated in the jejunum and ileum.

General Aspect of Management

Surgery

Surgery and endoscopic resection is the only available curative treatment and should always be considered. Solitary tumors (insulinomas, gastrinomas, gastrointestinal carcinoids) should be resected by laparotomy or endoscopy. Surgical management in patients with metastatic spread is not very well defined but should be an important treatment module of a multidisciplinary approach. Single and few liver metastases are exepted examples for palliative surgery. Many patients with slowly growing metastatic GEP tumors whether functionally active as some malignant insulinoma or functionally inactive have a benefit from this approach. Not well established is the place of surgery in patients with multiple, non-functioning endocrine pancreatic tumor as part of MEN-1 syndrome.

Liver transplantation should be considered in patients with resected primary tumorbut metastases only to the liver as shown by Octreoscan and other sensitive imaging methods and unresponsive to established medical and interventional treatment. However, reoccurrence of metastases in the transplanted liver has been observed as well as newly formed extra-hepatic metastases. Nevertheless, some patients have a significant benefitfrom liver transplantation with prolonged survival and quality of life.

Medical Treatment of Symptoms

The respective pharmaceutical principles have been in detail discussed elsewhere in this chapter.

Control of Tumor Growth in Malignant and Metastatic Tumors

Non-surgical control of tumor growth includes biotherapy with long-acting somatostatin analogues and α-interferon, systemic chemotherapy, ablative methods including chemoembolizaton, thermo- and alcohol treatment of liver lesions and tumor targeted radiotherapy.

Every therapeutic modality should recognize that well-differentiated endocrine GEP tumors are mostly slow growing and often exhibit phases of stable disease or such a slow growth that a significant increase in tumor size can only be substantiated with CT- or MRI scans performed in 6–12 months intervals. Therefore, non-surgical treatment options should not be considered in patients with stable disease and uncompromised life quality. Such patients should be offered regular control visits in 6 months intervals and treatment only offered in case of significant tumor growth (>20–25% increase in 3–6 months). Therefore, patients with newly-diagnosed metastatic disease from well-differentiated endocrine GEP-tumors and low mitotic activity should not be offered a specific treatment immediately.

Long Acting Somatostatin Analogs

Evidence for antiproliferative properties of somatostatin and its analogs derives from in vitro and in vivo studies. As discussed earlier, currently available somatostatin analogues bind preferentially to sst2 and sst5 receptors which mediate antimitogenic, antiproliferative and anti-apoptotic signals. Besides these receptor-dependent effects, somatostatin controls cell growth via receptor-independent effects. These include endocrine effects with inhibition of the release of circulating or paracrine tumor growth promoting factors, vascular antiangiogenetic effects and effects on the immune system.

Anecdotal reports of tumor regression in patients with metastatic endocrine tumors of the GI tract and of stable disease over a period of 4 years in two patients with carcinoid syndrome due to malignant metastatic neuroendocrine tumors of the lung are consistent with the above mentioned experimental data.

A retrospective report of the National Institute of Health on 96 patients with metastatic endocrine tumors showed a partial tumor response in 13%, stable disease in 63% and tumor progression in only 24%. However, partial tumor response was a very rare event in prospective studies and disease stabilization occurred in 36–70% of patients. However, even stable disease was short-lasting for a minimum of 2 months and a maximum of 60 months. In one study, patients were classified into those with rapidly-progressing tumors (increase >50% in 3 months) and slowly progressing tumors (increase >50% in 3 months). Inhibition of tumor growth occurred predominantly in slow-growing tumors [27]. All trials were uncontrolled. Regarding the unpredictable course of the disease and the moderate response to treatment for a relatively short period of time, it cannot be excluded that

the phases of stable disease and even partial response observed in a few patients reflect the natural course of the disease. Since long-term treatment with somatostatin analogs is expensive, placebo-controlled studies are now necessary to prove or to disprove the antiproliferative potency of long-acting somatostatin analogs in patients with metastatic endocrine GEP-tumors. Unresolved issues include the therapeutic dose, the equipotency of octreotide and lanreotide versus the longer acting release formulations and the treatment effect with regard to prolongation of live.

α-Interferon

Interferons affect tumor growth by blocking the cell cycle during the G_0-G_1 phase with prolongation of the S-phase. Experimental data suggest that α-interferon induce apoptosis and that tumor cells are replaced by fibrotic tissue. α-interferon, in addition, induces increased expression of class I antigens on the tumor cell surface which renders cells as targets for cytotoxic T-lymphocytes.

Several studies in metastatic endocrine GEP-tumors demonstrate both a symptomatic effect with improvement of flushing and diarrhea in patients with carcinoid syndrome, a concomitant decrease of biochemical markers and a stabilization of tumor growth in 20–40% and a reduction in tumor size in 12–20% of patients. As shown for long-acting somatostatin analogues, these effects are transient (6–20 months). Side effects of α-interferon treatment have a much greater impact on patients well-being and include flu-like symptoms, weight loss, fatigue, anemia, leukopenia, thrombocytopenia, autoimmune manifestations and psychiatric disturbances.

Combination Treatment with Octreotide and α-Interferon

In a prospective trial inhibition of tumor growth was observed in 67% of 21 patients with metastatic endocrine tumors of the GI-tract who were unresponsive to prior octreotide monotheraphy. Responders to combined treatment had a significant survival benefit. However, these data could not be confirmed by a prospective two arm study, comparing octreotide versus octreotide plus α-interferon in 105 patients performed by the authors of this chapter demonstrating the need for well-controlled and prospective trials in well-defined subgroups of patients with endocrine GEP-tumors.

Chemotherapy

Several chemotherapeutic agents have been used as single agents and as combinations in metastatic endocrine GEP tumors. The respective original data demonstate that chemotherapy is indicated only in patients with well-differentiated endocrine carcinomas of pancreatic origin and in rapidly growing undifferentiated and small cell endocrine carcinomas [8]. According to a 20 years old prospective study from the Eastern Cooperative Oncology Group (ECOG) which enrolled 125 patients with histologically proven unresectable islet-cell carcinomas streptozotocin plus doxorubicin was found superior to streptozotocin plus 5-fluouracil (5-FU) and to chlorozotocin. Tumor regression occurred in 69% of patients treated with streptozotocin plus doxorubicin and in 45% of patients treated with the combination of streptozotocin and 5-FU. Median duration of regression was 18 months for the doxorubicin combination and 14 months for the 5-FU combination. These beneficial effects influenced also survival. These favourable effects of tumor growth are contrasted by toxic reactions to treatment as nausea, vomiting, alopecia, hematologic and kidney toxicity. Heart failure has been observed in patients receiving the doxorubicin regimen and a cumulative dose of 400–500 mg/m² should, therefore, not be exceeded. In the institution of the authors patients are treated with the streptozotocin/doxorubicin combination. In case of response, treatment is changed to streptozotocin plus 5-FU if the limiting doxorubicin dose is reached. The dosages are summarized in table 16. Unfortunately it is impossible to identify patients responding to this treatment and patients not responding. In the latter situation one should try dacarbacine (150 mg/m²) as short infusion and repeat it every 28 days. At least 3 courses of treatment should be performed to prove or disprove success.

Importantly, streptozotocin combinations and dacarbacine are only effective in tumors of pancreatic origin. There is no established chemotherapy for malignant carcinoids of the stomach, small and large intestine. In patients with exploding undifferentiated tumors the fol-

lowing chemotherapeutic strategy should be offered: 130 mg/m² and day etoposide for 3 days plus 45 mg/m² and day cisplatin on days 2 and 3. Each agent is given by 24 hours infusion and cycles repeated every 6 weeks. These tumors may originate from the pancreas, stomach, small and large bowel and most patients with these rare undiffentiated tumors will respond at least for 2 to 3 cycles. Drug toxicity is a significant problem of these formulations requiring dosage reduction or cessation of treatment in some patients.

Ablative Measures

Ablative measures include transarterial chemoembolization and radio-frequency tissue or alcohol ablation. Transarterial chemoembolization [8] is based on the concept that arterial blood supply of metastases (via hepatic artery) from endocrine GEP tumors is more intense then that of other malignancies except primary hepatocellular carcinoma and that local ischemia induces cell necrosis. Unproven is the concept that the combination of desarterialization vial embolization and local cytotoxic chemotherapy increases response rates. Embolization is performed by selective injection of a mixture of iodinized oil plus doxorubicin followed by injection of gelatine sponge particles. Response rates with a decrease of symptoms in patients with carcinoid syndrome of 68–100%, decreases of hormone levels in 80% and a decrease in size of liver metastases in 37–100% of patients have been described by several institutions. Mean duration of response is approximately 24 months. In the own institution it has been shown that patients with a tumor burden >75% of the liver have not benefit from chemoembolization. Increased survival has been observed in patients with a tumor burden <50% and an accumulation of lipiodol in more then 50% of the tumor mass. Side effects include abdominal pain, nausea, vomiting and fever. Fatal complications are infection and sepsis and hepatic failure.

Radiofrequency treatment [33] is a novel advice to destroy liver metastases and can be performed percutaneously and intraoperatively using a cooled-tip needly applying 50 to 90 watts over 10–12 minutes under ultrasound control. This experimental procedure as well as ablation by injection of alcohol can be considered in case of few and small liver metastases.

Table 16
Chemotherapeutic protocols for well differentiated metastatic islet cell carcinoms

Streptozotocin plus 5-fluorouracil	500 mg/m² streptozotocin/day as intravenous injection for 5 consecutive days; *plus* 400 mg/m² 5-fluorouracil/day as intravenous injection for 5 consecutive days, repeated every 6 weeks
Streptozotocin plus doxorubcin	500 mg/m² streptozotocin/day as intravenous injection for 5 consecutive days; *plus* 50 mg/m² doxorubicin as intravenous injection on days 1 and 22; repeated every 6 weeks

Maximal dose of doxorubicin: 4500 mg/m². Reduced dosages of every drug in case of severe nausea, vomiting, stomatitis, diarrhea, leukopenia, thrombozytopenia. Reduced dosages of streptozotocin in case of elevated creatinine or proteinuria and discontinuation if abnormalities persist.

Radioligand Therapy

Tumor-targeted metabolic endo-radiotherapy using specific receptor ligands such as octreotide or lanreotide coupled to beta-emitting radionuclides is of special interest in endocrine malignancies since biotherapy is only effective in slow growing tumors and systemic chemotherapy in a subpopulation of patients with pancreatic tumors. Almost no treatment modality has been shown to affect bone metastases. Whereas radioiodinated somatostatin analogues usually show target-to-background ratios not high enough for therapeutic applications, which is mainly due to their lipophilicity and rapid intracellular degradability, radiometal-labelled analogues display excellent biodistribution properties. To bind radioisotopes such as ^{111}Indium, ^{90}Yttrium and ^{177}Lutetium tightly to somatostatin analogs, mono- and bifunctional chelators have been developed consisting of polyaminopolycarboxylic acids or their macrocyclic derivatives such as DTPA, TTHA, DFO, DOTA or TETA. To reach biofunctionality, aliphatic side chain, thiuronatobenzyl,-, acetamidobenzyl- or succinylbenzyl linkers connect the chelators with octreotide and related peptides. ^{111}In-labelled [DTPA]-octreotide has been shown to display an appropriate biodistribution profile in man.

Although generally regarded as mainly diagnostic, ^{111}-Indium emits Auger and conversion electrons, which display a tissue penetration of 0.02–10 µm and 200–500 µm respectively, and which can be used therapeutically. More therapeutic experience has been gained with ^{90}Yttrium, which is a classical β-particle emitter. To avoid dissociation of ^{90}Yttrium with a maximum path length of 9 mm from the chelated somatostatin analog, a stable [DOTA^0, Tyr^3]-octreotide complex has been developed. Replacement of phenylalanine at the 3-position of octreotide by tyrosine has been shown to even increase the affinity of this compound for the sst2 receptors. The effect of endo-radiotherapy depends on binding to the sst2 receptor and requires internalization of the radioligand. Therefore, a sufficient number of sst2 binding sites and rapid internalization are prerequisits for successful therapy. The principal efficacy of somatostatin receptor-mediated radiotherapy has been demonstrated in animal models. Recently, several studies including a limited number of patients with malignant endocrine tumors have been published, suggesting that radiotherapy with ^{111}In- and ^{90}-Y-labelled octreotide analogs is able to control symptoms in patients with functionally active endocrine tumors and to inhibit or even significantly decrease tumor load. Whether or not [^{177}Lu-DOTA, Typ^3]-Octreotate with a tissue penetration of 2 mm is superior to ^{111}Indium and ^{90}Yttrium labelled octreotide in the treatment of endocrine GEP tumors is currently under investigation.

How Should we Proceed in Patients with Metastatic Neuroendocrine GEP-Tumors?

Despite the availability of several antiproliferative strategies that can be offered to patients with metastatic disease, current recommendations how to start with non-surgical modalities are controversial and not supported by prospective and controlled studies. Therefore, therapeutic strategies for a single patient are based frequently on personal experiences of expert centers and vary, therefore, from center to center. To harmonize therapeutic ways of proceeding European experts in the field have founded the Eureopean Network for the Study of Endocrine GEP Tumors (ENET) to define the place of currently available and future diagnostic and therapeutic principles. According to the experiences of the authors of this chapter the following recommendations can be applied to patients with metastatic GEP tumors:

1. Surgery with curative resection of the primary in the absence of metastatic spread and tumor debulking in metastatic disease should be intended where ever possible.
2. Antiproliferative strategies should consider the growth characteristics and biology of a given tumor. Do not treat non-growing metastases which are stable by CT for 6 months and longer! It is questionable whether these patients have any benefit from anti-proliferative measures. Consider surgery or local ablative measures (radiofrequence ablation) in these patients.
3. In the case of moderately rapid progression chemotherapy should be offered in patients with tumors of pancreatic origin (streptozotocin combinations, dacarbacine). Chemotherapy should not be offered to patients with well-differentiated non-functional or functional tumors arising from the intestine (from stomach to rectum).
4. Offer chemotherapy (etoposid + cisplatin) in exploding tumors as small cell and undifferentiated neuroendocrine carcinomas.
5. Offer local irradiation in case of pain in patients with bone metastases since bone metastases do not respond to chemotherapy and biotherapy.
6. Offer octreotide to patients with well-differentiated slowly growing neuroendocrine tumors. In case of further growth add α-interferon.
7. Consider chemoembolization primarily in patients with liver metastases due to mid- and hindgut tumors since this group of patients does not respond to chemotherapy.
8. Consider radioligand therapy only within controlled and prospective studies since it is unsettled whether this modality should be offered to patients as first-line treatment or to patients unresponsive to other therapeutic alternatives.

