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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

_____ _____

Printed Name Signature

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant: NIYIKIZA Clet	
Title: NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.: X-14173B	

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Introductory Comments

Please amend the accompanying application as follows:

Amendments to the Specification are reflected on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims, which begins on page 3 of this paper.

Remarks/Arguments begin on page 6 of this paper.

Amendments to the Specification

At page 1, line 2, please insert the following replacement paragraph:

This application is a divisional of Application No. 11/288,807, filed 29 November 2005, which is a divisional of Application No. 10/297,821 filed 05 December 2002, now Patent Number 7,053,065, which claims priority under 35 USC 371, for PCT/US01/14860, filed 15 June 2001, which claims the priority of U.S. provisional applications No. 60/215,310, filed 30 June 2000, No. 60/235,859, filed 27 September 2000, and No. 60/284,448, filed 18 April 2001.

Please replace paragraph [0024], at page 6, lines 6-16, with the following amended paragraph:

[0024] The terms "antifolate" and "antifolate drug" refer to a chemical compound which inhibits at least one key folate-requiring enzyme of the thymidine or purine biosynthetic pathways, preferably thymidylate synthase ("TS"), dihydrofolate reductase ("DHFR"), or glycinamide ribonucleotide formyltransferase ("GARFT"), by competing with reduced folates for binding sites of these enzymes. Preferred examples of antifolates include ~~5-fluorouracil, as manufactured by Glaxo;~~ Tomudex®, as manufactured by Zeneca; Methotrexate®, as manufactured by Lederle; Lometrexol®, as manufactured by Tularik; pyrido[2,3-d]pyrimidine derivatives described by Taylor et al in U.S. Pat. Nos. 4,684,653, 4,833,145, 4,902,796, 4,871,743, and 4,882,334; derivatives described by Akimoto in U.S. Pat. No. 4,997,838; thymidylate synthase inhibitors as found in EPO application 239,362; and most preferred, Pemetrexed ~~Sodium~~ Disodium (ALIMTA), as manufactured by Eli Lilly & Co.

Amendments to the Claims

This listing of claims will replace all prior versions and listings of claims in the application.

Listing of Claims:

Claims 1-28. Cancelled

29. (New) An improved method for administering pemetrexed disodium to a patient in need of chemotherapeutic treatment, wherein the improvement comprises:

a) administration of between 350 μg and 1000 μg of folic acid, daily beginning approximately 1 to 3 weeks before treatment with pemetrexed disodium;

b) administration of a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12}, hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin, wherein the methylmalonic acid lowering agent is administered from about 1 to about 3 weeks prior to the first administration of pemetrexed disodium; and

c) administration of pemetrexed disodium in combination with between 350 μg and 1000μg of folic acid, daily, until administration of pemetrexed disodium is discontinued, and a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12}, hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin, wherein the methylmalonic acid lowering agent administration is repeated from about every 6 weeks to about every 12 weeks, until administration of pemetrexed disodium is discontinued.

30. (New) The improved method of **Claim 29** wherein the methylmalonic acid lowering agent is vitamin B_{12}.

31. (New) The improved method of Claim **30** wherein about 500μg to about 1500μg of vitamin B_{12} is administered.

32. (New) The improved method of **Claim 31** wherein about 1000 μg of vitamin B_{12} is administered.

33. (New) The improved method of **Claim 29** wherein the methylmalonic acid lowering agent is administered by an intramuscular injection, orally, or as a parenteral.

34. (New) The improved method of **Claim 33** wherein the methylmalonic acid lowering agent is administered by an intramuscular injection.

35. (New) The improved method of **Claim 34** wherein the methylmalonic acid lowering agent administration is repeated about every 9 weeks, until administration of pemetrexed disodium is discontinued.

36. (New) The improved method of **Claim 32** wherein vitamin B_{12} is administered by an intramuscular injection, orally, or as a parenteral.

37. (New) The improved method of **Claim 36** wherein vitamin B_{12} is administered by an intramuscular injection.

38. (New) The improved method of **Claim 37** wherein the methylmalonic acid lowering agent administration is repeated about every 9 weeks, until administration of pemetrexed disodium is discontinued.

39. (New) An improved method for administering pemetrexed disodium to a patient in need of chemotherapeutic treatment, wherein the improvement comprises:

a) administration of between 350 μg and 1000 μg of folic acid, daily beginning approximately 1 to 3 weeks before treatment with pemetrexed disodium;

b) administration of a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12}, hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin, wherein the methylmalonic acid lowering agent is administered from about 1 to about 3 weeks prior to the first administration of pemetrexed disodium; and

c) administration of pemetrexed disodium in combination with between 350 μg and 1000μg of folic acid, daily, until administration of pemetrexed disodium is discontinued, and a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12},

hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin, wherein the methylmalonic acid lowering agent is administered by an intramuscular injection and wherein administration is repeated from about every 24 hours to about every1680 hours, until administration of pemetrexed disodium is discontinued.

Remarks

Applicants submit this paper and request entry of the amendments herein.

The Specification has been amended to recite specific reference to earlier-filed applications from which this application claims priority. The Specification has also been amended to correct an obvious error in the name of the compound "Alimta," which is found on page 6, line 16. The name has been corrected to read "pemetrexed disodium." Support for the correction can be found at least on page 2, lines 6-7, where the correct name of the compound is recited.

Claims 1-28 have been cancelled, and new Claims 29-39 have been introduced. Support for new Claim 29-39 is generally found in the specification, at least on page 5, line 20 to page 6, line 5; page 6, line 19 to page 7, line 4; page 7, lines 5-8, and 18-27; page 12, lines 19-29; page 13, line 21 to page 14, line 6; as well as in the claims as originally filed. Support for the improved combination can be found at least on page 13, line 21 to page 14, line 6; as well as on page 16, lines 3-9, and Table 1. More specifically, support for each element of Claims 29-39 is listed in the table below.

Claim	Element	Basis at
29(a)	"administration of between 350μg and 1000μg of folic acid, daily beginning approximately 1 to 3 weeks before treatment with pemetrexed disodium"	Page 13, line 21 to 25.
29(b)	"administration of a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12}, hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin"	Page 7, lines 5-8; Originally filed Claim 7.
29(b)	"wherein the methylmalonic acid lowering agent is administered from about 1 to about 3 weeks prior to the first administration of pemetrexed disodium"	Page 7, lines 25-26.
29(c)	"administration of pemetrexed disodium in combination with"	Page 5, lines 20-21; Originally filed Claim 4.
29(c)	"between 350 μg and 1000μg of folic acid, daily, until administration of pemetrexed disodium is discontinued"	Page 13, line 21 to 25; Page 14, line 3.
29(c)	"a methylmalonic acid lowering agent selected from the group consisting of vitamin B_{12}, hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin and cobalamin"	Page 7, lines 5-8; Originally filed Claim 7.
29(c)	"wherein the methylmalonic acid lowering agent administration is repeated from about every 6 weeks to	Page 7, lines 26-27.

	about every 12 weeks, until administration of pemetrexed disodium is discontinued"	
30	"methylmalonic acid lowering agent is vitamin B_{12}	Page 6, lines 20-21.
31	"about 500μg to about 1500μg of vitamin B_{12}"	Page 7, lines 18-19.
32	"about 1000 μg of vitamin B_{12}"	Page 7, lines 24-25; Page 12, lines 21-24; Page 13, lines 27-28; Page 14, lines 3-4.
33/36	"administered by an intramuscular injection, orally, or as a parenteral"	Page 7, lines 9-13.
34/37	"administered by an intramuscular injection"	Page 7, lines 11-13, and 18-25; Page 12, lines 21-24; Page 13, lines 27-30; Page 14, lines 3-6.
35/38	"methylmalonic acid lowering agent administration is repeated about every 9 weeks, until administration of pemetrexed disodium is discontinued"	Page 7, lines 26-27; Page 12, lines 23-24; Page 13, lines 29-30; Page 14, lines 5-6.
39		See basis for elements of Claim 29; and Page 7, lines 18-22.

Applicants respectfully assert that no new matter has been introduced as a result of amendment of the Claims. Applicants request prompt consideration and allowance of the claimed subject matter. If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone her at the number provided.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney for Applicant
Registration No. 43,585
Phone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

July 11, 2007

Please type a plus sign (+) inside this box [+]

PTO/SB/01 (8-96) (MODIFIED)

Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

Attorney Docket Number	X-14173
First Named Inventor	Clet Niyikiza
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

[X] Declaration Submitted with Initial Filing

[ ] Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

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

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 plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NOVEL ANTIFOLATE COMBINATION THERAPIES

the specification of which
[ ] is attached hereto
OR
[X] was filed on (MM/DD/YYYY) 15 June 2001 as United States Application Number or PCT International Application Number PCT/US01/14860 and was amended on (MM/DD/YYYY) _____ (if applicable).

II hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or Inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached YES	Certified Copy Attached NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional applications(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)
60/215,310	30 June 2000
60/235,859	27 September 2000
60/284,448	18 April 2001

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

Please type a plus sign (+) inside this box [+]
□

PTO/SB/01 (8-96) (MODIFIED)

Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1 56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Attorney Name	Reg. No.
Arvie J. Anderson	45,263
Lynn D. Apelgren	45,341
Robert A Armitage	27,417
Brian P. Barrett	39,597
Michael T. Bates	34,121
Roger S. Benjamin	27,025
Gary M. Birch	48,881
William R. Boudreaux	35,796
Steven P. Caltrider	36,467
Paul R. Cantrell	36,470
John Cleveland	50,697
Charles E. Cohen	34,565
Donald L. Comeglio	30,741
Gregory A. Cox	47,504
Paula K. Davis	47,517
John C. Demeter	30,167
Manisha A. Desai	43,585
Paul J. Gaylo	36,808
Francis O. Ginah	44,712
Janet A. Gongola	48,436
Amy E. Hamilton	33,894
James A. Hoffmann	50,221
Danica Hostettler	51,820
Frederick D. Hunter	26,915
Thomas E. Jackson	33,064
Soonhee Jang	44,802
Charles Joyner	30,466
Gerald P. Keleher	43,707
James J. Kelley	41,888

Attorney Name	Reg. No.
Paul J. Koivuniemi	31,533
Thomas LaGrandeur	51,026
Robert E. Lee	27,919
Kirby Lee	47,744
James P. Leeds	35,241
Nelsen L Lentz	38,537
Elizabeth A. McGraw	44,646
Douglas K. Norman	33,267
Arleen Palmberg	40,422
Thomas G. Plant	35,784
Edward Prein	37,212
Grant E. Reed	41,264
James J Sales	33,773
Michael J. Sayles	32,295
David M. Stemerick	40,187
Mark J. Stewart	43,936
Robert D. Titus	40,206
Robert C. Tucker	45,165
Tina M. Tucker	47,145
MaCharri Vorndran-Jones	36,711
Gilbert T. Voy	43,972
Thomas D. Webster	39,872
Lawrence T. Welch	29,487
Alexander Wilson	45,782
MaryAnn Wiskerchen	45,511
Dan L. Wood	48,613

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

Direct all correspondence to

Name	ELI LILLY AND COMPANY				
Address	ATTN: Elizabeth A. McGraw				
Address	Patent Division, P.O. Box 6288				
City	INDIANAPOLIS	**State**	INDIANA	**ZIP**	46206-6288
Country		**Telephone**	(317) 277-7443	**Fax**	(317) 276-3861

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 Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A Petition has been filed for this unsigned inventor

Given Name	Clet	**Middle Name**		**Family Name**	Niyikiza	**Suffix e.g. Jr.**	
Inventor's Signature						**Date**	27 NOV. 2002
Residence: City	Indianapolis	**State**	IN	**Country**	US	**Citizenship**	US
Address	6802 Antietam Place						
Post Office Address	SAME AS ABOVE						
City	Indianapolis	**State**	IN	**Zip**	46278	**Country**	US

[X] Additional Inventors are being named on supplement sheet(s) attached hereto.

Please type a plus sign (+) inside this box ☐ [+]

PTO/SB/01 (8-96) (MODIFIED)

Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION

Name of Additional Joint Inventor, if any:	☐ A Petition has been filed for this unsigned inventor							
Given Name	Paolo	Middle Name		Family Name	Paoletti	Suffix e.g. Jr.		
Inventor's Signature	Paolo Paoletti					Date	Dec. 4, 2002	
Residence: City	Indianapolis	State	IN	Country	US	Citizenship	IT	
Address	8015 Hayward Drive							
Post Office Address	SAME AS ABOVE							
City	Indianapolis	State	IN	Zip	46240	Country	US	

Name of Additional Joint Inventor, if any:	☐ A Petition has been filed for this unsigned inventor							
Given Name	James	Middle Name	Jacob	Family Name	Rusthoven	Suffix e.g. Jr.		
Inventor's Signature	James Jacob Rusthoven					Date	16 November '02	
Residence: City	Ancaster	State	Ontario	Country	CA	Citizenship	US	
Post Office Address	15 Lovers Lane							
Post Office Address	SAME AS ABOVE							
City	Ancaster	State	Ontario	Zip	L9G 1G4	Country	CA	

Name of Additional Joint Inventor, if any:	☐ A Petition has been filed for this unsigned inventor							
Given Name		Middle Name		Family Name		Suffix e.g. Jr.		
Inventor's Signature						Date		
Residence: City		State		Country		Citizenship		
Post Office Address								
Post Office Address	SAME AS ABOVE							
City		State		Zip		Country		

Name of Additional Joint Inventor, if any:	☐ A Petition has been filed for this unsigned inventor							
Given Name		Middle Name		Family Name		Suffix e.g. Jr.		
Inventor's Signature						Date		
Residence: City		State		Country		Citizenship		
Post Office Address								
Post Office Address	SAME AS ABOVE							
City		State		Zip		Country		

PTO/SB/80 (12-03)
Approved for use through 11/30/2005. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby appoint:

☑ Practitioners associated with the Customer Number: 25885

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number

as attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(b).

Assignee Name and Address:

Eli Lilly and Company
Patent Division
PO Box 6288
Indianapolis, Indiana 46206-6288

A copy of this form, together with a statement under 37 CFR 3.73(b) (Form PTO/SB/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(b) may be completed by one of the practitioners appointed in this form if the appointed practitioner is authorized to act on behalf of the assignee, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Name	Douglas K. Norman		
Signature	Douglas K Norman	Date	10 August 2004
Title	Deputy General Counsel, General Patent Counsel	Telephone	317-433-1651

PTO/SB/96 (8-96) (MODIFIED)
Approved for use through 9/30/98, OMB 0651-0027
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

CERTIFICATE UNDER 37 CFR 3.73(b)

First Applicant: NIYIKIZA Clet

Entitled: NOVEL ANTIFOLATE COMBINATION THERAPIES

Docket No.: X-14173B

ELI LILLY AND COMPANY, an Indiana Corporation
(Name of Assignee) (Type of Assignee, e.g. corporation, partnership, university, government agency, etc.)
certifies that it is the assignee of the entire right, title and interest in the patent application identified above by virtue of either:

A. [X] An assignment from the inventor(s) of the patent application identified above.

[X] The assignment was recorded in the Patent and Trademark Office at Reel 014132, Frame 0597.
[ ] The assignment is being submitted separately for recordation; a copy of this assignment is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application identified above, to the current assignee as shown below:

1. From:_____ To:_____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame_____, or for which a copy thereof is attached.

2. From:_____ To:_____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame_____, or for which a copy thereof is attached.

3. From:_____ To:_____
The document was recorded in the Patent and Trademark Office at
Reel _____, Frame_____, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[ ] Copies of assignments or other documents in the chain of title are attached.

The undersigned (whose title is supplied below) is empowered to sign this certificate on behalf of the assignee.

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 are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

July 11, 2007
Date

/Manisha A. Desai/
Manisha A. Desai
Patent Counsel

Send to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

CERTIFICATION OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.

Type or print name of person signing certification

Signature Date

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant: NIYIKIZA Clet	
For: NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.: X-14173B	

AMENDMENT AND PETITION TO CORRECT INVENTORSHIP UNDER 37 C.F.R. 1.48(b)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. Amendment and Petition

This amendment and petition is to delete the names of the following persons originally named as inventors and who are not the inventors of the invention now being claimed: Paolo Paoletti, of Indianapolis, Indiana, and James Jacob Rusthoven, of Ancaster, Canada.

2. Claims Now On File

The claims in this application are as follows:

New claims 29-39 filed on July 11, 2007

3. Diligence

This amendment and petition is being filed diligently after discovery that any claims for which the above named inventors who are being deleted are now no longer the inventors of the subject matter being claimed.

4. Fee Payment

Please charge $130.00, the surcharge required by §1.17(i), and charge any additional fees which may be required by this or any other related paper, or credit any overpayment to Deposit Account No. 05-0840, in the name of Eli Lilly and Company. I enclose an original and two copies of this paper.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney for Applicant
Registration No. 43,585
Telephone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

July 11, 2007

"Express Mail" mailing label number _____

Date of Deposit _____

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Printed Name _____ Signature _____

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant: NIYIKIZA Clet	
Title: NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.: X-14173B	

INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

As a means of complying with the duty of disclosure, Applicants submit an "Information Disclosure Citation In An Application" on a Form PTO-1449 (modified) for consideration by the Examiner. As permitted by 37 C.F.R. §1.98(d), Applicants refer to application Serial No. 11/288,807, filed November 29, 2005, for copies of the listed documents. Since this Statement is being filed in accordance with 37 C.F.R. 1.97(b), Applicants submit that no additional fee is required.

Applicants request consideration of this information.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney for Applicant
Registration No. 43,585
Telephone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

July 11, 2007

FORM PTO 1449 (modified) INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X-14173B	Serial No
	First Applicant NIYIKIZA Clet	
	Filing Date	Group

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 Pages or Relevant Figures Appear
	AA	US 5,405,839	4/ 11/1995	Tetsuo, et al.	
	AB	US 5,431,925	07/00/1995	Ohmori, et al.	
	AC	US 5,563,126	10/8/1996	Allen, et al.	
	AD	US 5,736,402	4/7/1998	Francis, et al.	
	AE	US 6,207,651	3/27/2001	Allen, et al.	
	AF	US 6,297,224	10/2/2001	Allen, et al.	
	AG	US 6,528,496	3/4/2003	Allen, et al.	
	AH	US 03/0216350	11/20/2003	Allen, et al.	
	Al	US 03/0225030	12/4/2003	Allen, et al.	
	AJ	US 2,920,015	01/1960	Thompson, Robert E.	
	AK	US 2004/0005311 Al	01/2004	Pitman, Bradford D.	
	AL	US 5,344,932	09/1994	Taylor, Edward C.	
	AM	US 7,053,065	05/2006	Niyikiza, et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No. [1]	Foreign Patent Document Country Code [3]-Number [4]-Kind Code5 (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]
	BA	EP 0 546 870	6/16/1993	EPO		

Examiner Signature		Date Considered	

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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[6]
	CA	Calvert H.: "Folate status and the safety profile of antifolates", Seminars in Oncology, 2002, 29/2 Suppl. 5, pp. 3-7, XP008005755	
	CB	Calvert H.: "Future directions in the development of pemetrexed", Seminars in Oncology, 2002, 29/2 Suppl. 5, pp. 54-61, XP008005744	
	CC	Westerhof, et al: "Carrier-and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecularstructure and biological activity", Mol. Pharmacology, 1995, 48(3), pp. 459-71, XP008005762	
	CD	Worzalla, et a]: "Role of folic acid in modulating the toxicity and efficacy of the multitargeted antifolate, LY231514", Anticancer Research (1998), 18(5A), pp. 3235-3239, XP008005757	
	CE	Hanauske, et al: "Pemetrexed disodium: A novel antifolate clinically active against multiple solid tumors", Oncologist, Alphamed Press, US, Vol. 4, No. 6, 2001, pp. 363-373, XP008005751	
	CF	Bunn, et al: "Vitamin B 12 and folate reduce toxicity of Alimta (pemetrexed disodium, LY 231514, MTA), a novel antifolate/antimetabolite", Program/Proceedings - American Society of Clinical Oncology, the Society, US, Vol. 76A, No. 20, 2001, page 300, XPO08005885	
	CG	Dierkes, et al., Supplementation with Vitamin B12 Decreases Homocystein and Methylmalonic Acid but Also Serum Folate in Patients with End-Stage Renal Disease. Metabolism. May 1999. Vol. 48, No. 5, pages 631-635. See: abstract.	
	CH	Arsenyan et al. (Abstract: Onkol. Nauchn., (1978) 12(10):49-54	
	Cl	John, et al. (Cancer 2000, 88: 1807-13)	
	CJ	Poydock et al., "Growth-inhibiting effect of hydroxocobaltniin and L-ascorbic acid on two solid tumors in mce", IRCS Medical _Science, Vol. 12, No. 9, pp. 813 (1984).	
	CK	The Cecil Reference, TEXTBOOK of MEDICINE, 21st Edition (2000). Chapter 198. pps. 1060-1074.	

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Electronic Acknowledgement Receipt

EFS ID:	1962281
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Manisha Arvind Desai/Lisa Capps
Filer Authorized By:	Manisha Arvind Desai
Attorney Docket Number:	X-14173B
Receipt Date:	11-JUL-2007
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Time Stamp:	17:06:59
Application Type:	Utility under 35 USC 111(a)

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	Abstract		1	1	
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	Claims		17	21	
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	Multipart Description/PDF files in .zip description				
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	Specification		2	2	
	Claims		3	5	
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4	Oath or Declaration filed	X14173Declaration.pdf	180049 8f9e1f83c8bc87f9ce2800c6624c0dedd8f01b1a	no	3
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Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Filer:	Manisha Arvind Desai/Lisa Capps
Attorney Docket Number:	X-14173B

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UTILITY PATENT APPLICATION TRANSMITTAL

(Only) for new nonprovisional applications under 37 CFR 1.53(b)

Attorney Docket No.	X14173B
First Named Inventor or Application Identifier	NIYIKIZA Clet
Express Mail Label No.	

Application Elements

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Commissioner for Patents
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Alexandria, VA 22313-1450

1. [X] Fee Transmittal Form *(Submit an original, and a duplicate for fee processing)*

2. [X] Specification *(preferred arrangement set forth below)* [Total Pages 21]
- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claims
- Abstract of the Disclosure

3. [ ] Drawing(s) (35 USC 113) [Total Sheets]

4. Oath or Declaration [Total Pages 3]
   a. [ ] Newly executed (original or copy)
   b. [X] Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed) **[Note Box 5 below]**
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5. [X] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. [ ] Microfiche Computer Program (Appendix)

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
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ACCOMPANYING APPLICATION PARTS

8. [ ] Assignment Papers (cover sheet & document(s)

9. [X] 37 CFR 3.73(b) Statement *(when there is an assignee)* [X] Power of Attorney

10. [ ] English Translation Document *(if applicable)*

11. [X] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

12. [X] Preliminary Amendment

13. [ ] Return Receipt Postcard (MPEP 503) (Should be specifically itemized)

14. [ ] Small Entity Statement(s) [ ] Statement filed in prior application, Status still proper and desired

15. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. [X] Other:
Amendment and Petition to Correct Inventorship

17. **If a CONTINUING APPLICATION,** *check appropriate box and supply the requisite information:*
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18. CORRESPONDENCE ADDRESS

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(74) Agents: **DAWALT, Elizabeth, A.** et al.; Lilly Corporate Center, Indianapolis, IN 46285 (US).

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(54) Title: NOVEL ANTIFOLATE COMBINATION THERAPIES

(57) Abstract: A method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

NOVEL ANTIFOLATE COMBINATION THERAPIES

Potentially, life-threatening toxicity remains a major limitation to the optimal administration of antifolates. (see, generally, Antifolate Drugs in Cancer Therapy, edited by Jackman, Ann L., Humana Press, Totowa, NJ, 1999.) In some cases, a supportive intervention is routinely used to permit safe, maximal dosing. For example, steroids, such as dexamethone, can be used to prevent the formation of skin rashes caused by the antifolate. (Antifolate, pg 197.)

Antifolates represent one of the most thoroughly studied classes of antineoplastic agents, with aminopterin initially demonstrating clinical activity approximately 50 years ago. Methotrexate was developed shortly thereafter, and today is a standard component of effective chemotherapeutic regimens for malignancies such as lymphoma, breast cancer, and head and neck cancer. (Bonnadonna G, Zambetti M, Valagussa P. Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive nodes: Ten year results. JAMA 1995;273(7):542-547; Bonnadonna G, Valagussa P, Moliterni A, Zambetti M, Brambilla C. Adjuvant cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer: The results of 20 years of follow-up. N Engl J Med 1995;332(14):901-906; and Hong WK, Schaefer S, Issell B, et al. A prospective randomized trial of methotrexate versus cisplatin in the treatment of recurrent squamous cell carcinoma of the head and neck. Cancer 1983;52:206-210.) Antifolates inhibit one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), by competing with reduced folates for binding sites of these enzymes. (Shih C, Habeck LL, Mendelsohn LG, Chen VJ, Schultz RM. Multiple folate enzyme inhibition: Mechanism of a novel pyrrolopyrimidine-based antifolate LY231514 (MTA). Advan Enzyme Regul, 1998; 38:135-152 and Shih C, Chen VJ, Gossett LS, et al. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 1997;57:1116-1123.) Several antifolate drugs are currently in development. Examples of antifolates that have thymidylate

synthase inhibiting ("TSI") characteristics include 5-fluorouracil and Tomudex®. An example of an antifolate that has dihydrofolate reductase inhibiting ("DHFRI") characteristic is Methotrexate®. An example of an antifolate that has glycinamide ribonucleotide formyltransferase inhibiting ("GARFTI") characteristics is Lometrexol. Many of these antifolate drugs inhibit more than one biosynthetic pathway. For example Lometrexol is also an inhibitor of dihydrofolate reductase and pemetrexed disodium (Alimta®, Eli Lilly and Company, Indianapolis, IN) has demonstrated thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase inhibition.

A limitation to the development of these drugs is that the cytotoxic activity and subsequent effectiveness of antifolates may be associated with substantial toxicity for some patients. Additionally antifolates as a class are associated with sporadic severe mylosuppression with gastrointestinal toxicity which, though infrequent, carries a high risk of mortality. The inability to control these toxicities led to the abandonment of clinical development of some antifolates and has complicated the clinical development of others, such as Lometrexol and raltitrexed. (Jackman AL, Calvert AH Folate-Based Thymidylate Synthase Inhibitors as Anticancer Drugs. Ann Oncol 1995;6(9):871-881; Laohavinij S, Wedge SR, Lind MJ, et al. A phase I clinical study of the antipurine antifolate Lometrexol (DDATHF) given with oral folic acid. Invest New Drugs 1996;14:325-335; and Maughan TS, James RD, Kerr D, et al., on behalf of the British MRC Colorectal Cancer Working Party. Preliminary results of a multicenter randomized trial comparing 3 chemotherapy regimens (deGramont, Lokich, and raltitrexed) in metastatic colorectal cancer. Proc ASCO 1999;18:Abst 1007.)

Initially, folic acid was used as a treatment for toxicities associated with GARFTI see, e.g. U.S. Pat. No. 5,217,974. Folic acid has been shown to lower homocysteine levels (see e.g. Homocysteine Lowering Trialist's Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomized trials. BMJ 1998;316:894-898 and Naurath HJ, Joosten E, Riezler R, Stabler SP, Allen RH, Lindenbaum J. Effects of vitamin B12, folate and vitanin B6 supplements in elderly people with normal serum vitamin concentrations. Lancet 1995;346:85-89), and homocysteine levels have been shown to be a predictor of cytotoxic events related to the

use of GARFT inhibitors, see e.g. U.S. Pat. No. 5,217,974. However, even with this treatment, cytotoxic activity of GARFT inhibitors and antifolates as a class remains a serious concern in the development of antifolates as pharmaceutical drugs. The ability to lower cytotoxic activity would represent an important advance in the use of these agents.

Surprisingly and unexpectedly, we have now discovered that certain toxic effects such as mortality and nonhematologic events, such as skin rashes and fatigue, caused by antifolates, as a class, can be significantly reduced by the presence of a methylmalonic acid lowering agent, without adversely affecting therapeutic efficacy. The present invention thus provides a method for improving the therapeutic utility of antifolate drugs by administering to the host undergoing treatment with a methylmalonic acid lowering agent. We have discovered that increased levels of methylmalonic acid is a predictor of toxic events in patients that receive an antifolate drug and that treatment for the increased methylmalonic acid, such as treatment with vitamin B12, reduces mortality and nonhematologic events, such as skin rashes and fatigue events previously associated with the antifolate drugs.

Additionally, we have discovered that the combination of a methylmalonic acid lowering agent and folic acid synergistically reduces the toxic events associated with the administration of antifolate drugs. Although, the treatment and prevention of cardiovascular disease with folic acid in combination with vitamin B12 is known, the use of the combination for the treatment of toxicity associated with the administration of antifolate drugs was unknown heretofore.

The present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent, alone or in combination with a FBP binding agent, in the preparation of a medicament useful in lowering the mammalian toxicity of an antifolate. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate and a FBP binding agent.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the manufacture of a medicament for use in a method of inhibiting tumor growth in mammals, which method comprises administering said methylmalonic acid lowering agent in combination with an antifolate.

Furthermore, the present invention relates to a product containing a methylmalonic acid lowering agent, an antifolate and optionally a FBP binding agent as a combined preparation for the simultaneous, separate or sequential use in inhibiting tumour growth.

The current invention concerns the discovery that administration of a methylmalonic acid lowering agent in combination with an antifolate drug reduces the toxicity of the said antifolate drug.

The term "inhibit" as it relates to antifolate drugs refers to prohibiting, alleviating, ameliorating, halting, restraining, slowing or reversing the progression of, or reducing tumor growth.

As used herein, the term "effective amount" refers to an amount of a compound or drug, which is capable of performing the intended result. For example, an effective amount of an antifolate drug that is administered in an effort to reduce tumor growth is that amount which is required to reduce tumor growth.

As used herein, the term "toxicity" refers to a toxic event associated with the administration on an antifolate. Such events include, but are not limited to, neutropenia, thrombopenia, toxic death, fatigue, anorexia, nausea, skin rash, infection, diarrhea, mucositis, and anemia. For further explanation of the types of toxicity experienced by patients receiving antifolates, see, generally, Antifolate Drugs in Cancer Therapy. Preferably, toxicity refers to toxic death, fatigue, neutropenia, thrombopenia, and mucositis.