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http://www.springer.com/978-3-540-43462-7

Gastrointestinal and Liver Tumors
Scheppach, W.; Bresalier, R.S.; Tytgat, G.N.J. (Eds.)
2004, XVIII, 334 p., Hardcover
ISBN: 978-3-540-43462-7

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

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/S.J.L./	1	2264405	RU	C2	2005-11-20	Navarro et al.	Abstract only	☐

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
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	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

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EXAMINER SIGNATURE

Examiner Signature	/Samira Jean-louis/	Date Considered	05/05/2014

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter W. Marks
	Art Unit	1627
	Examiner Name	S. J. Jean-Louis
	Attorney Docket Number	PAT034678-US-PCT

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Stephen Johnson/	Date (YYYY-MM-DD)	2013-09-16
Name/Print	Stephen Johnson	Registration Number	45916

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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PTO/SB/08a (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 2006/071966	07-06-2006	Brigham & Women's Hospital		☐
						☐
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						☐
						☐

Examiner Signature	/Samira Jean-louis/	Date Considered	05/05/2014

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner
None			

SEARCH NOTES		
Search Notes	Date	Examiner
Palm Inventor Name Search	2/10/2011	SJL
STN-see enclosed search history	2/10/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	2/11/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub, Derwent)-see enclosed search notes	10/5/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	5/4/2014	SJL
STN-see enclosed search history	5/4/2014	SJL

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claim 1. (Currently Amended) A method for treating pancreatic endocrine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of 40-O-(2-hydroxyethyl)-rapamycin as a monotherapy and wherein the tumors are advanced tumors after failure of cytotoxic chemotherapy.

Claims 2. (Currently Amended) A The method of claim 1, ~~for treating pancreatic neuroendocrine tumor, comprising administering to a human subject in need thereof a therapeutically effective amount~~ wherein the unit dose of of 40-O-(2-hydroxyethyl)-rapamycin is 10 mg/day.

Claim 3. (Currently amended) The method of claim ~~2~~ 1, wherein the ~~unit dose of 40-O-(2-hydroxyethyl)-rapamycin administered is from 0.1 mg to 15 mg~~ tumor is islet cell tumor.

Claims 4-12. Cancelled.

CASE PAT034678-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Marks, Peter Wayne et al.
INTERNATIONAL APPLICATION NO: PCT/EP06/068656
FILED: November 20, 2006
U.S. APPLICATION NO: 12/094173
35 USC §371 DATE: May 19, 2008
FOR: Neuroendocrine Tumor Treatment

Art Unit: 1627
Examiner: Jean-Louis, Samira J
Conf. No.: 9572

MS: Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Sir:

This Reply is submitted in response to the Office Action mailed May 9, 2014. Reconsideration of the present rejections and withdrawal of the present rejections are respectfully requested.

Amendments to the Claims are reflected in the listing of the claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

Remarks/Arguments

Rejection Under 35 USC 103(c)

Claims 1-3 remain rejected under pre-AIA 35 USC 103(a) as allegedly being unpatentable over WO97/47317, as evidenced by Arnold et al. Reconsideration and withdrawal of this rejection is respectfully requested.

The difference between the mentioned prior art and the subject matter of the present claims is the use of one specific mTOR inhibitor (e.g. everolimus) as monotherapy in the treatment of pancreatic neuroendocrine tumors.

Each tumors type, including pancreatic neuroendocrine tumors, have their own specific biology, etiology and genetic features that result in different course of diseases.
It was known at the priority date of the present application that somatostatin and somatostatin analogs are the most promising drugs for the treatment of pancreatic neuroendocrine tumors, in particular when used in combination with interferon (see Warner 2005, p 1677, left column: "The single most important advance in the medical treatment of all GEP NETs has been the recognition of the effectiveness and use of somatostatin analogs in improving the symptoms of most of the functioning tumors, and in gastrinoma the introduction of proton pump inhibitors. P 1678, left column: " Biotherapy for antitumor effect uses somatostatin analogs or interferon alfa, alone or in combination. These drugs have been found to have tumoristatic effectiveness, particularly when combined, resulting in tumor stabilization of both functioning and nonfunctioning NETs).

Furthermore, somatostatin and its analogues inhibit the PI3K pathway, reduce the expression of tumor growth factors thereby the pNET tumor growth is arrested.

According to the present invention, it is proposed an alternative treatment for pNET tumor by using everolimus as monotherapy. As disclosed on page 37 of the application as originally filed, under the Experimental part D. Clinical Trials, the inventors proposed treatment of pNET by inhibition of S6K1 activity and a reduction of chromogranin A using everolimus monotherapy.

Based on the above, Applicant respectfully submits that the rejection has been overcome and that rejection, accordingly, should be withdrawn.

It is hereby requested that the term to respond to the Action of May 9. 2014 be extended pursuant to 37 C.F.R. § 1.136(a) for three (3) months, from August 9, 2014 to November 9, 2014. The Commissioner is hereby authorized to charge any fees required to Deposit Account No. **19-0134** in the name of Novartis.

Respectfully submitted,

/Gregory Ferraro/

Gregory Ferraro
Attorney for Applicant
Reg. No. 36,134

Novartis Pharmaceuticals Corporation
One Health Plaza, Bldg. 101
East Hanover, NJ 07936
+18627787831

Date: November 6, 2014

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory David Ferraro./Cindy Klepacky
Attorney Docket Number:	PAT034678-US-PCT

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**1400**

Electronic Acknowledgement Receipt	
EFS ID:	20634354
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Gregory David Ferraro./Cindy Klepacky
Filer Authorized By:	Gregory David Ferraro.
Attorney Docket Number:	PAT034678-US-PCT
Receipt Date:	07-NOV-2014
Filing Date:	19-MAY-2008
Time Stamp:	10:57:14
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1400
RAM confirmation Number	8709
Deposit Account	190134
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		PAT034678_US_PCT_2014_NOV7_Response.pdf	153307 dc6d26c0319df8c2a6383fe4af8b1a88b585c2b6	yes	4

	Multipart Description/PDF files in .zip description		
	Document Description	Start	End
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	4

Warnings:

Information:

2	Fee Worksheet (SB06)	fee-info.pdf	30528 c6bda02a910d50807111cfbf1f98bb58cb56bd2e	no	2

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New International Application Filed with the USPTO as a Receiving Office
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 12/094,173	Filing Date 05/19/2008	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	11/07/2014	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 3	Minus	** 20	= 0	X $80 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

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** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
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/LAJUAN HICKSON/

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Molecular and Cellular Endocrinology 194 (2002) 165–173

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Expression of chromogranin A and somatostatin receptors in pancreatic AR42J cells

Eva Hofsli [a,*], Liv Thommesen [a], Kristin Nørsett [a], Sture Falkmer [b], Unni Syversen [c], Arne K. Sandvik [a], Astrid Lægreid [a]

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Abstract

The exocrine pancreatic cell line AR42J is also known to display some neuroendocrine (NE) features. We have extended this fact by showing that AR42J cells express mRNA of chromogranin A (CgA), display immunoreactivity (IR) to CgA, and secrete its cleavage product pancreastatin. A sparse occurrence of typical NE secretion granules, together with only a faint IR to conventional NE markers, indicates that the NE cells are of a poorly differentiated type. CgA promoter reporter plasmid experiments showed that gastrin, epidermal growth factor, and phorbol 12-myristate 13-acetate, induce upregulation of CgA after 24 h. By RT-PCR, it was found that AR42J expresses all of the five subtypes of the somatostatin (SST) receptor (SSTR) family, except SSTR4. The existence of functional SSTRs was confirmed by showing that the SST analog octreotide could inhibit gastrin-induced proliferation. Thus, the AR42J cell line may function as a valuable experimental model to study the regulation of CgA and SSTRs in poorly differentiated NE tumor cells.

Keywords: Pancreastatin; Neuroendocrine features; Somatostatin receptors; AR42J; Reporter-gene

1. Introduction

The rat AR42J cell line is a widely used experimental model system for studies of pancreatic acinar cells (Rosewicz et al., 1992; Christophe, 1994). The cell line derives from a chemically induced pancreatic adenocarcinoma (Longnecker et al., 1979). In addition to its well-known exocrine properties, it also displays some neuroendocrine (NE) features such as the expression of the typical NE vesicle protein synaptophysin (Syn), the synaptic vesicle protein type 2 (SV2), voltage-activated ionic channels (Kusano and Gainer, 1991), as well as the neurotransmitters GABA, glutamate and glycine (Rosewicz et al., 1992; Christophe, 1994). AR42J cells also express some transcription factors typically found in NE cells (Palgi et al., 2000). Furthermore, upon treatment with betacellulin, a member of the epidermal growth factor family (EGF), or with hepatocyte growth factor, in combination with activin, AR42J cells differentiate into insulin-producing cells (Mashima et al., 1996a,b). This has also been shown after treatment with glucagon-like peptide 1 and exendin-4 (Zhou et al., 1999). These observations suggest that this cell line can serve as an experimental model to study the formation and differentiation of pancreatic endocrine cells.

Chromogranin A (CgA) belongs to the granin family of acidic secretory glycoproteins that are expressed in most types of normal NE cells and in the parenchyma of most NE tumors (c.f. Nobels et al., 1998; Wick, 2000). CgA is typically bound to the membrane of the NE secretion granules, but its precise biological functions are not fully elucidated. CgA is processed in a tissue specific manner into biologically active peptides with various functions. One of these peptides is pancreastatin, which has a regulatory effect on secretion from both endocrine and non-endocrine cells (Reinecke et al.,

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1991; Nobels et al. 1998). CgA itself appears to modulate the proteolytic processing of peptide hormones and neuropeptides, and to be involved in both the packing of peptides and in directing them to the regulated pathway of secretion. In a clinical setting, CgA seems to be one of the most important markers of NE tumors (Syversen et al., 1993; Nobels et al., 1998; Öberg, 2000). CgA is used both as a serum and tissue marker, and a future application may be its use in visualization of NE tumors in patients.

Somatostatin (SST) is a widely distributed peptide hormone which plays a pivotal regulatory role in multiple target organs (e.f. Patel, 1999). It inhibits secretion from a wide variety of both endocrine and exocrine cells, it functions as a neurotransmitter in the central nervous system (CNS), and it plays an important role in regulation of cell proliferation and differentiation. An antiproliferative effect of SST has been demonstrated both in normal cells, in malignant cell lines, and in tumors (Hofland and Lamberts, 1997; Patel, 1999). SST exerts its effects through binding to specific surface membrane receptors. Five different SST receptor (SSTR) subtypes have been characterized (SSTR1-5) (Schonbrunn et al., 1995; Hofland and Lamberts, 1997; Patel, 1999).

The majority of NE tumors express a high density of SSTRs and different SST analogs (e.g. octreotide) are widely used both in diagnosis and therapy of these tumors (Hofland and Lamberts, 1997; Wulbrand et al., 1998; Pollak and Schally, 1998; Öberg, 2000). Usually more than one subtype is expressed, and the general pattern of expression suggests a high frequency of SSTR2 mRNA. Interestingly, a specific loss of SSTR2 subtype gene expression has been observed in human pancreatic and advanced colorectal adenocarcinomas, and this has been suggested to represent a growth advantage in these tumors (Buscail et al., 1996). In fact, it has recently been shown that SSTR2 gene transfer mediates antitumor effect both in animals and in-vitro (Rochaix et al., 1999). Thus, SSTR2 gene transfer may represent a new therapy for cancer.

In spite of the extensive clinical use of SST analogs in the management of NE tumors, the exact functional significance of the presence of SSTRs, and how these are being regulated, have still not been fully established (Patel, 1999). The same is true concerning knowledge about the function and regulation of the NE cell marker CgA (Nobels et al., 1998). To this end, well-characterized in-vivo and in-vitro models are needed. The aim of this study was to investigate whether the AR42J cell line expresses CgA, and to characterize this cell line with respect to the expression of the five known SSTRs. Our findings lead us to propose that the AR42J cell line might be a valuable experimental model to study molecular mechanisms involved in the biology of NE tumor cells.

2. Materials and methods

2.1. Cells and reagents

AR42J (rat pancreatic acinar cell derived, ATCC, Rockville, MD, USA), Rat-2 (rat fibroblast, ATCC), NRK-52E (rat epithelial, ATCC) and PC-12 (rat pheochromocytoma, ATCC) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose (Gibco BRL, Life Technologies, Paisley, Scotland), 1 mM Na-pyruvate (Gibco), 0.1 mg/ml L-glutamine (Gibco), 10 U/ml penicillin/streptomycin (Gibco), 1 μg/ml fungizone (Gibco) supplemented with 15% (AR42J), 10% (PC-12) or 5% (Rat-2, NRK-52E) fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel). RIN-5F (rat insulinoma, ATCC) and RIN-14B (rat somatostatinoma, ATCC) cells were grown in RPMI 1640 (Gibco) with 2 g/l glucose, 0.1 mg/ml L-glutamine, 0.04 mg/ml garamycin (Schering-Plough Labo, Heist-op-den-Berg, Belgium), supplemented with 10% FCS.

Recombinant epidermal growth factor (EGF) (stored lyophilized) was purchased from Biomedical Technologies (Stoughton, MA, USA). Gastrin-17 (stored lyophilized) was obtained from Sigma (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) (Sigma) was dissolved in DMSO (1 mg/ml) and stored at −20°. Octreotide (Sandostatin) was purchased from Novartis (Oslo, Norway).

2.2. Northern blot analysis

Total RNA from AR42J, PC-12, RIN-5F, RIN-14B, Rat-2 and NRK-52E cells was isolated by phenol extraction as previously described (Liabakk et al., 1993). Twenty μg of each total RNA was electrophoresed on a formaldebyde agarose gel and blotted onto nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany). Plasmids containing cDNA fragments of CgA (Angelsen et al., 1997) or 18S (Bakke et al., 2000) were linearized, and antisense RNA probes labeled with ^{32}P were generated by in-vitro transcription according to standard protocols using SP6 or T7 RNA polymerases. Probes were purified on NucTrap columns (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were prehybridized for 4 h at 65 °C in 5 × sodium chloride–sodium-phosphate–EDTA buffer (SSPE; 0.75 M NaCl, 0.05 M sodium phosphate, and 5 mM EDTA, pH 7.4), containing 50% formamide, 5 × Denhardt's solution (0.1% bovine serum albumine (BSA), 0.1% polyvinylpyrrolidine, and 0.1% Ficoll 400; w/v), 0.5% sodium dodecyl sulfate (SDS) and 200 μg/ml sonicated salmon sperm DNA (Sigma), then hybridized in the same solution containing RNA probe (2×10^6 counts per min per ml) for a further 18 h at 65 °C.

After hybridization, membranes were washed twice at room temperature (RT) for 20 min with 2× SSPE containing 0.1% SDS and once at 65 °C for 20 min with 0.1 × SSPE containing 0.1% SDS. Washed membranes were exposed to a storage phosphor screen for 15 min (18S) or 18 h (CgA), and the screen was scanned on a Phosphorimager 425 (Molecular Dynamics, Sevenoaks, UK). Membranes were hybridized, first with the CgA riboprobe, then by 18S. Probes were removed between hybridizations by boiling in 0.1% SDS.