As used herein, the term "nonhematologic event" refers to the occurrence of skin rash or fatigue due to the administration of an antifolate.

As used herein, the term "in combination with" refers to the administration of the methylmalonic acid lowering agent, the antifolate drug, and optionally the folic acid; in any order such that sufficient levels of methylmalonic acid lowering agent and optionally folic acid are present to reduce the toxicity of an antifolate in a mammal. The administration of the compounds maybe simultaneous as a single composition or as two separate compositions or can be administered sequentially as separate compositions such that an effective amount of the agent first administered is in the patient's body when the second and/or third agent is administered. The antifolate drug may be administered to the mammal first, followed by treatment with the methylmalonic acid lowering agent. Alternatively, the mammal may be administered the antifolate drug simultaneously with the methylmalonic acid lowering agent. Preferably, the mammal is pretreated with the methylmalonic acid lowering agent and then treated with the antifolate. If folic acid is to

be administered in addition to the methylmalonic acid lowering agent, the folic acid may be administered at any time prior, post, or simultaneously to the administration of either the methylmalonic acid lowering agent or the antifolate. Preferably, the mammal is pretreated with the methylmalonic acid, and then treated with folic acid, followed by treatment with the antifolate compound.

The terms "antifolate" and "antifolate drug" refer to a chemical compound which inhibits at least one key folate-requiring enzyme of the thymidine or purine biosynthetic pathways, preferably thymidylate synthase ("TS"), dihydrofolate reductase ("DHFR"), or glycinamide ribonucleotide formyltransferase ("GARFT"), by competing with reduced folates for binding sites of these enzymes. Preferred examples of antifolates include 5-fluorouracil, as manufactured by Glaxo; Tomudex®, as manufactured by Zeneca; Methotrexate®, as manufactured by Lederle; Lometrexol®, as manufactured by Tularik; pyrido[2,3-d]pyrimidine derivatives described by Taylor et al. in U.S. Pat. Nos. 4684653, 4833145, 4902796, 4871743, and 4882,334; derivatives described by Akimoto in U.S. Pat. No. 4997838; thymidylate synthase inhibitors as found in EPO application 239,362; and most preferred, Pemetrexed Sodium (ALIMTA), as manufactured by Eli Lilly & Co.

The terms "methylmalonic acid" and "MMA" refer to a structural isomer of succinic acid present in minute amounts in healthy human urine.

The term "methylmalonic acid lowering agent" refers to a substrate, which lowers the concentration of methylmalonic acid in a mammal. A preferred example of such a substrate is vitamin B12. For methods of determining methylmalonic acid and substrates therefore, see, e.g., Matchar DB, Feussner JR, Millington DS, et al. Isotope dilution assay for urinary methylmalonic acid in the diagnosis of vitamin B12 deficiency. A prospective clinical evaluation. Ann Intern Med 1987; 106: 707-710; Norman EJ, Morrison JA. Screening elderly populations for cobalamin (vitamin B12) deficiency using the urinary methylmalonic acid assay by gas chromatography mass spectrometry. Am J Med 1993; 94: 589-594; Norman EJ. Gas Chromatography mass spectrometry screening of urinary methylmalonic acid: early detection of vitamin B12 (cobalamin) deficiency to prevent permanent neurologic disability. GC/MS News 1984; 12:120-129; Martin DC, Francis J, Protetch J, Huff FJ. Time dependency of cognitive recovery with cobalamin replacement: report of a pilot study. JAGS 1992; 40: 168-172; Norman EJ, Cronin C. Cobalamin

deficiency. Neurol 1996; 47: 310-311; Rasmussen K, Moelby I, Jensen MK. Studies on methylmalonic acid in humans; Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of methylmalonic acid and total homocysteine determination for diagnosing cobalamin and folate deficiency. Am J Med 1994; 96: 239-246.

The term "vitamin B12" refers to vitamin B12 and its pharmaceutical derivatives, such as hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin. Preferably the term refers to vitamin B12, cobalamin, and chlorocobalamin.

The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, such as a sustained release formulation, as an aqueous solution added to drinking water, or as an aqueous parenteral formulation. Preferably the methylmalonic acid lowering agent is administered as an intramuscular injection formulation. Such formulations are known in the art and are commercially available.

The skilled artisan will appreciate that the methylmalonic lowering agents are effective over a wide dosage range. For example, when cobalamin is used as the methylmalonic lowering agent, the dosage of cobalamin may fall within the range of about 0.2 μg to about 3000 μg of cobalamin from once daily for a month to once every nine weeks for a year. Preferably, cobalamin will be dosed as an intramuscular injection of about 500 μg to about 1500 μg administered from about every 24 hours to about every 1680 hours. Preferably, it is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to administration of the antifolate and repeated from about every 24 hours to about every 1680 hours, regardless of when treatment with the antifolate is started and continued until the administration of the antifolate is discontinued. Most preferred is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to the first administration of the antifolate and repeated every 6 to 12 weeks, preferably about every 9 weeks, and continued until the discontinuation of the antifolate administrations. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent

administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

The term "FBP binding agent" as used herein refers to a folic binding protein binding agent which includes folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof. This latter compound is the (6R)-isomer of leucovorin as disclosed in J. Am. Chem. Soc., 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position. They are 10-20 fold more efficient in binding the folate binding protein compared with their respective (6S)—isomer, see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21-22, 1991, Bethesda, MD. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques. See e.g. PCT Patent Application Publication WO 880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554. See, e.g. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline (2000), 8 Folate, pp. 196-305.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available...ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1-C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is converted to the parent acid in a biological system. The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, preferably as a

sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is usually administered to the subject mammal prior to treatment with the antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 0.1 mg to about 30 mg, most preferably about 0.3 mg to about 5 mg, of folic acid is administered orally to a mammal about 1 to 3 weeks post administration of the methylmalonic acid lowering agent and about 1 to about 24 hours prior to the parenteral administration of the amount of an antifolate. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

In general, the term "pharmaceutical" when used as an adjective means substantially non-toxic to living organisms.

Methods

To assess the effect of a methylmalonic acid lowering agent, alone or in combination with folic acid on the antitumor efficacy of an antifolate in a human tumor xenograft model, female nude mice bearing human MX-1 breast carcinoma were treated

with ALIMTA alone or along with super-physiologic doses of folic acid or vitamin B12 (cobalamin).

The animals were maintained on sterilized standard lab chow ad libitum and sterilized water ad libitum. The human MX-1 tumor cells (5×10^6) obtained from donor tumors were implanted subcutaneously in a thigh of female nude mice 8- to 10-weeks old. Beginning on day 7 post tumor cell implantation, the animals were treated with ALIMTA (100 mg/kg or 150 mg/kg) once daily on days 7 through 11 and 14 through 18 by intraperitoneal injection alone or along with folic acid (6 mg/kg or 60 mg/kg) and/or vitamin B12 (165 mg/kg) by intraperitoneal injection on the same schedule.

Tumor response was monitored by tumor volume measurements twice weekly over the course of the experiment. Toxicity was monitored by body weight measurements made at the same time as the tumor volume measurements. Tumor growth delay was the difference in days for the treated and the controls tumors to reach 1000 mm^3.

The human MX-1 breast carcinoma xenograft was responsive to treatment with ALIMTA with doses of 100 mg/kg and 150 mg/kg producing tumor growth delays of 17 days and 21 days, respectively. Folic acid was administered to the animals alone at two doses 6 mg/kg and 60 mg/kg on the same schedule as ALIMTA and produced tumor growth delays of 7 days and 12 days, respectively. Vitamin B12 administered alone at a dose of 165 mg/kg resulted in a tumor growth delay of 12 days.

Combinations of ALIMTA at each of the two doses were administered along with each of the vitamins as simultaneous combination regimens. Administration of folic acid (6 mg/kg) along with ALIMTA did not alter the tumor growth delay produced from that obtained with ALIMTA alone. The addition of folic acid at the higher dose (60 mg/kg) along with each dose of ALIMTA resulted in small increases in tumor growth delay to 22 days and 23 days at the ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively. The tumor growth delays with ALIMTA and vitamin B12 (165 mg/kg) treatment were 22 days and 24 days at ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively.

Body weight was used as a general measure of toxicity for each of the treatment regimens. The body weight loss pattern reflected the treatment regimens with weight decrease during the treatment times of days 7 through 11 and 14 through 18 with some

weight recovery during the intervening two days. The weight loss due to ALITMA was dose dependent but overall minor (3%). Folic acid alone at either 6 mg/kg or 60 mg/kg did not cause weight loss, in fact folic acid treated animals maintained weight and gained weight over the course of the experiment better than the control animals. The animals treated with ALIMTA (100 mg/kg) and folic acid (60 mg/kg) gained weight (about 20%) over the course of the experiment.

Administration of vitamin B12 did not prevent weight gain in the animals over the time course of the experiment. The animals treated with ALIMTA (100 mg/kg) along with vitamin B12 gained weight while those treated with ALIMTA (150 mg/kg) along with vitamin B12 maintained weight over the course of the experiment.

In conclusion, administration of super-physiologic but non-toxic doses of the vitamins, folic acid and vitamin B12, did not alter the antitumor activity of ALIMTA in the human MX-1 breast carcinoma xenograft tumor in nude mice and did not increase the toxicity of ALIMTA as determined by body weight measurements of the animals.

The effect of vitamin B12, alone or in combination with folic acid, on antifolates can be demonstrated in standard tests commonly utilized to determine the antitumor activity and toxic effects of the antifolates themselves. In one such test, mice are inoculated with the C3H strain of mammary adenocarcinoma by inserting a 2 mm by 2 mm section of tumor into the axillary region of the mice by trocar. The timing of administering the methylmalonic acid lowering agent, alone or in combination with the folic acid, and the antifolate may be varied. Ten animals are used at each dosage level. Antitumor activity is assessed on day ten (when day one is first dosage of antifolate) by measuring the length and width of the tumor growth using vernier calipers, and the activity is expressed as a percent inhibition of tumor growth.

When the antifolate is administered to infected mice which are maintained on a diet totally free of vitamin B12 and optionally folic acid for two weeks prior to and during treatment, it exhibits moderated antitumor activity at very low doses, but also causes severe toxicity at a very low dose (measured as death of mice).

A test group of mice are maintained on a vitamin B12 and optionally folic acid free diet for two weeks before treatment. Vitamin B12 and optionally folic acid is then

administered during the treatment by intramuscular injection of 0.0003% vitamin B12 (weight/volume) and optionally providing the animals drinking water containing 0.0003% folic acid (weight/volume). This concentration translates to about 1.75 mg of vitamin B12 and optionally folic acid per square meter of body surface per day. As the foregoing results indicate, addition of the indicated level of vitamin B12 to the diet of a subject receiving an antifolate results in excellent antitumor activity at low doses, with little or no toxic effects.

The foregoing tests establish that for tumor bearing mice maintained on a vitamin B12 and optionally folic acid free diet prior to and during treatment with an antifolate, the toxicity of the antifolate is very large, with 1 mg/kg/day being lethal to the majority of the mice, and lower antitumor activity is observed at non-toxic drug doses. Very low doses of vitamin B12 partially reverses drug toxicity and improved antitumor activity. Larger doses of vitamin B12 reduce antifolate toxicity even more significantly. Pretreatment of the mouse with vitamin B12 and then administering folic acid prior to administering the antifolate demonstrates a striking reduction in toxicity, almost eliminating the antifolate toxicity completely. Thus, the use of vitamin B12 in combination with an antifolate reduces drug toxicity without adversely affecting antitumor activity, and the use of vitamin B12 in conjunction with folic acid synergistically reduces drug toxicity.

In a typical clinical evaluation involving cancer patients, all of whom have histologically or cytologically confirmed diagnosis of cancer, an antifolate is administered in combination with vitamin B12. Vitamin B12 is administered as a 1000 μg intramuscular injection 1-3 weeks prior to treatment with the antifolate, and 1000 μg intramuscular injection of vitamin B12 is made approximately every 9 weeks until the patient discontinues from therapy. The antifolate is administered in four doses over a two week period by rapid intravenous injection, followed by two weeks of non-therapy. Dosing is made on days 1, 4, 8 and 11 of any two week period. Patients will have an initial course of therapy at a dose of 5 mg/m^2/dose, and depending upon the toxic effects observed in the initial course, their subsequent courses may be at the same dose, or may be escalated to 6 mg/m^2, or may be attenuated to 4 mg/m^2.

In preparation for the foregoing clinical study, pilot studies in humans have established that vitamin B12 given to patients receiving Alimta has effected reduced side effects due to the Alimta. One to two weeks prior to administration of ALIMTA urine is collected and blood is drawn from a human subject; and vitamin metabolite levels, methylmalonic acid and homocysteine, are determined. Homocysteine levels are determined in blood by a fluorescent polarization immunoassay kit manufactured by Abbot Laboratories. Methylmalonic acid levels are determined by urine levels using a 24 hour urine collection kit available from Biolab Medical Unit (a United Kingdom company). Additionally urine and blood may be collected one week prior to administration of ALIMTA (after at least 5 days of folic acid supplementation and at least 1 week vitamin B12 supplementation), and up to 4 days prior to every cycle.

Method of administration and dosing procedures:

1. Folic Acid:

Folic acid will be supplied as one of the following options, with preference in order from option #1 to option #3:

1. 350 - 600 μg folic acid.
2. A multivitamin containing folic acid in the range of 350 μg to 600 μg is acceptable if option #1 is not available.
3. A dose of folic acid between 350 μg and 1000 μg is acceptable if neither option #1 or option # 2 is available.

For purposes of this study, patients should take oral folic acid daily beginning approximately 1 to 3 weeks before treatment with ALIMTA plus cisplatin or cisplatin alone and continuing daily until discontinuation from study therapy.

2. Vitamin B12

Vitamin B12 will be obtained and administered as a 1000 μg intramuscular injection. A vitamin B12 injection must be administered approximately 1 to 3 weeks before treatment with ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Folic acid supplementation, 350 – 600 μg or equivalent should be taken orally daily beginning approximately 1 to 3 weeks prior to the first dose of MTA plus cisplatin and continue daily until the patient discontinues from study therapy. A vitamin B12 injection, 1000 μg, must be given intramuscularly approximately 1 to 3 weeks prior to the first dose of ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Compare presupplementation homocysteine and methylmalonic acid levels to a) the level immediately prior to the initial dose of study drug, and b) to the level immediately prior to the second dose of study drug (i.e., after a full cycle of supplementation), and compare the prevalence of specific toxicities experienced in up to the first seven cycles of therapy in patients who have been supplemented from baseline to the prevalence seen in the earlier patients (n = 246) who were not supplemented (Farber et al.)

Toxicity may be compared in specific patients in non-supplemented cycles versus supplemented cycles (cross-over patients).

The data to be compared are:

1) Patient numbers and baseline demographic data for those supplemented from baseline.
2) Homocysteine and methylmalonic acid levels, levels at baseline, prior to first dose, prior to second dose, and prior to each therapy cycle depending of the type of cancer under study.
3) Grade 3 and 4 hematologic toxicity in these fully supplemented patients.
4) Grade 3 and 4 nonhematologic toxicity in these fully supplemented patients.

The grading of toxicities in chemotherapuetic clinical trials is well known to a person of skill in the art. Examples of fatigue and skin rash grading are provided below.

Fatigue Grading --

	Neuromotor
Grade 0	none or no change
Grade 1	subjective weakness; no objective findings
Grade 2	mild objective weakness without significant impairment of function
Grade 3	objective weakness with impairment of function
Grade 4	paralysis

Rash Grading --

	Skin
Grade 0	none or no change
Grade 1	scattered macular or papular eruption or erythema that is asymptomatic
Grade 2	scattered macular or papular eruption or erythema with pruritus or other associated eruption symptoms
Grade 3	generalized symptomatic macular, papular, or vesicular eruption
Grade 4	exfoliative dermatitis or ulcerating dermatitis

The vitamins (both folic acid and B12) to be used in the following studies may be obtained from Zenith Gold Line, Centrum, Folvite, or in Canada Apo-Folic. Cyanocobalamin is used as the methylmalonic acid lowering agent in these studies.

Current and past clinical trials show a 4% drug-related death total, 50% grade 3/4 neutropenia, 7% grade 4 thrombocytopenia, and 10% grade 3/4 diarrheas and mucositis in patients administered ALIMTA and folic acid as described in U.S. Pat. No. 5,217,974. Vitamin B12 supplementation with ALIMTA has a moderate effect on drug related toxicity, lowering drug related deaths to 3% and severe toxicities by about 25%. The combination of vitamin B12 and folic acid with ALIMTA has lowered the drug related deaths to <1% in over 480 so treated. The combination of vitamin B12 and folic acid has lowered the drug related grade 3/4 toxic events, see Table 1.

Table 1

	Percent of occurrences prior to B12/folic acid treatment (N=246)	Percent of occurrences post B12/folic acid treatment (N=78)
Hematologic Toxicity/Non-Hematologic Toxicity	37%	6.4%
Neutropenia	32%	2.6%
Mucositis	5%	1.3%
Diarrhea	6%	2.6%
Neutropenia and Mucositis	3%	0%
Neutropenia and Diarrhea	3%	0%
Neutropenia and Infection	2%	0%

Additionally, sixty-two chemonaive patients requiring chemotherapeutic treatment were divided into two groups. Seventeen of these patients received ALIMTA, but did not receive vitamin B12 or folic acid, as described *supra*. The remaining patients received treatment with vitamin B12, folic acid, and ALIMTA, as described *supra*. Of patients who received the combination treatment, 8 out of 45 responded to the chemotherapy. Of patients who did not receive the combination treatment, but rather, received only treatment with ALIMTA, only 1 out of 17 patients responded.

We Claim:

1. A method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

2. A method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

3. A method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent.

4. A method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and FBP binding agent.

5. A method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and FBP binding agent.

6. A method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent and FBP binding agent.

7. A method of any one of claims 1-6 wherein the methylmalonic acid lowering agent is selected from the group consisting of hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin.

8. A method of any one of claims 4-6 wherein the FBP binding agent is selected from the group consisting of folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

9. A method of any one of claims 1-8 wherein the antifolate is ALIMTA.

10. A method of any one of claims 1-9 wherein the mammal is pretreated with methylmalonic acid lowering agent.

11. The use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate.

12. The use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate and a FBP binding agent.

13. The use any one of claims 11-12 wherein the methylmalonic acid lowering agent is selected from the group consisting of hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin.

14. The use of any one of claims 11-13 wherein the FBP binding agent is selected from the group consisting of folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

15. The use of any one of claims 11-14 wherein the antifolate is ALIMTA.

16. The use of any one of claims 11-15 wherein the mammal is pretreated with methylmalonic acid lowering agent.

17. Use of a methylmalonic acid lowering agent in the manufacture of a medicament for lowering the mammalian toxicity associated with administration of an antifolate wherein said methylmalonic acid lowering agent is administered in combination with said antifolate.

18. Use of a methylmalonic acid lowering agent in the manufacture of a medicament for use in a method of inhibiting tumor growth in mammals, which method comprises administering said methylmalonic acid lowering agent in combination with an antifolate.

19. Use according to claim 17 or 18 wherein a FBP binding agent is also administered in combination with said methylmalonic acid lowering agent and antifolate.

20. Use according to any one of claims 17-19 wherein the methylmalonic acid lowering agent, antifolate and optionally FBP binding agent is administered simultaneously, separately or sequentially of one another.

21. The use any one of claims 17-20 wherein the methylmalonic acid lowering agent is selected from the group consisting of hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin.

22. The use of any one of claims 19-21 wherein the FBP binding agent is selected from the group consisting of folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

23. The use of any one of claims 17-22 wherein the antifolate is ALIMTA.

24. The use of any one of claims 17-23 wherein the mammal is pretreated with the methylmalonic acid lowering agent.

25. A product containing a methylmalonic acid lowering agent, an antifolate and optionally a FBP binding agent as a combined preparation for the simultaneous, separate or sequential use in inhibiting tumour growth.

26. A product according to claim 25 wherein the methylmalonic acid lowering agent is selected from the group consisting of hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin.

27. A product according to claim 25 or 26 wherein the antifolate is ALIMTA.

28. A product according to anyone of claims 25-27 wherein the FBP binding agent is selected from the group consisting of folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

Electronic Acknowledgement Receipt

EFS ID:	1962281
Application Number:	11776329
International Application Number:	
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First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Manisha Arvind Desai/Lisa Capps
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	Specification		2	2	
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	Applicant Arguments/Remarks Made in an Amendment		6	7	
Warnings:					
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4	Oath or Declaration filed	X14173Declaration.pdf	180049 8f9e1f83c8bc87f9ce2800c6624c0dedd8f01b1a	no	3
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5	Power of Attorney	X14173BPOA.pdf	317670 06c7d70ef336416e59316cc6408d288ee9cdeea2	no	1

Warnings:					
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7	Miscellaneous Incoming Letter	X14173BCorrectInventorship.pdf	82734 5d0fd58fa29a8f476a4e8e9945ca5bfa512f128e	no	2
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8	Information Disclosure Statement (IDS) Filed	X14173BIDS.pdf	72699 8b14cc73cae338f95afeb5c7c94ee7db0494793a	no	1
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9	Information Disclosure Statement (IDS) Filed	X14173B1449.pdf	86170 24dd6c5e029b6f159c7b50f5ad7bd1c0de0182ab	no	2
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11/776,329

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))							300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))							500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))							200
TOTAL CLAIMS (37 CFR 1.16(i))	11 minus 20 =		X 25=			X 50=	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =		X 100=			X 200=	
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))			N/A			N/A	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1000

APPLICATION AS AMENDED – PART II

		(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 ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
AMENDMENT A	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))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT B	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))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T 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.

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.**

7/11/07

PTO/SB/06 (12-04)
Approved for use through 7/31/2006. 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	11/776,329

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY			OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))							300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))							500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))							200
TOTAL CLAIMS (37 CFR 1.16(i))	11 minus 20 =		X 25=		OR	X 50=	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =		X 100=			X 200=	
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))			N/A			N/A	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1000

APPLICATION AS AMENDED – PART II

7/11/07

	(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)		RATE ($)	ADDI-TIONAL FEE ($)
AMENDMENT A Total (37 CFR 1.16(i))	* 11	Minus	** 20	= 0	X =		OR	X =	0
Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	X =		OR	X =	0
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
					TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	0

	(Column 1)		(Column 2)	(Column 3)			OR		
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)		RATE ($)	ADDI-TIONAL FEE ($)
AMENDMENT B 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))					N/A		OR	N/A	
					TOTAL ADD'T FEE		OR	TOTAL ADD'T 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.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO
11/776,329	07/11/2007		1000	X14173B

CONFIRMATION NO. 6568

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN46206-6288

FILING RECEIPT

Date Mailed: 07/18/2007

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 write to the Office of Initial Patent Examination's Filing Receipt Corrections. 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)

Clet Niyikiza, Indianapolis, IN;
Paolo Paoletti, Indianapolis, IN;
James Jacob Rusthoven, Ancaster, CANADA;

Power of Attorney: The patent practitioners associated with Customer Number 25885

Domestic Priority data as claimed by applicant

This application is a DIV of 11/288,807 11/29/2005
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

Foreign Applications

If Required, Foreign Filing License Granted:

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL ANTIFOLATE COMBINATION THERAPIES

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.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

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).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/776,329	07/11/2007	Clet Niyikiza	X14173B

CONFIRMATION NO. 6568

FORMALITIES LETTER

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

Date Mailed: 07/18/2007

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- A substitute specification excluding claims in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 is required. The substitute specification must be submitted with markings and be accompanied by a clean version (without markings) as set forth in 37 CFR 1.125(c) and a statement that the substitute specification contains no new matter (see 37 CFR 1.125(b)). Since a preliminary amendment was present on the filing date of the application and such amendment is part of the original disclosure of the application, the substitute specification must include all of the desired changes made in the preliminary amendment. See 37 CFR 1.115 and 1.215.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies should be mailed to: Mail Stop Missing Parts
Commissioner for Patents
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Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

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If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199
PART 3 - OFFICE COPY

Electronic Acknowledgement Receipt

EFS ID:	2057405
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Manisha Arvind Desai/Lisa Capps
Filer Authorized By:	Manisha Arvind Desai
Attorney Docket Number:	X14173B
Receipt Date:	07-AUG-2007
Filing Date:	11-JUL-2007
Time Stamp:	16:30:00
Application Type:	Utility under 35 USC 111(a)

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	Applicant Response to Pre-Exam Formalities Notice	X14173BResptoRequestforCorrectedFiling.pdf	150572 54fd6d75d68eb420aff19840ee863d0c5f3aaf09	no	3
Warnings:					

Information:					
2		X14173BAmendedSpecMarkedupcopy.pdf	162063 3054d6e3790327768bd692b0332775634e562f3c	yes	17

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	16
	Abstract	17	17

Warnings:					
Information:					
3		X14173BAmendedSpecCleancopy.pdf	161578 419cb785313f9b017f2bb89ace30db3e3d20d404	yes	17

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	16
	Abstract	17	17

Warnings:					
Information:					
4	Request for Corrected Filing Receipt	X14173BFinalCorrectedFilingReceipt.pdf	266851 e1daad260634970264bef9d76d4602f9781c02b6	no	5
Warnings:					
Information:					
		Total Files Size (in bytes):	741064		

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.

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant: NIYIKIZA Clet	
Serial No.: 11/776,329	
Application Date: 7/11/2007	Conf No.: 6568
For: NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.: X14173B	

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

Commissioner for Patents
Mail Stop Missing Parts
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is in response to a "Notice to File Corrected Application Papers," dated July 18, 2007, noting the absence of a marked up and clean copy of a substitute specification, excluding claims.

Enclosed herewith are: 1) a copy of the Notice; 2) a marked up copy of the specification, excluding claims, in compliance with 37 CFR 1.115 and 37 CFR 1.125; and 3) a clean copy of the specification, excluding claims, in compliance with 37 CFR 1.125(c).

Applicants assert that the substitute specification contains no new matter.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney for Applicant
Registration No. 43,585
Phone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

August 6, 2007

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/776,329	07/11/2007	Clet Niyikiza	X14173B

CONFIRMATION NO. 6568

FORMALITIES LETTER

25885
ELI LILLY & COMPANY
PATENT DIVISION
P O. BOX 6288
INDIANAPOLIS, IN 46206-6288

Response Due
18 SEP 2007

Date Mailed. 07/18/2007

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a)

The required item(s) identified below must be timely submitted to avoid abandonment

- A substitute specification excluding claims in compliance with 37 CFR 1 52, 1 121(b)(3), and 1 125 is required The substitute specification must be submitted with markings and be accompanied by a clean version (without markings) as set forth in 37 CFR 1.125(c) and a statement that the substitute specification contains no new matter (see 37 CFR 1.125(b)). Since a preliminary amendment was present on the filing date of the application and such amendment is part of the original disclosure of the application, the substitute specification must include all of the desired changes made in the preliminary amendment. See 37 CFR 1 115 and 1.215.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies should be mailed to: Mail Stop Missing Parts
Commissioner for Patents
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If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199
PART 1 - ATTORNEY/APPLICANT COPY

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant: NIYIKIZA Clet	
Serial No.: 11/776,329	
Application Date: July 11, 2007	Conf No.: 6568
For: NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.: X14173B	

REQUEST FOR CORRECTED FILING RECEIPT

Commissioner for Patents
Office of Initial Patent Examination
Customer Service Center
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant requests correction of the filing receipt for this application. A copy of the receipt, with the corrections noted, is enclosed.

With the transmittal of this application, an Amendment and Petition to Correct Inventorship under 37 CFR 1.48(b) was also submitted. The filing receipt does not reflect the corrected inventorship.

Applicant believes no fees are due; however, if any fees are due, please charge any fees that may be required by this or related papers, or credit any overpayment, to Deposit Account No. 05-0840 in the name of Eli Lilly and Company. Applicant therefore requests that the filing receipt be corrected.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney/Agent for Applicant
Registration No. 43,585
Phone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288
August 7, 2007

Enclosure: Copy of Filing Receipt with the changes noted thereon.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPL NO	FILING OR 371(c) DATE	ART UNIT	FIL FEE REC'D	ATTY DOCKET NO	TOT CLMS	IND CLMS
11/776,329	07/11/2007		1000	X14173B	11	2

CONFIRMATION NO. 6568

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

FILING RECEIPT

OC000000024887418

Date Mailed: 07/18/2007

Receipt is acknowledged of this nonprovisional 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 write to the Office of Initial Patent Examination's Filing Receipt Corrections. 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 (if appropriate).**

Applicant(s)

Clet Niyikiza, Indianapolis, IN;
~~Paolo Paoletti, Indianapolis, IN~~;
~~James Jacob Rusthoven, Ancaster, CANADA~~;

Power of Attorney: The patent practitioners associated with Customer Number **25885**.

Domestic Priority data as claimed by applicant

This application is a DIV of 11/288,807 11/29/2005
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

Foreign Applications

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL ANTIFOLATE COMBINATION THERAPIES

Preliminary Class

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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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NOVEL ANTIFOLATE COMBINATION THERAPIES

This application is a divisional of Application No. 11/288,807, filed 29 November 2005, which is a divisional of Application No. 10/297,821 filed 12 May 2002, now Patent Number 7,053,065, which claims priority under 35 USC 371, for PCT/US01/14860, filed 15 June 2001, which claims the priority of U.S. provisional applications No. 60/215,310, filed 30 June 2000, No. 60/235,859, filed 27 September 2000, and No. 60/284,448, filed 18 April 2001.

Potentially, life-threatening toxicity remains a major limitation to the optimal administration of antifolates. (see, generally, Antifolate Drugs in Cancer Therapy, edited by Jackman, Ann L., Humana Press, Totowa, NJ, 1999.) In some cases, a supportive intervention is routinely used to permit safe, maximal dosing. For example, steroids, such as dexamethone, can be used to prevent the formation of skin rashes caused by the antifolate. (Antifolate, pg 197.)

Antifolates represent one of the most thoroughly studied classes of antineoplastic agents, with aminopterin initially demonstrating clinical activity approximately 50 years ago. Methotrexate was developed shortly thereafter, and today is a standard component of effective chemotherapeutic regimens for malignancies such as lymphoma, breast cancer, and head and neck cancer. (Bonnadonna G, Zambetti M, Valagussa P. Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive nodes: Ten year results. JAMA 1995;273(7):542-547; Bonnadonna G, Valagussa P, Moliterni A, Zambetti M, Brambilla C. Adjuvant cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer: The results of 20 years of follow-up. N Engl J Med 1995;332(14):901-906; and Hong WK, Schaefer S, Issell B, et al. A prospective randomized trial of methotrexate versus cisplatin in the treatment of recurrent squamous cell carcinoma of the head and neck. Cancer 1983;52:206-210.) Antifolates inhibit one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), by competing with reduced folates for binding sites of these enzymes. (Shih C, Habeck LL, Mendelsohn LG, Chen

VJ, Schultz RM. Multiple folate enzyme inhibition: Mechanism of a novel pyrrolopyrimidine-based antifolate LY231514 (MTA). Advan Enzyme Regul, 1998; 38:135-152 and Shih C, Chen VJ, Gossett LS, et al. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 1997;57:1116-1123.) Several antifolate drugs are currently in development. Examples of antifolates that have thymidylate synthase inhibiting ("TSI") characteristics include 5-fluorouracil and Tomudex®. An example of an antifolate that has dihydrofolate reductase inhibiting ("DHFRI") characteristic is Methotrexate®. An example of an antifolate that has glycinamide ribonucleotide formyltransferase inhibiting ("GARFTI") characteristics is Lometrexol. Many of these antifolate drugs inhibit more than one biosynthetic pathway. For example Lometrexol is also an inhibitor of dihydrofolate reductase and pemetrexed disodium (Alimta®, Eli Lilly and Company, Indianapolis, IN) has demonstrated thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase inhibition.

A limitation to the development of these drugs is that the cytotoxic activity and subsequent effectiveness of antifolates may be associated with substantial toxicity for some patients. Additionally antifolates as a class are associated with sporadic severe mylosuppression with gastrointestinal toxicity which, though infrequent, carries a high risk of mortality. The inability to control these toxicities led to the abandonment of clinical development of some antifolates and has complicated the clinical development of others, such as Lometrexol and raltitrexed. (Jackman AL, Calvert AH Folate-Based Thymidylate Synthase Inhibitors as Anticancer Drugs. Ann Oncol 1995;6(9):871-881; Laohavinij S, Wedge SR, Lind MJ, et al. A phase I clinical study of the antipurine antifolate Lometrexol (DDATHF) given with oral folic acid. Invest New Drugs 1996;14:325-335; and Maughan TS, James RD, Kerr D, et al., on behalf of the British MRC Colorectal Cancer Working Party. Preliminary results of a multicenter randomized trial comparing 3 chemotherapy regimens (deGramont, Lokich, and raltitrexed) in metastatic colorectal cancer. Proc ASCO 1999;18:Abst 1007.)

Initially, folic acid was used as a treatment for toxicities associated with GARFTI see, e.g. U.S. Pat. No. 5,217,974. Folic acid has been shown to lower homocysteine levels (see e.g. Homocysteine Lowering Trialist's Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomized trials. BMJ

1998;316:894-898 and Naurath HJ, Joosten E, Riezler R, Stabler SP, Allen RH, Lindenbaum J. Effects of vitamin B12, folate and vitamin B6 supplements in elderly people with normal serum vitamin concentrations. Lancet 1995;346:85-89), and homocysteine levels have been shown to be a predictor of cytotoxic events related to the use of GARFT inhibitors, see e.g. U.S. Pat. No. 5,217,974. However, even with this treatment, cytotoxic activity of GARFT inhibitors and antifolates as a class remains a serious concern in the development of antifolates as pharmaceutical drugs. The ability to lower cytotoxic activity would represent an important advance in the use of these agents.

Surprisingly and unexpectedly, we have now discovered that certain toxic effects such as mortality and nonhematologic events, such as skin rashes and fatigue, caused by antifolates, as a class, can be significantly reduced by the presence of a methylmalonic acid lowering agent, without adversely affecting therapeutic efficacy. The present invention thus provides a method for improving the therapeutic utility of antifolate drugs by administering to the host undergoing treatment with a methylmalonic acid lowering agent. We have discovered that increased levels of methylmalonic acid is a predictor of toxic events in patients that receive an antifolate drug and that treatment for the increased methylmalonic acid, such as treatment with vitamin B12, reduces mortality and nonhematologic events, such as skin rashes and fatigue events previously associated with the antifolate drugs.

Additionally, we have discovered that the combination of a methylmalonic acid lowering agent and folic acid synergistically reduces the toxic events associated with the administration of antifolate drugs. Although, the treatment and prevention of cardiovascular disease with folic acid in combination with vitamin B12 is known, the use of the combination for the treatment of toxicity associated with the administration of antifolate drugs was unknown heretofore.

The present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent, alone or in combination with a FBP binding agent, in the preparation of a medicament useful in lowering the mammalian toxicity of an antifolate. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate and a FBP binding agent.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the manufacture of a medicament for use in a method of inhibiting tumor growth in mammals, which method comprises administering said methylmalonic acid lowering agent in combination with an antifolate.

Furthermore, the present invention relates to a product containing a methylmalonic acid lowering agent, an antifolate and optionally a FBP binding agent as a combined preparation for the simultaneous, separate or sequential use in inhibiting tumour growth.

The current invention concerns the discovery that administration of a methylmalonic acid lowering agent in combination with an antifolate drug reduces the toxicity of the said antifolate drug.

The term "inhibit" as it relates to antifolate drugs refers to prohibiting, alleviating, ameliorating, halting, restraining, slowing or reversing the progression of, or reducing tumor growth.

As used herein, the term "effective amount" refers to an amount of a compound or drug, which is capable of performing the intended result. For example, an effective amount of an antifolate drug that is administered in an effort to reduce tumor growth is that amount which is required to reduce tumor growth.

As used herein, the term "toxicity" refers to a toxic event associated with the administration on an antifolate. Such events include, but are not limited to, neutropenia, thrombopenia, toxic death, fatigue, anorexia, nausea, skin rash, infection, diarrhea, mucositis, and anemia. For further explanation of the types of toxicity experienced by patients receiving antifolates, see, generally, Antifolate Drugs in Cancer Therapy. Preferably, toxicity refers to toxic death, fatigue, neutropenia, thrombopenia, and mucositis.

As used herein, the term "nonhematologic event" refers to the occurrence of skin rash or fatigue due to the administration of an antifolate.

As used herein, the term "in combination with" refers to the administration of the methylmalonic acid lowering agent, the antifolate drug, and optionally the folic acid; in any order such that sufficient levels of methylmalonic acid lowering agent and optionally folic acid are present to reduce the toxicity of an antifolate in a mammal. The administration of the compounds maybe simultaneous as a single composition or as two separate compositions or can be administered sequentially as separate compositions such that an effective amount of the agent first administered is in the patient's body when the second and/or third agent is administered. The antifolate drug may be administered to the mammal first, followed by treatment with the methylmalonic acid lowering agent.

Alternatively, the mammal may be administered the antifolate drug simultaneously with the methylmalonic acid lowering agent. Preferably, the mammal is pretreated with the methylmalonic acid lowering agent and then treated with the antifolate. If folic acid is to be administered in addition to the methylmalonic acid lowering agent, the folic acid may be administered at any time prior, post, or simultaneously to the administration of either the methylmalonic acid lowering agent or the antifolate. Preferably, the mammal is pretreated with the methylmalonic acid, and then treated with folic acid, followed by treatment with the antifolate compound.

The terms "antifolate" and "antifolate drug" refer to a chemical compound which inhibits at least one key folate-requiring enzyme of the thymidine or purine biosynthetic pathways, preferably thymidylate synthase ("TS"), dihydrofolate reductase ("DHFR"), or glycinamide ribonucleotide formyltransferase ("GARFT"), by competing with reduced folates for binding sites of these enzymes. Preferred examples of antifolates include ~~5-fluorouracil, as manufactured by Glaxo;~~ Tomudex®, as manufactured by Zeneca; Methotrexate®, as manufactured by Lederle; Lometrexol®, as manufactured by Tularik; pyrido[2,3-d]pyrimidine derivatives described by Taylor et al in U.S. Pat. Nos. 4,684,653, 4,833,145, 4,902,796, 4,871,743, and 4,882,334; derivatives described by Akimoto in U.S. Pat. No. 4,997,838; thymidylate synthase inhibitors as found in EPO application 239,362; and most preferred, Pemetrexed ~~Sodium~~ Disodium (ALIMTA), as manufactured by Eli Lilly & Co.

The terms “methylmalonic acid” and “MMA” refer to a structural isomer of succinic acid present in minute amounts in healthy human urine.

The term “methylmalonic acid lowering agent” refers to a substrate, which lowers the concentration of methylmalonic acid in a mammal. A preferred example of such a substrate is vitamin B12. For methods of determining methylmalonic acid and substrates therefore, see, e.g., Matchar DB, Feussner JR, Millington DS, et al. Isotope dilution assay for urinary methylmalonic acid in the diagnosis of vitamin B12 deficiency. A prospective clinical evaluation. Ann Intern Med 1987; 106: 707-710; Norman EJ, Morrison JA. Screening elderly populations for cobalamin (vitamin B12) deficiency using the urinary methylmalonic acid assay by gas chromatography mass spectrometry. Am J Med 1993; 94: 589-594; Norman EJ. Gas Chromatography mass spectrometry screening of urinary methylmalonic acid: early detection of vitamin B12 (cobalamin) deficiency to prevent

permanent neurologic disability. GC/MS News 1984; 12:120-129; Martin DC, Francis J, Protetch J, Huff FJ. Time dependency of cognitive recovery with cobalamin replacement: report of a pilot study. JAGS 1992; 40: 168-172; Norman EJ, Cronin C. Cobalamin deficiency. Neurol 1996; 47: 310-311; Rasmussen K, Moelby I, Jensen MK. Studies on methylmalonic acid in humans; Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of methylmalonic acid and total homocysteine determination for diagnosing cobalamin and folate deficiency. Am J Med 1994; 96: 239-246.

The term "vitamin B12" refers to vitamin B12 and its pharmaceutical derivatives, such as hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin. Preferably the term refers to vitamin B12, cobalamin, and chlorocobalamin.

The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, such as a sustained release formulation, as an aqueous solution added to drinking water, or as an aqueous parenteral formulation. Preferably the methylmalonic acid lowering agent is administered as an intramuscular injection formulation. Such formulations are known in the art and are commercially available.

The skilled artisan will appreciate that the methylmalonic lowering agents are effective over a wide dosage range. For example, when cobalamin is used as the methylmalonic lowering agent, the dosage of cobalamin may fall within the range of about 0.2 μg to about 3000 μg of cobalamin from once daily for a month to once every nine weeks for a year. Preferably, cobalamin will be dosed as an intramuscular injection of about 500 μg to about 1500 μg administered from about every 24 hours to about every 1680 hours. Preferably, it is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to administration of the antifolate and repeated from about every 24 hours to about every 1680 hours, regardless of when treatment with the antifolate is started and continued until the administration of the antifolate is discontinued. Most preferred is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to the first administration of the antifolate and repeated every 6 to 12 weeks, preferably about every 9 weeks, and continued until the discontinuation of the antifolate administrations. However, it will be

understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

The term "FBP binding agent" as used herein refers to a folic binding protein binding agent which includes folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof. This latter compound is the (6R)-isomer of leucovorin as disclosed in J. Am. Chem. Soc., 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position. They are 10-20 fold more efficient in binding the folate binding protein compared with their respective (6S)—isomer, see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21-22, 1991, Bethesda, MD. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques. See e.g. PCT Patent Application Publication WO 880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554. See, e.g. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline (2000), 8 Folate, pp. 196-305.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available…ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1-C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is

converted to the parent acid in a biological system. The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, preferably as a sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is usually administered to the subject mammal prior to treatment with the antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 0.1 mg to about 30 mg, most preferably about 0.3 mg to about 5 mg, of folic acid is administered orally to a mammal about 1 to 3 weeks post administration of the methylmalonic acid lowering agent and about 1 to about 24 hours prior to the parenteral administration of the amount of an antifolate. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

In general, the term “pharmaceutical” when used as an adjective means substantially non-toxic to living organisms.

Methods

To assess the effect of a methylmalonic acid lowering agent, alone or in combination with folic acid on the antitumor efficacy of an antifolate in a human tumor

xenograft model, female nude mice bearing human MX-1 breast carcinoma were treated with ALIMTA alone or along with super-physiologic doses of folic acid or vitamin B12 (cobalamin).

The animals were maintained on sterilized standard lab chow ad libitum and sterilized water ad libitum. The human MX-1 tumor cells (5×10^6) obtained from donor tumors were implanted subcutaneously in a thigh of female nude mice 8- to 10-weeks old. Beginning on day 7 post tumor cell implantation, the animals were treated with ALIMTA (100 mg/kg or 150 mg/kg) once daily on days 7 through 11 and 14 through 18 by intraperitoneal injection alone or along with folic acid (6 mg/kg or 60 mg/kg) and/or vitamin B12 (165 mg/kg) by intraperitoneal injection on the same schedule.

Tumor response was monitored by tumor volume measurements twice weekly over the course of the experiment. Toxicity was monitored by body weight measurements made at the same time as the tumor volume measurements. Tumor growth delay was the difference in days for the treated and the controls tumors to reach 1000 mm^3.

The human MX-1 breast carcinoma xenograft was responsive to treatment with ALIMTA with doses of 100 mg/kg and 150 mg/kg producing tumor growth delays of 17 days and 21 days, respectively. Folic acid was administered to the animals alone at two doses 6 mg/kg and 60 mg/kg on the same schedule as ALIMTA and produced tumor growth delays of 7 days and 12 days, respectively. Vitamin B12 administered alone at a dose of 165 mg/kg resulted in a tumor growth delay of 12 days.

Combinations of ALIMTA at each of the two doses were administered along with each of the vitamins as simultaneous combination regimens. Administration of folic acid (6 mg/kg) along with ALIMTA did not alter the tumor growth delay produced from that obtained with ALIMTA alone. The addition of folic acid at the higher dose (60 mg/kg) along with each dose of ALIMTA resulted in small increases in tumor growth delay to 22 days and 23 days at the ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively. The tumor growth delays with ALIMTA and vitamin B12 (165 mg/kg) treatment were 22 days and 24 days at ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively.

Body weight was used as a general measure of toxicity for each of the treatment regimens. The body weight loss pattern reflected the treatment regimens with weight

decrease during the treatment times of days 7 through 11 and 14 through 18 with some weight recovery during the intervening two days. The weight loss due to ALITMA was dose dependent but overall minor (3%). Folic acid alone at either 6 mg/kg or 60 mg/kg did not cause weight loss, in fact folic acid treated animals maintained weight and gained weight over the course of the experiment better than the control animals. The animals treated with ALIMTA (100 mg/kg) and folic acid (60 mg/kg) gained weight (about 20%) over the course of the experiment.

Administration of vitamin B12 did not prevent weight gain in the animals over the time course of the experiment. The animals treated with ALIMTA (100 mg/kg) along with vitamin B12 gained weight while those treated with ALIMTA (150 mg/kg) along with vitamin B12 maintained weight over the course of the experiment.

In conclusion, administration of super-physiologic but non-toxic doses of the vitamins, folic acid and vitamin B12, did not alter the antitumor activity of ALIMTA in the human MX-1 breast carcinoma xenograft tumor in nude mice and did not increase the toxicity of ALIMTA as determined by body weight measurements of the animals.

The effect of vitamin B12, alone or in combination with folic acid, on antifolates can be demonstrated in standard tests commonly utilized to determine the antitumor activity and toxic effects of the antifolates themselves. In one such test, mice are inoculated with the C3H strain of mammary adenocarcinoma by inserting a 2 mm by 2 mm section of tumor into the axillary region of the mice by trocar. The timing of administering the methylmalonic acid lowering agent, alone or in combination with the folic acid, and the antifolate may be varied. Ten animals are used at each dosage level. Antitumor activity is assessed on day ten (when day one is first dosage of antifolate) by measuring the length and width of the tumor growth using vernier calipers, and the activity is expressed as a percent inhibition of tumor growth.

When the antifolate is administered to infected mice which are maintained on a diet totally free of vitamin B12 and optionally folic acid for two weeks prior to and during treatment, it exhibits moderated antitumor activity at very low doses, but also causes severe toxicity at a very low dose (measured as death of mice).

A test group of mice are maintained on a vitamin B12 and optionally folic acid free diet for two weeks before treatment. Vitamin B12 and optionally folic acid is then

administered during the treatment by intramuscular injection of 0.0003% vitamin B12 (weight/volume) and optionally providing the animals drinking water containing 0.0003% folic acid (weight/volume). This concentration translates to about 1.75 mg of vitamin B12 and optionally folic acid per square meter of body surface per day. As the foregoing results indicate, addition of the indicated level of vitamin B12 to the diet of a subject receiving an antifolate results in excellent antitumor activity at low doses, with little or no toxic effects.

The foregoing tests establish that for tumor bearing mice maintained on a vitamin B12 and optionally folic acid free diet prior to and during treatment with an antifolate, the toxicity of the antifolate is very large, with 1 mg/kg/day being lethal to the majority of the mice, and lower antitumor activity is observed at non-toxic drug doses. Very low doses of vitamin B12 partially reverses drug toxicity and improved antitumor activity. Larger doses of vitamin B12 reduce antifolate toxicity even more significantly. Pretreatment of the mouse with vitamin B12 and then administering folic acid prior to administering the antifolate demonstrates a striking reduction in toxicity, almost eliminating the antifolate toxicity completely. Thus, the use of vitamin B12 in combination with an antifolate reduces drug toxicity without adversely affecting antitumor activity, and the use of vitamin B12 in conjunction with folic acid synergistically reduces drug toxicity.

In a typical clinical evaluation involving cancer patients, all of whom have histologically or cytologically confirmed diagnosis of cancer, an antifolate is administered in combination with vitamin B12. Vitamin B12 is administered as a 1000 μg intramuscular injection 1-3 weeks prior to treatment with the antifolate, and 1000 μg intramuscular injection of vitamin B12 is made approximately every 9 weeks until the patient discontinues from therapy. The antifolate is administered in four doses over a two week period by rapid intravenous injection, followed by two weeks of non-therapy. Dosing is made on days 1, 4, 8 and 11 of any two week period. Patients will have an initial course of therapy at a dose of 5 mg/m^2/dose, and depending upon the toxic effects observed in the initial course, their subsequent courses may be at the same dose, or may be escalated to 6 mg/m^2, or may be attenuated to 4 mg/m^2.

In preparation for the foregoing clinical study, pilot studies in humans have established that vitamin B12 given to patients receiving Alimta has effected reduced side

effects due to the Alimta. One to two weeks prior to administration of ALIMTA urine is collected and blood is drawn from a human subject; and vitamin metabolite levels, methylmalonic acid and homocysteine, are determined. Homocysteine levels are determined in blood by a fluorescent polarization immunoassay kit manufactured by Abbot Laboratories. Methylmalonic acid levels are determined by urine levels using a 24 hour urine collection kit available from Biolab Medical Unit (a United Kingdom company). Additionally urine and blood may be collected one week prior to administration of ALIMTA (after at least 5 days of folic acid supplementation and at least 1 week vitamin B12 supplementation), and up to 4 days prior to every cycle.

Method of administration and dosing procedures:

1. Folic Acid:

Folic acid will be supplied as one of the following options, with preference in order from option #1 to option #3:

1. 350 - 600 μg folic acid.
2. A multivitamin containing folic acid in the range of 350 μg to 600 μg is acceptable if option #1 is not available.
3. A dose of folic acid between 350 μg and 1000 μg is acceptable if neither option #1 or option # 2 is available.

For purposes of this study, patients should take oral folic acid daily beginning approximately 1 to 3 weeks before treatment with ALIMTA plus cisplatin or cisplatin alone and continuing daily until discontinuation from study therapy.

2. Vitamin B12

Vitamin B12 will be obtained and administered as a 1000 μg intramuscular injection. A vitamin B12 injection must be administered approximately 1 to 3 weeks before treatment with ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Folic acid supplementation, 350 – 600 μg or equivalent should be taken orally daily beginning approximately 1 to 3 weeks prior to the first dose of MTA plus cisplatin and continue daily until the patient discontinues from study therapy. A vitamin B12

injection, 1000 μg, must be given intramuscularly approximately 1 to 3 weeks prior to the first dose of ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Compare presupplementation homocysteine and methylmalonic acid levels to a) the level immediately prior to the initial dose of study drug, and b) to the level immediately prior to the second dose of study drug (i.e., after a full cycle of supplementation), and compare the prevalence of specific toxicities experienced in up to the first seven cycles of therapy in patients who have been supplemented from baseline to the prevalence seen in the earlier patients (n = 246) who were not supplemented (Farber et al.)

Toxicity may be compared in specific patients in non-supplemented cycles versus supplemented cycles (cross-over patients).

The data to be compared are:

1) Patient numbers and baseline demographic data for those supplemented from baseline.
2) Homocysteine and methylmalonic acid levels, levels at baseline, prior to first dose, prior to second dose, and prior to each therapy cycle depending of the type of cancer under study.
3) Grade 3 and 4 hematologic toxicity in these fully supplemented patients.
4) Grade 3 and 4 nonhematologic toxicity in these fully supplemented patients.

The grading of toxicities in chemotherapuetic clinical trials is well known to a person of skill in the art. Examples of fatigue and skin rash grading are provided below.

Fatigue Grading --

	Neuromotor
Grade 0	none or no change
Grade 1	subjective weakness; no objective findings
Grade 2	mild objective weakness without significant impairment of function
Grade 3	objective weakness with impairment of function
Grade 4	paralysis

Rash Grading --

	Skin
Grade 0	none or no change
Grade 1	scattered macular or papular eruption or erythema that is asymptomatic
Grade 2	scattered macular or papular eruption or erythema with pruritus or other associated eruption symptoms
Grade 3	generalized symptomatic macular, papular, or vesicular eruption
Grade 4	exfoliative dermatitis or ulcerating dermatitis

The vitamins (both folic acid and B12) to be used in the following studies may be obtained from Zenith Gold Line, Centrum, Folvite, or in Canada Apo-Folic. Cyanocobalamin is used as the methylmalonic acid lowering agent in these studies.

Current and past clinical trials show a 4% drug-related death total, 50% grade 3/4 neutropenia, 7% grade 4 thrombocytopenia, and 10% grade 3/4 diarrheas and mucositis in patients administered ALIMTA and folic acid as described in U.S. Pat. No. 5,217,974. Vitamin B12 supplementation with ALIMTA has a moderate effect on drug related toxicity, lowering drug related deaths to 3% and severe toxicities by about 25%. The combination of vitamin B12 and folic acid with ALIMTA has lowered the drug related deaths to <1% in over 480 so treated. The combination of vitamin B12 and folic acid has lowered the drug related grade 3/4 toxic events, see Table 1.

Table 1

	Percent of occurrences prior to B12/folic acid treatment (N=246)	Percent of occurrences post B12/folic acid treatment (N=78)
Hematologic Toxicity/Non-Hematologic Toxicity	37%	6.4%
Neutropenia	32%	2.6%
Mucositis	5%	1.3%
Diarrhea	6%	2.6%
Neutropenia and Mucositis	3%	0%
Neutropenia and Diarrhea	3%	0%
Neutropenia and Infection	2%	0%

Additionally, sixty-two chemonaive patients requiring chemotherapeutic treatment were divided into two groups. Seventeen of these patients received ALIMTA, but did not receive vitamin B12 or folic acid, as described *supra*. The remaining patients received

treatment with vitamin B12, folic acid, and ALIMTA, as described *supra*. Of patients who received the combination treatment, 8 out of 45 responded to the chemotherapy. Of patients who did not receive the combination treatment, but rather, received only treatment with ALIMTA, only 1 out of 17 patients responded.

Abstract

A method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

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NOVEL ANTIFOLATE COMBINATION THERAPIES

This application is a divisional of Application No. 11/288,807, filed 29 November 2005, which is a divisional of Application No. 10/297,821 filed 12 May 2002, now Patent Number 7,053,065, which claims priority under 35 USC 371, for PCT/US01/14860, filed 15 June 2001, which claims the priority of U.S. provisional applications No. 60/215,310, filed 30 June 2000, No. 60/235,859, filed 27 September 2000, and No. 60/284,448, filed 18 April 2001.

Potentially, life-threatening toxicity remains a major limitation to the optimal administration of antifolates. (see, generally, Antifolate Drugs in Cancer Therapy, edited by Jackman, Ann L., Humana Press, Totowa, NJ, 1999.) In some cases, a supportive intervention is routinely used to permit safe, maximal dosing. For example, steroids, such as dexamethone, can be used to prevent the formation of skin rashes caused by the antifolate. (Antifolate, pg 197.)

Antifolates represent one of the most thoroughly studied classes of antineoplastic agents, with aminopterin initially demonstrating clinical activity approximately 50 years ago. Methotrexate was developed shortly thereafter, and today is a standard component of effective chemotherapeutic regimens for malignancies such as lymphoma, breast cancer, and head and neck cancer. (Bonnadonna G, Zambetti M, Valagussa P. Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive nodes: Ten year results. JAMA 1995;273(7):542-547; Bonnadonna G, Valagussa P, Moliterni A, Zambetti M, Brambilla C. Adjuvant cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer: The results of 20 years of follow-up. N Engl J Med 1995;332(14):901-906; and Hong WK, Schaefer S, Issell B, et al. A prospective randomized trial of methotrexate versus cisplatin in the treatment of recurrent squamous cell carcinoma of the head and neck. Cancer 1983;52:206-210.) Antifolates inhibit one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), by competing with reduced folates for binding sites of these enzymes. (Shih C, Habeck LL, Mendelsohn LG, Chen

VJ, Schultz RM. Multiple folate enzyme inhibition: Mechanism of a novel pyrrolopyrimidine-based antifolate LY231514 (MTA). Advan Enzyme Regul, 1998; 38:135-152 and Shih C, Chen VJ, Gossett LS, et al. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 1997;57:1116-1123.) Several antifolate drugs are currently in development. Examples of antifolates that have thymidylate synthase inhibiting ("TSI") characteristics include 5-fluorouracil and Tomudex®. An example of an antifolate that has dihydrofolate reductase inhibiting ("DHFRI") characteristic is Methotrexate®. An example of an antifolate that has glycinamide ribonucleotide formyltransferase inhibiting ("GARFTI") characteristics is Lometrexol. Many of these antifolate drugs inhibit more than one biosynthetic pathway. For example Lometrexol is also an inhibitor of dihydrofolate reductase and pemetrexed disodium (Alimta®, Eli Lilly and Company, Indianapolis, IN) has demonstrated thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase inhibition.

A limitation to the development of these drugs is that the cytotoxic activity and subsequent effectiveness of antifolates may be associated with substantial toxicity for some patients. Additionally antifolates as a class are associated with sporadic severe mylosuppression with gastrointestinal toxicity which, though infrequent, carries a high risk of mortality. The inability to control these toxicities led to the abandonment of clinical development of some antifolates and has complicated the clinical development of others, such as Lometrexol and raltitrexed. (Jackman AL, Calvert AH Folate-Based Thymidylate Synthase Inhibitors as Anticancer Drugs. Ann Oncol 1995;6(9):871-881; Laohavinij S, Wedge SR, Lind MJ, et al. A phase I clinical study of the antipurine antifolate Lometrexol (DDATHF) given with oral folic acid. Invest New Drugs 1996;14:325-335; and Maughan TS, James RD, Kerr D, et al., on behalf of the British MRC Colorectal Cancer Working Party. Preliminary results of a multicenter randomized trial comparing 3 chemotherapy regimens (deGramont, Lokich, and raltitrexed) in metastatic colorectal cancer. Proc ASCO 1999;18:Abst 1007.)

Initially, folic acid was used as a treatment for toxicities associated with GARFTI see, e.g. U.S. Pat. No. 5,217,974. Folic acid has been shown to lower homocysteine levels (see e.g. Homocysteine Lowering Trialist's Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomized trials. BMJ

1998;316:894-898 and Naurath HJ, Joosten E, Riezler R, Stabler SP, Allen RH, Lindenbaum J. Effects of vitamin B12, folate and vitamin B6 supplements in elderly people with normal serum vitamin concentrations. Lancet 1995;346:85-89), and homocysteine levels have been shown to be a predictor of cytotoxic events related to the use of GARFT inhibitors, see e.g. U.S. Pat. No. 5,217,974. However, even with this treatment, cytotoxic activity of GARFT inhibitors and antifolates as a class remains a serious concern in the development of antifolates as pharmaceutical drugs. The ability to lower cytotoxic activity would represent an important advance in the use of these agents.

Surprisingly and unexpectedly, we have now discovered that certain toxic effects such as mortality and nonhematologic events, such as skin rashes and fatigue, caused by antifolates, as a class, can be significantly reduced by the presence of a methylmalonic acid lowering agent, without adversely affecting therapeutic efficacy. The present invention thus provides a method for improving the therapeutic utility of antifolate drugs by administering to the host undergoing treatment with a methylmalonic acid lowering agent. We have discovered that increased levels of methylmalonic acid is a predictor of toxic events in patients that receive an antifolate drug and that treatment for the increased methylmalonic acid, such as treatment with vitamin B12, reduces mortality and nonhematologic events, such as skin rashes and fatigue events previously associated with the antifolate drugs.

Additionally, we have discovered that the combination of a methylmalonic acid lowering agent and folic acid synergistically reduces the toxic events associated with the administration of antifolate drugs. Although, the treatment and prevention of cardiovascular disease with folic acid in combination with vitamin B12 is known, the use of the combination for the treatment of toxicity associated with the administration of antifolate drugs was unknown heretofore.

The present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of administering an antifolate to a mammal in need thereof, comprising administering an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent and a FBP binding agent. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent, alone or in combination with a FBP binding agent, in the preparation of a medicament useful in lowering the mammalian toxicity of an antifolate. A preferred FBP binding agent is folic acid.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the preparation of a medicament useful in lowering the mammalian toxicity associated with an antifolate, and the medicament is administered in combination with an antifolate and a FBP binding agent.

Furthermore, the present invention relates to the use of a methylmalonic acid lowering agent in the manufacture of a medicament for use in a method of inhibiting tumor growth in mammals, which method comprises administering said methylmalonic acid lowering agent in combination with an antifolate.