2.3. RT-PCR

AR42J was seeded out in growth medium at 0.9×10^6 cells per well in six-well plates and cultivated for 3 days (subconfluent). Then, the cells were washed twice with phosphate-buffered saline (PBS), and 500 μl lysis/binding buffer (100 mM Tris, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol) was added. DNA was sheared by forcing the lysate five times through a 21 gauge needle by a 1–2 ml syringe. PolyA+ RNA was isolated from lysate (2.5×10^5 cells) with 125 μl oligo dT Dynabeads (Dynal, Oslo, Norway) according to the protocol of the manufacturer, and eluted from the beads in 20 μl Tris–HCl (10 mM, pH 7.5). Total RNA from rat cerebral cortex was isolated and prepared as previously described (Sandvik et al., 1995).

RT-PCR CgA was performed with 0.5 μl eluate with 1.25 U rTth DNA polymerase (Roche) according to the procedure recommended by the manufacturer. cDNA synthesis was performed at 61 °C for 40 min, followed by PCR with 300 μM dNTP (Roche), 500 nM primers and 3.0 mM $Mn(OAc)_2$. PCR amplification was run for 28 cycles at 94 °C for 15 s, 55 °C for 15 s, and at 72 °C for 30 s, followed by a final extension step for 3 min at 72 °C. The following PCR primers were used (S; sense, AS; antisense): **CgA-S**: 5′-TCC ATG AAG CTC TCC TTC-3′ and **CgA-AS**: 5′-AGA AAG CTG CCT GTG TTC-3′. The number of PCR-cycles was selected on the basis of experiments with 28, 30, 32, 34 and 36 cycles, which showed that 28 cycles yield quantitative results within the linear range.

RT-PCR for SSTRs were performed by a two step procedure. For reverse transcription, 5 μl of polyA+ RNA eluate or total RNA in a final volume of 30 μl, containing 150 U MuLV reverse transcriptase (Roche), 3 μl of 10 × PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 60 U RNAsin (Promega), 250 μM dNTPs (Boehringer Mannheim), 5 μM oligo-d(T) (Amersham Pharmacia Biotech) and 5 mM $MgCl_2$ (Roche), was incubated at 42 °C for 60 min, and then the enzyme was denatured at 95 °C for 2 min. To ensure that the specific RT-PCR products were exclusively dependent on mRNA transcripts present, controls were performed without reverse transcriptase.

For PCR amplification, 1 μl of cDNA was incubated in a final volume of 20 μl with 1 U of AmpliTaqGold (Roche), 2 mM $MgCl_2$, 500 nM of each sense- and antisense primer, 300 μM dNTP, and 2 μl GeneAmp 10 × PCR Buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) (Roche). PCR amplifications were run for 28 (SSTR1), 35 (SSTR2), 34 (SSTR3), 40 (SSTR4) and 42 (SSTR5) cycles, respectively, at 94 °C for 15 s, 66 °C for 15 s, and at 72 °C for 30 s, followed by a final extension step for 7 min at 72 °C. The following primers were used: **SSTR1-S**: 5′ ATG GTG GCC CTC AAG GCC GG 3′, **SSR1-AS**: 5′GGC AGT GGC GTA GTA GTC AA 3′, **SSTR2-S**: 5′ TCA TCA AGG TGA AGT CCT CTG G 3′, **SSTR2-AS**: 5′ AGA TAC TGG TTT GGA GGT CTC CA 3′, **SSTR3-S**: 5′ TGC CAG TGG GTA CAG GCA CC 3′, **SSTR3-AS**: 5′ CTG GAG GGC CAG ACC CTG GC 3′, **SSTR4-S**: 5′ TGC GGG CTG GCT GGC AAC AA 3′, **SSTR4-AS**: 5′ GTA GTC CAG GGG CTC TTC CT 3′, **SSTR5-S**: 5′ CCT TTC CTG GCC ACG CAG AAC GC 3′, **SSTR5-AS**: 5′ GGC CAG GTT GAC GAT GTT GAC 3′.

To check whether comparable amounts of polyA+ RNA from each sample were used, RT-PCR reactions for the house-keeping gene GAPDH were performed using the following primers: **GAPDH-S**: 5′-CCCATCACCATCTTCCAG-3′ and **GAPDH-AS**: 5′-ACAGTCTTCTGAGTGGCA-3′. PCR was run for 28 cycles at 94 °C for 15 s, 50 °C for 15 s and at 72 °C for 30 s, followed by a final extension step for 3 min at 72 °C.

The identity of the SSTR PCR products was checked by informative restriction analysis using one or two different restriction enzymes (Table 1). In each case, 8 μl of the PCR product was treated at 37 °C for 2 h with the appropriate enzymes (9-16 U) and restriction enzyme buffers in a total volume of 20 μl.

2.4. Reporter plasmid experiments

The plasmid pXp100Luc containing 100 bp of the proximal CgA promoter (Wu et al., 1995) was a generous gift from Dr D. O'Connor (University of California, San Diego, CA, USA). Cells (2×10^4) per well were seeded out in 96-well plates and transfected after 24 h with 0.12 μg luciferase reporter plasmid DNA per well, using 0.35 μl Fugene transfection reagent (Roche). After culture for 2 days in the presence of plasmid and transfection agent, cells were treated with agonists for 24 h followed by PBS wash (twice) and lysis in 15 μl Promega lysis buffer. Luciferase activity was measured by Turner Luminometer model TD-20/20 (Turner Designs, Sunnyvale, CA, USA) using the Luciferase reporter Assay System (Promega Corp., Madison, WI, USA) as recommended by the manufacturer.

Table 1
Restriction enzymes used, and expected post-cleavage product length (bp), for verification of amplified SSTR (SSTR1-5) products

PCR-product	Expected length (bp)	Restriction enzymes	Expected post-cleavage length (bp)
SSTR1	318	BsaHI	149+169
		PvuII	269+49
SSTR2	414	BamHI	22+392
		PvuII	220+194
SSTR3	328	BamHI	269+59
		PvuII	209+119
SSTR4	311	PvuII	184+127
SSTR5	549	BsaHI	174+375
		SphI	426+123

2.5. Detection of pancreastatin

AR42J cells were grown in 75 cm^2 culture flasks for 4 days to reach confluence. The cells were then cultivated for 24 h in serum free media (3 ml), before medium was collected, centrifuged, and kept frozen at −80 °C until assay. Cell lysates were prepared by lysis in distilled water after one wash in PBS. Determination of rat pancreastatin was performed by using a commercial RIA kit (Peninsula Laboratories, Inc., San Carlos, CA, USA) as described by the manufacturer. Briefly, primary antibody (rabbit anti-peptide serum) was added to standards and unknown samples, followed by incubation overnight (4 °C). On the next day, ^{125}I-peptide was added and incubated for 24 h (4 °C). Then, addition of goat anti-rabbit IgG, incubation 90 min at RT and admixture of RIA buffer followed. The samples were centrifuged for 20 min, supernatant aspirated, and assay tubes counted. The detection limit of the assay was 5 pg/ml, and the intra- and inter-assay variations were 4.9 and 3.8%, respectively, (Syversen et al., 1993).

2.6. Light-microscopical, immunohistochemical, and ultrastructural examinations

Trypsinized AR42J cells were centrifuged at 4.000 × *g*. For the light-microscopical (LM) and immunohistochemical (IHC) investigations, the pellet was conventionally fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections, about 4-5 micron thick, on poly-L-lysine-coated slides, were used for the IHC examinations. They were performed both by means of the conventional avidin-biotin peroxidase procedure, using the Vectastain ABC kit (Vector Lab., Burlingame, CA, USA), and by applying the Tyramid Signal Amplification (TSA) technique, using the 'TSA indirect kit' (NEN LifeSci. Products, Boston, MA, USA), as recently described (Qvigstad et al., 1999). The CgA antiserum was provided by Incstar (Stillwater, MN, USA), known to be immunoreactive (IR) in rat NE cells. It was applied at a dilution of 1/500. For the Syn IHC examination, the monoclonal mouse anti-Syn antiserum, provided by Dako (Glostrup, Denmark) was employed with the dilution 1/20. For the neuron-specific enolase (NSE) IHC studies, the 'anti rat NSE' antiserum, provided by Polysciences (Warrington, PA, USA) was used, dilution 1/500.

For the electron microscopical (EM) investigations, the pellet was fixed in 2% neutral glutaraldehyde, post-fixed in 2% osmium tetroxide, contrasted with 1% lead citrate and 4% uranyl acetate, and conventionally embedded in Epon. Semi-thin sections were cut and stained with toluidine blue for orientation and trimming of the blocks. Finally, from the areas selected, conventional ultrathin sections were cut and analyzed by means of our transmission EMs (JEOL 100CX and Phillips SEI Tecnai 12).

2.7. Proliferation assay

Proliferation rate was determined by measuring DNA synthesis using the Cell proliferation ELISA BrdU (5-bromo-2′-deoxyuridine) kit (Roche). AR42J cells (2×10^3) were seeded out in 96-well microtiter plates in 150 μl serum-containing medium. After 24 h the cells were washed with 200 μl serum-free medium before treatment with gastrin and/or octreotide in a final volume of 100 μl. After 24 h, BrdU-labeling solution (10 μl per well) was added, and the cells were cultured for an additional 18 h before incorporation of BrdU was measured as described by the manufacturer. Briefly, after removing the labeling medium, the cells were fixed and DNA denatured in one step by adding 200 μl FixDenat-solution per well for 30 min at RT After removing FixDenat-solution, 100 μl anti-BrdU-POD working solution was added to each well, and incubated at RT for 90 min. The cells were then rinsed three times with 200 μl washing solution before 100 μl substrate solution was added to each well. After 3 min the light emission of the samples (RLU = relative luminiscence units) was measured in a microplate luminometer (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland).

2.8. Statistics

Statistical analysis were performed using the Student's *t*-test.

3. Results

3.1. AR42J cells express CgA mRNA

To determine whether AR42J cells express the NE cell marker CgA, we used Northern blot analysis. As shown in Fig. 1, a distinct hybridization band of a size corresponding to the reported length of rat CgA mRNA (2.1 kb) was detected in AR42J. This was confirmed by RT-PCR (data not shown). The rat NE cell lines PC-12 (pheochromocytoma) and RIN-5F (insulinoma) were used as positive controls, as these cell lines have been shown to express CgA mRNA (Rausch et al., 1988; Swarovsky et al., 1994). The level of CgA in AR42J was comparable to the level in PC-12. We found CgA gene expression also in the somatostatinoma cell line RIN-14B (Fig. 1). This has not been reported earlier. The rat fibroblast Rat-2 and the rat epithelial NRK-52E cell lines were used as negative controls (Fig. 1).

3.2. Gastrin and EGF upregulate CgA gene expression

In order to study the regulation of CgA gene expression in AR42J, we performed CgA promoter reporter plasmid experiments. Gastrin, EGF and PMA induced a moderate (136, 77 and 114%, respectively) transcriptional upregulation of CgA after 24 h ($P < 0.001$) (Fig. 2). Gastrin and EGF are both known to

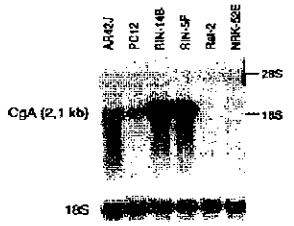

Fig. 1. Northern blot analysis of CgA. 20 μg of total RNA from AR42J, and from the NE cell lines RIN-5F (insulinoma), PC-12 (pheochromocytoma) and RIN-14B (somatostatinoma) were electrophoresed in 1% agarose-formaldehyde gels, electroblotted onto nylon membranes, and hybridized with RNA probes for CgA and 18S. The indicated size (kb) of CgA was obtained by comparison with the sizes of 18S and 28S rRNA. As negative controls were used the Rat-2 (fibroblast) and NRK-52E (epithelial) cell lines. The results shown are representative for three independent experiments.

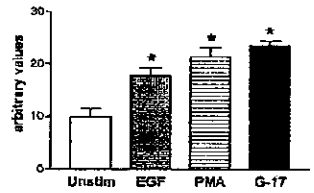

Fig. 2. Activation of the CgA reporter plasmid pXp100 AR42J cells were transfected with CgA promoter reporter plasmid pXp100 (Wu et al., 1995) and treated with either 50 ng/ml EGF, 50 nM gastrin (G-17) or 100 ng/ml PMA for 24 h with quadriplicate parallels per condition. Results are shown as mean value±S.E.M. of one representative experiment, and are expressed as fold induction compared with untreated cells (* indicates significant difference with untreated cells; $P < 0.001$). Similar results were obtained in two other experiments.

activate protein kinase C (PKC) in AR42J cells (Stepan et al., 1999). Since PMA is known to exert its effect via both classical and novel diacylglycerol (DAG)-responsive PKCs (Newton and Johnson, 1998), the PMA-induced increase (114%) in CgA promotor activation suggests that PKC is involved in mediating CgA promoter transactivation in AR42J cells.

3.3. AR42J cells secrete pancreastatin

In order to confirm translation of CgA mRNA into protein, we looked for the cleavage product pancreastatin in the cell medium. After incubation of confluent AR42J cells (grown in 75 cm^2 culture flasks in a volume of 3 ml) in serum free media for 24 h, the concentrations of pancreastatin were found to be in the range of 69-162 pg/ml, with a mean level of 96.5±30.5 pg/ml ($n = 6$). These ranges lie within the steep part of the standard curve. We also detected pancreastatin in AR42J cell lysates (data not shown).

3.4. Some AR42J cells are equipped with NE secretion granules and are faintly IR to NE markers

In order to examine whether our AR42J cells fulfil the classical histopathologicaI criteria of being a NE parenchymal cell, we examined the LM, IHC, and EM features of the cells. We focused our attention on four major NE criteria, namely the LM/IHC detection of CgA, Syn and NSE IR, as well as the actual EM demonstration of typical NE secretion granules in the cytoplasm of clear cells of NE appearance.

Only a faint IR could be discerned in a small minority of the AR42J cells when antisera against CgA were used. After the application of the TSA technique, a more convincing IR was seen. However, the CgA/TSA IR was still confined only to a small minority of the cells. This was also the case when antisera against Syn and NSE

were used. The Syn IR was more distinct than those of CgA and NSE.

EM revealed that only a minority population of AR42J displayed a fine structure, compatible with the idea that they might be of NE nature (Fig. 3). Like the clear cells of the 'Helle-Zellen-System' originally discovered by Friedrich Feyrter in the 1930's in the gastro-entero-pancreatic (GEP) organs (Falkmer, 1993; Falkmer and Wilander, 1995), their cytoplasm was found to be less electron dense than that of their non-NE counterparts. They were observed to be well equipped with cytoplasmic organelles, mainly mitochondria, lysosomal bodies, and an endoplasmic reticulum (ER) of both rough and smooth type. In addition, a few typical NE secretion granules appeared (Fig. 3; inset). They were displaying a fine structure that was rather commonplace, an electron dense, homogeneous core of non-crystalline shape, surrounded by a moderately broad halo and a thin granule membrane. Practically no cells were found containing numerous NE secretion granules. The overall structure of the AR42J cells was that of cells from a poorly differentiatied neoplastic parenchyma with numerous mitotic figures. The absence of any densely granulated NE cells indicated that the cells with NE features were rather immature.

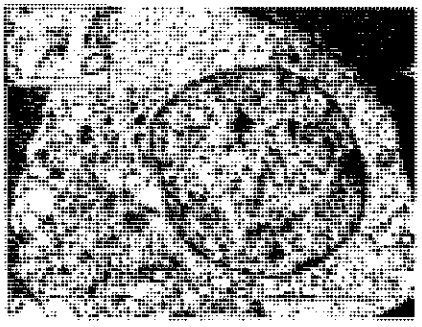

Fig. 3. Fine structure of an AR42J cell and one of its secretion granules (inset). Low-power EM, showing the ultrastructural features of an AR42J cell equipped with secretion granules of NE type ('SG'; inset; upper left corner). The cytoplasm of the cells of this minority population cell type was found to be of lower electron density than that of the adjacent AR42J cells in the pellet (upper right corner), forming the majority cell population of the pellet. In addition to mitochondria, a well developed endoplasmic reticulum ('ER'; inset, upper left corner), both of smooth and rough type, and several kinds of lysosomal bodies occurred. The secretion granules of NE type formed only a small minority of the cytoplasmic organelles. The bars give the actual lengths of 5 μm (main electron micrograph) and 0.5 μm (inset), respectively. Thus, the diameter of the secretion granule in the inset amounts to approximately 136 nm.

3.5. AR42J cells express SSTR subtype 1, 2, 3 and 5

There are conflicting results as to the question of which SSTR subtypes AR42J cells express (Vidal et al., 1994; Froidevaux et al., 1999). By RT-PCR analysis, we could show the presence of mRNA of the four SSTR subtypes 1, 2, 3 and 5 in AR42J cells (Fig. 4). This is the first report of SSTR5 expression in AR42J. SSTR4 mRNA was not detected. Each analysis was repeated at least five times. Contamination with genomic DNA was ruled out by performing PCR on samples where reverse transcriptase had been omitted in the cDNA synthesis step. The PCR reaction for each of the five SSTR subtypes was verified by using rat cerebral cortex as positive control, as this tissue has been shown to express transcripts of all the five SSTRs (Bruno et al., 1993; Thoss et al., 1995). The specificity of each PCR product was verified by informative restriction analysis. Fig. 5 shows that all specific SSTR PCR products exhibited the expected restriction fragment sizes (see Table 1).

3.6. Proliferation studies

Expression of SSTR mRNA in cancer cell lines is not always coupled with the expression of functional cell surface receptors, as assessed by classic competitive binding or proliferation studies (Fisher et al., 1998). In order to confirm the existence of functional SSTRs in AR42J cells, we examined the effect of the SST analog octreotide on gastrin-induced proliferation (Seemama et al., 1987; Seva et al., 1990; Watson et al., 1992). Octreotide binds with a high affinity to SSTR2, with a moderate affinity to SSTR3 and 5, and with a very low affinity to SSTR1 and 4. Octreotide, at a concentration of 0.1 nM, strongly inhibited gastrin-induced proliferation (Fig. 6). The effect was highly significant ($P = 0.0011$) at 0.4 nM gastrin. Our finding confirms the expression of functional SSTRs in AR42J.

Fig. 4. RT-PCR analysis of the five SSTR subtypes (SSTR1-5) in AR42J cells. RT-PCR of polyA+RNA from AR42J was performed with (+) or without (−) reverse transcriptase (RT), to rule out contamination with genomic DNA. RT-PCR of total RNA from rat cerebral cortex (c) was used as positive control. PCR products were visualized in ethidium bromide stained 1.2% agarose gels. The marker (M) is a 100 bp DNA ladder molecular weight standard (Gibco), and the estimated PCR products are the following: SSTR1: 318 bp, SSTR2: 414 bp, SSTR3: 328 bp, SSTR4: 311 bp, SSTR5: 549 bp. To ensure that comparable amounts of RNA were used in RT$^+$ and RT$^-$ PCR, GAPDH RT-PCR was performed. The results shown are representative of at least five experiments.

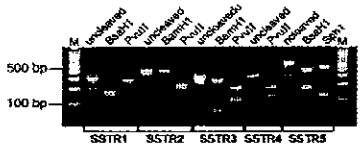

Fig. 5. Informative restriction enzyme analysis of amplified SSTR (SSTR1-5) products. Reactions were performed as described in Section 2. M: 100 bp ladder. Shown are uncleaved PCR product of each receptor, and the restriction enzyme pattern obtained with the different enzymes used. See Table 1 for expected post-cleavage product length (bp).

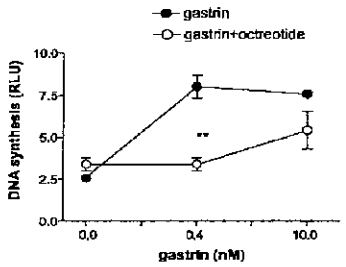

Fig. 6. Effect of octreotide on gastrin-induced proliferation in AR42J cells. Proliferation was measured by using the BrdU proliferation kit, as described in Materials and methods. AR42J cells were stimulated with gastrin (0.4 and 10 nM G-17) in the absence (●–●) or presence of octreotide (0.1 nM) (○–○) with quadriplicate parallels per condition. Results are shown as the mean value±S.E.M. of one representative experiment, and are expressed as relative light units (RLU). Similar results were obtained in two other experiments. $^{*}P = 0.0011$.

4. Discussion

Here we demonstrate, for the first time, that pancreatic acinar AR42J cells express the rather specific NE marker CgA, and that this expression is transcriptionally regulated by the growth factors gastrin and EGF. The sparse occurrence of typical NE secretion granules, supposed to contain CgA, combined with the faint CgA, Syn, and NSE IR, indicate that the cells are of a rather poorly differentiated type. Furthermore, we show that AR42J cells express mRNA of all SSTR subtypes, except SSTR4. Our findings indicate that the AR42J cell line could serve as a valuable experimental model to study the regulation of CgA and SSTRs in poorly differentiated NE tumor cells.

The fact that Rosewicz et al. (1992) were unable to detect CgA in AR42J cells by immunoblotting may be due to the low sensitivity of the immunoblotting method. Our analyses show that the CgA mRNA levels in AR42J cells are comparable to the levels in the NE pheochromocytoma cell line PC-12 (Fig. 1). We also detected CgA mRNA in the rat somatostatinoma cell line RIN-14B. To the best of our knowledge, this is the first report of CgA expression in RIN-14B. However, the finding was not surprising, since others (Funakoshi et al., 1990) have reported that CgA is expressed in the human somatostatinoma cell line QGP-1.

We found a moderate transcriptional activation of CgA in AR42J upon treatment with gastrin and EGF (Fig. 2). Gastrin-induced regulation of CgA promotor activity has previously been shown in gastric carcinoma cells (Höcker et al., 1998). However, Weiss et al. (2001) could not detect any upregulation of neither CgA mRNA (Northern blot), nor CgA protein (RIA), upon treatment with EGF in neuroblastoma cell lines. This may be due to a lower sensitivity of the Northern blot method as compared with reporter plasmid analysis used in the present study. However, it may also indicate that CgA expression is not inducible by EGF in all NE cell types. In conclusion, the demonstration that CgA gene expression is regulated by gastrin and EGF, and that CgA mRNA is translated into protein in AR42J, strongly suggest a physiologically important role of CgA in this pancreatic cell line.

Even though AR42J cells are known to express both functional SSTRs (Viguerie et al., 1988) and their mRNAs (Taylor et al., 1994; Vidal et al., 1994; Froidevaux et al., 1999), there are conflicting results as to which subtypes these cells express. Vidal et al. (1994) found high levels of SSTR2 PCR products, and only low levels of SSTR1 and 3. Froidevaux et al. (1999), on the other hand, were unable to detect SSTR3 or SSTR5 mRNA, and concluded that AR42J cells can be considered to be cells expressing exclusively SSTR2. Their study was, however, limited to examining the expression of just the three octreotide-binding receptor subtypes, SSTR2, SSTR3, and SSTR5. Our finding that the AR42J cells express four of the five SSTRs has, as far as we know, not been reported previously. At least any SSTR5 expression has not previously been detected in AR42J cells. We were unable to detect SSTR4 mRNA, which has not been reported investigated by others.

Wulbrand et al. (1998) found a widely varying expression pattern of the five SSTR subtypes in different types of NE gastro-entero-pancreatic (GEP) tumors. However, almost 20% of the GEP tumors were found to express four or five SSTR subtypes. Thus, it is obviously of great importance in investigations of the exact functional significance of SSTR expression to have access to experimental models in which the NE tumor cells express several SSTR subtypes. The AR42J cell line fulfils these criteria, and thus has an advantage over SSTR transfected cell lines which in most cases express only one receptor subtype.

SSTR scintigraphy is a well-established diagnostic tool for staging of NE tumors, and may indicate sensitivity to treatment with SST analogs (Chiti et al., 2000). More recently, radiotherapy of SSTR positive tumors with radiolabeled SST analogs has been carried out with survival benefit (McCarthy et al., 2000). In addition, because of their nearly universal inhibitory actions on the release of peptide hormones and growth factors, SST analogs are regarded as the main choice for symptomatic treatment of hormone-related syndromes often related to NE tumors (Hofland and Lamberts, 1997; Öberg, 2000). In light of this extensive clinical use of SST analogs, it will be of great interest to elucidate molecular mechanisms involved in regulation of the expression of the different SSTR subtypes, since this may provide strategies to upregulate SSTRs in vivo. Our results lead us to suggest that the AR42J cell line could be a valuable experimental model for this purpose.

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	Art Unit	1627
	Examiner Name	Jean-Louis, Samira JM
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Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory David Ferraro./Cindy Klepacky
Attorney Docket Number:	PAT034678-US-PCT

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First Named Inventor/Applicant Name:	Peter Wayne Marks
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
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NOTICE OF ALLOWANCE AND FEE(S) DUE

1095 7590 01/30/2015

NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

EXAMINER
JEAN-LOUIS, SAMIRA JM

ART UNIT	PAPER NUMBER
1627	

DATE MAILED: 01/30/2015

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT034678-US-PCT	9572

TITLE OF INVENTION: Neuroendocrine Tumor Treatment

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	04/30/2015

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

1095 7590 01/30/2015
NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT034678-US-PCT	9572

TITLE OF INVENTION: Neuroendocrine Tumor Treatment

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	04/30/2015

EXAMINER	ART UNIT	CLASS-SUBCLASS
JEAN-LOUIS, SAMIRA JM	1627	514-183000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature Date

Typed or printed name _____ Registration No. _____

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT034678-US-PCT	9572

1095 7590 01/30/2015

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EXAMINER	
JEAN-LOUIS, SAMIRA JM	
ART UNIT	**PAPER NUMBER**
1627	

DATE MAILED: 01/30/2015

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 12/094,173	Applicant(s) MARKS ET AL.	
	Examiner SAMIRA JEAN-LOUIS	Art Unit 1627	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *11/07/14*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1-3*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov .
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 10/07/11
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date *1/21/15* .
5. ☒ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/SAMIRA JEAN-LOUIS/
Primary Examiner, Art Unit 1627

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Gregory Ferraro on January 22, 2015.

The application has been amended as follows:

1. In claim 1, line 1, after “treating pancreatic" delete “endocrine” and insert “neuroendocrine”.
2. In claim 2, line 1, delete “Claims 2” and insert “Claim 2”.
3. In claim 2, line 3, after "wherein" delete "the unit dose" and insert "a unit dose".

EXAMINER'S STATEMENT OF REASONS FOR ALLOWANCE

Applicant's amendment to the claims filed November 07, 2015 has been fully considered. In light of Applicant's amendment and the Examiner's amendment, claims 1-3.

In light of Applicant's amendment and remarks, the 103 (a) rejection over Weckbecker (WO 97/47317) as evidenced by Arnold et al. (Gastrointestinal and Liver Tumors, 2004) A1 is withdrawn.

The following is an examiner's statement of reasons for allowance: Claims 1-3 are drawn to a method of treating pancreatic neuroendocrine tumors, comprising administering to a human subject in need thereof a therapeutically effective amount of Everolimus as a monotherapy and wherein the tumors are advanced tumors after failure of cytotoxic chemotherapy. There is no prior art disclosing applicant's method of treatment, particularly with Everolimus as a monotherapy in advanced pancreatic neuroendocrine tumors that have failed after cytotoxic chemotherapy as disclosed in claim 1. Importantly, applicant's affidavit teaches that the instant claimed invention provided unexpected results wherein Everolimus administered as a monotherapy was effective in doubling the time without tumor growth and in reducing the risk of tumor progression by 65% as compared to placebo. Since the present claims require the use of Everolimus as a monotherapy and Weckbecker alone does not render obvious the particular method of claim 1, claims 1-3 are therefore allowable.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Claims 1-3 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samira Jean-Louis whose telephone number is 571-270-3503. The examiner can normally be reached on 7:30-6 PM EST M-Th.
If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SAMIRA JEAN-LOUIS/

Primary Examiner, Art Unit 1627

01/23/2015

Examiner-Initiated Interview Summary	Application No.	Applicant(s)	
	12/094,173	MARKS ET AL.	
	Examiner	Art Unit	
	SAMIRA JEAN-LOUIS	1627	

All participants (applicant, applicant's representative, PTO personnel):

(1) *SAMIRA JEAN-LOUIS*. (3)_____.

(2) *Gregory Ferraro*. (4)_____.

Date of Interview: *22 January 2015*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1-3*.

Identification of prior art discussed: *N/A*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The examiner contacted Attorney Ferraro to indicate that applicant possesses potential allowable subject matter. The examiner however contends that because applicant demonstrated treatment of pancreatic neuroendocrine tumors, applicant needed to claim such tumors. Additinoally, the term "Claims 2" needed to be changed to "Claim 2". Finally, claim 2, line 2, refers to "the unit dose" and such terms need to be changed to "a unit dose". Applicant agreed to such amendment and for such amendment to be done via an examiner's amendment. Claims are therefore allowed..