Furthermore, the present invention relates to a product containing a methylmalonic acid lowering agent, an antifolate and optionally a FBP binding agent as a combined preparation for the simultaneous, separate or sequential use in inhibiting tumour growth.

The current invention concerns the discovery that administration of a methylmalonic acid lowering agent in combination with an antifolate drug reduces the toxicity of the said antifolate drug.

The term "inhibit" as it relates to antifolate drugs refers to prohibiting, alleviating, ameliorating, halting, restraining, slowing or reversing the progression of, or reducing tumor growth.

As used herein, the term "effective amount" refers to an amount of a compound or drug, which is capable of performing the intended result. For example, an effective amount of an antifolate drug that is administered in an effort to reduce tumor growth is that amount which is required to reduce tumor growth.

As used herein, the term "toxicity" refers to a toxic event associated with the administration on an antifolate. Such events include, but are not limited to, neutropenia, thrombopenia, toxic death, fatigue, anorexia, nausea, skin rash, infection, diarrhea, mucositis, and anemia. For further explanation of the types of toxicity experienced by patients receiving antifolates, see, generally, Antifolate Drugs in Cancer Therapy. Preferably, toxicity refers to toxic death, fatigue, neutropenia, thrombopenia, and mucositis.

As used herein, the term "nonhematologic event" refers to the occurrence of skin rash or fatigue due to the administration of an antifolate.

As used herein, the term "in combination with" refers to the administration of the methylmalonic acid lowering agent, the antifolate drug, and optionally the folic acid; in any order such that sufficient levels of methylmalonic acid lowering agent and optionally folic acid are present to reduce the toxicity of an antifolate in a mammal. The administration of the compounds maybe simultaneous as a single composition or as two separate compositions or can be administered sequentially as separate compositions such that an effective amount of the agent first administered is in the patient's body when the second and/or third agent is administered. The antifolate drug may be administered to the mammal first, followed by treatment with the methylmalonic acid lowering agent.

Alternatively, the mammal may be administered the antifolate drug simultaneously with the methylmalonic acid lowering agent. Preferably, the mammal is pretreated with the methylmalonic acid lowering agent and then treated with the antifolate. If folic acid is to be administered in addition to the methylmalonic acid lowering agent, the folic acid may be administered at any time prior, post, or simultaneously to the administration of either the methylmalonic acid lowering agent or the antifolate. Preferably, the mammal is pretreated with the methylmalonic acid, and then treated with folic acid, followed by treatment with the antifolate compound.

The terms "antifolate" and "antifolate drug" refer to a chemical compound which inhibits at least one key folate-requiring enzyme of the thymidine or purine biosynthetic pathways, preferably thymidylate synthase ("TS"), dihydrofolate reductase ("DHFR"), or glycinamide ribonucleotide formyltransferase ("GARFT"), by competing with reduced folates for binding sites of these enzymes. Preferred examples of antifolates include Tomudex®, as manufactured by Zeneca; Methotrexate®, as manufactured by Lederle; Lometrexol®, as manufactured by Tularik; pyrido[2,3-d]pyrimidine derivatives described by Taylor et al in U.S. Pat. Nos. 4,684,653, 4,833,145, 4,902,796, 4,871,743, and 4,882,334; derivatives described by Akimoto in U.S. Pat. No. 4,997,838; thymidylate synthase inhibitors as found in EPO application 239,362; and most preferred, Pemetrexed Disodium (ALIMTA), as manufactured by Eli Lilly & Co.

The terms “methylmalonic acid” and “MMA” refer to a structural isomer of succinic acid present in minute amounts in healthy human urine.

The term “methylmalonic acid lowering agent” refers to a substrate, which lowers the concentration of methylmalonic acid in a mammal. A preferred example of such a substrate is vitamin B12. For methods of determining methylmalonic acid and substrates therefore, see, e.g., Matchar DB, Feussner JR, Millington DS, et al. Isotope dilution assay for urinary methylmalonic acid in the diagnosis of vitamin B12 deficiency. A prospective clinical evaluation. Ann Intern Med 1987; 106: 707-710; Norman EJ, Morrison JA. Screening elderly populations for cobalamin (vitamin B12) deficiency using the urinary methylmalonic acid assay by gas chromatography mass spectrometry. Am J Med 1993; 94: 589-594; Norman EJ. Gas Chromatography mass spectrometry screening of urinary methylmalonic acid: early detection of vitamin B12 (cobalamin) deficiency to prevent permanent neurologic disability. GC/MS News 1984; 12:120-129; Martin DC, Francis J,

Protetch J, Huff FJ. Time dependency of cognitive recovery with cobalamin replacement: report of a pilot study. JAGS 1992; 40: 168-172; Norman EJ, Cronin C. Cobalamin deficiency. Neurol 1996; 47: 310-311; Rasmussen K, Moelby I, Jensen MK. Studies on methylmalonic acid in humans; Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of methylmalonic acid and total homocysteine determination for diagnosing cobalamin and folate deficiency. Am J Med 1994; 96: 239-246.

The term "vitamin B12" refers to vitamin B12 and its pharmaceutical derivatives, such as hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin. Preferably the term refers to vitamin B12, cobalamin, and chlorocobalamin.

The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, such as a sustained release formulation, as an aqueous solution added to drinking water, or as an aqueous parenteral formulation. Preferably the methylmalonic acid lowering agent is administered as an intramuscular injection formulation. Such formulations are known in the art and are commercially available.

The skilled artisan will appreciate that the methylmalonic lowering agents are effective over a wide dosage range. For example, when cobalamin is used as the methylmalonic lowering agent, the dosage of cobalamin may fall within the range of about 0.2 μg to about 3000 μg of cobalamin from once daily for a month to once every nine weeks for a year. Preferably, cobalamin will be dosed as an intramuscular injection of about 500 μg to about 1500 μg administered from about every 24 hours to about every 1680 hours. Preferably, it is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to administration of the antifolate and repeated from about every 24 hours to about every 1680 hours, regardless of when treatment with the antifolate is started and continued until the administration of the antifolate is discontinued. Most preferred is an intramuscular injection of about 1000 μg administered initially from about 1 to about 3 weeks prior to the first administration of the antifolate and repeated every 6 to 12 weeks, preferably about every 9 weeks, and continued until the discontinuation of the antifolate administrations. However, it will be understood that the amount of the methylmalonic acid lowering agent actually

administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

The term "FBP binding agent" as used herein refers to a folic binding protein binding agent which includes folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof. This latter compound is the (6R)-isomer of leucovorin as disclosed in J. Am. Chem. Soc., 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position. They are 10-20 fold more efficient in binding the folate binding protein compared with their respective (6S)—isomer, see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21-22, 1991, Bethesda, MD. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques. See e.g. PCT Patent Application Publication WO 880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554. See, e.g. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline (2000), 8 Folate, pp. 196-305.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available…ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1-C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is converted to the parent acid in a biological system. The dosage generally will be

provided in the form of a vitamin supplement, namely as a tablet administered orally, preferably as a sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is usually administered to the subject mammal prior to treatment with the antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 0.1 mg to about 30 mg, most preferably about 0.3 mg to about 5 mg, of folic acid is administered orally to a mammal about 1 to 3 weeks post administration of the methylmalonic acid lowering agent and about 1 to about 24 hours prior to the parenteral administration of the amount of an antifolate. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

In general, the term “pharmaceutical” when used as an adjective means substantially non-toxic to living organisms.

Methods

To assess the effect of a methylmalonic acid lowering agent, alone or in combination with folic acid on the antitumor efficacy of an antifolate in a human tumor xenograft model, female nude mice bearing human MX-1 breast carcinoma were treated

with ALIMTA alone or along with super-physiologic doses of folic acid or vitamin B12 (cobalamin).

The animals were maintained on sterilized standard lab chow ad libitum and sterilized water ad libitum. The human MX-1 tumor cells (5×10^6) obtained from donor tumors were implanted subcutaneously in a thigh of female nude mice 8- to 10-weeks old. Beginning on day 7 post tumor cell implantation, the animals were treated with ALIMTA (100 mg/kg or 150 mg/kg) once daily on days 7 through 11 and 14 through 18 by intraperitoneal injection alone or along with folic acid (6 mg/kg or 60 mg/kg) and/or vitamin B12 (165 mg/kg) by intraperitoneal injection on the same schedule.

Tumor response was monitored by tumor volume measurements twice weekly over the course of the experiment. Toxicity was monitored by body weight measurements made at the same time as the tumor volume measurements. Tumor growth delay was the difference in days for the treated and the controls tumors to reach 1000 mm^3.

The human MX-1 breast carcinoma xenograft was responsive to treatment with ALIMTA with doses of 100 mg/kg and 150 mg/kg producing tumor growth delays of 17 days and 21 days, respectively. Folic acid was administered to the animals alone at two doses 6 mg/kg and 60 mg/kg on the same schedule as ALIMTA and produced tumor growth delays of 7 days and 12 days, respectively. Vitamin B12 administered alone at a dose of 165 mg/kg resulted in a tumor growth delay of 12 days.

Combinations of ALIMTA at each of the two doses were administered along with each of the vitamins as simultaneous combination regimens. Administration of folic acid (6 mg/kg) along with ALIMTA did not alter the tumor growth delay produced from that obtained with ALIMTA alone. The addition of folic acid at the higher dose (60 mg/kg) along with each dose of ALIMTA resulted in small increases in tumor growth delay to 22 days and 23 days at the ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively. The tumor growth delays with ALIMTA and vitamin B12 (165 mg/kg) treatment were 22 days and 24 days at ALIMTA doses of 100 mg/kg and 150 mg/kg, respectively.

Body weight was used as a general measure of toxicity for each of the treatment regimens. The body weight loss pattern reflected the treatment regimens with weight decrease during the treatment times of days 7 through 11 and 14 through 18 with some

weight recovery during the intervening two days. The weight loss due to ALITMA was dose dependent but overall minor (3%). Folic acid alone at either 6 mg/kg or 60 mg/kg did not cause weight loss, in fact folic acid treated animals maintained weight and gained weight over the course of the experiment better than the control animals. The animals treated with ALIMTA (100 mg/kg) and folic acid (60 mg/kg) gained weight (about 20%) over the course of the experiment.

Administration of vitamin B12 did not prevent weight gain in the animals over the time course of the experiment. The animals treated with ALIMTA (100 mg/kg) along with vitamin B12 gained weight while those treated with ALIMTA (150 mg/kg) along with vitamin B12 maintained weight over the course of the experiment.

In conclusion, administration of super-physiologic but non-toxic doses of the vitamins, folic acid and vitamin B12, did not alter the antitumor activity of ALIMTA in the human MX-1 breast carcinoma xenograft tumor in nude mice and did not increase the toxicity of ALIMTA as determined by body weight measurements of the animals.

The effect of vitamin B12, alone or in combination with folic acid, on antifolates can be demonstrated in standard tests commonly utilized to determine the antitumor activity and toxic effects of the antifolates themselves. In one such test, mice are inoculated with the C3H strain of mammary adenocarcinoma by inserting a 2 mm by 2 mm section of tumor into the axillary region of the mice by trocar. The timing of administering the methylmalonic acid lowering agent, alone or in combination with the folic acid, and the antifolate may be varied. Ten animals are used at each dosage level. Antitumor activity is assessed on day ten (when day one is first dosage of antifolate) by measuring the length and width of the tumor growth using vernier calipers, and the activity is expressed as a percent inhibition of tumor growth.

When the antifolate is administered to infected mice which are maintained on a diet totally free of vitamin B12 and optionally folic acid for two weeks prior to and during treatment, it exhibits moderated antitumor activity at very low doses, but also causes severe toxicity at a very low dose (measured as death of mice).

A test group of mice are maintained on a vitamin B12 and optionally folic acid free diet for two weeks before treatment. Vitamin B12 and optionally folic acid is then administered during the treatment by intramuscular injection of 0.0003% vitamin B12

(weight/volume) and optionally providing the animals drinking water containing 0.0003% folic acid (weight/volume). This concentration translates to about 1.75 mg of vitamin B12 and optionally folic acid per square meter of body surface per day. As the foregoing results indicate, addition of the indicated level of vitamin B12 to the diet of a subject receiving an antifolate results in excellent antitumor activity at low doses, with little or no toxic effects.

The foregoing tests establish that for tumor bearing mice maintained on a vitamin B12 and optionally folic acid free diet prior to and during treatment with an antifolate, the toxicity of the antifolate is very large, with 1 mg/kg/day being lethal to the majority of the mice, and lower antitumor activity is observed at non-toxic drug doses. Very low doses of vitamin B12 partially reverses drug toxicity and improved antitumor activity. Larger doses of vitamin B12 reduce antifolate toxicity even more significantly. Pretreatment of the mouse with vitamin B12 and then administering folic acid prior to administering the antifolate demonstrates a striking reduction in toxicity, almost eliminating the antifolate toxicity completely. Thus, the use of vitamin B12 in combination with an antifolate reduces drug toxicity without adversely affecting antitumor activity, and the use of vitamin B12 in conjunction with folic acid synergistically reduces drug toxicity.

In a typical clinical evaluation involving cancer patients, all of whom have histologically or cytologically confirmed diagnosis of cancer, an antifolate is administered in combination with vitamin B12. Vitamin B12 is administered as a 1000 μg intramuscular injection 1-3 weeks prior to treatment with the antifolate, and 1000 μg intramuscular injection of vitamin B12 is made approximately every 9 weeks until the patient discontinues from therapy. The antifolate is administered in four doses over a two week period by rapid intravenous injection, followed by two weeks of non-therapy. Dosing is made on days 1, 4, 8 and 11 of any two week period. Patients will have an initial course of therapy at a dose of 5 mg/m^2/dose, and depending upon the toxic effects observed in the initial course, their subsequent courses may be at the same dose, or may be escalated to 6 mg/m^2, or may be attenuated to 4 mg/m^2.

In preparation for the foregoing clinical study, pilot studies in humans have established that vitamin B12 given to patients receiving Alimta has effected reduced side effects due to the Alimta. One to two weeks prior to administration of ALIMTA urine is

collected and blood is drawn from a human subject; and vitamin metabolite levels, methylmalonic acid and homocysteine, are determined. Homocysteine levels are determined in blood by a fluorescent polarization immunoassay kit manufactured by Abbot Laboratories. Methylmalonic acid levels are determined by urine levels using a 24 hour urine collection kit available from Biolab Medical Unit (a United Kingdom company). Additionally urine and blood may be collected one week prior to administration of ALIMTA (after at least 5 days of folic acid supplementation and at least 1 week vitamin B12 supplementation), and up to 4 days prior to every cycle.

Method of administration and dosing procedures:

1. Folic Acid:

Folic acid will be supplied as one of the following options, with preference in order from option #1 to option #3:

1. 350 - 600 μg folic acid.
2. A multivitamin containing folic acid in the range of 350 μg to 600 μg is acceptable if option #1 is not available.
3. A dose of folic acid between 350 μg and 1000 μg is acceptable if neither option #1 or option # 2 is available.

For purposes of this study, patients should take oral folic acid daily beginning approximately 1 to 3 weeks before treatment with ALIMTA plus cisplatin or cisplatin alone and continuing daily until discontinuation from study therapy.

2. Vitamin B12

Vitamin B12 will be obtained and administered as a 1000 μg intramuscular injection. A vitamin B12 injection must be administered approximately 1 to 3 weeks before treatment with ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Folic acid supplementation, 350 – 600 μg or equivalent should be taken orally daily beginning approximately 1 to 3 weeks prior to the first dose of MTA plus cisplatin and continue daily until the patient discontinues from study therapy. A vitamin B12 injection, 1000 μg, must be given intramuscularly approximately 1 to 3 weeks prior to the

first dose of ALIMTA and should be repeated approximately every 9 weeks until the patient discontinues from study therapy.

Compare presupplementation homocysteine and methylmalonic acid levels to a) the level immediately prior to the initial dose of study drug, and b) to the level immediately prior to the second dose of study drug (i.e., after a full cycle of supplementation), and compare the prevalence of specific toxicities experienced in up to the first seven cycles of therapy in patients who have been supplemented from baseline to the prevalence seen in the earlier patients (n = 246) who were not supplemented (Farber et al.)

Toxicity may be compared in specific patients in non-supplemented cycles versus supplemented cycles (cross-over patients).

The data to be compared are:

1) Patient numbers and baseline demographic data for those supplemented from baseline.
2) Homocysteine and methylmalonic acid levels, levels at baseline, prior to first dose, prior to second dose, and prior to each therapy cycle depending of the type of cancer under study.
3) Grade 3 and 4 hematologic toxicity in these fully supplemented patients.
4) Grade 3 and 4 nonhematologic toxicity in these fully supplemented patients.

The grading of toxicities in chemotherapuetic clinical trials is well known to a person of skill in the art. Examples of fatigue and skin rash grading are provided below.

Fatigue Grading --

	Neuromotor
Grade 0	none or no change
Grade 1	subjective weakness; no objective findings
Grade 2	mild objective weakness without significant impairment of function
Grade 3	objective weakness with impairment of function
Grade 4	paralysis

Rash Grading --

Skin

Grade 0 none or no change

Grade 1 scattered macular or papular eruption or erythema that is asymptomatic

Grade 2 scattered macular or papular eruption or erythema with pruritus or other associated eruption symptoms

Grade 3 generalized symptomatic macular, papular, or vesicular eruption

Grade 4 exfoliative dermatitis or ulcerating dermatitis

The vitamins (both folic acid and B12) to be used in the following studies may be obtained from Zenith Gold Line, Centrum, Folvite, or in Canada Apo-Folic. Cyanocobalamin is used as the methylmalonic acid lowering agent in these studies.

Current and past clinical trials show a 4% drug-related death total, 50% grade 3/4 neutropenia, 7% grade 4 thrombocytopenia, and 10% grade 3/4 diarrheas and mucositis in patients administered ALIMTA and folic acid as described in U.S. Pat. No. 5,217,974. Vitamin B12 supplementation with ALIMTA has a moderate effect on drug related toxicity, lowering drug related deaths to 3% and severe toxicities by about 25%. The combination of vitamin B12 and folic acid with ALIMTA has lowered the drug related deaths to <1% in over 480 so treated. The combination of vitamin B12 and folic acid has lowered the drug related grade 3/4 toxic events, see Table 1.

Table 1

	Percent of occurrences prior to B12/folic acid treatment (N=246)	Percent of occurrences post B12/folic acid treatment (N=78)
Hematologic Toxicity/Non-Hematologic Toxicity	37%	6.4%
Neutropenia	32%	2.6%
Mucositis	5%	1.3%
Diarrhea	6%	2.6%
Neutropenia and Mucositis	3%	0%
Neutropenia and Diarrhea	3%	0%
Neutropenia and Infection	2%	0%

Additionally, sixty-two chemonaive patients requiring chemotherapeutic treatment were divided into two groups. Seventeen of these patients received ALIMTA, but did not receive vitamin B12 or folic acid, as described *supra*. The remaining patients received treatment with vitamin B12, folic acid, and ALIMTA, as described *supra*. Of patients

who received the combination treatment, 8 out of 45 responded to the chemotherapy. Of patients who did not receive the combination treatment, but rather, received only treatment with ALIMTA, only 1 out of 17 patients responded.

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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
11/776,329	07/11/2007	1751	1000	X14173B	11	2

CONFIRMATION NO. 6568

UPDATED FILING RECEIPT

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN46206-6288

Date Mailed: 08/31/2007

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 write to the Office of Initial Patent Examination's Filing Receipt Corrections. 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)

Clet Niyikiza, Indianapolis, IN;
Paolo Paoletti, Indianapolis, IN;
James Jacob Rusthoven, Ancaster, CANADA;

Power of Attorney: The patent practitioners associated with Customer Number 25885

Domestic Priority data as claimed by applicant

This application is a DIV of 11/288,807 11/29/2005
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

Foreign Applications

If Required, Foreign Filing License Granted: 08/31/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US11/776,329**

Projected Publication Date: 12/13/2007

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL ANTIFOLATE COMBINATION THERAPIES

Preliminary Class

510

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.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

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 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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APPLICATION NUMBER	FILING OR 371(c) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/776,329	07/11/2007	Clet Niyikiza	X14173B

CONFIRMATION NO. 6568

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN46206-6288

Date Mailed. 11/23/2007

NOTICE OF NEW OR REVISED PROJECTED PUBLICATION DATE

The above-identified application has a new or revised projected publication date. The current projected publication date for this application is 02/07/2008. If this is a new projected publication date (there was no previous projected publication date), the application has been cleared by Licensing & Review or a secrecy order has been rescinded and the application is now in the publication queue.

If this is a revised projected publication date (one that is different from a previously communicated projected publication date), the publication date has been revised due to processing delays in the USPTO or the abandonment and subsequent revival of an application. The application is anticipated to be published on a date that is more than six weeks different from the originally-projected publication date.

More detailed publication information is available through the private side of Patent Application Information Retrieval (PAIR) System. The direct link to access PAIR is currently http://pair.uspto.gov. Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Questions relating to this Notice should be directed to the Office of Patent Publication at 1-888-786-0101.

PART 1 - ATTORNEY/APPLICANT COPY

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APPLICATION NUMBER	FILING OR 371(c) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/776,329	07/11/2007	Clet Niyikiza	X14173B

CONFIRMATION NO. 6568

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN46206-6288

Title: NOVEL ANTIFOLATE COMBINATION THERAPIES

Publication No. US-2008-0032948-A1
Publication Date: 02/07/2008

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.

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Pre-Grant Publication Division, 703-605-4283

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	Clet Niyikiza	Conf No.: 6568
Serial No.:	11/776,329	
Application Date:	July 11, 2007	
For:	NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.:	X-14173B	

SECOND PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Introductory Comments

Please amend the accompanying application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

Listing of Claims:

Claims 1-39 (Cancelled)

40. (New) A method for administering pemetrexed disodium to a patient in need thereof comprising administering an effective amount of pemetrexed disodium in combination with a methylmalonic acid lowering agent, wherein:

the methylmalonic lowering agent is selected from the group consisting of vitamin B_{12}, hydroxycobolamin, cyano-10-chlorocobolamin, aquocobolamin perchlorate, aquo-10-cobolamin perchlorate, azidocobolamin or chlorocobolamin;

the methylmalonic acid lowering agent is administered from about 1 week to about 3 weeks prior to the first administration of the pemetrexed disodium; and

the methylmalonic acid administration is repeated about every 6 to about every 12 weeks until administration of the pemetrexed disodium is discontinued.

41. (New) The method of claim 40, wherein the methylmalonic lowering agent is vitamin B_{12}.

42. (New) The method of claim 41, wherein the vitamin B_{12} is administered as an intramuscular injection of about 500 μg to about 1500 μg.

43. (New) The method of claim 42, wherein the vitamin B_{12} is administered as an intramuscular injection of about 1000 μg.

44. (New) The method of claim 41, 42 or 43, wherein the vitamin B_{12} administration is repeated about every 9 weeks until the administration of the pemetrexed disodium is discontinued.

45. (New) The method of claim 44, further comprising administering a folic-binding-protein binding agent to the patient.

46. (New) The method of claim 45 wherein the folic-binding-protein binding agent is folic acid and the folic acid is administered prior to the first administration of the pemetrexed disodium.

47. (New) The method of claim 46 wherein the folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

48. (New) The method of claim 47wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

49. (New) The method according to any one of claims 46-48, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

50. (New) The method of claim 49 wherein about 350μg to about 1000 μg of folic acid is administered.

51. (New) The method of claim 50 wherein 350 μg to 600 μg of folic acid is administered.

52. (New) The method of claim 40 or 45 further comprising the administration of cisplatin to the patient.

Remarks

Applicants submit this paper and request entry of the amendments herein. Claims 1-39 are hereby cancelled and new Claims 40-52 are introduced. Support for new Claims 40-52 is found in the specification, as well as in the claims as originally filed. Applicants respectfully assert that no new matter has been introduced as a result of the amendments to the claims.

Applicants request prompt consideration and allowance of the claimed subject matter. If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

Respectfully submitted,

/John A. Cleveland, Jr./
John A. Cleveland, Jr., Ph.D.
Attorney for Applicant
Registration No. 50,697
Phone: (317) 276-0307

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, IN 46206-6288
December 8, 2008

Electronic Patent Application Fee Transmittal

Application Number:	11776329
Filing Date:	11-Jul-2007
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Filer:	John A. Cleveland/Lisa Capps
Attorney Docket Number:	X14173B

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Multiple dependent claims	1203	1	390	390
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**390**

Electronic Acknowledgement Receipt

EFS ID:	4418432
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	John A. Cleveland/Lisa Capps
Filer Authorized By:	John A. Cleveland
Attorney Docket Number:	X14173B
Receipt Date:	09-DEC-2008
Filing Date:	11-JUL-2007
Time Stamp:	10:37:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$390
RAM confirmation Number	6258
Deposit Account	050840
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		X14173BUSPreliminaryAmendment.pdf	86772 7939711f9c3fb4f3ab7acf30c9f7c8c20351c515	yes	4

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Claims	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	4

Warnings:

Information:

2	Fee Worksheet (PTO-06)	fee-info.pdf	30193 62164f53fae261e03c8ca115834309e18a655863	no	2

Warnings:

Information:

Total Files Size (in bytes):	116965

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.

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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

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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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/776,329	07/11/2007	

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 12/09/2008

	(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))	* 16	Minus	** 20	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 1	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

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		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:
/YOLANDA CHADWICK/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 02/02/2009

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	
ART UNIT	**PAPER NUMBER**
1614	

NOTIFICATION DATE	DELIVERY MODE
02/02/2009	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):

patents@lilly.com

Interview Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KEVIN WEDDINGTON*. (3)*MR. WILLIAM McMILLEN*.

(2) *DR. JOHN A. CLEVELAND, JR.*. (4)_____.

Date of Interview: *27 January 2009*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☒ applicant's representative]

Exhibit shown or demonstration conducted: d)☒ Yes e)☐ No.
If Yes, brief description: *Binder with related applications*.

Claim(s) discussed: *The claims in general*.

Identification of prior art discussed: *NONE*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *The attorney of record, Dr. Cleveland, explained the importance of the present application and its related patent application. Upon examination of the present application, the Examiner will inform the attorney of any critical problems*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Kevin E Weddington/ Primary Examiner, Art Unit	

Notice of References Cited	Application/Control No. 11/776,329	Applicant(s)/Patent Under Reexamination NIYIKIZA ET AL.	
	Examiner Kevin E. Weddington	Art Unit 1614	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-4,140,707	02-1979	Cleare et al.	556/137
	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					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	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.

FORM PTO 1449 (modified) INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X-14173B	Serial No
	First Applicant NIYIKIZA Clet	
	Filing Date	Group

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 Pages or Relevant Figures Appear
/KW/	AA	US 5,405,839	4/ 11/1995	Tetsuo, et al.	
	AB	US 5,431,925	07/00/1995	Ohmori, et al.	
	AC	US 5,563,126	10/8/1996	Allen, et al.	
	AD	US 5,736,402	4/7/1998	Francis, et al.	
	AE	US 6,207,651	3/27/2001	Allen, et al.	
	AF	US 6,297,224	10/2/2001	Allen, et al.	
	AG	US 6,528,496	3/4/2003	Allen, et al.	
	AH	US 03/0216350	11/20/2003	Allen, et al.	
	Al	US 03/0225030	12/4/2003	Allen, et al.	
	AJ	US 2,920,015	01/1960	Thompson, Robert E.	
	AK	US 2004/0005311 Al	01/2004	Pitman, Bradford D.	
	AL	US 5,344,932	09/1994	Taylor, Edward C.	
/KW/	AM	US 7,053,065	05/2006	Niyikiza, et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No. [1]	Foreign Patent Document Country Code [3]-Number [4]-Kind Code5 (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]
/KW/	BA	EP 0 546 870	6/16/1993	EPO		

Examiner Signature	/Kevin Weddington/ (02/11/2009)	Date Considered	02/11/2009

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2] See Kinds 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.

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[6]
/KW/	CA	Calvert H.: "Folate status and the safety profile of antifolates", Seminars in Oncology, 2002, 29/2 Suppl. 5, pp. 3-7, XP008005755	
	CB	Calvert H.: "Future directions in the development of pemetrexed", Seminars in Oncology, 2002, 29/2 Suppl. 5, pp. 54-61, XP008005744	
	CC	Westerhof, et al: "Carrier-and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecularstructure and biological activity", Mol. Pharmacology, 1995, 48(3), pp. 459-71, XP008005762	
	CD	Worzalla, et a]: "Role of folic acid in modulating the toxicity and efficacy of the multitargeted antifolate, LY231514", Anticancer Research (1998), 18(5A), pp. 3235-3239, XP008005757	
	CE	Hanauske, et al: "Pemetrexed disodium: A novel antifolate clinically active against multiple solid tumors", Oncologist, Alphamed Press, US, Vol. 4, No. 6, 2001, pp. 363-373, XP008005751	
	CF	Bunn, et al: "Vitamin B 12 and folate reduce toxicity of Alimta (pemetrexed disodium, LY 231514, MTA), a novel antifolate/antimetabolite", Program/Proceedings - American Society of Clinical Oncology, the Society, US, Vol. 76A, No. 20, 2001, page 300, XPO08005885	
	CG	Dierkes, et al., Supplementation with Vitamin B12 Decreases Homocystein and Methylmalonic Acid but Also Serum Folate in Patients with End-Stage Renal Disease. Metabolism. May 1999. Vol. 48, No. 5, pages 631-635. See: abstract.	
	CH	Arsenyan et al. (Abstract: Onkol. Nauchn., (1978) 12(10):49-54	
	Cl	John, et al. (Cancer 2000, 88: 1807-13)	
	CJ	Poydock et al., "Growth-inhibiting effect of hydroxocobaltniin and L-ascorbic acid on two solid tumors in mce", IRCS Medical _Science, Vol. 12, No. 9, pp. 813 (1984).	
/KW/	CK	The Cecil Reference, TEXTBOOK of MEDICINE, 21st Edition (2000). Chapter 198. pps. 1060-1074.	

Examiner Signature	/Kevin Weddington/ (02/11/2009)	Date Considered	02/11/2009

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2] See Kinds 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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 02/18/2009

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	

ART UNIT	PAPER NUMBER
1614	

NOTIFICATION DATE	DELIVERY MODE
02/18/2009	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):

patents@lilly.com

Office Action Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	Kevin E. Weddington	1614	

-- 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 *09 December 2008*.