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/SAMIRA JEAN-LOUIS/ Primary Examiner, Art Unit 1627	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	2	WO-2005064343-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:02
S2	1	WO-02080975-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:49
S3	2	WO-2005082411-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 17:57
S4	1	WO-02098416-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:01
S5	1	("5538739").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/10 18:04
S6	2	WO-9705167-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:05
S7	0	EP-0462071-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 18:06
S8	2	EP-462071-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	ADJ	ON	2011/02/10 18:06

			DERWENT; IBM_TDB			
S9	3	"2002257123"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:03
S10	7	"20020198137"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:05
S11	3	"20040176339"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:08
S12	2	"20040258662"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:09
S13	2	"20070105887"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2011/02/10 19:09
S14	4250	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:44
S15	26943	(endocrine tumor) or (neuroendocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic tumor) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (neuroendocrine carcinoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:51
S16	0	S14 near3 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S17	0	S14 near30 S15	US-PGPUB;	ADJ	ON	2011/02/10

			USPAT; USOCR; DERWENT			20:52
S18	0	S14 near300 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S19	27	S14 same3 S15	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/10 20:52
S20	1	("5665772").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/11 10:34
S21	23453	(endocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:20
S22	4250	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:21
S23	4250	S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:21
S24	0	S21 near3 S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:22
S25	0	S21 near30 S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:22
S26	0	S21 near300 S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:22
S27	0	S21 with S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:23
S28	0	S21 adj S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:38
S29	5	S21 same S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:38

S30	237	S21 and S22	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 17:45
S31	3	"20070104721"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:21
S32	2	"20070185069"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:25
S33	7	"20020198137"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:47
S34	1	WO-2004004644-$.did.	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/02/11 18:55
S35	1	2004-091226.NRAN.	DERWENT	ADJ	ON	2011/02/11 18:57
S36	1	("6573285").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/11 22:00
S37	1	("5538739").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2011/02/13 23:25
S38	6	"20020183240"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 00:11
S39	1	"20050187184"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:19
S40	6	"20020183239"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:20
S41	6	"20030008923"	US-PGPUB; USPAT; USOCR	ADJ	ON	2011/02/14 01:21
S42	4865	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 18:54
S43	29693	(endocrine tumor) or (neuroendocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic tumor) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (neuroendocrine carcinoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26

S44	0	S42 near3 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S45	0	S42 near30 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S46	0	S42 near300 S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S47	345	S42 and S43	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:26
S48	18514	somatostatin	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:27
S49	65	S47 and S48	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2011/10/05 19:27
S50	2	WO-2005080593-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2012/01/17 21:05
S51	2	WO-2004004644-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2012/01/17 21:10
S52	2	WO-2006065780-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2012/01/17 21:13
S53	0	WO-2002066019-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2012/01/17 21:15
S54	1	WO-02066019-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2012/01/17 21:15
S55	8276	Everolimus or (RAD-001) or (SDZ-RAD)	US-PGPUB;	ADJ	ON	2014/05/04

		or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	USPAT; USOCR; DERWENT			22:12
S56	36271	(endocrine tumor) or (carcinoid tumor) or (islet cell tumor) or (APUDomas) or (pancreatic neuroendocrine tumor) or (insulinoma) or (glucagonoma) or (nonfunctioning pancreatic neuroendocrine tumor) or (gastrinoma) or (VIPoma) or (somtostatinoma) or (GRFoma) or (adrenal gland tumor) or (Merkel cell cancer) or (pheochromocytoma) or (parathyroid tumor) or (parathyroid cancer) or (thyroid tumor) or (thyroid cancer) or (pituitary gland tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:12
S57	38	S55 same S56	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:12
S58	2	S55 near3 S56	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:13
S59	3	S55 near30 S56	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:25
S60	4	S55 near300 S56	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:25
S61	1017	S55 and S56	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:25
S62	105	(pancreatic neuroendocrine tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:26
S63	16	S61 and S62	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/04 22:26
S64	3	WO-9747317-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/05/04 22:45
S65	4	WO-2006071986-$.did.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2014/05/05 05:03
S66	4	WO-2006071966-$.did.	US-PGPUB; USPAT;	ADJ	ON	2014/05/05 05:04

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S67	692	(pancreatic endocrine tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S68	8276	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S69	0	S67 same S68	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S70	0	S67 near3l2	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S71	0	S67 near3 S68	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S72	0	S67 near30 S68	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S73	0	S67 near300 S68	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:15
S74	3055	(islet tumor) or (islet cell tumor)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S75	0	S68 same S74	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S76	0	S68 near3 S74	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S77	0	S68 near30 S74	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S78	0	S68 near300 S74	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S79	169	S68 and S74	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:20
S80	4721	(neuroendocrine tumor) or (endocrine tumor)	US-PGPUB; USPAT;	ADJ	ON	2014/05/05 06:21

			USOCR; DERWENT			
S81	31	S79 and S80	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2014/05/05 06:21
S82	9539	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 13:54
S83	10406	(pancreatic neuroendocrine tumor) or (islet cell tumor) or (pancreatic islect cell tumor) or (APUDomas) or insulinoma or glucagonoma or gastrinoma	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 14:07
S84	35	S82 same S83	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 14:09
S85	4	S82 near3 S83	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 14:10
S86	364293	"I3" or "I4"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:50
S87	9539	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:50
S88	10406	(pancreatic neuroendocrine tumor) or (islet cell tumor) or (pancreatic islect cell tumor) or (APUDomas) or insulinoma or glucagonoma or gastrinoma	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:50
S89	4	S87 near3 S88	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:50
S90	35	S87 same S88	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:50
S91	35	S89 or S90	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:51
S92	3	"20080255029"	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 16:51
S93	3	S91 AND ((A61K31/436 OR A61K31/00 OR A61K31/58 OR A61K31/675).CPC.)	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/21 17:01
S94	9555	Everolimus or (RAD-001) or (SDZ-RAD) or Zortress or Certican or Afinitor or (40-O-(2-hydroxyethyl)-rapamycin)	US-PGPUB; USPAT; USOCR;	ADJ	ON	2015/01/22 13:31

			DERWENT			
S95	10415	(pancreatic neuroendocrine tumor) or (islet cell tumor) or (pancreatic islect cell tumor) or (APUDomas) or insulinoma or glucagonoma or gastrinoma	US-PGPUB; USPAT; USOCR; DERWENT	ADJ	ON	2015/01/22 13:31
S101	3	"20080255029"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2015/01/23 09:34

1/23/2015 5:15:54 PM
C:\Users\sjeanlouis\Documents\EAST\Workspaces\12094173-neuroendocrine tumor treatment.wsp

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	**Art Unit**
	SAMIRA JEAN-LOUIS	1627

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	11/26/2010	02/11/2011	10/05/2011	05/04/2014	01/23/2015				
1	1	÷	✓	✓	✓	=				
2	2	÷	✓	✓	✓	=				
3	3	÷	✓	✓	✓	=				
	4	÷	N	-	-	-				
	5	÷	N	-	-	-				
	6	÷	N	-	-	-				
	7	÷	N	-	-	-				
	8	÷	✓	✓	-	-				
	9	÷	✓	✓	-	-				
	10	÷	✓	-	-	-				
	11	÷	N	-	-	-				
	12	÷	✓	-	-	-				

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	**Art Unit**
	SAMIRA JEAN-LOUIS	1627

CPC							
Symbol						**Type**	**Version**
A61K		38	/	31		F	2013-01-01
A61K		31	/	436		I	2013-01-01
A61K		45	/	06		I	2013-01-01
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CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
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			/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	3	
/SAMIRA JEAN-LOUIS/ Primary Examiner.Art Unit 1627	01/23/2015	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION										
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED					
		A	6	1	K	31 / 436 (2006.01.01)						
CROSS REFERENCE(S)												
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)											

NONE (Assistant Examiner)	(Date)	Total Claims Allowed: 3	
/SAMIRA JEAN-LOUIS/ Primary Examiner.Art Unit 1627 (Primary Examiner)	01/23/2015 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	**Art Unit**
	SAMIRA JEAN-LOUIS	1627

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1														
2	2														
3	3														

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 3	
/SAMIRA JEAN-LOUIS/ Primary Examiner.Art Unit 1627 (Primary Examiner)	01/23/2015 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
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		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO2005/080593 A2	09/01/2005	Novartis Pharma GmbH		☐
		WO2004/004644 A2	01/15/2004	Neel, Benjamin G		☐
		WO2006/065780	06-22-2006	Novartis AG		☐
		WO2002/066019	08-29-2002	Novartis AG		☐
						☐
						☐

Examiner Signature		Date Considered	

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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.J.L.

PTO/SB/08b (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	12/094173
Filing Date	November 20, 2006
First Named Inventor	Marks, Peter Wayne et al.
Art unit	1627
Examiner Name	Jean-Louis, Samira J
Attorney Docket Number	PAT034678-US-PCT

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Database Medline: Canobbio L. et al: "Use of long-acting somatostatin analog, lanreotide, in neuro-endocrine tumors." Oncology reports, vol. 1, no.1 Jan. 1994 p. 129- 131	☐
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Examiner Signature	/Samira Jean-louis/	Date Considered	01/26/2015

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw a line through citation if not in conformance and not considered. Include copy of this form with the next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12094173	MARKS ET AL.
	Examiner	Art Unit
	SAMIRA JEAN-LOUIS	1627

CPC- SEARCHED		
Symbol	Date	Examiner
	1/21/2015	SJL
A61K 31/436		

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner
None			

SEARCH NOTES		
Search Notes	Date	Examiner
Palm Inventor Name Search	2/10/2011	SJL
STN-see enclosed search history	2/10/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	2/11/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub, Derwent)-see enclosed search notes	10/5/2011	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	5/4/2014	SJL
STN-see enclosed search history	5/4/2014	SJL
East (U.S. Pat. Full, USOCR, PgPub)-see enclosed search notes	1/23/2015	SJL

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
A61K 31	436	1/23/2015	SJL

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	1627
	Examiner Name	Jean-Louis, Samira
	Attorney Docket Number	PAT034678-US-PCT

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	Dorlands Illustrated Medical Dictionary, "Dorlands Illustrated Medical Dictionary", Elsevier Saunders, 2012, Ed. 32nd 955	☐
	2	The Merck Index; 15th edition; 2013; page 718	☐
	3	Hanin et al., "Effect of Interferon-a Treatment...."; The Journal of Nuclear Medicine, No. 52, 2011; pp. 580-585	☐

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Gregory Ferraro/	Date (YYYY-MM-DD)	2015-02-03
Name/Print	Gregory Ferraro	Registration Number	36134

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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D3

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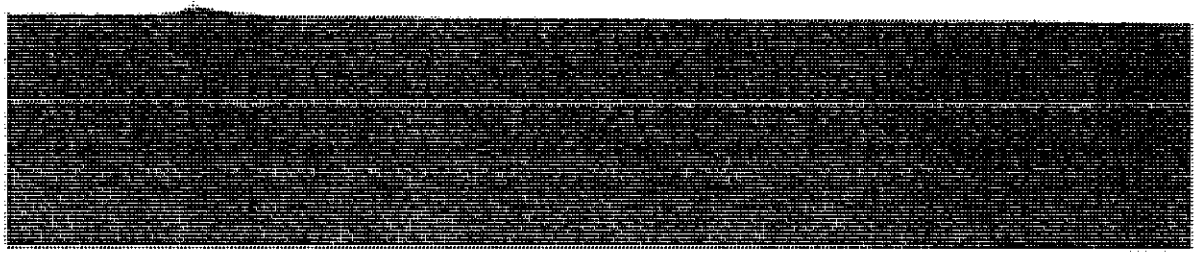


Europic chloride. $EuCl_3$. Greenish-yellow needles, mp 623° in nitrogen (in a closed tube), d 4.471. Prepd by passing sulfur chloride over the heated oxide at [illegible]. LD in [illegible] 550 mg/kg i.p.; 5 g/kg orally (Haley).

Europous chloride. $EuCl_2$. Prepd by reduction of $EuCl_3$ with hydrogen at 600°. White amorphous powder, sol in water.

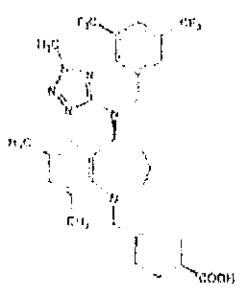
Europic sulfate. $Eu_2(SO_4)_3$. Octahydrate, a pinkish cryst solid, prepd by dissolving the oxide in sulfuric acid. Soly in water: 2.56 parts per 100 parts at 20°, 1.93 parts per 100 parts at 40°. [illegible] at 375° yields the anhydr sulfate.

Europic nitrate. $Eu(NO_3)_3$. Hexahydrate, mp [illegible] in its water of crystallization (sealed tube). LD_{50} in rats (mg/kg): 210 i.p.; >5000 orally (Haley).

Europous sulfate. $EuSO_4$. Colorless crystals. Insol in [illegible] in dil acids. Prepd by electrolytic reduction of europic salts.

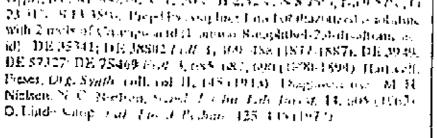
USE: The salts in cathode ray tube coatings for color television receivers. Eu has a very high cross section for the capture of thermal neutrons which is of value in the construction of electric atomic power stations. Organic derivs as shift reagents in NMR spectroscopy: C. C. Hinckley, *J. Am. Chem. Soc.* 91, 5160 (1969); R. E. Sievers, *Nuclear Magnetic Resonance Shift Reagents* (Academic Press, New York, 1973).

3947. **Evacetrapib.** [illegible] LY-2484595. [illegible] mol wt 638.66. C [illegible] Cholesteryl ester transfer protein (CETP) inhibitor. Prepn: [illegible] *et al.*, WO 06002342; [illegible] *et al.*, US 7786163; [illegible] *et al.*, US 10031309 (2006, 2010, 2010 all to Lilly). Pharmacology: G. Cao *et al.*, *J. Lipid Res.* 52, 2169 (2011). Clinical effect on serum cholesterol levels in patients with dyslipidemia: S. J. Nicholls *et al.*, *J. Am. Med. Assoc.* 306, 2099 (2011).



White amorphous solid or hydrated crystals from water.
THERAP CAT: Antilipemic.

3948. **Evan's Blue.** [illegible] 6,6'-[(3,3'-Dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid] tetrasodium salt; [illegible] C.I. Direct Blue 53; [illegible] Azovan Blue. [illegible] mol wt 960.79. C 42.50%, H 2.52%, N 8.75%, Na 9.57%, O [illegible] S 13.35%. Prepd by coupling 1 mol of diazotized [illegible] with 2 mols of [illegible] acid [illegible] DE 35341; DE 38802 [illegible] 1887), DE 39949, DE 57327; DE 75469 [illegible] 1899). [illegible] Fieser, *Org. Synth.* coll. vol. II, 145 (1943). [illegible] M. H. Nielsen, N. C. Nielsen, *Scand. J. Clin. Lab. Invest.* 13, 605 (1961); O. Linde-[illegible] 125, 115 (197?).



Blue crystals with bronze to green luster. Sol in water, alcohol, acids, alkalies. [illegible] changing color near pH 10. Destroyed by strong oxidizing and reducing agents and precipitated from soln by strong [illegible] of neutral salts. Rather stable in aq soln, and may be autoclaved at 15 lbs pressure for 30 min. Dye made up in physiological saline should not be autoclaved.

THERAP CAT: Diagnostic aid (blood volume determination).

3949. **Evening Primrose Oil.** EPO. Seed oil of the evening primrose, *Oenothera biennis* L., [illegible], which contains approx 72% linoleic acid and approx 9% γ-linolenic acid, *q.v.*, as the two main constituents. Unique among vegetable oils because of its high content of γ-linolenic acid. Effect on prostaglandin biosynthesis in rats: [illegible] *et al.*, *Prostaglandins Leukotrienes Med.* 8, 275 (1982). Clinical studies in atopic eczema: C. R. Lovell *et al.*, *Lancet* 1, 278 (1981); S. Wright, J. L. Burton, *ibid.* 2, 1120 (1982); [illegible] Ingredient in cosmetics for [illegible] skin: J. P. Morin, DE 3447628 (1985 to [illegible]), *C.A.* 103, 110058 (1985). Brief review including discussion of [illegible]: A. J. Barber, *Pharm. J.* 240, 723-725 (1988).

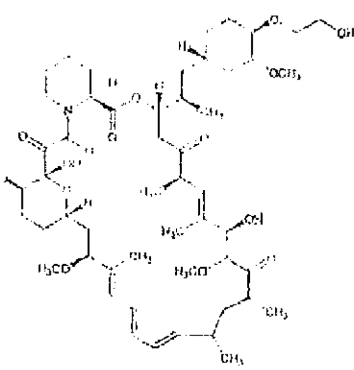
Clear, golden yellow oil. d_{15} 0.9258; $n_D^{?}$ 1.4792. [illegible] 787.8. Iodine no. 154.8.

Note: Evening primrose oil products include *Efamol, Efamast, Epogam.*

USE: Dietary supplement.

THERAP CAT: In treatment of atopic eczema and mastalgia.

3950. **Everolimus.** [159351-69-6] [illegible] 40-O-(2-hydroxyethyl)rapamycin; RAD 001; SDZ RAD; Afinitor; Certican; Votubia; Zortress. [illegible] mol wt 958.22. C 66.33%, H 8.73%, N 1.46%, O 23.37%. Macrolide immunosuppressant; derivative of rapamycin, *q.v.* Inhibitor of the [illegible] which suppresses cytokine-mediated lymphocyte proliferation. Prepn: S. Cottens, R. Sedrani, WO 9409010; *eidem*, US 5665772 (1994, 1997 both to Sandoz). Pharmacology: W. Schuler *et al.*, *Transplantation* 64, 36 (1997). Whole blood determn by LC-MS: N. Brignol *et al.*, *Rapid Commun. Mass Spectrom.* 15, 898 (2001); by HPLC: S. Baldelli *et al.*, *J. Chromatogr. B* 816, 99 (2005). Clinical pharmacokinetics in combination with cyclosporine: J. M. Kovarik *et al.*, *Clin. Pharmacol. Ther.* 69, 48 (2001). Clinical study in prevention of cardiac-allograft vasculopathy: H. J. Eisen *et al.*, *N. Engl. J. Med.* 349, 847 (2003); in advanced renal cell carcinoma: R. J. Motzer *et al.*, *Lancet* 372, 449 (2008). Clinical trial for treatment of subependymal giant cell astrocytomas (SEGA) in tuberous sclerosis: D. A. Krueger *et al.*, *N. Engl. J. Med.* 363, 1801 (2010). *Reviews:* F. J. Dumont *et al.*, *Curr. Opin. Invest. Drugs* 2, 1220-1234 (2001); B. Nashan, *Ther. Drug Monit.* 24, 53-58 (2002).



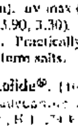
THERAP CAT: Immunosuppressant; antineoplastic.

3951. **Evodiamine.** [518-17-2] (13bS)-8,13,13b,14-Tetrahydro-14-methylindolo[2',3':3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one.

$C_{19}H_{17}N_3O$; mol wt 303.37. [illegible]
5.27%. From *Evodia rutaecarp*[illegible]
thoxylum rhetsa DC., Rutace[illegible]
Pharm. Soc. Jpn. 1915, 1293, [illegible]
Tetrahedron 8, 293 (1960). S[illegible]
Jpn. 1924, 1; Chu, *J. Pharm.* [illegible]
(1951). Synthesis: Asahina, [illegible]
[illegible] *et al.*, *J. Am. Chem. Soc.* 9[illegible]
d, 23 (1976). Biosynthesis: *M*[illegible]
1966, 3221; *ibid.* 1967, 3317, [illegible]
Chim. Acad. Sci. Hung. 89, 85 [illegible]

Yellow plates from alc, mp [illegible]
[illegible]440° (chloroform). uv max ([illegible]
(log ε 4.06, 4.02, 3.90, 3.30). [illegible]
ether, chloroform. Practically [illegible]
Does not seem to form salts.

3952. **Exaltolide®.** [illegible]
15-hydroxypentadecanoic [illegible]
240.39. C 74.95%, H [illegible]
for the pleasant odor of angelic[illegible]
Helv. Chim. Acta 11, 1159 (19[illegible]
don) 1960, 559; Dhekne *et al*[illegible]
Bhattacharya, *ibid.* 1963, 35[illegible]
54, 2889 (1971). Alternate syn[illegible]
Lett. 1977, 441. K. Narasaka *e*[illegible]
M. Kellogg, *Chem. Commun.* [illegible]



Thick oil. Odor of amber a[illegible]
0.9564; n_D^{25} 1.4708. When su[illegible]
Sol in alc.

USE: As a fixative in perfum[illegible]

3953. **Exametazime.** [illegible]
3,3'-[(2,2-Dimethyl-1,3-propa[illegible]
dioxime; (*RR,SS*)-4,8-diaza-3,6[illegible]
bisoxime; (±)-(3*RS*,3'*RS*)-3,[illegible]
ino)]di-2-butanone dioxime; *d*,[illegible]
d,l-HM-PAO; hexametazime; C[illegible]
C 57.32%, H 10.36%, N 20.57[illegible]
derivative that forms a neutral, [illegible]
diffuses across cellular membr[illegible]
Used in regional cerebral blood [illegible]
et al., EP 194843; *eidem*, US [illegible]
sham). Prepn and radiopharma[illegible]
Neirinckx *et al.*, *Nucl. Med. Com*[illegible]
et al., *J. Nucl. Med.* 28, 191 (1[illegible]
P. F. Sharp *et al.*, *ibid.* 27, 17[illegible]
K. Nakamura *et al.*, *Eur. J.* [illegible]
evaluation in single photon em[illegible]
ingiomas: S. Nakano *et al.*, *J.* [illegible]
radiolabeling of human platel[illegible]
Med. Commun. 9, 267 (1986). [illegible]
et al., *ibid.* 465. Clinical stud[illegible]
inflammatory lesions: J. H. Re[illegible]

D1

DORLAND'S ILLUSTRATED MEDICAL DICTIONARY

32nd EDITION

ELSEVIER
SAUNDERS

...vening (sequence) + -on] in eukaryotic genes, a ...sequence separating two coding sequences (exons), it is ...into heterogeneous nuclear RNA (hnRNA) as part of ...script, but is spliced out in the generation of mature mes... (mRNA), prior to translation. By extension, the term also ...sequences in the primary RNA transcript. Called also ...

...(Roferon) trademark for a preparation of interferon alfa-2b.

...(Ro-hyp-nin) trademark for a preparation of dopamine hydro...

...(in"tro-spek'shan) [*intro-* + L. *specere* to look] the ...observation of one's own thoughts and feelings, self anal...

...sception (in"tro-sep'shan) intussusception.

...version (in"tro-ver'zhan) [*intro-* + *vertere*] 1. the turning out... or less completely, of an organ, or the resulting condi... turning inward to the self of one's interest, with lack of ... external world. 3. introversion.

...vert (in'tro-vert) 1. a person whose interest is turned inward to... to turn one's interest inward to the self. 3. a structure that ... or drawn inwards. 4. to turn a part or organ inward upon...

...vert (in"tro-vert'shan) in orthodontic therapy, a technique of de... teeth back into the occlusal plane in an effort to prevent its ... elongation during the correction of an excessive overbite. ... *deep bite*. Cf. *extrusion* (def. 3).

...vert (in'tro-vert) to treat by intubation.

...vation (in"tro-va'shan) [L. *in* into + *tubus* tube] the insertion of a ... body canal or cavity; see also *extubation* and *catheterization*.

...cheal i. insertion of an endotracheal tube; see under *tube*.

... insertion of a tube through the nose, such as a nasogastric or ... al tube.

...cheal i. insertion of a nasotracheal tube.

... insertion of a tube through the mouth.

...cheal i. insertion of an orotracheal tube.

...tionist (in"tro-va'shan-ist) an individual who performs in...

...tor (in'tro-va'tor) an instrument used in intubation.

...esce (in"tu-mes'ĕ) to swell up.

...escence (in"tu-mes'ens) [L. *intumescentia*] 1. a swelling. ... stromal. 2. the process of swelling.

...escent (in"tu-mes'ent) [L. *intumescens*] swelling or becoming ...

...tion (in"tu-mes'shun) [L. *intumescentia* rise] an ... or swelling.

...dis [TA] cervical enlargement, the enlargement of the cervical ... at the level of attachment of the nerves to the upper limbs, ... extending from the third to the second thoracic vertebra.

...sacra'lis [TA] lumbosacral enlargement, the enlargement of ... spinal cord at the level of attachment of the nerves to the lower ... usually extending from the first lumbar to the third sacral vertebra.

...nica [TA] Sympathetic enlargement, a pseudoganglion on the ... branch (nerve) of the glossopharyngeal nerve; called also *ganglion* ... TA alternative, *retropharyngeal ganglion*, *tympanic ganglion of Valentin*, ... *parapharyngeal*.

...ception (in"tus-sep'shan) [L. *intus* within + *suscipere* to ... receive] see *in...*. 2. prolapse of one part of the intestine ... of an immediately adjoining part. It is most common in ... adults, it is often associated with a *neoplasm*. Symptoms ... intestinal obstruction, palpable abdominal mass, and abdominal ... cramping.

...cephalic i. invagination of one part of the vermiform appendix into ... tion, ranging in severity from invagination of the tip into the ... lumen to prolapse of the entire appendix into the cecum.

...colonic i. intussusception involving two segments of the colon, ... the enveloping of one intussusception by an even larger one.

... intussusception involving two segments of the small intestine.

... intussusception involving two segments of the ileum.

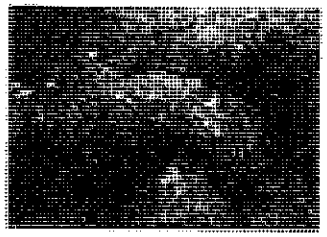
... intussusception at the ileocecal junction, with the cecum ... back into the ileum. Cf. *ileocecal*.

... intussusception at the ileocecal junction, with the distal ... drawn into the colon. Cf. *ileocecal*.

... [illegible] sometimes seen after gastrojejunostomy.

... [illegible] duodenum prolapsing into the stomach.

... [illegible] distal part of the intestine into a prox...



■ Enteric intussusception.

in•tus•sus•cep•tum (in"tə-sə-sep'təm) [L.] in intussusception, the portion of intestine that has been invaginated within another part.

in•tus•sus•cip•i•ens (in"tə-sə-sip'ē-ənz) [L.] in intussusception, the portion of intestine into which another portion has invaginated.

In•u•la (in'u-lə) [L.] a genus of composite flowered plants (family Compositae), whose rhizomes contain inulin. The root has numerous uses in folk medicine.

in•u•lase (in'u-lās) inulinase.

in•u•lin (in'u-lin) an indigestible polysaccharide vegetable starch found in the rhizome of certain plants (Compositae). It is a polymer of fructofuranose, yields fructose on hydrolysis, and is used in a test for determining glomerular filtration rate. See *inulin clearance*, under *clearance*.

in•u•lin•ase (in'u-lin-ās) [EC 3.2.1.7] an enzyme of the hydrolase class that catalyzes the cleavage of specific linkages between fructose residues in inulin, releasing fructose. The enzyme occurs in a variety of fungi and in higher plants.

in•unc•tion (in-ungk'shən) [*in-*[2] + *unction*] the act of anointing or of applying an ointment with rubbing.

in utero (in u'tər-o) [L.] inside the uterus.

Inv [from the name of the propositus] see *Km allotypes*, under *allotype*.

in vac•uo (in vak'u-o) [L.] in a vacuum.

in•vag•i•nate (in-vaj'i-nāt) to unfold one portion of a structure within another portion.

in•vag•i•na•tion (in vaj"i-na'shən) [L. *invaginatus*, from *in* within + *vagina* sheath] 1. the state of being or the process of becoming invaginated. 2. in embryology, a process by which (*a*) one region of a hollow, single-walled, spherical blastula caves in to form and line a new cavity in the now cup-shaped, double-walled gastrula, or (*b*) an ever-deepening pit develops into a diverticulum or tube from the surface into the tissues below. 3. intussusception.

basilar i. a developmental deformity of the occipital bone and upper end of the cervical spine in which the latter appears to have pushed the floor of the occipital bone upward; see also *platybasia*. Called also *basilar impression*.

in•va•lid (in'və-lid) [L. *invalidus*, *in* not + *validus* strong] 1. not well and strong. 2. a person who is disabled by illness or infirmity.

In•vanz (in'vanz) trademark for a preparation of ertapenem sodium.

in•va•sin (in-va'zin) hyaluronidase.

in•va•sion (in-va'zhən) [L. *invasio*, from *in* into + *vadere* to go] 1. the attack or onset of a disease. 2. the infiltration and active destruction of surrounding tissue by a malignant tumor.

in•va•sive (in-va'siv) 1. pertaining to or characterized by invasion. 2. involving puncture or incision of the skin or insertion of an instrument or foreign material into the body; said of diagnostic techniques.

in•va•sive•ness (in-va'siv-nis) 1. the ability of a pathogenic microorganism to enter and spread throughout the body. 2. the ability of a malignant tumor to be invasive.

in•ven•to•ry (in'vən-tor"ē) a comprehensive list of personality traits, aptitudes, and interests.

Beck Depression I. a self-report questionnaire for measuring the symptoms of depression, focusing on the cognitive symptoms.