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) *40-52* 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) *40-52* is/are rejected.

7)☐ Claim(s) _____ 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 *7-11-07*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Claim 40-52 are presented for examination.

Applicants' preliminary amendment filed December 9, 2008; and the information disclosure statement filed July 11, 2007 have been received and entered.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 45 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

This is a written description rejection.

A lack of adequate written description issue arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process. See, e.g., Fujikawa v. Wattanasin, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); In re Ruschig, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967).

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that

applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

In particular, the specification as original filed fails to provide sufficient written bases of any of the agents demonstrating wherein possession of use of the broad term: **a folic-binding-protein agent**. The mere fact that Applicant may have discovered one type of folic-binding-protein agent is combined with the composition comprising pemetrexed disodium and a methylmalonic acid lowering agent is not sufficient to claim the entire genus.

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. See Eli Lilly, 119 F.3d at 1568, 43 USPQ2d at 1406.

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. The disclosure of only one species encompassed within a genus adequately describes a claim directed to that genus only if

the disclosure "indicates that the patentee has invented species sufficient to constitute the gen[us]."

Claim 45 is not allowed.

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 40-52 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.

Claim 40 is rendered indefinite because the phrase “methylmalonic acid”, located in line 9. The Examiner thinks the applicants left out some important words such as “lowering agent”. The remaining claims 41-52 are rendered indefinite to the extent that they incorporate the above terminology.

Claims 40-52 are not allowed.

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.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

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).

Claims 40-52 are rejected under 35 U.S.C. 103(a) as being unpatentable over Taylor (5,344,932) of PTO-1449 in view of Poydock et al., IRCS Medical Science, Vol. 12, No. 9, pp. 813 (1984) of PTO-1449, further in view of Worzalla et al., Anticancer Research, Vol. 18, No. 5, pp. 3235-3239 of PTO-1449, and further in view of Cleare et al. (4,149,707).

Taylor teaches N-(pyrrolo(2,3-D)pyrimidin-3-ylacyl)-glutamic acid derivatives which includes LY 2315 (pemetrexe) and LY 231514-disodium, (pemetrexed disodium) are effective as antineoplastic agents to inhibit the growth of tumors (see column 8, lines 57-63). Note particularly column 8, lines 64-68 states that other antineoplastic agents can be combined with LY 231514. Note particularly column 9, line 1 shows the various modes of administration such as parenteral routes (intramuscular) and oral.

The instant invention differs from the cited reference in that the cited reference does not teach the addition of a methylmalonic acid lowering agent . However, the secondary reference, Poydock et al., teaches a methylmalonic acid lowering agent such as hydroxocobalamin is effective by inhibiting tumors implanted in mice (see the abstract).

The instant invention differs from the cited references in that the cited references do not teach the addition of a folic-binding-protein agent. However, the tertiary reference, Worzalla et al., teaches the supplementation of folic acid with LY 231514 to enhance LY 231514 antitumor activity.

The instant invention differs from the cited references in that the cited references do not teach the addition of cisplatin. However, the quaternary reference, Cleare et al., teaches malonato platinum anti-tumor compounds such as cisplatin to treat malignant tumors (see the abstract).

Clearly, one skilled in the art would have assumed the combination of three antineoplastic agents into a single composition would give an additive effect in the absence of evidence to the contrary.

The instant invention differs from the cited references in that the cited references do not teach the applicants' preferred dosage range for the methylmalonic acid lowering agent. However, those skilled in the art would have been readily optimized effective dosages and concurrent administration dosage forms as determined by good medical practice and the clinical condition of the individual patient. Regardless of the manner of administration, the specific dose may be calculated according to body weight, body

surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those skilled in the art and is within the ability of tasks routinely performed by them without undue experimentation.

Claims 40-52 are not allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kevin E. Weddington whose telephone number is (571)272-0587. The examiner can normally be reached on 12:30 pm-9:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on (571)272-0718. 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.

Kevin E. Weddington
Primary Examiner
Art Unit 1614

/Kevin E. Weddington/
Primary Examiner, Art Unit 1614

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BIB DATA SHEET

CONFIRMATION NO. 6568

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/776,329	07/11/2007	510	1614	X14173B

APPLICANTS

Clet Niyikiza, Indianapolis, IN;
Paolo Paoletti, Indianapolis, IN;
James Jacob Rusthoven, Ancaster, CANADA;

**** CONTINUING DATA ***************************

This application is a DIV of 11/288,807 11/29/2005 ABN
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

08/31/2007

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /KEVIN E WEDDINGTON/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		IN	0	11	2

ADDRESS

ELI LILLY & COMPANY
PATENT DIVISION
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UNITED STATES

TITLE

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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	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	02/11/2009								
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	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	02/11/2009								
	37									
	38									
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	40	✓								
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	48	✓								
	49	✓								
	50	✓								
	51	✓								
	52	✓								

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	Art Unit
	Kevin E Weddington	1614

SEARCHED

Class	Subclass	Date	Examiner
514	52	2/11/09	KEW
514	77	2/11/09	KEW
514	249	2/11/09	KEW
514	251	2/11/09	KEW
514	265.1	2/11/09	KEW

SEARCH NOTES

Search Notes	Date	Examiner
Consultation with parent applications, 10/297,821 and 11/288,807	2/11/09	KEW
EAST and PALM for Inventors' Names	2/11/09	KEW

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
5			

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* * * * * * * * * *   Welcome to STN International   * * * * * * * * * *

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                   substances identified in English-, French-, German-,
                   and Japanese-language basic patents from 2004-present
 NEWS  3  NOV 26   MARPAT enhanced with FSORT command
 NEWS  4  NOV 26   CHEMSAFE now available on STN Easy
 NEWS  5  NOV 26   Two new SET commands increase convenience of STN
                   searching
 NEWS  6  DEC 01   ChemPort single article sales feature unavailable
 NEWS  7  DEC 12   GBFULL now offers single source for full-text
                   coverage of complete UK patent families
 NEWS  8  DEC 17   Fifty-one pharmaceutical ingredients added to PS
 NEWS  9  JAN 06   The retention policy for unread STNmail messages
                   will change in 2009 for STN-Columbus and STN-Tokyo
 NEWS 10  JAN 07   WPIDS, WPINDEX, and WPIX enhanced Japanese Patent
                   Classification Data
 NEWS 11  FEB 02   Simultaneous left and right truncation (SLART) added
                   for CERAB, COMPUAB, ELCOM, and SOLIDSTATE
 NEWS 12  FEB 02   GENBANK enhanced with SET PLURALS and SET SPELLING
 NEWS 13  FEB 06   Patent sequence location (PSL) data added to USGENE
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FILE 'HOME' ENTERED AT 18:11:42 ON 11 FEB 2009

=> file reg
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       0.44       0.44

FILE 'REGISTRY' ENTERED AT 18:12:43 ON 11 FEB 2009
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STRUCTURE FILE UPDATES:   10 FEB 2009  HIGHEST RN 1104141-78-7
DICTIONARY FILE UPDATES:  10 FEB 2009  HIGHEST RN 1104141-78-7

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TSCA INFORMATION NOW CURRENT THROUGH July 5, 2008.

  Please note that search-term pricing does apply when
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conducting SmartSELECT searches.

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http://www.cas.org/support/stngen/stndoc/properties.html

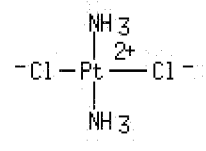
```
=> e cisplatin/cn
E1            1     CISPENTACIN/CN
E2            1     CISPERMETHRIN/CN
E3            1 --> CISPLATIN/CN
E4            1     CISPLATIN ADDUCT EXCISION NUCLEASE/CN
E5            1     CISPLATIN RESISTANCE ASSOCIATED (MOUSE STRAIN FVB/N-3 CLONE
                    MGC:59008 IMAGE:6486043)/CN
E6            1     CISPLATIN RESISTANCE ASSOCIATED ALPHA PROTEIN (HUMAN CELL LI
                    NE CISPLATIN RESISTANT CELL A2780 E(80) DERIVED FROM A2780 (
                    HUMAN OVARIAN CARCINOMA CELL LINE) GENE HCRA ALPHA)/CN
E7            1     CISPLATIN RESISTANCE PROTEIN (HUMAN PRECURSOR SEQUENCE HOMOL
                    OG)/CN
E8            1     CISPLATIN RESISTANCE RELATED PROTEIN CRR9P (HUMAN CLONE MGC:
                    39275 IMAGE:3051368)/CN
E9            1     CISPLATIN RESISTANCE RELATED PROTEIN CRR9P (MOUSE STRAIN MIX
                     FVB/N, C57BL/6J CLONE MGC:36304 IMAGE:5028264)/CN
E10           1     CISPLATIN RESISTANCE-ASSOCIATED OVEREXPRESSED PROTEIN (HUMAN
                     CELL LINE ACHN/CDDP GENE CROP/LUC7A)/CN
E11           1     CISPLATIN RESISTANCE-ASSOCIATED OVEREXPRESSED PROTEIN (HUMAN
                     GENE LUC7A)/CN
E12           1     CISPLATIN RESISTANCE-ASSOCIATED OVEREXPRESSED PROTEIN (MOUSE
                     STRAIN FVB/N CLONE MGC:7100 IMAGE:3157532)/CN

=> s e3
L1            1 CISPLATIN/CN

=> d

L1   ANSWER 1 OF 1  REGISTRY  COPYRIGHT 2009 ACS on STN
RN   15663-27-1  REGISTRY
ED   Entered STN:  16 Nov 1984
CN   Platinum, diamminedichloro-, (SP-4-2)-  (CA INDEX NAME)
OTHER CA INDEX NAMES:
CN   Platinum, diamminedichloro-, cis- (8CI)
OTHER NAMES:
CN   Abiplatin
CN   Biocisplatinum
CN   Briplatin
CN   CACP
CN   CDDP
CN   cis-DDP
CN   cis-Diaminedichloroplatinum(II)
CN   cis-Diaminodichloroplatinum(II)
CN   cis-Diamminedichloroplatinum
CN   cis-Diamminedichloroplatinum(II)
CN   cis-Dichlorodiamineplatinum(II)
CN   cis-Dichlorodiammineplatinum
CN   cis-Dichlorodiammineplatinum(II)
CN   cis-Platin
CN   cis-Platine
CN   cis-Platinous diaminodichloride
CN   cis-Platinum
CN   cis-Platinum diaminodichloride
CN   cis-Platinum II
CN   cis-Platinum(II) diaminodichloride
CN   cis-Platinum(II) diamminedichloride
CN   cis-Platinumdiamine dichloride
CN   cis-Platinumdiammine dichloride
CN   Cismaplat
CN   Cisplatin
CN   Cisplatino
CN   Cisplatinum
CN   Cisplatyl
```

CN Citoplatino
CN CPDC
CN CPDD
CN CPPD
CN DDP
CN DDP (antitumor agent)
CN Fauldiscipla
CN Lederplatin
CN Lipoplatin
CN Neoplatin
CN NSC 119875
CN Platamine
CN Platiblastin
CN Platidiam
CN Platinex
CN Platinol
CN Platinol AQ
CN Platinoxan
CN Platistin
CN Platosin
CN Rand
ADDITIONAL NAMES NOT AVAILABLE IN THIS FORMAT - Use FCN, FIDE, or ALL for
    DISPLAY
DR 936542-99-3, 96081-74-2
MF Cl2 H6 N2 Pt
CI CCS, COM
LC STN Files: ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, AQUIRE, BIOSIS,
      BIOTECHNO, CA, CAPLUS, CASREACT, CBNB, CHEMCATS, CHEMLIST, CIN, CSCHEM,
      CSNB, DDFU, DRUGU, EMBASE, GMELIN*, HSDB*, IFICDB, IFIPAT, IFIUDB,
      IMSPATENTS, IMSPRODUCT, IMSRESEARCH, IPA, MEDLINE, MRCK*, MSDS-OHS,
      PATDPASPC, PHAR, PIRA, PROMT, PROUSDDR, PS, RTECS*, SYNTHLINE,
      TOXCENTER, USAN, USPAT2, USPATFULL, VETU
        (*File contains numerically searchable property data)
    Other Sources: EINECS**, NDSL**, TSCA**, WHO
        (**Enter CHEMLIST File for up-to-date regulatory information)



PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

          23661 REFERENCES IN FILE CA (1907 TO DATE)
            755 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
          23758 REFERENCES IN FILE CAPLUS (1907 TO DATE)

=> file merck
COST IN U.S. DOLLARS   SINCE FILE   TOTAL
                                                      ENTRY   SESSION
FULL ESTIMATED COST   7.88   8.32

FILE 'MRCK' ENTERED AT 18:13:09 ON 11 FEB 2009
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FILE COVERS FROM LATE 19TH CENTURY TO PRESENT. LAST UPDATE: AUGUST 2008

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=> s l2
L2 NOT FOUND
The L-number entered has not been defined in this session, or it
has been deleted. To see the L-numbers currently defined in this
session, enter DISPLAY HISTORY at an arrow prompt (=>).

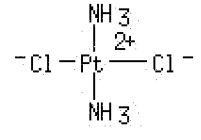
=> s l1

L2 1 L1

=> d all

L2 ANSWER 1 OF 1 MRCK COPYRIGHT (C) 2009 Merck and Co., Inc., Whitehouse Station, New Jersey, USA. All rights reserved. on STN

MERCK Number (MNO): 1402317
CAS Registry No. (RN): **15663-27-1**
MERCK Index Name (MIN): Cisplatin
CA Index Name (CN): (SP-4-2)-Diamminedichloroplatinum
Synonym(s) (CN): Cis-diamminedichloroplatinum; Cis-platinum II; Cis-DDP; CACP; CPDC; DDP
Drug Code(s) (CN): NSC-119875
Trade Name(s) (CN): Blastolem (Lemery); Briplatin (Bristol-Myers Squibb Co.; BMS); Cisplatyl (Sanofi-Aventis Group; Sanofi-Aventis); Neoplatin (Bristol-Myers Squibb Co.; BMS); Platamine (Pfizer, Inc.; Pfizer); Platinex (Bristol-Myers Squibb Co.; BMS); Platiblastin (Pfizer, Inc.; Pfizer); Platinol (Bristol-Myers Squibb Co.; BMS); Platosin (Pharmachemie); Randa (Nippon Kayaku Co., Ltd.; Nippon Kayaku)
File Segment. (FS): Active Monographs
Molecular Form. (MF): Cl2 H6 N2 Pt
Wgt Composition (COMP): Cl 23.63%, H 2.02%, N 9.34%, Pt 65.02%.
Molecular Weight (MW): 300.05
References (RE): Antitumor platinum coordination complex. Originally known as Peyrone's salt or Peyrone's chloride; of interest in the development of coordination theory. Prepn: M. Peyrone, Ann. 51, 1 (1845); G. B. Kauffman, D. O. Cowan, Inorg. Synth. 7, 239 (1963); S. C. Dhara, Indian J. Chem. 8, 193 (1970). Early structural studies: R. Werner, Z. Anorg. Chem. 3, 267 (1893); H. D. K. Drew et al., J. Chem. Soc. 1932, 988. Discovery of anti-tumor activity: B. Rosenberg et al., Nature 205, 698 (1965); 222, 385 (1972). Use as neoplasm inhibitor: M. L. Tobe et al., DE 2318020 (1972 to Rustenburg Platinum Mines Ltd.), C.A. 80, 55897e (1974); M. J. Cleare et al., DE 2329485 (1972 to Research Corp.), C.A. 81, 21172v (1974). X-ray structure of cisplatin-DNA adduct: S. E. Sherman et al., Science 230, 412 (1985). Inhibition of in vitro DNA synthesis: A. L. Pinto, S. J. Lippard, Proc. Natl. Acad. Sci. USA 82, 4616 (1985). Pharmacology: A. Sirica et al., Proc. Am. Assoc. Cancer Res. 12, 4 (1971); C. L. Litterst et al., Cancer Res. 36, 2340 (1976); N. P. Johnson et al., Chem. Biol. Interact. 23, 267 (1978). Metabolism: R. C. Lange et al., J. Nucl. Med. 14, 191 (1973). Clinical studies: J. J. Ochs et al., Cancer Treat. Rep. 62, 239 (1978); H. M. Pinedo et al., Eur. J. Cancer 14, 1149 (1978). Toxicology: R. L. Dixon, Proc. 7th Int. Congr. Chemother. Vol. 2 (University Park Press, Baltimore, 1972) pp 241-243; R. W. Fleishman et al., Toxicol. Appl. Pharmacol. 33, 320 (1975). Review of carcinogenicity studies: IARC Monographs 26, 154-161 (1981); of neurotoxicity: R. J. Cersosimo, Cancer Treat. Rev. 16, 195-211 (1989). Comprehensive description: C. M. Riley, L. M. Sternson, Anal. Profiles Drug Subs. 14, 77-105 (1985). Book: Cisplatin, Current Status and New Developments, A. W. Prestayko et al., Eds. (Academic Press, New York, 1980) 527 pp. Review of mechanism of action: M. A. Fuertes et al., Curr. Med. Chem. 10, 257-266 (2003); Z. H. Siddik, Oncogene 22, 7265-7279 (2003).



Toxicity (TOX):
LD50 in guinea pigs: 9.7 mg/kg i.p. (Fleishman).
Other Properties (OCPP):
Yellow to orange crystalline powder. Soly in water 0.253 g/100 g at 25°; slowly changes to trans-form in aq soln. Insol in most common solvents. Sol in DMF. LD50 in guinea pigs: 9.7 mg/kg i.p. (Fleishman) .
Notes (NTE):
Caution: This substance is reasonably anticipated to be a human carcinogen: Report on Carcinogens, Eleventh Edition (PB2005-104914, 2004) p III-67.

Therapeutic Codes (THER):
     Antineoplastic.
Therapeutic Codes (Veterinary) (VTHER):
     Antineoplastic.
Other Sources (OS):
     CA 80:55897; CA 81:21172
Referenced Patent (RPN):
     DE2318020; DE2329485

=> file ca
COST IN U.S. DOLLARS   SINCE FILE ENTRY   TOTAL SESSION
FULL ESTIMATED COST   3.62   11.94

FILE 'CA' ENTERED AT 18:15:10 ON 11 FEB 2009
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FILE COVERS 1907 - 8 Feb 2009 VOL 150 ISS 7
FILE LAST UPDATED: 8 Feb 2009 (20090208/ED)

CA now includes complete International Patent Classification (IPC)
reclassification data for the third quarter of 2008.

CAS Information Use Policies apply and are available at:

http://www.cas.org/legal/infopolicy.html

This file contains CAS Registry Numbers for easy and accurate
substance identification.

=> s l1
L3   23661 L1

=> s (cancer or tumor?)
        360427 CANCER
        526372 TUMOR?
L4   720567 (CANCER OR TUMOR?)

=> s l3 and l4
L5   14478 L3 AND L4

=> d 14400-14478

L5   ANSWER 14400 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN   89:157244 CA
OREF 89:24255a,24258a
TI   Platinum complexes as radiosensitizers of hypoxic mammalian cells
AU   Douple, E. B.; Richmond, R. C.
CS   Norris Cotton Cancer Cent., Dartmouth, NH, USA
SO   British Journal of Cancer, Supplement (1978), 37(3), 98-102
     CODEN: BJCSB5; ISSN: 0306-9443
DT   Journal
LA   English

L5   ANSWER 14401 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN   89:140347 CA

OREF 89:21617a,21620a
TI Evaluation of single-agent therapy in human colorectal **tumor** xenografts
AU Houghton, P. J.; Houghton, J. A.
CS Dep. Radiopharmacol., Inst. Cancer Res., Sutton, UK
SO British Journal of Cancer (1978), 37(5), 833-40
CODEN: BJCAAI; ISSN: 0007-0920
DT Journal
LA English

L5 ANSWER 14402 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:140186 CA
OREF 89:21585a,21588a
TI Distribution of a platinum anti-**tumor** drug in HeLa cells by analytical electron microscopy
AU Khan, M. U. A.; Sadler, P. J.
CS Chem. Dep., Birkbeck Coll., London, UK
SO Chemico-Biological Interactions (1978), 21(2-3), 227-32
CODEN: CBINA8; ISSN: 0009-2797
DT Journal
LA English

L5 ANSWER 14403 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:99746 CA
OREF 89:15115a,15118a
TI A general mechanism for microsomal activation of quinone anticancer agents to free radicals
AU Bachur, Nicholas R.; Gordon, Sandra L.; Gee, Malcolm V.
CS Baltimore Cancer Res. Cent., Natl. Cancer Inst., Baltimore, MD, USA
SO Cancer Research (1978), 38(6), 1745-50
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

L5 ANSWER 14404 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:99480 CA
OREF 89:15047a,15050a
TI Variation in response of xenografts of colorectal carcinoma to chemotherapy
AU Nowak, K.; Peckham, M. J.; Steel, G. G.
CS Div. Radiotherap. Biophys., Inst. Cancer Res., Sutton, UK
SO British Journal of Cancer (1978), 37(4), 576-84
CODEN: BJCAAI; ISSN: 0007-0920
DT Journal
LA English

L5 ANSWER 14405 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:84661 CA
OREF 89:12869a
TI Chemotherapy of transplantable mouse **tumors** with cis-dichlorodiammineplatinum(II) alone and in combination with sarcolysin
AU Presnov, M. A.; Konovalova, A. L.; Romanova, L. F.; Sofina, Z. P.; Stetsenko, A. I.
CS Lab. Exp. Cancer Chemother., Cancer Res. Cent., Moscow, USSR
SO Cancer Treatment Reports (1978), 62(5), 705-12
CODEN: CTRRDO; ISSN: 0361-5960
DT Journal
LA English

L5 ANSWER 14406 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:70802 CA
OREF 89:10819a,10822a
TI Evaluation of single agents and combinations of chemotherapeutic agents in mouse colon carcinomas
AU Corbett, T. H.; Griswold, D. P., Jr.; Roberts, B. J.; Peckham, J. C.; Schabel, F. M., Jr.
CS Southern Res. Inst., Birmingham, AL, USA
SO Cancer (New York, NY, United States) (1977), 40(5, Suppl.), 2660-80

CODEN: CANCAR; ISSN: 0008-543X
DT Journal
LA English

L5 ANSWER 14407 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:36513 CA
OREF 89:5535a,5538a
TI Differential chemotherapeutic susceptibility of human T-lymphocytes and B-lymphocytes in culture
AU Ohnuma, Takao; Arkin, Hadara; Minowada, Jun; Holland, James F.
CS Dep. Neoplast. Dis., Mt. Sinai Sch. Med., New York, NY, USA
SO Journal of the National Cancer Institute (1940-1978) (1978), 60(4), 749-52
CODEN: JNCIAM; ISSN: 0027-8874
DT Journal
LA English

L5 ANSWER 14408 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 88:569 CA
OREF 88:119a,122a
TI Treating viral infections
IN Davidson, James P.; Rosenberg, Barnett; Hinz, Ronald W.
PA Research Corp., USA
SO U.S., 5 pp.
CODEN: USXXAM
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 4053587	A	19771011	US 1975-540109	19750110
	US 4258051	A	19810324	US 1977-773216	19770301
	US 4440782	A	19840403	US 1980-188343	19800918
PRAI	US 1973-350924	A1	19730413		
	US 1973-350929	A1	19730413		
	US 1975-540109	A3	19750110		
	US 1977-773216	A3	19770301		

L5 ANSWER 14409 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:193675 CA
OREF 87:30527a,30530a
TI Effects of cytotoxic agents on 3H-thymidine incorporation and growth delay in human colonic **tumor** xenografts
AU Houghton, P. J.; Houghton, J. A.; Taylor, D. M.
CS Dep. Radiopharmacol., R. Marsden Hosp., Sutton, UK
SO British Journal of Cancer (1977), 36(2), 206-14
CODEN: BJCAAI; ISSN: 0007-0920
DT Journal
LA English

L5 ANSWER 14410 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:127357 CA
OREF 87:20161a,20164a
TI Intravesical and systemic chemotherapy of murine bladder **cancer**
AU Soloway, Mark S.
CS Dep. Urol., Univ. Tennessee Cent. Health Sci., Memphis, TN, USA
SO Cancer Research (1977), 37(8, Pt. 2), 2918-29
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

L5 ANSWER 14411 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:111354 CA
OREF 87:17585a,17588a
TI Mutagenicity of **cancer** chemotherapeutic agents in the Salmonella/microsome test
AU Benedict, William F.; Baker, Mary S.; Haroun, Lynne; Choi, Edmund; Ames, Bruce N.

CS Dep. Med., Child. Hosp., Los Angeles, CA, USA
SO Cancer Research (1977), 37(7, Pt. 1), 2209-13
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

L5 ANSWER 14412 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:78571 CA
OREF 87:12437a,12440a
TI High dose cis-platinumdiamminedichloride. Amelioration of renal toxicity by mannitol diuresis
AU Hayes, Daniel M.; Cvitkovic, Esteban; Golbey, Robert B.; Scheiner, Ellen; Helson, Lawrence; Krakoff, Irwin H.
CS Mem. Sloan-Kettering Cancer Cent., New York, NY, USA
SO Cancer (New York, NY, United States) (1977), 39(4), 1372-81
CODEN: CANCAR; ISSN: 0008-543X
DT Journal
LA English

L5 ANSWER 14413 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:78408 CA
OREF 87:12401a,12404a
TI Origin of giant cells in regressing sarcoma-180 after cis-dichlorodiammine platinum(II) treatment: a fine structural study
AU Sodhi, Ajit
CS Dep. Zool., Banaras Hindu Univ., Varanasi, India
SO Journal of Clinical Hematology and Oncology (1977), 7(2), 569-79
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14414 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:78193 CA
OREF 87:12353a,12356a
TI Phase I study of high-dose cis-dichlorodiammineplatinum(II) with forced diuresis
AU Chary, Kandala K.; Higby, Donald J.; Henderson, Edward S.; Swinerton, Kenneth D.
CS Dep. Med. A, Roswell Park Mem. Inst., Buffalo, NY, USA
SO Cancer Treatment Reports (1977), 61(3), 367-70
CODEN: CTRRDO; ISSN: 0361-5960
DT Journal
LA English

L5 ANSWER 14415 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:68321 CA
OREF 87:10885a,10888a
TI Phosphorus-nitrogen compounds. 30. Synthesis of platinum derivatives of polymeric and cyclic phosphazenes
AU Allcock, Harry R.; Allen, Robert W.; O'Brien, John P.
CS Dep. Chem., Pennsylvania State Univ., University Park, PA, USA
SO Journal of the American Chemical Society (1977), 99(12), 3984-7
CODEN: JACSAT; ISSN: 0002-7863
DT Journal
LA English

L5 ANSWER 14416 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:62655 CA
OREF 87:9887a,9890a
TI Therapeutic potentiation in a mouse mammary **tumor** and an intracerebral rat brain **tumor** by combined treatment with cis-dichlorodiammineplatinum(II) and radiation
AU Douple, Evan B.; Richmond, Robert C.; Logan, Mark E.
CS Dep. Ther. Radiol., Dartmouth-Hitchcock Med. Cent., Hanover, NH, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(2), 585-603
CODEN: JCHODP; ISSN: 0162-9360
DT Journal

LA English

L5 ANSWER 14417 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:62521 CA
OREF 87:9855a,9858a
TI Analog comparison, combination chemotherapy, and combined modality studies with cis-platinum(II) diamminedichloride (NSC 119875) using in vivo animal **tumor** models
AU Merker, P. C.; Wodinsky, I.; Mabel, J.; Branfman, A.; Venditti, J. M.
CS Life Sci. Div., Arthur D. Little, Inc., Cambridge, MA, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 301-21
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14418 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:47932 CA
OREF 87:7531a,7534a
TI Antineoplastic effect of complex platinum(IV) compounds
AU Konovalova, A. L.; Presnov, M. A.; Zheligovskaya, N. N.; Treshchalina, E. M.
CS Onkol. Nauchn. Tsentr., Moscow, USSR
SO Doklady Akademii Nauk SSSR (1977), 234(1), 223-6 [Biochem.]
CODEN: DANKAS; ISSN: 0002-3264
DT Journal
LA Russian

L5 ANSWER 14419 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:33558 CA
OREF 87:5237a,5240a
TI Spermine-platinum(II) chloride as a potential anti-**tumor** agent
AU Tsou, K. C.; Yip, K. F.; Lo, K. W.; Ahmad, S.
CS Sch. Med., Univ. Pennsylvania, Philadelphia, PA, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 322-9
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14420 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:33557 CA
OREF 87:5237a,5240a
TI The enhanced antitumor activity of cis-diamminedichloroplatinum(II) against murine **tumors** when combined with other agents
AU Page, R. H.; Talley, R. W.; Buhagiar, J.
CS Div. Oncol., Henry Ford Hosp., Detroit, MI, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 96-104
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14421 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:15862 CA
OREF 87:2433a,2436a
TI The effect of cis-diamminedichloroplatinum(II) and cyclophosphamide on immune response and **tumor** rejection in BALBc and PL/Jax mice
AU Page, R. H.; Talley, R. W.; Livermore, D. H.
CS Div. Oncol., Henry Ford Hosp., Detroit, MI, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 105-13
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14422 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 87:299 CA
OREF 87:55a,58a
TI Sulfato 1,2-diaminocyclohexane platinum(II): a potential new antitumor

agent
AU Speer, Robert J.; Ridgway, Helen; Stewart, David P.; Hall, Larry M.; Zapata, Alba; Hill, Joseph M.
CS Wadley Inst. Mol. Med., Dallas, TX, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 210-19
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14423 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:183312 CA
OREF 86:28685a,28688a
TI Response of transferrin bound iron to treatment of rat lymphosarcoma with cis-dichlorodiammineplatinum(II)
AU Warner, F. W.; Demanuelle, M.; Stjernholm, R.; Cohn, I.; Baddley, W. H.
CS Div. Eng. Res., Louisiana State Univ., Baton Rouge, LA, USA
SO Journal of Clinical Hematology and Oncology (1977), 7(1), 180-9
CODEN: JCHODP; ISSN: 0162-9360
DT Journal
LA English

L5 ANSWER 14424 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:165238 CA
OREF 86:25889a,25892a
TI Comparative nephrotoxicity of platinum **cancer** chemotherapeutic agents
AU Ward, J. M.; Young, D. M.; Fauvie, K. A.; Wolpert, M. K.; Davis, R.; Guarino, A. M.
CS Lab. Toxicol., Natl. Cancer Inst., Bethesda, MD, USA
SO Cancer Treatment Reports (1976), 60(11), 1675-8
CODEN: CTRRDO; ISSN: 0361-5960
DT Journal
LA English

L5 ANSWER 14425 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:150511 CA
OREF 86:23571a,23574a
TI cis-Dichlorodiammineplatinum(II) chemotherapy in experimental murine myeloma MOPC 104E
AU Ghanta, Vithal K.; Jones, M. Terry; Woodard, Dolores A.; Durant, John R.; Hiramoto, Raymond N.
CS Comprehensive Cancer Cent., Univ. Alabama, Birmingham, AL, USA
SO Cancer Research (1977), 37(3), 771-4
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

L5 ANSWER 14426 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:115133 CA
OREF 86:18129a,18132a
TI Antineoplastic activity of cis-diamminedichloroplatinum(II)
AU Nikolin, V. P.; Gruntenko, E. V.; Mal'chikov, G. D.; Sysoeva, G. M.
CS Inst. Tsitol. Genet., Novosibirsk, USSR
SO Voprosy Onkologii (1976), 22(12), 73-5
CODEN: VOONAW; ISSN: 0507-3758
DT Journal
LA Russian

L5 ANSWER 14427 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:83786 CA
OREF 86:13189a,13192a
TI Effects of the cis-dichlorodiamminoplatinum(II)-deoxyribonucleic acid complex on normal and **cancer** cells
AU Heinen, E.; Desaive, C.; Houssier, C.; Gillet, M. C.; Chevremont, M.
CS Inst. Histol., Liege, Belg.
SO Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales (1976), 170(4), 919-21
CODEN: CRSBAW; ISSN: 0037-9026

DT Journal
LA French

L5 ANSWER 14428 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:312 CA
OREF 86:55a,58a
TI Ultrastructural changes of sarcoma-180 cells after treatment with cis-dichlorodiammine platinum(II), in vivo and in vitro
AU Sodhi, Ajit
CS Dep. Zool., Banaras Hindu Univ., Banaras, India
SO Indian Journal of Experimental Biology (1976), 14(4), 383-90
CODEN: IJEBA6; ISSN: 0019-5189
DT Journal
LA English

L5 ANSWER 14429 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 85:186584 CA
OREF 85:29765a,29768a
TI Mode of action of cis-dichloro-diammine platinum(II) on mouse Ehrlich ascites **tumor** cells
AU Heinen, Ernst; Bassleer, Roger
CS Inst. Histol., Univ. Liege, Liege, Belg.
SO Biochemical Pharmacology (1976), 25(16), 1871-5
CODEN: BCPCA6; ISSN: 0006-2952
DT Journal
LA English

L5 ANSWER 14430 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 85:171668 CA
OREF 85:27365a,27368a
TI Effects of dinitrato(1,2-diaminocyclohexane)platinum (NSC 239851) on murine myeloma and hemopoietic precursor cells
AU Ogawa, Makio; Gale, Glen R.; Meischen, Sandra J.; Cooke, Victoria A.
CS Dep. Med., Med. Univ. South Carolina, Charleston, SC, USA
SO Cancer Research (1976), 36(9, Pt. 1), 3185-8
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

L5 ANSWER 14431 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 85:137309 CA
OREF 85:21951a,21954a
TI Synthesis, in vivo and in vitro studies on the antineoplastic effect of cis-dichloro-dipeptide ester-platinum(II) complexes
AU Beck, Wolfgang; Purucker, Bernhard; Girnth, Michael; Schoenenberger, Helmut; Seidenberger, Horst; Ruckdeschel, Gotthard
CS Inst. Anorg. Chem., Univ. Muenchen, Munich, Fed. Rep. Ger.
SO Zeitschrift fuer Naturforschung, Teil B: Anorganische Chemie, Organische Chemie (1976), 31B(6), 832-45
CODEN: ZNBAD2; ISSN: 0340-5087
DT Journal
LA German

L5 ANSWER 14432 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 85:103109 CA
OREF 85:16457a,16460a
TI Platinum complexes and **cancer**
AU Koros, Endre
CS Budapest, Hung.
SO Termeszet Vilaga (1976), 107(4), 170-2
CODEN: TEVIAS; ISSN: 0040-3717
DT Journal; General Review
LA Hungarian

L5 ANSWER 14433 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 85:40874 CA

OREF 85:6598h,6599a
TI Effects of cis-dichlorodiammine platinum(II) on DNA synthesis in kidney and other tissues of normal and **tumor**-bearing rats
AU Taylor, David M.; Tew, Kenneth D.; Jones, Julie D.
CS Radiopharmacol. Dep., Inst. Cancer Res., Sutton/Surrey, UK
SO European Journal of Cancer (1965-1981) (1976), 12(4), 249-54
CODEN: EJCAAH; ISSN: 0014-2964
DT Journal
LA English

L5 ANSWER 14434 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 84:130173 CA
OREF 84:21093a
TI Inhibition by caffeine of post-replication repair in Chinese hamster cells treated with cis platinum(II) diamminedichloride: the extent of platinun binding to template DNA in relation to the size of low molecular weight nascent DNA
AU Van den Berg, H. W.; Roberts, J. J.
CS Inst. Cancer Res., R. Cancer Hosp., Chalfont St. Giles/Bucks, UK
SO Chemico-Biological Interactions (1976), 12(3-4), 375-90
CODEN: CBINA8; ISSN: 0009-2797
DT Journal
LA English

L5 ANSWER 14435 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 84:38769 CA
OREF 84:6319a,6322a
TI Combined radiotherapy and chemotherapy of P388 leukemia in vivo
AU Wodinsky, I.; Kensler, C. J.; Venditti, J. M.
CS Arthur D. Little, Inc., Cambridge, MA, USA
SO Prog. Chemother. (Antibacterial, Antiviral, Antineoplast.), Proc. Int. Congr. Chemother., 8th (1974), Meeting Date 1973, Volume 3, 95-100. Editor(s): Daikos, George K. Publisher: Hell. Soc. Chemother., Athens, Greece.
CODEN: 31TFAO
DT Conference
LA English

L5 ANSWER 14436 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 83:172656 CA
OREF 83:27049a,27052a
TI Single and combination chemotherapy for primary murine bladder **cancer**
AU Soloway, Mark S.
CS Dep. Surg., Univ. Hosp., Cleveland, OH, USA
SO Cancer (New York, NY, United States) (1975), 36(2), 333-40
CODEN: CANCAR; ISSN: 0008-543X
DT Journal
LA English

L5 ANSWER 14437 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 83:108573 CA
OREF 83:16985a,16988a
TI Platinum-pyrimidine blues and related complexes. New class of potent antitumor agents
AU Davidson, James P.; Faber, Paula J.; Fischer, Robert G., Jr.; Mansy, Samir; Peresie, Henry J.; Rosenberg, Barnett; VanCamp, Loretta
CS Dep. Biophys., Michigan State Univ., East Lansing, MI, USA
SO Cancer Chemotherapy Reports, Part 1 (1975), 59(2), 287-300
CODEN: CCROBU; ISSN: 0576-6559
DT Journal
LA English

L5 ANSWER 14438 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 83:3770 CA
OREF 83:695a,698a
TI Platinum-195m, a new radionuclide. Its application to the monitoring of **cancer** chemotherapeutic agents

AU Wolf, W.; Berman, J.; Leh, F.; Poggenburg, Ken
CS Radiopharm. Program, Univ. South California, Los Angeles, CA, USA
SO Recent Adv. Nucl. Med., Proc. World Congr. Nucl. Med., 1st (1974), 944-5
Publisher: Jpn. Radioisot. Assoc., Tokyo, Japan.
CODEN: 30HHAX
DT Conference
LA English

L5 ANSWER 14439 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 83:572 CA
OREF 83:111a,114a
TI Inhibition of cytokinesis in mammalian cells by cis-dichlorodiammineplatinum (II)
AU Aggarwal, S. K.
CS Dep. Zool., Michigan State Univ., East Lansing, MI, USA
SO Cytobiologie (1974), 8(3), 395-402
CODEN: CYTZAM; ISSN: 0070-2463
DT Journal
LA English

L5 ANSWER 14440 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 82:132827 CA
OREF 82:21171a,21174a
TI Chemical and biological effects of cis-dichlorodiammineplatinum (II), an antitumor agent, on DNA
AU Munchausen, Linda L.
CS Biol. Div., Oak Ridge Natl. Lab., Oak Ridge, TN, USA
SO Proceedings of the National Academy of Sciences of the United States of America (1974), 71(11), 4519-22
CODEN: PNASA6; ISSN: 0027-8424
DT Journal
LA English

L5 ANSWER 14441 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 82:132786 CA
OREF 82:21163a,21166a
TI Renaturation effects of cis- and trans-platinum II and IV compounds on calf thymus deoxyribonucleic acid
AU Harder, Harold C.
CS Sch. Med., Yale Univ., New Haven, CT, USA
SO Chemico-Biological Interactions (1975), 10(1), 27-39
CODEN: CBINA8; ISSN: 0009-2797
DT Journal
LA English

L5 ANSWER 14442 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:145909 CA
OREF 81:22739a,22742a
TI Effects of cis-dichlorodiammineplatinum(II) in the regression of Sarcoma 180. Fine structural study
AU Sodhi, Ajit; Aggarwal, Surinder K.
CS Dep. Zool., Michigan State Univ., East Lansing, MI, USA
SO Journal of the National Cancer Institute (1940-1978) (1974), 53(1), 85-101
CODEN: JNCIAM; ISSN: 0027-8874
DT Journal
LA English

L5 ANSWER 14443 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:58218 CA
OREF 81:9231a,9234a
TI Role of host defenses in cis-dichlorodiammineplatinum(II)-mediated regressions of Sarcoma 180 in mice
AU Conran, Philip B.
CS Michigan State Univ., East Lansing, MI, USA
SO (1973) 119 pp. Avail.: Univ. Microfilms, Ann Arbor, Mich., Order No. 74-6025
From: Diss. Abstr. Int. B 1974, 34(9), 4469

DT Dissertation
LA English

L5 ANSWER 14444 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:45355 CA
OREF 81:7205a,7208a
TI Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo
AU Wodinsky, Isidore; Swiniarski, Joseph; Kensler, Charles J.; Venditti, John M.
CS Arthur D. Little, Inc., Cambridge, MA, USA
SO Cancer Chemotherapy Reports, Part 2 (1974), 4(1), 73-97
CODEN: CCSUBJ; ISSN: 0069-0120
DT Journal
LA English

L5 ANSWER 14445 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:45352 CA
OREF 81:7205a,7208a
TI Potentially useful combinations of chemotherapy detected in mouse **tumor** systems
AU Kline, Ira
CS Microbiol. Assoc., Inc., Bethesda, MD, USA
SO Cancer Chemotherapy Reports, Part 2 (1974), 4(1), 33-43
CODEN: CCSUBJ; ISSN: 0069-0120
DT Journal
LA English

L5 ANSWER 14446 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:45271 CA
OREF 81:7189a,7192a
TI Fine structural analysis of Sarcoma-180 before and after cis-dichlorodiammineplatinum(II) in Swiss white mice, in vivo and in vitro studies
AU Sodhi, Ajit
CS Michigan State Univ., East Lansing, MI, USA
SO (1973) 137 pp. Avail.: Univ. Microfilms, Ann Arbor, Mich., Order No. 74-6135
From: Diss. Abstr. Int B 1974, 34(9), 4759
DT Dissertation
LA English

L5 ANSWER 14447 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:21172 CA
OREF 81:3384h,3385a
TI Platinum coordination compounds
IN Cleare, Michael J.; Hoeschele, James D.; Rosenberg, Barnett; Van Camp, Loretta L.
PA Research Corp.
SO Ger. Offen., 23 pp.
CODEN: GWXXBX
DT Patent
LA German
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	DE 2329485	A1	19731220	DE 1973-2329485	19730608
	DE 2329485	B2	19791122		
	DE 2329485	C3	19800731		
	CH 588505	A5	19770615	CH 1973-7999	19730604
	CH 605550	A5	19780929	CH 1977-2036	19730604
	CA 1023759	A1	19780103	CA 1973-173182	19730605
	NL 7307863	A	19731211	NL 1973-7863	19730606
	NL 183724	B	19880801		
	NL 183724	C	19890102		
	FR 2187345	A1	19740118	FR 1973-20788	19730607
	GB 1380228	A	19750108	GB 1973-27304	19730607
	SE 415182	B	19800915	SE 1973-8050	19730607

	SE 415182	C	19810115		
	JP 49048621	A	19740511	JP 1973-64636	19730608
	JP 56029676	B	19810709		
	US 4140707	A	19790220	US 1977-778955	19770318
	SE 7810577	A	19781010	SE 1978-10577	19781010
	US 4140707	B1	19891219	US 1989-90001716	19890214
PRAI	US 1972-260989	A	19720608		
	CH 1973-7999		19730604		
	US 1977-778955	A	19770318		

OS MARPAT 81:21172

L5 ANSWER 14448 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:141013 CA
OREF 80:22713a,22716a
TI Effects of cis-dichlorodiammine platinum(II) on the fine structure of the mammalian cells in vitro
AU Aggarwal, S. K.; Sodhi, A.
CS Dep. Zool., Michigan State Univ., East Lansing, MI, USA
SO Proceedings - Annual Meeting, Electron Microscopy Society of America (1973), 31, 546-7
CODEN: EMSPAR; ISSN: 0424-8201
DT Journal
LA English

L5 ANSWER 14449 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:128231 CA
OREF 80:20617a,20620a
TI Effect of chemotherapeutic agents on bladder **cancer**. New animal model
AU Soloway, Mark S.; DeKernion, Jean B.; Rose, Daniel; Persky, Lester
CS Sch. Med., Case West. Reserve Univ., Cleveland, OH, USA
SO Surgical Forum (1973), 24, 542-4
CODEN: SUFOAX; ISSN: 0071-8041
DT Journal
LA English

L5 ANSWER 14450 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:128133 CA
OREF 80:20597a,20600a
TI Fine structural analysis of sarcoma-180 **tumor** before and after cis-platinum(II) diamminodichloride
AU Aggarwal, S. K.; Sodhi, A.; Van Camp, L.
CS Dep. Zool., Michigan State Univ., East Lansing, MI, USA
SO Proceedings - Annual Meeting, Electron Microscopy Society of America (1971), 29, 386-7
CODEN: EMSPAR; ISSN: 0424-8201
DT Journal
LA English

L5 ANSWER 14451 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:55897 CA
OREF 80:9065a,9068a
TI Antitumorous diamminedichloroplatinum complexes
IN Tobe, Martin L.; Khokhar, Abdul R.; Braddock, Peter D. M.
PA Rustenburg Platinum Mines Ltd.
SO Ger. Offen., 13 pp.
CODEN: GWXXBX
DT Patent
LA German
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	DE 2318020	A1	19731108	DE 1973-2318020	19730410
	NL 7304882	A	19731012	NL 1973-4882	19730409
	FR 2182943	A1	19731214	FR 1973-12664	19730409
	JP 49013316	A	19740205	JP 1973-40779	19730410
PRAI	GB 1972-16350	A	19720410		
	GB 1972-21389	A	19720508		

L5 ANSWER 14452 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:43984 CA
OREF 80:7135a,7138a
TI Drug-induced inhibition of hematogeneously spread metastases
AU Hellmann, Kurt; Salsbury, Allen, J.; Burrage, Karen S.; Le Serve, A. W.; James, Sandra E.
CS Cancer Chemother. Dep., Imp. Cancer Res. Fund, London, UK
SO Chemother. Cancer Dissemination Metastasis (1973), 355-9. Editor(s): Garattini, Silvio. Publisher: Raven, New York, N. Y.
CODEN: 27IMAL
DT Conference
LA English

L5 ANSWER 14453 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:33650 CA
OREF 80:5503a
TI Platinum coordination complexes in **cancer** chemotherapy
AU Rosenberg, Barnett
CS Dep. Biophys., Mich. State Univ., East Lansing, MI, USA
SO Naturwissenschaften (1973), 60(9), 399-406
CODEN: NATWAY; ISSN: 0028-1042
DT Journal; General Review
LA English

L5 ANSWER 14454 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:73858 CA
OREF 79:11889a,11892a
TI Enhanced antigenicity as a possible mode of action of platinum antitumor drugs
AU Rosenberg, B.
CS Biophys. Dep., Michigan State Univ., East Lansing, MI, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 101-2. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md.
CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14455 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:73783 CA
OREF 79:11876h,11877a
TI Cis-platinum(II) diamminedichloride (PDD) in combined therapy of leukemia L1210
AU Speer, R. J.; Lapis, S.; Ridgeway, H.; Meyers, T. D.; Hill, J. M.
CS Wadley Inst. Mol. Med., Dallas, TX, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 253-4. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md.
CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14456 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:73779 CA
OREF 79:11873a,11876a
TI Cis-platinum diamminedichloride(II)-induced regression of carcinogen-induced rat mammary **tumors**
AU Welsch, C. W.
CS Dep. Anat., Michigan State Univ., East Lansing, MI, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 231-2. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md.
CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14457 OF 14478 CA COPYRIGHT 2009 ACS on STN

Full Text
AN 79:73541 CA
OREF 79:11821a,11824a
TI Cis-dichlorodiammineplatinum(II). Irreversible inhibition of DNA synthesis and cell growth in tissue culture and inhibition of chick embryo cell transformation by Rous sarcoma virus
AU Kara, J.; Svoboda, J.; Drobnik, J.
CS Inst. Exp. Biol. Genet., Czech. Acad. Sci., Prague, Czech.
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 205-7. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md. CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14458 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:38643 CA
OREF 79:6255a,6258a
TI Whole-body counting and the distribution of platinum-195m-labeled cis-dichlorodiammineplatinum(II) in the major organs of Swiss white mice
AU Hoeschele, J. D.; VanCamp, Loretta
CS Biophys. Dep., Michigan State Univ., East Lansing, MI, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 241-2. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md. CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14459 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:38642 CA
OREF 79:6255a,6258a
TI Combination therapy of cis-dichlorodiammineplatinum(II) with cytoxan against the sarcoma 180 **tumor** in Swiss white mice
AU VanCamp, Loretta; Rosenberg, B.
CS Dep. Biophys., Michigan State Univ., East Lansing, MI, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 239-40. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md. CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14460 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:38641 CA
OREF 79:6255a,6258a
TI Role of host defenses in the regression of sarcoma-180 in mice treated with cis-dichlorodiammineplatinum(II)
AU Conran, P. B.; Rosenberg, B.
CS Biophys. Dep., Michigan State Univ., East Lansing, MI, USA
SO Advan. Antimicrob. Antineoplastic Chemother., Proc. Int. Congr. Chemother., 7th (1972), Meeting Date 1971, Volume 2, 235-6. Editor(s): Hejzlar, Miroslav. Publisher: Univ. Park Press, Baltimore, Md. CODEN: 26QZAP
DT Conference
LA English

L5 ANSWER 14461 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 79:15069 CA
OREF 79:2427a,2430a
TI Antitumor agent cis-diamminedichloroplatinum. Distribution studies and dose calculations for platinum-193m and platinum-195m
AU Lange, Robert C.; Spencer, Richard P.; Harder, Harold C.
CS Sch. Med., Yale Univ., New Haven, CT, USA
SO Journal of Nuclear Medicine (1973), 14(4), 191-5 CODEN: JNMEAQ; ISSN: 0161-5505
DT Journal
LA English

L5 ANSWER 14462 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 78:105913 CA
OREF 78:16927a,16930a
TI Regression of sarcoma-180 after cis-dichlorodiammineplatinum (II). Fine-structural study
AU Sodhi, Ajit
CS Dep. Zool., Michigan State Univ., East Lansing, MI, USA
SO Proceedings - Annual Meeting, Electron Microscopy Society of America (1972), 30, 68-9
CODEN: EMSPAR; ISSN: 0424-8201
DT Journal
LA English

L5 ANSWER 14463 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 78:105899 CA
OREF 78:16923a,16926a
TI Antitumor platinum compounds. Relation between structure and activity
AU Cleare, Michael J.; Hoeschele, J. D.
CS Johnson Matthey and Co., Ltd., London, UK
SO Platinum Metals Review (1973), 17(1), 2-13
CODEN: PTMRA3; ISSN: 0032-1400
DT Journal
LA English

L5 ANSWER 14464 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 78:79753 CA
OREF 78:12657a,12660a
TI New platinum complexes with antitumour activity
AU Connors, T. A.; Jones, M.; Ross, W. C. J.; Braddock, P. D.; Khokhar, A. R.; Tobel, M. L.
CS Chester Beatty Res. Inst., Cancer Hosp., London, UK
SO Chemico-Biological Interactions (1972), 5(6), 415-24
CODEN: CBINA8; ISSN: 0009-2797
DT Journal
LA English

L5 ANSWER 14465 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 78:67164 CA
OREF 78:10619a,10622a
TI Suppression of lymphocyte blastogenesis in man following cis-platinous diaminodichloride administration
AU Khan, Amanullah; Hill, Joseph M.
CS Wadley Inst. Mol. Med., Dallas, TX, USA
SO Proceedings of the Society for Experimental Biology and Medicine (1973), 142(1), 324-6
CODEN: PSEBAA; ISSN: 0037-9727
DT Journal
LA English

L5 ANSWER 14466 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 77:124670 CA
OREF 77:20561a,20564a
TI Effect of cis-platinous diamminodichloride on graft rejection. Prolonged survival of skin grafts against H2 histocompatibility
AU Khan, Amanullah; Albayrak, Aydogan; Hill, Joseph M.
CS Dep. Immunother., Wadley Inst. Mol. Med., Dallas, TX, USA
SO Proceedings of the Society for Experimental Biology and Medicine (1972), 141(1), 7-9
CODEN: PSEBAA; ISSN: 0037-9727
DT Journal
LA English

L5 ANSWER 14467 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 77:83330 CA
OREF 77:13689a,13692a

TI Chemistry of complexes related to cis-dichlorodiamine platinum(II). Antitumor drug
AU Thomson, A. J.; Williams, R. J. P.; Reslova, S.
CS Sch. Chem. Sci., Univ. East Anglia, Norwich/Norfolk, UK
SO Structure and Bonding (Berlin, Germany) (1972), 11, 1-46 CODEN: STBGAG; ISSN: 0081-5993
DT Journal; General Review
LA English

L5 ANSWER 14468 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 77:59271 CA
OREF 77:9805a,9808a
TI Synthesis and distribution of a radiolabeled antitumor agent: cis-diamminedichloroplatinum(II)
AU Lange, Robert C.; Spencer, Richard P.; Harder, Harold C.
CS Sch. Med., Yale Univ., New Haven, CT, USA
SO Journal of Nuclear Medicine (1972), 13(5), 328-30 CODEN: JNMEAQ; ISSN: 0161-5505
DT Journal
LA English

L5 ANSWER 14469 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 76:148785 CA
OREF 76:24163a,24166a
TI Cross-linking of complementary strands of DNA in mammalian cells by antitumor platinum compounds
AU Roberts, J. J.; Pascoe, J. M.
CS Chester Beatty Res. Inst., R Cancer Hosp., London, UK
SO Nature (London, United Kingdom) (1972), 235(5336), 282-4 CODEN: NATUAS; ISSN: 0028-0836
DT Journal
LA English

L5 ANSWER 14470 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 76:108073 CA
OREF 76:17385a,17388a
TI Suppression of graft-versus-host reaction by cis-platinum(II) diaminodichloride
AU Khan, Amanullah; Hill, Joseph M.
CS Dep. Immunother., Wadley Inst. Mol. Med., Dallas, TX, USA
SO Transplantation (1972), 13(1), 55-7 CODEN: TRPLAU; ISSN: 0041-1337
DT Journal
LA English

L5 ANSWER 14471 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 76:94747 CA
OREF 76:15213a,15216a
TI Growth inhibition of rat mammary carcinoma induced by cis-platinum diamminodichloride-II
AU Welsch, Clifford W.
CS Dep. Anat., Michigan State Univ., East Lansing, MI, USA
SO Journal of the National Cancer Institute (1940-1978) (1971), 47(5), 1071-8 CODEN: JNCIAM; ISSN: 0027-8874
DT Journal
LA English

L5 ANSWER 14472 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 76:81035 CA
OREF 76:12993a,12996a
TI Effect of cis-diaminoplatinum chloride in viruses and virus-cell relations
AU Popescu, M.; Pascaru, Adina; Nicolau, Cl.
CS Inst. Virusol. "St. S. Nicolau", Bucharest, Rom.
SO Studii si Cercetari de Inframicrobiologie (1971), 22(4), 383-9 CODEN: SCIBAJ; ISSN: 0039-3975
DT Journal
LA Romanian

L5 ANSWER 14473 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 75:117024 CA
OREF 75:18477a,18480a
TI Distribution and histopathological effects of cis-platinum(II)diamminodichloride on nontumored and **tumored** (sarcoma 180) Swiss white mice
AU Toth-Allen, Jean E.
CS Michigan State Univ., East Lansing, MI, USA
SO (1970) 130 pp. Avail.: Univ. Microfilms, Ann Arbor, Mich., Order No. 71-11,774
From: Diss. Abstr. Int. B 1971, 31(11), 6445-6
DT Dissertation
LA English

L5 ANSWER 14474 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 75:74445 CA
OREF 75:11797a,11800a
TI **Cancer** chemotherapeutic properties and toxicologic effects of cis-platinum(II) diammino dichloride
AU Kociba, Richard J.
CS Michigan State Univ., East Lansing, MI, USA
SO (1970) 87 pp. Avail.: Univ. Microfilms, Ann Arbor, Mich., Order No. 71-2097
From: Diss. Abstr. Int. B 1971, 31(8), 4804
DT Dissertation
LA English

L5 ANSWER 14475 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 74:40885 CA
OREF 74:6585a,6588a
TI Inhibition of Dunning ascitic leukemia and Walker 256 carcinosarcoma with cis-diamminedichloroplatinum (NSC-119875)
AU Kociba, Richard J.; Sleight, Stuart D.; Rosenberg, B.
CS Pathol. Dep., Michigan State Univ., East Lansing, MI, USA
SO Cancer Chemotherapy Reports, Part 1 (1970), 54(5), 325-8
CODEN: CCROBU; ISSN: 0576-6559
DT Journal
LA English

L5 ANSWER 14476 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 73:129299 CA
OREF 73:21081a,21084a
TI Cis-dichlorodiammineplatinum(II). Persistent and selective inhibition of deoxyribonucleic acid synthesis in vivo
AU Howle, Jerry A.; Gale, Glen R.
CS Veterans Adm. Hosp., Charleston, SC, USA
SO Biochemical Pharmacology (1970), 19(10), 2757-62
CODEN: BCPCA6; ISSN: 0006-2952
DT Journal
LA English

L5 ANSWER 14477 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 73:118796 CA
OREF 73:19349a,19352a
TI Inhibitory effects of antitumor platinum compounds on DNA, RNA, and protein syntheses in mammalian cells in vitro
AU Harder, Harold C.; Rosenberg, Barnett
CS Biophys. Dep., Michigan State Univ., East Lansing, MI, USA
SO International Journal of Cancer (1970), 6(2), 207-16
CODEN: IJCNAW; ISSN: 0020-7136
DT Journal
LA English

L5 ANSWER 14478 OF 14478 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 73:86239 CA

OREF 73:14103a,14106a
TI Successful regression of large solid sarcoma 180 **tumors** by platinum compounds
AU Rosenberg, Barnett; VanCamp, Loretta
CS Biophys. Dep., Michigan State Univ., East Lansing, MI, USA
SO Cancer Research (1970), 30(6), 1799-802
CODEN: CNREA8; ISSN: 0008-5472
DT Journal
LA English

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COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	105.09	117.03

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PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	Clet Niyikiza	Group Art Unit: 1614
Serial No.:	11/776,329	Examiner: Weddington, Kevin
Application Date:	July 11, 2007	Conf No.: 6568
For:	NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.:	X14173B	

INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Under the guidelines of 37 C.F.R. 1.97, Applicant submits a copy of each of the documents listed on the attached Form PTO-1449 (modified) for consideration by the Examiner.

Since this Statement is being filed after the period specified in §1.97(b), but before the mailing date of a final action or a notice of allowance, please charge the fee under 37 C.F.R. 1.17(p), and charge any additional fees which may be required by this or any other related paper, or credit any overpayment to Deposit Account No. 05-0840.

Applicant requests consideration of this information.

Respectfully submitted,

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John A. Cleveland, Jr.
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Phone: 317-276-0307

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Patent Division
P.O. Box 6288
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May 4, 2009

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	Clet Niyikiza	Group Art Unit: 1614	
Serial No.:	11/776,329	Examiner: Weddington, Kevin	
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Docket No.:	X14173B		

COMMUNICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Office Action dated February 18, 2009, Applicants submit the following remarks in connection with the above-identified patent application:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

Amendments to the Claims

The following listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claims 1-39 (Cancelled)

40. (Currently amended) A method for administering pemetrexed disodium to a patient in need thereof comprising administering an effective amount of pemetrexed disodium in combination with a methylmalonic acid lowering agent, wherein:

the methylmalonic acid lowering agent is selected from the group consisting of vitamin B_{12}, hydroxycobolamin, cyano-10-chlorocobolamin, aquocobolamin perchlorate, aquo-10 cobolamin perchlorate, azidocobolamin or chlorocobolamin;

the methylmalonic acid lowering agent is administered from about 1 week to about 3 weeks prior to the first administration of the pemetrexed disodium; and

the methylmalonic acid lowering agent administration is repeated about every 6 to about every 12 weeks until administration of the pemetrexed disodium is discontinued.

41. (previously presented) The method of claim 40, wherein the methylmalonic lowering agent is vitaminB_{12}.

42. (previously presented) The method of claim 41, wherein the vitamin B_{12} is administered as an intramuscular injection of about 500 μg to about 1500 μg.

43. (previously presented) The method of claim 42, wherein the vitamin B_{12} is administered as an intramuscular injection of about 1000 μg.