California Personality I. (CPI) a self-report, true-false test designed to measure aspects of personality style; generally used in counseling situations or for less than severe psychopathology.

Millon Clinical Multiaxial I. (MCMI) a self-report inventory designed to produce a profile of the personality style and structure underlying mental disorders.

Minnesota Multiphasic Personality I. (MMPI) a self-report, true-false test designed to evaluate personality and particularly to assess psychopathology.

D4

The Journal of NUCLEAR MEDICINE

Effect of Interferon-α Treatment on [^{68}Ga-DOTA,Tyr3,Thre8]Octreotide Uptake in CA20948 Tumors: A Small-Animal PET Study

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Effect of Interferon-α Treatment on [^{68}Ga-DOTA,Tyr3,Thre8] Octreotide Uptake in CA20948 Tumors: A Small-Animal PET Study

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In peptide receptor radionuclide therapy of neuroendocrine tumors, improvements have been made by increasing the affinity for receptors and by protecting critical organs (e.g., kidneys). However, tumor parameters involved in radiopeptide uptake are still under investigation. Interferon-α (IFNα) is used as biotherapy for neuroendocrine tumors. Several mechanisms of action are described, but the potential effect of IFNα on tumor uptake of labeled peptide has not been studied in vivo yet. **Methods:** Twenty-six male CA20948 tumor-bearing Lewis rats were imaged before and during IFNα treatment using quantitative small-animal PET with [^{68}Ga-DOTA,Tyr^3,Thre^8]octreotide. Imaging was performed at days 0, 3, and 7. Animals were divided into 3 groups according to the treatment: control (injected daily with saline), half (4 d of IFNα treatment from day 0 to day 3, then saline), and full (7 d of IFNα). A daily dose of IFNα (1.5 mIU) was administered subcutaneously. Quantitative PET results are expressed as percentage injected dose per cm^3 and normalized to baseline (day 0) values. Tumor size was monitored by PET and caliper measurements. **Results:** Gross tumor uptake and tumor volumes increased in all groups over the 7-d period. On day 3, mean ± SD ratios to day 0 were 1.2 ± 0.2, 1.3 ± 0.5, and 1.2 ± 0.4, respectively, for control, half, and full groups. On day 7, respective values were 1.1 ± 0.2, 1.3 ± 0.6, and 1.5 ± 0.4. At day 3, a comparison among groups showed no statistically significant difference. At day 7, the full group showed a significantly higher ratio in activity concentration than the control group ($P = 0.021$). A good correlation was found between tumor volumes assessed by small-animal PET and caliper measurements ($R = 0.89$, $P < 0.0001$). **Conclusion:** As expected, over a period of 7 d, both tumor volumes and radiopeptide uptake increased in all animals. However, the activity concentration increased significantly more at day 7 in animals treated for 7 d with IFNα, compared with controls. This is the first, to our knowledge, in vivo indication that IFNα is able to increase tumor uptake of the labeled analog in a small-animal model of neuroendocrine tumors. The mechanisms underlying this effect (flow, vascular permeability, receptor upregulation) remain unknown and need to be further investigated.

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Most neuroendocrine tumors express a high density of somatostatin receptors (sstrs), allowing the use of this property for diagnostic and therapeutic purposes. Current therapeutic approaches toward gastroenteropancreatic neuroendocrine tumors include surgery, chemoembolization, chemotherapy, and biotherapy using somatostatin analogs or interferon-α (IFNα) (*1*,*2*).

Peptide receptor radionuclide therapy (PRRT) is another therapeutic opportunity that takes advantage of the high sstr expression of tumors to target them with β-emitter–labeled somatostatin analogs. Clinical results showed response in 6%–30% of the patients (*3*). Several procedures have been developed to increase therapy efficiency by protecting critical organs, mainly the kidneys (*4*). However, tumor parameters involved in radiolabeled analog uptake and their potential modulation remain to be investigated.

Introduced in 1983 as therapy for neuroendocrine tumors (*5*), IFNα proved effective, with a symptomatic response in 40%–60% of the patients. Tumor reduction, however, was obtained in only 10%–15% of the patients (*6*). Although some data suggest a cumulative effect of IFNα and somatostatin analogs, because of the lack of evidence current clinical practice does not recommend a combination treatment (*7*). Some data suggest that IFNα could induce an upregulation of sstrs (*7*,*8*), but, to the best of our knowledge, no original article has clearly demonstrated this effect.

Assuming that the amount of receptors at the cell surface limits the peptide uptake, further upregulation of receptors on tumor cells could be a key for optimization of PRRT.

This study aimed to evaluate the effect of IFNα on tumor uptake of the ^{68}Ga-labeled analog [^{68}Ga-DOTA,Tyr^3,Thre^8] octreotide using small-animal PET in the CA20948 rat model of pancreatic neuroendocrine tumor. The ability of

PET quantitative imaging to follow the same animal over time was exploited to compare the tumor uptake in each animal after experimental treatment with its own baseline uptake values.

MATERIALS AND METHODS

Study Design

To compare the increase of [^{68}Ga-DOTA,Tyr^3,Thre^8]octreotide uptake in tumors, animals were randomly assigned to 3 groups, according to the IFNα treatment: control (no IFNα treatment, subcutaneous injection of 100 μL of saline daily), half (subcutaneous injection of IFNα for 4 d, then saline for 3 d), and full (subcutaneous injection of IFNα for 7 d). Imaging was performed on days 0 (baseline imaging just before the first injection of IFNα or saline), 3, and 7. The general design of the treatment and imaging studies is depicted in Figure 1.

Animals and Tumor Inoculation

Twenty-eight male Lewis rats (age, 7 wk) were subcutaneously injected with 1 mL (~10^8 cells/mL) of tumor cell suspension prepared with crude unfrozen CA20948 tumor tissue in Dulbecco's modified Eagle's medium plus GlutaMax-1 (Invitrogen Corp.).

The CA20948 neuroendocrine exocrine pancreatic tumor cell line was previously well characterized (*9*). It was shown to strongly express sstr 2 at the cell surface; this expression was quantitated, and the binding of sstr analogs in vivo was shown to be receptor-specific (*9,10*). In addition, this cell line internalizes several ^{111}In-labeled somatostatin analogs and was used previously to demonstrate the effect of PRRT (*11*).

Rats were purchased from Charles River Ltd. Tumors were allowed to grow for 15–20 d after inoculation before study. Animals were housed 2 or 3 per cage and fed ad libitum. All imaging procedures were performed under continuous isoflurane anesthesia (induction, 3%; maintenance, 1.5%; Forene [Abbott Laboratories Ltd.]). Animals were housed in a facility approved by the Belgian Ministry of Agriculture in accordance with current regulations and standards. The experimental design was approved by the Ethics Committee on animal experimentations of the Medical School of the Université Catholique de Louvain. The principles of laboratory animal care (*12*) were strictly followed.

[^{68}Ga-DOTA,Tyr^3,Thre^8]Octreotide

[DOTA,Tyr^3,Thre^8]octreotide was provided by Biosynthema. The ^{68}Ge/^{68}Ga generator was obtained from Cyclotron Ltd. Elution was performed with 0.1 M ultrapure HCl (prepared from ultrapure HCl 30% TraceSelectUltra and Ultrapure water; Fluka). Labeling was performed by adding 2 μg of peptide and 8 μL of 2.5 M Na acetate to 150 μL of eluate containing 30–90 MBq of ^{68}Ga activity and by heating the solution for 10 min at 80°C, as described previously (*13*). The reaction solution was then cooled in ice-cold water for 5 min. Five microliters of ethylenediaminetetraacetic acid (5 mM) were added to chelate any residual ^{68}Ga. Saline was added to reach a final volume of 600 μL; the resulting solution was used for 2 animals, each of which received 1 μg of peptide and approximately 15–45 MBq of ^{68}Ga. Radiopharmaceutical purity was assessed by high-performance liquid chromatography (HPLC) using a C18, 150/4.6 Nucleosil 100-5 column and HPLC-grade acetonitrile as a solvent (Chromanorm for HPLC—Gradient Grade; Macherey-Nagel) and was in excess of 90% for all experiments.

IFNα Treatment

IFNα (Intron A; Schering-Plough) was injected subcutaneously once a day at 1.5 mIU (~4–5 mIU/kg). All animals, including those in the control group, received paracetamol in drinking water (1 mg/mL). Rats were weighed daily, and tumor dimensions were measured by the caliper method (product of 2 largest diameters). The surface measurements (S) obtained using the caliper method were fitted with:

$$S = aV^b, \qquad \text{Eq. 1}$$

where V is the volume of interest (VOI; as determined by PET), and parameters a and b are not constrained.

Image Acquisition

PET images were acquired with the Mosaic (Philips) scanner. Activity was measured in all syringes before and after injection using a dose calibrator. Immediately after tracer injection, a transmission scan was acquired in single mode using a 370-MBq ^{137}Cs source for attenuation correction. A short scan for emission contamination correction was obtained thereafter. A 15-min emission scan was started about 25 min after injection of [^{68}Ga-DOTA, Tyr^3,Thre^8]octreotide. At the end of the emission scan, recovery from anesthesia was reached within 5 min in all cases.

Reconstruction and Quantification of Uptake

Transmission data were reconstructed after emission contamination correction. Raw data were corrected for attenuation, random, and scatter coincidences and for system dead-time. All images were reconstructed with a fully 3-dimensional row-action maximum likelihood algorithm. Each reconstructed matrix was composed of 120 transverse 128 × 128 images with voxels of 1 mm^3.

VOIs were drawn manually on tumors by the same operator using PMOD software (version 2.75; PMOD Technologies Ltd.). VOI statistics were decay-corrected and converted to injected dose

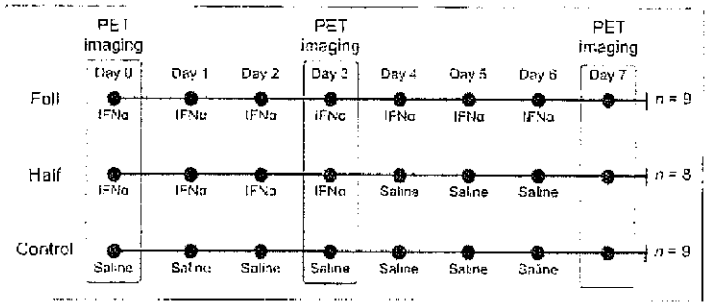

FIGURE 1. Design of study.

using an external standard for calculation of percentage injected dose (%ID) in tumors. Results are expressed as %ID/cm^3 after correction for VOI. Uptake data were normalized to day 0 for each animal.

Statistical Analysis

Unless otherwise stated, data are presented as mean ± SD. The Student *t* test, paired Student *t* test, Pearson correlation test, or nonlinear regression was used, as appropriate. The Shapiro–Wilk normality test was applied to assess gaussian distribution in small groups. All tests were performed using Prism software (version 5.0; GraphPad Software).

RESULTS

Effect of IFNα Treatment

No rat receiving IFNα treatment presented limiting toxicity that precluded the continuation of the study. One rat was excluded from the control group because of technical failure, and 1 rat from the full group was excluded because of death due to tumor growth. In the control, half, and full groups, 9, 8, and 9 rats, respectively, could be fully analyzed. Over a period of 7 d, rats in all groups showed a reduction in body weight ranging between 0.5% and 16%. The mean rat weight on day 0 was 340.0 ± 34.5, 318.8 ± 27.6, and 331.3 ± 31.7 g, respectively, in control, half, and full groups. On day 7, weight was 321.9 ± 39.5, 296.4 ± 34.3, and 317.9 ± 31.0 g, respectively. No statistically significant difference was found among the groups at any day (Student *t* test, $P > 0.11$; all groups successfully passed the Shapiro–Wilk normality test).

Tumor Uptake After IFNα Treatment

All tumors displayed a relatively heterogeneous uptake at all times. Tumor uptake, expressed as %ID/cm^3, is reported in Table 1. No statistically significant difference among the groups for %ID/cm^3 values was found at any time. In the control group, a statistically significant difference was noted between day 0 and day 3 (paired *t* test, $P = 0.007$), whereas in the fully treated group, a statistically significant difference was observed between day 0 and day 7 (Wilcoxon matched-pair test, $P = 0.01$) and between day 3 and day 7 (Wilcoxon matched-pair test, $P = 0.02$).

TABLE 1
%ID/cm^3 of Tumor Tissue Measured by PET in 3 Groups at Days 0 (baseline), 3, and 7

Group	Day 0	Day 3	Day 7
Control	0.50 ± 0.11	0.60 ± 0.17*	0.55 ± 0.11
Half	0.50 ± 0.13	0.58 ± 0.16	0.60 ± 0.17
Full	0.45 ± 0.17	0.50 ± 0.10	0.64 ± 0.17*†

*$P < 0.05$ vs. day 0.
†$P < 0.05$ vs. day 3.
Data are mean ± SD.

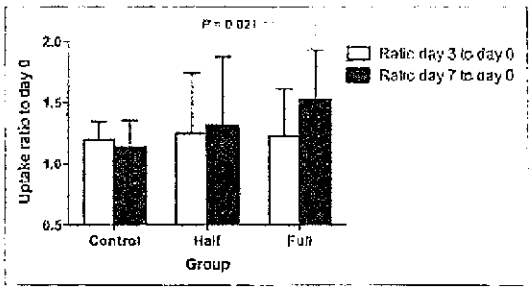

FIGURE 2. Mean ± SD ratios to day 0 of activity concentration (%ID/cm^3) at days 3 and 7. Statistically significant difference between control and full groups is observed at day 7.

The ratio of uptake to day 0 was computed to compare each animal with its own baseline values. On day 3, ratios to day 0 were 1.2 ± 0.2, 1.3 ± 0.5, and 1.2 ± 0.4, respectively, for control, half, and full groups. On day 7, respective values were 1.1 ± 0.2, 1.3 ± 0.6, and 1.5 ± 0.4. All groups passed the Shapiro–Wilk normality test. At day 3, the comparison among groups showed no significant differences. At day 7, a statistically significant difference was found between the control group and the fully treated group ($P = 0.021$, Fig. 2). In addition, only the full group showed a statistically significant increase in concentration between day 3 and day 7, as assessed by the respective ratios to day 0 ($P = 0.013$).