44. (previously presented) The method of claim 41, 42 or 43, wherein the vitamin B_{12} administration is repeated about every 9 weeks until the administration of the pemetrexed disodium is discontinued.

45. (currently amended) The method of claim 44, further comprising administering a folic-binding protein binding agent to the patient, wherein the folic-binding protein binding agent is selected from the group consisting of folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid or (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically available salt or ester therof.

46. (previously presented) The method of claim 45 wherein the folic-binding-protein binding agent is folic acid and the folic acid is administered prior to the first administration of the pemetrexed disodium.

47. (previously presented) The method of claim 46 wherein the folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

48. (previously presented) The method of claim 47 wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

49. (previously presented) The method according to any one of claims 46-48, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

50. (previously presented) The method of claim 49 wherein about 350μg to about 1000 μg of folic acid is administered.

51. (previously presented) The method of claim 50 wherein 350 μg to 600 μg of folic acid is administered.

52. (previously presented) The method of claim 40 or 45 further comprising the administration of cisplatin to the patient.

Remarks

Claims 40-52 are pending in the application. No Claims are allowed. Claim 45 is rejected under 35 U.S.C. § 112, 1st paragraph. Claims 40-52 are rejected under 35 U.S.C. § 112, second paragraph and 35 U.S.C. 103(a).

In view of the present amendment and reasons set forth below, it is submitted that the rejections are improper and should be withdrawn. Reconsideration and reexamination of the present application is respectfully requested.

Rejection Under 35 USC §112, first paragraph

Claim 45 is stands rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The Office Action asserts that the specification as originally filed fails to provide sufficient written bases of any of the agents demonstrating wherein possession of use of the broad term: "folic-binding-protein binding agents." In response, Claim 45 has been amended to disclose specific folic-binding-protein binding agent species recited in the specification. In light of this amendment, reconsideration and withdrawal of the rejection is respectfully requested.

Rejection Under 35 USC §112, second paragraph

Claims 40-52 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite. The office action points out that the phrase "methylmalonic acid" appears to be missing the phrase "lowering agent" in one of the recitations of claim 40. In response, Claim 40 has been amended to add the inadvertently omitted phrase "lowering agent." In light of this amendment, reconsideration and withdrawal of the rejection is respectfully requested.

Rejection Under 35 USC §103(a)

Claims 40-52 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Taylor (5,344,932) of PTO-1449 in view of Poydock et al., IRCS Medical Science, Vol. 12, No. 9, pp. 813 (1984) of PTO-1449, further in view of Worzalla et al., Anticancer Research, Vol. 18, No. 5, pp. 3235-3239 of PTO-1449, and further in view of Cleare et al. (4,149,707).

Specifically, the Office Action asserts that: "[t]he instant invention differs from the cited reference in that the cited reference does not teach the addition of a methylmalonic acid lowering agent. However, the secondary reference, Poydock et al., teaches a methylmalonic acid lowering agent such as hydroxocobalamin is effective by inhibiting tumors implanted in mice (see the abstract)."

Applicants note at the outset that independent Claim 40 comprises administration of pemetrexed disodium with a methylmalonic acid lowering agent (e.g., vitamin B12). Applicants assert that since Poydock et al. was discredited prior to the present application's priority date, it cannot even be used to support an assertion that methylmalonic acid lowering agent (e.g., hydroxocobalamin) is effective at inhibiting tumors implanted in mice.

Poydock et al. teaches that mice given a mixture containing L-ascorbic acid, hydroxocobalamin (a methylmalonic acid lowering agent), and Na ascorbate is effective at inhibiting tumors implanted in mice. Shortly after this abstract was published, however, it was discovered that the antitumor activity was not associated with the L-ascorbic acid, the hydroxocobalamin (a methylmalonic acid lowering agent), or the Na ascorbate. In fact, the researchers found that the L-ascorbic acid which they had used had oxidized to dehydroascorbic acid (see, e.g., Toohey, John I., Cancer Letters (Shannon, Ireland) (2008), 263(2), 164-169). In subsequent research with authentic materials, it was discovered that it was in fact the dehydroascorbic acid which was the active factor in the mixture (see Poydock et al., Experimental Cell Biology (1982), 50(2), 88-91; Poydock et al., American Journal of Clinical Oncology 8 (1985) 266-269; and particularly Poydock et al., American Journal of Clinical Nutrition 54 (1991) 1261S-1265S).

In addition, Poydock himself demonstrated that "[i]njections of ascorbic acid or of vitamin B_{12} alone had no effect on mitotic activity…" (see Poydock et al., American Journal of Clinical Nutrition 54 (1991) 1261S-1265S page 1262S 3rd paragraph) Moreover, in addition to reviewing the discovery of the antitumor activity of dehydroascorbic acid, Toohey, John I., Cancer Letters (Shannon, Ireland) (2008), 263(2), 164-169) also discusses the use of Vitamin B_{12} (a methylmalonic acid lowering agent) in studies by Poydock (see footnote page 164):

> "It should be noted that Poydock continued to add Vitamin B_{12} to most treatment protocols although her own data showed that it was not needed and there was no good rationale for adding it.…To this day there is no rationale for giving B_{12} and no known reaction between B_{12} and ascorbic acid or dehydroascorbic acid which could explain her result."

These clarification studies (at least those published prior to Applicant's priority date) demonstrate that vitamin B12 does, in fact, not possess anti-tumor activity, contrary to the teaching of Poydock et al. Therefore, Poydock et al. cannot be used to support the assertion in the Office Action that one skilled in the art would have combined pemetrexed disodium with vitamin B12 because both are anti-neoplastic agents. For the same reason, since Claims 41-52 depend from Claim 40, which contains the methylmalonic acid lowering agent limitation, the combination with folic-binding protein binding agent and/or cisplatin would not be obvious.

In view of the foregoing remarks, Applicants respectfully assert that the rejection is improper and should be withdrawn. Reconsideration is, therefore, kindly solicited. For at least the reasons set forth above, it is respectfully submitted that the above identified application is in condition for allowance. Favorable reconsideration and prompt allowance of the claims are respectfully requested.

Respectfully submitted,

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May 4, 2009

Am J Clin Oncol (CCT) 8: 266–269, 1985.

Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin

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S. Harguindey, M.D.
T. Hart, Ph.D.
H. Takita, M.D.
D. Kelly, B.S.

THE PRESENT STUDY WAS DESIGNED to test the effect of a combination of dehydroascorbic acid (DHA) and hydroxycobalamin (vitamin B_{12}) on the survival of mice bearing L1210 leukemia. Results showed a significant increase in survival of treated mice compared with controls ($p \leq 0.0001$) (Student's *t*-test). This positive effect was significantly lost when DHA was substituted by ascorbic acid (AA) in the same experimental conditions. *In vitro* findings also revealed that the DHA–B_{12} combination specifically inhibited mitoses of L1210 cells while non-neoplastic L929 cells were not affected.

From the Cancer Research Unit, Mercyhurst College, Erie, Pennsylvania (MEP, DK); Department of Thoracic Surgery, Roswell Park Memorial Institute, Buffalo, New York (TH, HT); and Department of Oncology, Clinica Universitaria, Pamplona, Spain (SH).

Write for reprints to: Salvador Harguindey, M.D., Dpto. Quimioterapia, Clinica Universitaria, Pamplona, Spain.

Growth suppression of human leukemic cells *in vitro* by L-ascorbic acid (AA) has been recently reported.[4] Preliminary *in vivo and in vitro* experiments from our laboratory have previously shown that a mixture of L-ascorbic acid (AA) and hydroxycobalamin inhibited mitoses of several transplanted ascites tumors.[6] Subsequent studies disclosed that the survival rate of mice bearing P388 leukemia and Ehrlich carcinoma was significantly increased after treatment with L-ascorbic acid and hydroxycobalamin buffered with Na^+ ascorbate.[7] DHA has been advanced by several authors to potentially offer a more potent antitumor activity than AA. Cell uptake of DHA is much higher than AA in normal and leukemic leucocytes as well as in HeLa cells and erythrocytes.[1,2] DHA uptake also seems to be related to the concentration of the vitamin in the medium.[2] Previous work from this laboratory has shown that DHA completely inhibited mitoses in Ehrlich ascites tumor and P388 leukemia in mice.[8] The authors thought it advisable, therefore, to test the effect of dehydroascorbic acid and hydroxycobalamin on the highly resistant L1210 mouse leukemia.

Material and methods

Preparation of vitamin mixture

Using aseptic technique, the vitamin combination, in crystalline form, was prepared by introducing into a small, sterile tube, 0.01 g hydroxycobalamin, 0.02 g dehydroascorbic acid (or 0.02 g ascorbic acid (AA)) buffered to pH 5.2 with 0.02 g sodium ascorbate, as previously reported.[7] In our hands, this dosage has proven

to be the highest tolerated dose which does not induce weight loss in the treated mice. A sufficient number of tubes with dry crystals were prepared in advance. Immediately before injection 1 ml of sterile normal saline at 60°C was added to the tube. To avoid denaturization, only the amount needed (1 ml) to treat five mice was diluted at one time. Hydroxycobalamin was obtained from Merck Chemical Division (West Point, PA). Ascorbic acid and sodium ascorbate were purchased from Sigma Chemical Co. (St. Louis, MO). DHA was obtained from ICN Nutritional Biochemicals (Cleveland, OH).

In vivo experiments

L1210 leukemia was supplied in DBA/2 mice from Roswell Park Memorial Institute, Buffalo, NY. This was the standard line used at the Institute for a number of years and it has been largely validated in a multiplicity of chemotherapy testing programs. Three experiments were tried using a total of 140 mice. In each of the two experiments involving DHA 40 female DBA/2 mice, 10 weeks old, within a 2-gram weight range, were used. Another 60 mice of the same characteristics (30 treated and 30 controls) were used for the AA experiment. All mice were fed normal laboratory food *ad libitum*. The tested mice were injected (i.p.) with 10^5 washed L1210 cells, suspended in 0.1 ml Tyrode's solution, producing an ascitic leukemia that later progressed to generalized disease. Mice were then randomized into equally fed groups. Starting 24 hours after implantation, test mice were injected i.p. with 10 mg/0.2 ml (0.4 g/kg body weight) DHA–B_{12} or AA–B_{12} mixture for 8 successive days in sterile conditions, after passing it through a 0.22 μ Millipore system. Two days later they were given, on alternate days, two more treatments, making a total of 10 treatments. Control mice were injected i.p. in the same conditions with 0.2 ml of a saline solution of the same pH (pH: 5.2) and osmolarity (305 mosm/liter).

In vitro experiments

Cells used in this study were propagated in RPMI medium 1640, supplemented with 10% calf serum plus 1% garamycin. Viable L1210 was the neoplastic tissue tested; whereas L929 fibroblasts (Microbiological Associates, suspension in frozen ampule) were the non-neoplastic cells used. For testing the effect of DHA–B_{12} on L1210 cells *in vitro* the ascites exudate was removed from tumor-bearing mice. It was washed with Tyrodes' solution, centrifuged at 500 rpm for 10 minutes, and the pellet resuspended in RPMI medium 1640 to yield the initial inoculum of 1×10^6 cells/ml. Then 0.4 ml of the L1210 cell suspension was introduced into each of four Falcon Flasks containing 4 ml RPMI medium. After an initial mitotic count was made, the flasks were divided into two test and two control, and 0.62 mg/0.05 ml of the vitamin mixture was added to each of the test flasks. They were incubated at 37.5°C. At 2, 4, 6, and 24 hours, mitotic counts were made by withdrawing a drop of the cell suspension with a Pasteur pipette and staining with acetocarmine, using the procedure described previously.[6] For estimating the effect of DHA–B_{12} on mitoses of L929 fibroblasts the procedure was the same except that counts were made in 10 fields of the monolayer. An inverted microscope with phase contrast optics at 300× was used. This experiment was repeated, following the same procedure mentioned above, and the results were totally reproducible. The effect of AA–B_{12} on L1210 and L929 cells has been reported before.[6]

Results

Figure 1 demonstrates the survival curve for 40 DBA/2 mice bearing L1210 leukemia after treatment with DHA–B_{12}. Two identical experiments were tried, and the results were identical. Results revealed a pronounced increase in the survival rate of the treated mice compared with the control group ($p \leq 0.0001$). The median survival time of the treated mice was greater than 60 days, and for the control group it was just under 12 days. The experiment was continued for 60 days after tumor implantation, then terminated. Postmortem and histological examination of untreated mice revealed disseminated disease to liver, spleen, and lungs. Treated mice sacrificed after day 60 revealed no evidence of disease. An incidental finding in some of the dead treated mice was "cloromization" (formation of solid tumors) of L1210 in the peritoneal cavity.

Table 1 compares the *in vitro* results of L1210 neoplastic cells with viable non-neoplastic L929 fibroblasts. This table represents the average of two experiments, two test and two control flasks in each. Results indicated that the vitamin mixture inhibited mitoses in

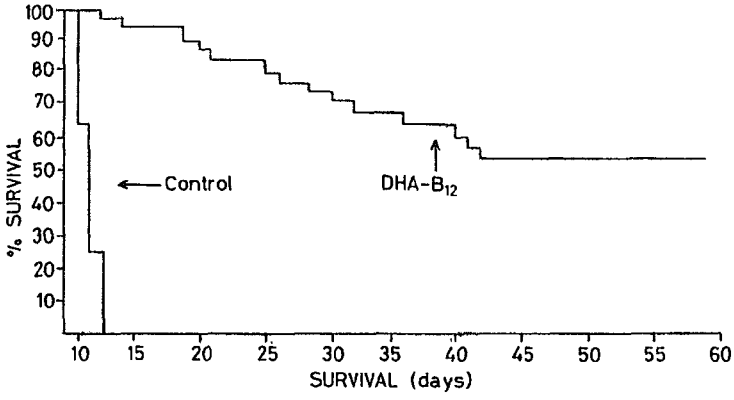

FIGURE 1. Survival of mice bearing leukemia L1210 after treatment with a mixture of hydroxycobalamin and dehydroascorbic acid. I.p. doses, 10 mg/0.2 ml on days 1–8, 11, and 13 were given. Eighty mice were sampled (40 treated and 40 control).

L1210 cells, but mitotic division of the nonneoplastic L929 fibroblasts was not affected.

Figure 2 shows the packed cell volume of the L1210 exudate taken from a treated DBA/2 (TEST) and control (CON) mouse after five treatments with DHA–B_{12}. A microscopic scrutiny of the acetocarmine smears, prepared from the ascitic fluid taken from the treated mice only revealed disintegrating tumor cells. Also, an increase in lymphocytes, neutrophils, and monocytes (some of which were dividing) was noted in the sample treated with DHA–B_{12}. Viability tests with trypan blue showed that these white blood cells were all viable.

Figure 3 shows the survival curve for 60 (30 test and 30 treated) mice bearing L1210 leukemia after treatment with AA–B_{12}. This experiment demonstrates that the substitution of DHA by AA in the same experimental conditions abolishes most of the antitumoral effect of the vitamin C–B_{12} combination.

TABLE 1

DHA and Vitamin B_{12}

Tissue Tested	Incubation Time (hr.)	Mitotic Index	
		Test	Control
L1210	0	46	46
	2	0	40
	4	0	42
	6	0	48
	24	0	42
L929	0	52	52
	2	50	48
	4	62	60
	6	64	62
	24	56	58

Comparison of *in vitro* inhibitory effect of a mixture of hydroxycobalamin and dehydroascorbic acid on mitoses of L 1210 cells and L929 fibroblasts. (Dosage 0.62 mg/0.05 ml).

Discussion

The results of these investigations show that the DHA–B_{12} mixture significantly increases the survival rate of mice bearing the classic L1210 leukemia. We have previously reported that vitamin C (L-ascorbic) and B_{12} administered separately at the same dosage as the mixed preparation to tumor bearing mice had no apparent effect on mitoses of Ehrlich ascites

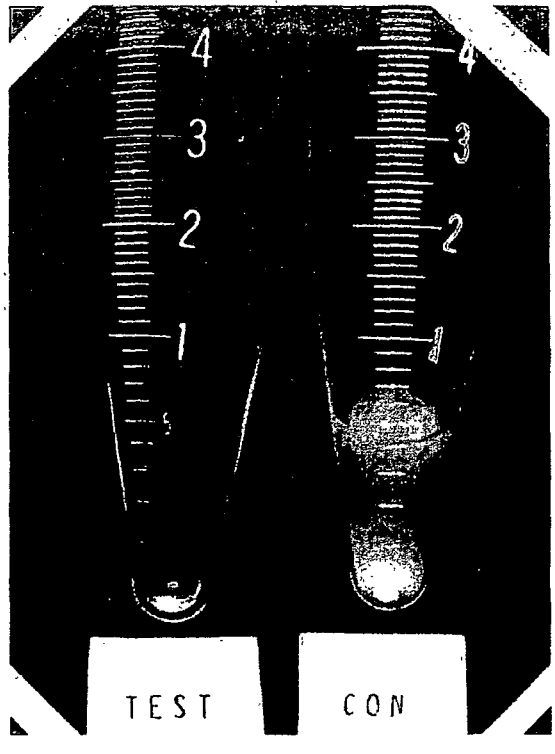

FIGURE 2. Packed cell volume of the L1210 i.p. exudate taken from a treated DBA/2 (TEST) and control (CON) mouse after five treatments with DHA–B_{12}.

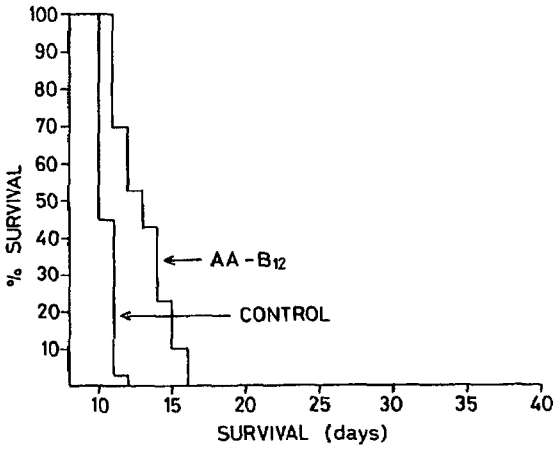

FIGURE 3. Survival of mice bearing leukemia L1210 after treatment with a mixture of hydroxycobalamin and ascorbic acid. Dosages and conditions were exactly the same as shown in the experiment in Figure 1. Sixty mice (30 treated and 30 control) were sampled.

tumor.[6] We have also demonstrated the effect of dehydroascorbic acid alone on mitoses in two murine ascites tumors (Ehrlich and P388 leukemia) and the stained ascitic fluid revealed no mitotic figures.[8] The mechanism by which the mixture of hydroxycobalamin and dehydroascorbic acid seems to act selectively on tumor cells and inhibits mitoses has not yet been resolved, nor the reason for the apparent potentiating effect of B_{12} on vitamin C derivates.[6,7] Work done by others in the past has shown that high doses of B_{12} alone caused a decrease in the growth of certain transplanted mouse tumors and the results exhibited a selective action of vitamin B_{12} for the specific tumor studied.[9] Several possible mechanisms may explain the suppressive effect of ascorbic acid derivatives on tumor cells.[5,10–13] It has been suggested that glutathione may be involved in the modulation of mitosis by ascorbic acid derivatives.[11,13] It has also been known for some time that the level of cAMP is increased by L-ascorbic acid; therefore cell division may indirectly be suppressed by a mixture of vitamin C derivatives and B_{12}.[12] Mitotic inhibition may also be mediated by a membrane redox phenomenon, an antioxidant effect produced by dehydroascorbic acid,[10] an oxidation of SH groups of nucleic acid DNA polymerase,[5] or even a combination of two or more of these mechanisms. Although the mechanism of action that halts cell division of neoplastic cells is still controversial and a direct toxic effect can not be ruled out, a mixture of dehydroascorbic acid and hydroxycobalamin, given in sustained doses, is highly cytotoxic for L1210 cells, nontoxic for L929 non-neoplastic cells, and can reach a cure rate of at least 50% of the animals transplanted with this resistant leukemia. At the present time, a phase I–II human study using this kind of DHA–B_{12} combinations is in progress. ℭ

ACKNOWLEDGMENT

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References

1. Basu, B., Bhattacharaya, D. K., and Chatterjee, G. C.: Uptake of ascorbic acid by human leukocytes under normal and leukemic conditions. *Indian J Exp Biol 15: 352–354, 1977.*
2. Okamura, M.: Uptake of L-ascorbic acid and L-dehydroascorbic-acid by human erythrocytes and Hela cells. *J Nutr Sci Vitaminol 25: 269–280, 1979.*
3. Omura, H., Tomita, Y., Nakamura, Y., and Murakami, H.: Antitumoric potentiality of some ascorbate derivatives. *J Fac Agr Kyushu Univ 18: 181–189, 1974.*
4. Park, C. H., Amare, M., Savin, M. A., and Hougstraten, B.: Growth suppression of human leukemic cells *in vitro* by L-ascorbic acid. *Cancer Res 40: 1062–1065, 1980.*
5. Pethig, R., Gascoyne, P. R., McLaughlin, J. A., and Szent-Gyorgyi, A.: Ascorbate–quinone interactions: Electrochemical, free radical, and cytotoxic properties. *Proc Natl Acad Sci USA 80: 129–132, 1983.*
6. Poydock, M. E., Fardon, J. C., Gallina, D., Ferro, V., and Heher, C.: Inhibiting effect of vitamins C and B_{12} on the mitotic activity of ascites tumors. *Exp Cell Biol 47: 210–217, 1979.*
7. Poydock, M. E., Reikert, D., and Rice, J.: Influence of vitamins C and B_{12} on the survival rate of mice bearing ascites tumors. *Exp Cell Biol 50: 88–91, 1982.*
8. Poydock, M.E., Reikert, D., Rice, J., and Aleandri, L.: Inhibiting effect of dehydroascorbic acid on cell division in ascites tumors in mice. *Exp Cell Biol 50: 34–38, 1982.*
9. Rigby, C. C. and Bodian, M.: Experimental study of the relationship between vitamin B_{12} and two animal tumor systems. *Br J Cancer 17: 90–99, 1963.*
10. Stankova, L., Gerhardt, N. B., Nagel, L., and Bigley, R. H.: Ascorbate and phagocyte function. *Infection and Immunity 12: 252–256, 1975.*
11. Stern, H.: Sulfhydryl groups and cell division. *Science 124: 1292–1293, 1956.*
12. Tisdale, M. J.: Inhibition of cyclic 3′, 5′-monophosphate phosphodiesterase from Walker carcinoma by ascorbic and dehydroascorbic acids. *Biochem Biophys Res Commun 62: 877–882, 1975.*
13. Woodward, G. E.: Glutathione and ascorbic acid in tissues of normal and tumor-bearing albino rats. *Biochem J 29: 2405–2412, 1975.*

Intrathecal Methotrexate–Induced Megaloblastic Anemia in Patients With Acute Leukemia

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• ***Objective.***—**To evaluate the occurrence of megaloblastic anemia induced by the infusion of therapeutic or prophylactic methotrexate in patients with acute leukemia.**

Design.—**Data on 3 patients with acute leukemia receiving intrathecal methotrexate were prospectively analyzed.**

Setting.—**Large tertiary-care center.**

Results.—**All 3 patients with acute leukemia developed megaloblastic anemia confirmed by examination of the bone marrow aspirate and biopsy. Two of the 3 patients had low folic acid levels, while all patients had normal serum B_{12} levels. All patients responded favorably to a therapeutic trial of folic acid. The median time for recovery of the hematologic parameters in these patients was 7 days.**

Conclusions.—**Intrathecally administered methotrexate may result in megaloblastic changes in the bone marrow of leukemic patients. The morphologic clues suggestive of folate deficiency in patients with acute leukemia may be masked by coexisting factors, such as the effects of cytotoxic treatment, prior transfusions, or persistent changes from the leukemic clone itself. Caution should be exercised to avoid attributing these changes to the neoplastic process, since the prognosis and treatment for the conditions involved are totally different. Repeat examination of the bone marrow, obtaining folic acid and vitamin B_{12} levels, and a therapeutic trial of folic acid may help identify and reverse these changes.**

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By binding critical enzymes, folate acts as a cofactor and functions to receive a single-carbon fragment from selected enzymatic reactions and transfer this carbon unit to other molecules important for de novo synthesis of purines and pyrimidines, precursors of DNA.[1] To form an active compound, folate must be reduced to tetrahydrofolate (FH_4). The enzyme dihydrofolate reductase, a critical enzyme in intracellular methylation reactions, catalyzes the reduction of folic acid to dihydrofolate and FH_4.[1,2]

Methotrexate (MTX), an inhibitor of dihydrofolate reductase, is often used in the treatment of hematologic malignancies, as well as for prophylaxis and treatment of the central nervous system in patients with acute leukemia or lymphoma. When used intravenously or orally, MTX can cause depletion of intracellular tetrahydrofolate, leading to megaloblastic anemia.

In MTX-treated cells, inhibition of dihydrofolate reductase results in the entrapment of folate as inactive dihydrofolate with subsequent suppression of thymidylate synthesis, leading to a reduction in the rate of DNA synthesis.[3,4] The slowing of DNA synthesis in combination with normal cytoplasmic RNA synthesis leads, over several cell divisions, to asynchrony between the rates of cytoplasmic and nuclear maturation. This unbalanced growth affects all proliferating cells, but is most easily observed in bone marrow cells, which morphologically appear to be megaloblastic.

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REPORT OF CASES

The following is a critical description of the courses of 3 patients with acute leukemia and megaloblastic anemia. The Table provides a detailed laboratory analysis of each case before and after folic acid supplementation and lists each patient's MTX, folic acid, and vitamin B_{12} level at the time megaloblastic anemia was diagnosed.

Case 1

A 64-year-old woman with acute myelomonocytic leukemia (French-American-British grade M4), presented 2 months after the end of her consolidation treatment with headache, confusion, and increasing fatigue. Cerebrospinal fluid (CSF) cytology revealed numerous blasts consistent with leukemic involvement. Bone marrow aspirate and biopsy revealed no evidence of leukemia. Methotrexate at a dose of 15 mg was administered intrathecally via lumbar puncture and then through ventricular reservoir for 2 additional doses. Repeat CSF analysis showed normal protein and glucose levels and no blasts. Two weeks later, the patient presented with increasing fatigue and mild chest discomfort. Laboratory analysis at the time demonstrated an absolute neutrophil count of 310/μL; platelets, 14×10^9/L; hemoglobin, 88 g/L; and mean cell volume, 95.5 fL. Review of the blood film revealed nuclear hypersegmentation of neutrophils, as depicted in Figure 1. Repeat examination of the bone marrow aspirate and biopsy showed megaloblastic erythroid hyperplasia and large metamyelocytes (Figure 2). The patient's lactate dehydrogenase (LDH) level was 296 U/L (normal, 122–240 U/L). After obtaining folic acid and vitamin B_{12} levels, the patient was given a therapeutic trial of 5 mg folic acid daily; white blood cell and platelet counts recovered on day 7, and LDH levels normalized by day 21.

Case 2

A 20-year-old woman with acute lymphoblastic leukemia underwent induction treatment with L-asparaginase, doxorubicin,

Characteristics and Laboratory Results of 3 Patients With Acute Leukemia and Megaloblastic Anemia Before and 2 Weeks After Replacement With Folic Acid*

Patient No.	Diagnosis	Before Replacement							After Replacement			
		Hb, g/L	MCV, fL	ANC, /μL	Platelets, × 10⁹/L	B_{12}, pmol/L	Folates, Serum/RBC, nmol/L	MTX, μmol/L	Hb, g/L	MCV, fL	ANC, /μL	Platelets, ×10⁹/L
1	AML	88	96	310	14	494	13.8/543 8	<0.05	98	85	3200	130
2	ALL	74	112	1210	19	472	2.2/244 7	<0.05	90	92	3900	122
3	ALL	79	103	940	22	542	2.7/233.3	<0.05	88	89	3600	127

* AML indicates acute myeloid leukemia, ALL, acute lymphoid leukemia; Hb, hemoglobin; MCV, mean cell volume; ANC, absolute neutrophil count; RBC, red blood cell; and MTX, methotrexate Normal values: Hb, 120–160 g/L, MCV, 80–100 fL; ANC, 2500–4500 neutrophils/μL; platelets, ×10⁹/L; serum folate, 11–57 nmol/L; RBC folate, 376–1450 nmol/L; serum B_{12}, 247–911 pmol/L; and MTX, lower limit of detection, 0.05 μmol/L.