Tumor Volume After IFNα Treatment

Tumor volume data are shown in Figure 3. Tumor volumes increased significantly in all groups over time (paired Student *t* test, $P < 0.01$ in all cases). On day 0, VOIs were 10.9 ± 3.7, 10.8 ± 5.1, and 13.4 ± 3.5 cm^3, respectively, in control, half, and full groups. On day 3, volumes were 16.8 ± 6.5, 14.8 ± 4.0, and 19.0 ± 5.4 cm^3, respectively, and on day 7, volumes were 24.8 ± 11.5, 19.7 ± 7.3, and 25.8 ± 8.5 cm^3, respectively. No statistical difference in VOIs was

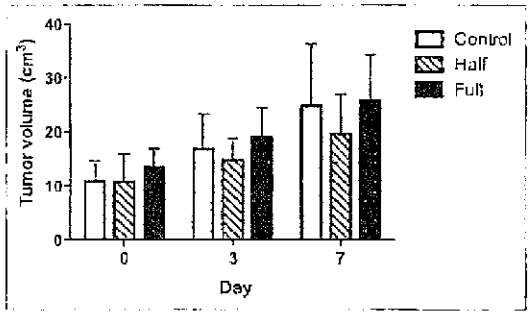

FIGURE 3. Mean ± SD tumor volumes assessed by small-animal PET VOIs at each imaging session. No statistically significant difference between groups was found.

noted among groups, whatever the day (Student t test, $P > 0.09$ in all cases).

A nonlinear regression correlation between volume drawing (VOIs) and tumor surface determined by the caliper method was fitted to Equation 1 and gave a value for S of $1.28\ V^{0.65}$ ($R = 0.89$, Fig. 4).

DISCUSSION

This study demonstrates that IFNα administered over 7 d affects the uptake of the ^{68}Ga-labeled somatostatin analog [DOTA,Tyr^3,Thre^8]octreotide in the CA20948 animal model of pancreatic neuroendocrine tumors. The quantitative capability of PET allowed uptake in the animal to be followed over time, using day 0 imaging of each animal as its own baseline value.

Although this kind of experiment cannot fully explain all mechanisms involved, it has the double advantage of reduction of the number of animals in a longitudinal pharmacologic study and direct observation of the sum of all potential effects. Conversely, it does not allow the dissection of the possible involved mechanisms, which requires appropriate additional investigations.

Because of the significant variance in tumor size at the 3 studied time points (i.e., days 0 [baseline], 3, and 7), the gross uptake (expressed as %ID/cm^3) did not differ with statistical significance among groups. However, when rescaling all data to the baseline value (which is somehow an intrinsic tumor feature unaffected by the potential therapeutic effect of IFNα), we noted a significant increase in uptake at day 7, but only in the fully treated group.

Effects of IFNα have been previously studied, and different mechanisms of action were reported. IFNα interacts with specific cell-surface receptors inducing transcription of several genes (*14–17*). Observed effects of IFNα are fibrosis of the tumor (*18*), antiangiogenic effect by inhibiting transcription of the vascular endothelial growth factor gene (*19,20*), induction of apoptosis (*6,21*), and blockade of cell cycle (*22*). IFNα was also reported to increase the expression of some receptors, such as class I antigens (*23*), urokinase-type plasminogen activator receptor (*24*), and epidermal growth factor receptor (*25*), at the cell surface.

In clinical practice, combination of biotherapies using IFNα and somatostatin analogs is somewhat controversial. In vitro, a clear additive inhibitory effect was demonstrated on pituitary adenoma cells (*26*). In patients, although some studies do not show evidence of improved response by combination therapies (*6,7,27*), others advocate such combinations in individual cases (*2,23,28,29*).

In this study, we investigated IFNα used alone with the goal of optimizing PRRT, by potentially increasing the analog uptake in tumor cells. We cannot definitely infer from these data that the expression of sstrs is upregulated, but the final objective—that is, increased uptake of the labeled analog—was observed.

A few tumor parameters involved in peptide uptake have been studied so far. In vitro, upregulation of sstrs was observed after chronic exposure to cold octreotide (*30*). This effect was confirmed by preclinical studies and proved to be present only after prolonged or chronic exposure and not after single administration of the analog (*31,32*). In addition, upregulation of sstrs was shown in rats treated with suboptimal doses of ^{177}Lu-[DOTA,Tyr^3,Thre^8]octreotide (*33*). IFNα was described as increasing the density of sstrs at the cell surface (*7,8*), but—to the best of our knowledge (PubMed search)—this increase was reported only as a cumulative effect of IFNα and somatostatin analogs on tumor cells. We found no original article demonstrating specifically the upregulation of sstrs by IFNα treatment either in vivo or in vitro. Further, the cellular or biochemical mechanisms involved remain entirely unexplained.

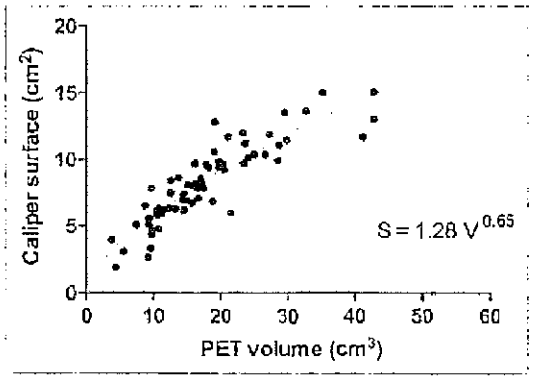

FIGURE 4. Nonlinear correlation between tumor volumes assessed by small-animal PET and caliper measurements (2 largest perpendicular diameters). $R = 0.89$.

We can conclude from the current study that ours is the first data indicating that IFNα increases somatostatin analog uptake in a model of neuroendocrine tumors. As for every experimental protocol, potential biases inherent to the animal model should be discussed.

First, it is impossible to predict with certainty to what extent the animal data can be extrapolated to humans. Clinical studies should be undertaken to address this issue by comparing the uptake of the radiolabeled analog in IFNα-treated patients before and during treatment. In addition, human-recombinant IFNα was used here to treat rats, and species dependency may influence results observed in humans in a similar setting. Third, uptake was assessed by the concentration of labeled peptide in tumors, namely, the activity divided by the volume (%ID/cm^3). Tumor volumes were determined by manually drawn VOIs around the tumor, and this bias might be a source of error. However, as a double-check test, we found an excellent correlation ($R = 0.89$) between the PET volume and 2-dimensional caliper measurements used as an accepted reference method. More convincing, however, was the fact that the best fit gave a value of b close to two thirds, which is the theoretic value

to translate a volume into a surface. Fourth, the dosage was based on doses used in rats for pharmacologic studies as reported in the literature (*34*). The duration of the treatment was intrinsically limited by the natural growth of CA20948 tumors. As a matter of fact, baseline imaging has to be performed on tumors that have already reached a sufficient volume for accurate delineation and reduction of the partial-volume effect (i.e., tumors ~1 cm in diameter); conversely, the last imaging session has to be performed before tumors reach unacceptable sizes according to the basic standards for humane handling of laboratory animals (i.e., ~3–4 cm in largest diameter) (*35*).

Cellular or vascular microenvironment mechanisms involved in this increased uptake concentration are yet unknown. Further research is required to understand the relationship between IFNα treatment and increased uptake, and several hypotheses have to be investigated.

First, whether the expression of sstrs is upregulated after treatment with IFNα can be evaluated by binding assays ex vivo on tumors grown in vivo. This procedure is arduous because in such a model, the role of heterogeneity and necrosis must be considered for data analysis as they may be confounding. Second, increased uptake after IFNα could be specific to this tumor model, and repeating such experiments with another tumor line would be justified. The ideal tumor line would be one with neuroendocrine features expressing high levels of sstrs, but with a slower growing time that would allow a longer follow-up. Third, increased flow might be speculated to be a way to enhance peptide delivery to the tumor. However, an antiangiogenic effect of IFNα was demonstrated in several animal models (*36–38*) and in neuroendocrine tumors in humans (*20*). In addition, no increased blood flow was shown in patients with carcinoid tumors receiving chronic IFNα treatment (*39*). Conversely, IFNα was shown to increase blood flow only within 60–90 min after injection in a model of melanoma (*40,41*); this finding does not apply to our studies, in which the last IFNα injection was performed 24 h before the last imaging session, as illustrated in Figure 1. Finally, we have previously shown that blood flow in this tumor model cannot be investigated accurately by PET, because it proved to be low (*32*), thus definitively limiting the exploration of this avenue using noninvasive methods.

CONCLUSION

IFNα given daily for 7 d was shown to further increase [^{68}Ga-DOTA,Tyr^3,Thre^8]octreotide uptake in CA20948 tumors, as compared with control groups. This study can be considered as a proof of concept for future studies on the influence of IFNα on somatostatin analog tumor uptake and tumor-absorbed doses. The cellular mechanisms involved in this effect remain undetermined, and studies are needed to assess the dose–response relationship, effect on tumors in terms of absorbed doses in a therapeutic setting, and, finally, indication for IFNα before PRRT for neuroendocrine tumors using somatostatin analogs.

DISCLOSURE STATEMENT

The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

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Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory David Ferraro./Cindy Klepacky
Attorney Docket Number:	PAT034678-US-PCT

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt	
EFS ID:	21393000
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Gregory David Ferraro./Cindy Klepacky
Filer Authorized By:	Gregory David Ferraro.
Attorney Docket Number:	PAT034678-US-PCT
Receipt Date:	04-FEB-2015
Filing Date:	19-MAY-2008
Time Stamp:	10:23:18
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	18398
Deposit Account	190134
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	PAT034678_US_PCT_2015_Feb3_IDS.pdf	678948 1cd06b75e4027a2ea1b14c520119e7721b937c83	no	4
Warnings:					
Information:					
A U.S. Patent Number Citation is required in the Information Disclosure Statement (IDS) form. You may remove the form to add the required data in order to correct the Informational Message or if you chose not to, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.					
2	Non Patent Literature	D1Dorlands_Dictionary.pdf	243723 7254f92fae52a7e82ab3898222d0b2c5ab6b2aa5	no	2
Warnings:					
Information:					
3	Non Patent Literature	D3Merck_Index_Fifteenth_Edition.pdf	270771 cdf1f94afe7efe33e6a310a95fff23e89a1096fa	no	2
Warnings:					
Information:					
4	Non Patent Literature	D4_Hanin.pdf	432024 52742b5ca407d48ff39a1c0c3d7a92fb694160f4	no	7
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30610 4b5914aaacf4cc4cdbf9fc409abd1fdea6165 8cc	no	2
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or Fax (571)-273-2885

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1095 7590 01/30/2015
NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT03-4678-US-PCT	9572

TITLE OF INVENTION: Neuroendocrine Tumor Treatment

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	04/30/2015

EXAMINER	ART UNIT	CLASS-SUBCLASS
JEAN-LOUIS, SAMIRA JM	1627	514-183000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" Indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. Use of a Customer Number is required.

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 Gregory Ferraro
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Novartis AG
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Basel, Switzerland

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies

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5. Change in Entity Status (from status indicated above)
☐ Applicant certifying micro entity status. See 37 CFR 1.29
NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
☐ Applicant asserting small entity status. See 37 CFR 1.27
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
☐ Applicant changing to regular undiscounted fee status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

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Authorized Signature Gregory Ferraro Date 2/5/15
Typed or printed name Gregory Ferraro Registration No. 36134

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Electronic Patent Application Fee Transmittal

Application Number:	12094173
Filing Date:	19-May-2008
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Filer:	Gregory David Ferraro./Cindy Klepacky
Attorney Docket Number:	PAT034678-US-PCT

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Utility Appl Issue Fee	1501	1	960	960
Publ. Fee- Early, Voluntary, or Normal	1504	1	0	0
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**960**

Electronic Acknowledgement Receipt	
EFS ID:	21407050
Application Number:	12094173
International Application Number:	
Confirmation Number:	9572
Title of Invention:	Neuroendocrine Tumor Treatment
First Named Inventor/Applicant Name:	Peter Wayne Marks
Customer Number:	1095
Filer:	Gregory David Ferraro./Cindy Klepacky
Filer Authorized By:	Gregory David Ferraro.
Attorney Docket Number:	PAT034678-US-PCT
Receipt Date:	05-FEB-2015
Filing Date:	19-MAY-2008
Time Stamp:	11:18:40
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$960
RAM confirmation Number	30963
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Authorized User	
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	PAT034678_US_PCT_2015_FEB5_Issue_Fee_Payment.pdf	53813 8a85567e054c1e0413cc2c2fb8facf81d5b038680	no	1

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2	Fee Worksheet (SB06)	fee-info.pdf	32346 fdd6367a289a0f8780108933d8bf34cbff341fd	no	2

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Total Files Size (in bytes):	86159

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	05/19/2008	Peter Wayne Marks	PAT034678-US-PCT	9572

1095 7590 02/23/2015

NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
JEAN-LOUIS, SAMIRA JM	

ART UNIT	PAPER NUMBER
1627	

NOTIFICATION DATE	DELIVERY MODE
02/23/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

phip.patents@novartis.com

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
12/094,173	19 May, 2008	MARKS ET AL.	PAT034678-US-PCT

NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

EXAMINER	
SAMIRA JEAN-LOUIS	
ART UNIT	PAPER
1627	20150213-A

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The IDS submitted on 01/26/14 and 02/04/15 have been considered and are entered into record.

/SAMIRA JEAN-LOUIS/
Primary Examiner, Art Unit 1627

PTO-90C (Rev.04-03)

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	1627
	Examiner Name	Jean-Louis, Samira JM
	Attorney Docket Number	PAT034678-US-PCT

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	1627
	Examiner Name	Jean-Louis, Samira JM
	Attorney Docket Number	PAT034678-US-PCT

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/S.J.L./	1	Hofsli, Eva et al., "Expression of Chromogranin A and Somatostatin Receptors in pancreatic AR42J Cells", Molecular and Cellular Endocrinology, Vol. 194, pp. 165-173 2002	☐

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Examiner Signature	/Samira Jean-louis/	Date Considered	02/13/2015

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	1627
	Examiner Name	Jean-Louis, Samira JM
	Attorney Docket Number	PAT034678-US-PCT

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Gregory Ferraro/	Date (YYYY-MM-DD)	2015-01-26
Name/Print	Gregory Ferraro	Registration Number	36134

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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	Application Number	12094173
	Filing Date	2008-05-19
	First Named Inventor	Peter Wayne Marks
	Art Unit	1627
	Examiner Name	Jean-Louis, Samira
	Attorney Docket Number	PAT034678-US-PCT

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/S.J.L./	1	Dorlands Illustrated Medical Dictionary, "Dorlands Illustrated Medical Dictionary", Elsevier Saunders, 2012, Ed. 32nd 955	☐
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Examiner Signature	/Samira Jean-louis/	Date Considered	02/13/2015

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Gregory Ferraro/	Date (YYYY-MM-DD)	2015-02-03
Name/Print	Gregory Ferraro	Registration Number	36134

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/094,173	04/14/2015	9006224	PAT034678-US-PCT	9572

1095 7590 03/25/2015

NOVARTIS PHARMACEUTICAL CORPORATION
INTELLECTUAL PROPERTY DEPARTMENT
ONE HEALTH PLAZA 433/2
EAST HANOVER, NJ 07936-1080

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 589 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Peter Wayne Marks, Woodbridge, CT;
David Lebwohl, Madison, NJ;