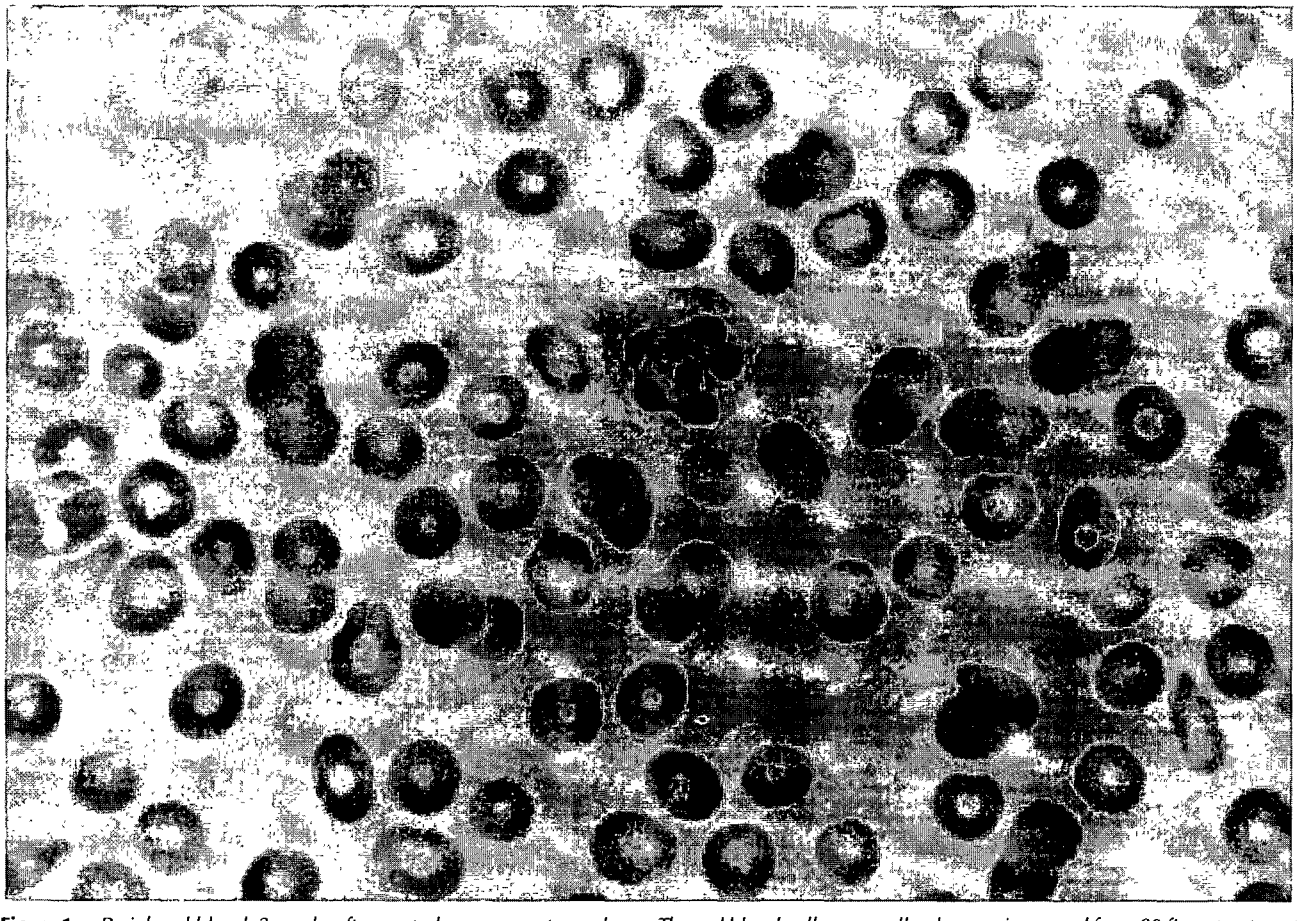

Figure 1. *Peripheral blood, 2 weeks after central nervous system relapse. The red blood cell mean cell volume is increased from 88 fL pretreatment with methotrexate to 96 fL (normal, 80–100 fL). Note the large 6-lobed neutrophil and profound thrombocytopenia (Wright-Giemsa, original magnification ×630).*

vincristine, prednisone, cytosine-arabinoside, and cyclophosphamide. Bone marrow aspirate and biopsy following treatment showed no evidence of leukemia. Two weeks later, consolidation treatment with vincristine and doxorubicin was started, and prophylactic intrathecal MTX was administered at a dose of 12 mg via lumbar puncture twice weekly. Repeat complete blood counts following the third dose of MTX showed an absolute neutrophil count of 1210/μL; platelets, 19 × 10⁹/L; hemoglobin, 74 g/L; and mean cell volume, 112 fL. Few ovalocytes and anisopoikilocytosis were present in the peripheral blood smear. This degree of pancytopenia was disproportionate to what was expected from this particular chemotherapy regimen. Therefore, we examined the bone marrow aspirate and biopsy, which revealed megaloblastic erythroid hyperplasia and giant metamyelocytes. The patient's LDH level at this presentation was 310 U/L. After obtaining folic acid and vitamin B_{12} levels, a therapeutic trial of 2-mg daily doses of folic acid was started. No complete blood count was available on this patient on day 7; however, repeat laboratory analysis showed improvement in both the hematologic parameters and LDH by day 14 of folic acid replacement.

Case 3

The patient, a 29-year-old man with acute lymphoblastic leukemia in remission, verified by bone marrow examination, was started on prophylactic administration of MTX at a dose of 12 mg through ventricular reservoir. Following the third dose, the patient presented with increasing fatigue and shortness of breath. Laboratory findings at the time of presentation showed the pa-

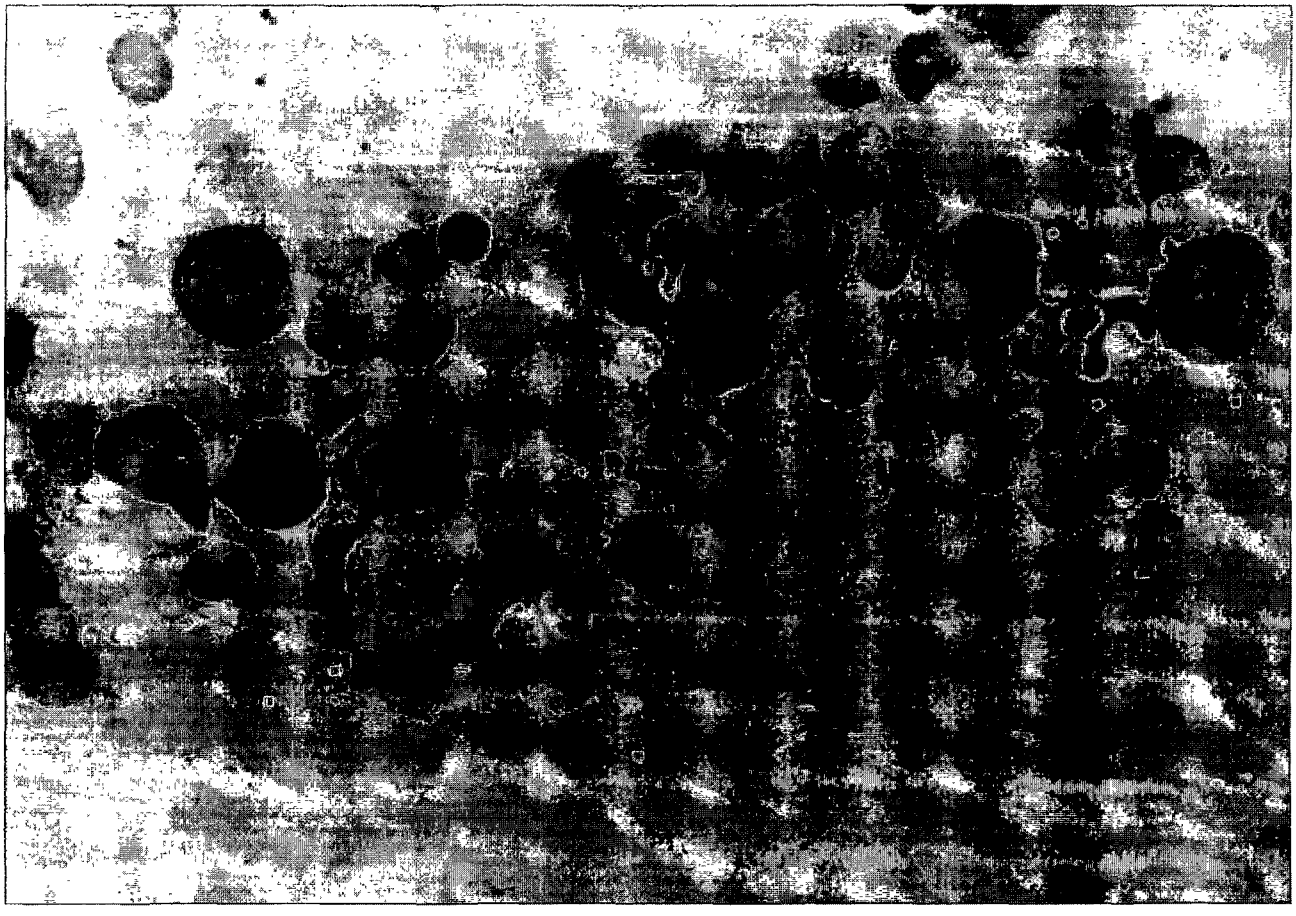

Figure 2. *Marrow aspirate at the time of central nervous system relapse. Erythroid hyperplasia with megaloblastic changes is evident. Note the large megaloblasts and metamyelocytes, as well as nuclear-cytoplasmic dysynchrony. At this time, the myeloid-erythroid ratio was 1:2 and myeloblasts were 4% of the nucleated cells (Wright-Giemsa, original magnification ×630)*

tient's absolute neutrophil count to be 940/μL; platelets, 22 × 10⁹/L; hemoglobin, 79 g/L; and mean cell volume, 103 fL. Mild anisocytosis, poikilocytosis, and few tear drops were evident in the blood film. Examination of the bone marrow aspirate and biopsy showed megaloblastic erythroid hyperplasia. The patient's LDH level was 276 U/L. After obtaining folic acid and B_{12} levels, a daily replacement with 2 mg folic acid was administered, resulting in improvement of the blood counts by day 7 and normalization of LDH by day 14.

MATERIALS AND METHODS

Folate and Vitamin B_{12}

Folate and vitamin B_{12} were measured on a Technicon Immuno 1 (Technicon, Tarrytown, NY) system using competitive magnetic separation immunoassays. Folate and vitamin B_{12} were first released from serum binders with a pretreatment. The released vitamins were reacted with a binding protein reagent (folate binding protein and intrinsic factor for folate and vitamin B_{12}, respectively) and incubated at 37°C. Folate or B_{12} conjugated to an alkaline phosphatase enzyme was then added to compete with the sample for the binding protein. A monoclonal immunomagnetic particle was incubated with the reagents, washed, and paranitrophenyl phosphate substrate was added. Absorbance at 405 nm due to the formation of paranitrophenoxide is inversely proportional to the amount of vitamin in the sample. The folate assay is linear from 0.7 to 34 nmol/L (0.3–15 ng/mL), with a normal range of 5.9 to 39.2 nmol/L (2.6–17.3 ng/mL) for serum folate and 362.6 to 1602.1 nmol/L (160–707 ng/mL) for red blood cell folate; the vitamin B_{12} assay is linear from 19 to 1106 pmol/L (26–1500 pg/mL), with a normal range of 182 to 672 pmol/L (247–911 pg/mL). These tests were performed after examining the bone marrow aspirate and biopsy, and before replacement with folic acid.

Methotrexate

Methotrexate was measured on a TDX analyzer (Abbott Laboratories USA, Abbott Park, Ill) using a fluorescence polarization immunoassay. This technique adds a pretreatment reagent to free any bound analyte from serum proteins. A specific monoclonal antibody is added together with a tracer-labeled MTX that competes for the antibody with the MTX in the sample. The intensity of the fluorescent signal varies inversely with the amount of MTX in the sample. The assay range is 0 to 1000 μmol/L with a lower limit of detection of 0.05 μmol/L. Methotrexate concentrations were measured 48 to 72 hours after treatment.

Bone Marrow Preparation

Bone marrow biopsies were fixed for 2 hours in 10% buffered formalin, decalcified for 30 minutes in Baxter Decal, and processed routinely for surgical pathology. Biopsies were evaluated for cellularity; cellular composition, including the presence or absence of clusters of leukemic blasts; and the overall adequacy of megakaryocytes. Aspirate particle smears and biopsy touch preparations were stained with Wright-Giemsa stain and were examined to determine the myeloid-erythroid ratio, evaluate trilineage hematopoiesis, and evaluate the presence or absence of leukemic blasts. Storage iron was evaluated with Prussian blue stain. Special stains for myeloperoxidase, chloroacetate esterase,

and α-naphthyl butyrate esterase and immunophenotyping by flow cytometry were performed as needed to determine leukemic lineage.

Follow-up Studies

Follow-up complete blood count and LDH values were performed on a weekly basis after the administration of folic acid supplements until normalization of these parameters and then as dictated by each patient's treatment plan. Laboratory data at 1 week posttreatment were not available for patient 2, owing to noncompliance with our instructions.

RESULTS

Intrathecally administered MTX resulted in megaloblastic anemia in 3 patients with acute leukemia. None of these patients had morphologic evidence of relapsed leukemia in their bone marrow biopsies at the time of assessment.

Serum folate levels were below normal in patients 2 and 3, but were normal in patient 1. Serum B_{12} levels were normal in all patients. Methotrexate concentrations were below detectable limits in all patients. Replacement with folic acid led to improvement of the hematologic parameters and LDH in all patients. None of the patients had evidence of systemic toxicity or end-organ damage at the time of administration.

COMMENT

Methotrexate is an antineoplastic agent used in the treatment of several malignancies. Through its tight binding of dihydrofolate reductase, MTX inhibits the production of tetrahydrofolates necessary for tumor cell growth. Methotrexate exhibits activity in leukemia and lymphoma, two malignancies with a propensity for sequestration in the central nervous system.

Owing to its low lipid solubility, diffusion of MTX into the CSF in concentrations necessary for tumor cell kill (usually 1 μmol/L) are not achievable at "conventional" doses in the range of 30 to 100 mg/m^2 (CSF-plasma ratio, 0.03). This obstacle may be overcome by (1) administering the agent in higher doses (eg, ≥1 g/m^2) or (2) direct administration into the CSF. The expected corollary is that intrathecally administered MTX can slowly diffuse from the CSF into the systemic circulation, yielding prolonged elevated plasma levels and potential toxicities.[5]

Elimination from the central nervous system is primarily by bulk resorption of CSF. Jacobs and colleagues[6] compared the pharmacokinetics of orally, intravenously, and intrathecally administered MTX in 2 patients with acute lymphocytic leukemia. Their results suggest that MTX given intrathecally slowly diffuses into the plasma, leading to concentrations greater than 10^{-8} mol/L for twice as long as expected for the same dose given orally or intravenously. A patient with high-grade lymphoma and central nervous system involvement who developed tumor lysis after 1 dose of intrathecal MTX has also been described.[7]

In this short series, 3 patients received intrathecal MTX and subsequently developed megaloblastic bone marrow changes and peripheral cytopenias that recovered following replacement with folic acid. The course of improvement of both the peripheral counts (average, 7 days) and LDH (average, 14 days) corresponds well with the reversion of the effects of folic acid deficiency following replacement. All the patients had serum MTX levels below the limits of detection, suggesting that there was no laboratory evidence of systemic toxicity. In support of this finding is the absence of clinical symptoms or signs related to MTX toxicity, such as nausea, vomiting, and mucositis.

As was noted, patient 1 had normal folic acid and vitamin B_{12} values. However, examination of the bone marrow and peripheral blood smear in this patient demonstrated findings consistent with megaloblastic anemia, including the multilobated neutrophils depicted in Figure 1. In addition, the patient had a well-documented response to a therapeutic trial of folic acid.

It is important to realize that none of the patients had renal insufficiency or third space fluid collection, factors that may prolong the exposure to MTX and lead to systemic toxicity. Also, none of the patients at the time of their evaluation was receiving other medications that might have induced folic acid deficiency.

It appears that intrathecally administered MTX may have resulted in megaloblastic bone marrow changes by a diffusion mechanism from the central nervous system to the systemic circulation in amounts not sufficient to cause systemic toxicity. An alternative explanation is that our 3 previously treated patients had borderline low folic acid levels, and that even a small leakage of MTX to the systemic circulation led to megaloblastic anemia.

Also noteworthy were the extreme neutropenia and thrombocytopenia observed in the 3 patients, as well as the lack of the typical hypercellular marrow characteristic of nutritional megaloblastic anemia.[8] These findings may be explained by prior exposure to systemic chemotherapy and the persistence of a subclinical leukemic clone.

It is crucial not to assume automatically that pancytopenia in the peripheral blood or megaloblastic erythropoiesis in the bone marrow of patients receiving intrathecal MTX is due to relapsed leukemia, since these findings may also be due to MTX administration and may respond favorably to folic acid therapy.

References

1. Person AG, Turner A. Folate-dependent 1-carbon transfer to biogenic amines mediated by methylene-tetrahydro-folate reductase. *Nature.* 1975;258:173–174.
2. Steinberg SE, Fonda S, Campbell CL, Hillman RS. Cellular abnormalities of folate deficiency. *Br J Haematol.* 1983;54:605–612.
3. Jolivet J, Chabner BA. Intracellular pharmacokinetics of methotrexate polyglutamates in human breast cancer cells: selective retention and less dissociable bindings of 4-N-10-CH_3-pterolglutamate$_4$ and 4-NH_2-10-CH_3-pterolglutamate$_5$ to dihydrofolate reductase. *J Clin Invest.* 1983;72:773–778.
4. Bleyer WA. The clinical pharmacology of methotrexate. new applications of an old drug. *Cancer* 1978;41:36–51.
5. Bleyer WA, Nelson A, Kamen BK. Accumulation of methotrexate in systemic tissues after intrathecal administration. *J Pediatr Hematol Oncol.* 1997;19.530–532.
6. Jacobs SA, Bleyer WA, Chabner BA, Johns DG. Altered plasma pharmacokinetics of methotrexate administered intrathecally. *Lancet.* 1975;1:455–456.
7. Simmons ED, Somberg KA. Acute tumor lysis syndrome after intrathecal methotrexate administration. *Cancer* 1991;57:2062–2065.
8. Easton DJ. Severe thrombocytopenia associated with acute folic acid deficiency and severe hemorrhage in two patients. *Can Med Assoc J* 1984;130:418–420.

The Inhibition and Promotion of Cancers by Folic Acid, Vitamin B_{12}, and Their Antagonists

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For the past two years, our group has been collaborating with that of Dr. Ludwik Gross in the area of these studies, attempting to "switch off" the oncogene for guinea pig leukemia/lymphoma (1). The concept involved is that DNA methylation, and specifically methylation of cytosine in higher eukaryotes can directly suppress gene expression. This concept has been elaborated in several reviews, of which the most recent is by Eick et al. (2) in Analytical Biochemistry 135:165-171, 1983. The first dramatic presentation of possible clinical value of being able to demethylate a gene was the study by Heller and his associates in Chicago suggesting that they could "switch on" the fetal hemoglobin gene using 5-azacytidine, presumably by hypomethylating the fetal globin gene. They collaborated with Ley et al. in a study strongly suggesting that they could, in fact, switch on fetal hemoglobin synthesis with 5-azacytidine (3). This was confirmed by Charache and Dover and their associates at Johns Hopkins University (4) but Nathan and Lethvin and their associates showed that two other S-phase specific cytotoxic agents, hydroxyurea and cytosine arabinoside, could also increase fetal hemoglobin synthesis, Stanatiannopoulos and Poppianopoulou found that cytosine arabinoside can produce identical response in baboons to 5-azacytidine, and Nathan was quoted as concluding that the three drugs "probably act in the same way. Methylation has nothing to do with it" (5).

However, W. French Anderson and his associates were able to show directly in the cell culture system that 5-azacytidine does in fact selectively hypomethylate fetal globin genes, but that other genes, including an oncogene, are remethylated shortly after losing their methyl groups. These superficially divergent results can be reconciled by the concept that hypomethylation causes the persistent hemoglobin, whereas other mechanisms produce the acute increased production of fetal hemoglobin which occurs after 5-azacytidine (or hydroxyurea or cytosine arabinoside (5).

Similarly, different acute and persistent effects may explain

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why the same antifolate, methotrexate, which can shut down a lympho-proliferative malignancy, may result years later in the development of a second malignancy (1). One can speculate that an acute toxic effect kills the tumor but that the same methotrexate which acutely toxically kills the tumor, in a long period of time will result in demethylation of an oncogene, which can then be expressed as second malignancy.

Other evidence that demethylation can cause the expression of malignancies comes from studies by Feinberg and Vogelstein at Johns Hopkins (6) at the Institute Pasteur by Bourgeois and her associates, who found that glucocorticoids can cause expression of murine mammary tumor virus (MMTV) by glucocorticoid-induced methylation of long-terminal repeat sequences (7). Similarly, Proirier and his associates published a number of studies delineating the ability of methyl-deficient, amino acid-defined diets to produce liver tumors in rats treated both with and without initiating doses of diethylnitrosamine (8, 9). Their studies indicate that diethylmethyl deficiency markedly promotes liver carcinogenesis and exhibits complete carcinogenetic activity in this organ in the rat. Rogers and Newberne had shown that dietary methyl deficiency enhances the activities of a number of hepatocarcinogens, and Shinizuka and Lombardi have found that choline deficiency enhanced the hepatocarcinogenic activities of several agents.

The concept that deficiency of folate or vitamin B-12, or any other cause of failure to methylate DNA and/or RNA can activate malignancy by hypomethylation or oncogenes, and that methylating oncogenes can inhibit malignancy by making them dormant, is similar to the concept of "relaxed control" of RNA synthesis. In the '50s, Mendel and Borek (10) had noted that when an organism autotrophic from methionine is deprived of methionine, it loses its ability to suppress synthesis of RNA, which is then synthesized more rapidly. It was speculated that deficiency of B-12 or folate could produce similar "relaxed control". It is possible that some forms of vitamin B-12 and of folic acid may act as inhibitors of methylation and other forms as promoters of methylation of RNA and DNA (1). Reduced forms of folic acid are metabolically active; oxidized forms may be antimetabolites (1). Hydroxocobalamin is metabolically active; cyanocobalamin can be a B-12 antimetabolite (1).

Leuchtenberger et al. had reported that inositol inhibited animal tumor growth but various B vitamins did not (11, 12). This requires reinvestigation to determine whether inositol can methylate oncogenes. Oxidized folate monoglutamate, which is not a metabolically active form of the vitamin, not only did not inhibit spontaneous breast cancer in mice but actually produced a more rapid growth of the primary tumors and a significant increase in lung metastases. This work requires repeating today, particularly from the point of view of whether metabolically

inactive folate, that is, oxidized folate, can promote tumor development but metabolically active folate, particularly the very active triglutamates, can promote methylation of oncogenes and thereby inhibit their expression.

For many years, a number of workers have been exploring the question of whether one form of a vitamin can be a growth promoter by acting as a coenzyme (i.e., a promoter of normal and tumor cell growth) while another form of the same vitamin can attach to the same apoenzyme or other ligand (such as a vitamin transporting protein) and then jam the machinery, just as a key with a tooth missing can fit into a lock and then not turn. The answer to that question is clearly yes. Slight to major differences in the same vitamin structure (i.e., analogues and congeners) both exist in nature and are synthesizable; some of them are antagonists or anti-vitamins which can be created from vitamins by only slightly warping their structure (1).

Farber et al. (13) reported from Harvard giving pteroyltriglutamic acid (teropterin) and pteroyldiglutamic acid (diopterin), both synthesized by U. SubbaRow and his associates at Lederle Laboratories, to 90 patients with various malignancies, noting that "in general, adult patients experienced improvement in energy, appetite, sense of well being...might be ascribed to improved morale resulting from frequent visits, more medical attention..." They also reported inconstant temporary decreases in size of metastases in some tumors and degeneration and necrosis in others.

The apocryphal story is that Dr. Farber was also giving folic acid (the oxidized, stable pharmaceutical form of the vitamin) to children with lymphoproliferative malignancies (lymphocytic leukemia and lymphoma) until one of his residents collected sufficient data to suggest that the children receiving this new vitamin were dying faster than those children not receiving it. This observation allegedly led Dr. Farber to ask the Lederle people to create a warped folic acid molecule which would interfere with folate metabolism in the malignant cells, and this was done by adding an NH_2 group, thereby creating aminopterin. A second alteration, metyhylation in the 10-position, created methotrexate, still one of our most potent anti-cancer agents, particularly effective against childhood lymphoproliferative disorders and trophoblastic malignancies.

There is considerable evidence that rapidly growing neoplastic tissue consumes folate at so rapid a rate that folate deficiency megaloblastosis can occur in the host cells (14, 15). There is also evidence that vitamin B-12 deficiency may slow tumor growth, whether that deficiency results from inadequate absorption or elevated levels in serum of a vitamin B-12 binder which does not deliver the vitamin to tumor tissue (16), but will deliver it to the liver in a calcium-dependent fashion (14, 17-20). Interestingly, granulocytes and liver are a major source

of serum binding proteins for both bitamin B-12 and folic acid (21), and malignancies of granulocytes and liver may partly control themselves by releasing into the serum large amounts of binders for vitamin B-12 and folic acid which bind those vitamins and thereby prevent delivery to, and nourishment of, the tumor.

Oxidized folate is not only metabolically dead buyt may even be neurotoxic. For example, a patient with epilepsy who has not had a convulsion in years because dilantin has produced complete control, can be thrown into an immediate convulsion with a megadose of folic acid, because folic acid and dilantin compete for absorption at the brain cell surface, and too much oxidized folic acid will block the ability of the brain cell to take up dilantin, similar to the competition between dilantin and folic acid for uptake by the gutcell (22).

There appear to be one-way transport systems to remove oxidized folates from the nervous system (22, 23) and to remove vitamin B-12 analogues from the body via the bile (24). Some of the B-12 analogues present in multivitamin/mineral preparations may block mammalian cell metabolism (25) and since they block normal cell metabolism, possibly may block malignant cell metabolism. For a number of years, Russian workers have been feeding analogues of vitamin B-12 to normal and malignant cells and showing that these analogues will knock out B-12 metabolism (25a).

Do the B-12 analogues (which have now been found in human serum, liver, bile red cells, and brain) (26) play any role in the promotion or inhibition of carcinogenesis in humans? Levels of analogue in serum are elevated in some malignancies (1). Levels of methylated bases in urine are elevated in some hematologic melignancies (1). In the same hematological malignancies in which methylated bases are elevated in the urine, B-12 analogue is elevated in the blood serum (1). We have recently found enormous quantities of analogues in human stool, and have been studying whether the analogue in human colon bacteria is the source of the analogue in human tissues (27). In preliminary studies, we found two large analogue peaks in human bile and two similar large analogue peaks in human stool. We are now attempting to find out whether these peaks are the same analogue (27). If they are, then the analogue in bile would have come from the analogue in stool, because the quantity of analogue present in food is tiny compared to the quantity present in human stool.

Working with Dr. Ludwik Gross in our first attempts to methylate oncogenes, we gave 5-methylcytidine (5mC) to guinea pigs in whom was transplanted guinea pig leukemia/lymphoma (1). The experiments over a six-month period were unsuccessful in showing any dramatic inhibition, although there was a non-statistically significant inhibition. We subsequently began giving 5-iodocytidine (5IC) to these guinea pigs, having switched from 5MC because of the evidence that the methyl group is taken

off on passage through the liver, resulting in cytidine alone being incorporated into the DNA and RNA of the oncogene. We though that perhaps iodine would not be removed from the cytidine as easily, and iodocytidine would be incorporated intact into RNA, with the iodine perceived by the cell as if it were a methyl group, as is true for iododeoxyuridine being perceived by cells as if it were methyldeoxyuridine (i.e., thymidine) (1). These studies are not yet completed; preliminary results have been equivocal but teasing.

Evidence that methylation can suppress normal and malignant gene expression, and demethylation can bring about expression, continues to build (28-30), although expression is not always related to state of methylation (31, 32).

Literature Cited

1. Herbert, V. In "Nutritional Factors in the Induction and Maintenance of Malignancy"; Butterworth, C.E., and Hutchinson, M.L., Eds.; Academic Press: New York, 1983, pp. 273-287.
2. Eick, D., Fritz, H.-J., Doerfler, W.: Analyt. Biochem. 1983, 135, 165-171.
3. Ley, T.J., DeSimone, J., Noguchi, C.T., Turner, P. H., Schechter, A.N., Heller, P., Nienhuis, A.W., Blood 1983, 62, 370-380.
4. Charache, S., Dover, G., Smith, K., Talbot, C., Conover, C., Jr., Moyer, M., Boyer, S. Proc. Natl. Acad. Sci. 1983, 80, 4842-6.
5. Kolata, G. Science 1984, 223, 470-1.
6. Feinberg, A.P., Vogelstein, B. Nature 1983, 301, 89.
7. Mermod, J.-J., Bourgeois, S., Defer, N., Crepin, M. Proc. Natl. Acad. Sci. 1983, 80, 110-114.
8. Brown, J.D., Wilson, M.J., Poirier, L.A. Carcinogenesis 1983, 4, 173-177.
9. Mikol, Y.B., Hoover, K.L., Creasia, D., Poirier, L.A. Carcinogenesis 1983, 4.
10. Mandel, L.R., Borek, E. Biochem. Biophys. Res. Comm. 1961, 6, 138.
11. Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., Lewisohn, R.
Science 1945, 101, 46.
12. Leuchtenberger, C., Leuchtenberger, R. In: "Nutritional Factors in the Induction and Maintenance of Malignancy"; Butterworth, C.E., and Hutchinson, M.L., Eds.; Academic Press: New York, 1983, pp. 131-148.
13. Farber, S., Cutler, E.C., Hawkins, J.W., Harrison, J.H., Peirce, E.C., Lenz, G.G. Science 1947, 106, 619-621.
14. Herbert, V. "The Megaloblastic Anemias." Grune and Stratton, New York, 1959.

15. Chanaran, I. "The Megalblastic Anemias." Blackwell Scientific, St. Louis, 1979.
16. Corcino, J., Zalusky, R., Greenberg, M., Herbert, V. Brit. J. Haemat. 1971, 20, 511-520.
17. Herbert, V. J. Clin. Invest. 1958, 37, 646-650.
18. Herbert, V., Spaet, T.H. Amer. J. Physiol. 1958, 195, 194-196.
19. Allen, R.H. Prog. Hemat. 1975, 9, 57.
20. Beck, W.S. In: "B-12"; Dolphin, D., Ed. John Wiley and Sons: New York, 1982, pp
21. Herbert, V., Colman, N. In "Lithium Effects on Granulopoiesis and Immune Function"; Rossof, A.H., Robinson, W.A., Eds. Plenum Publishing: New York, 1980, pp. 61-78.
22. Colman, N., Herbert, V. In: "Biochemistry of Brain"; Kumar, S., Ed. Pergamon Press: New York, 1980, pp. 103, 125.
23. Poncz, M., Colman, N., Herbert, V., Schwartz, E., Cohen, A.R. J. Ped. 1981, 98, 76-79.
24. Kanazawa, S., Herbert, V. Trans. Assoc. Amer. Phys. 1984, 96, 336-344.
25. Kondo, H., Binder, M.J., Kolhouse, J.F., Smythe, W.R., Podell, E.R., Allen, R.H. J. Clin. Invest. 1982,k 70, 889-898.
25a. Myashcheva, N.W., Quadros, E.V., Matthews, D.M., Linnell, J.C. Biochim. Biophys. Acta 1979, 588, 81-88.
26. Kanazawa, S., Herbert, V. Clin. Res. 1982, 30, 540A.
27. Herbert, V., Drivas, G., Manusselis, C., Mackler, B., Eng, J., Schwarts, E. Trans. Assoc. Amer. Phys. 1984, 97.
28. Wilson, V.L., Jones, P.A. Cell 1983, 32, 239-246.
29. Christman, J.K., Mendolsohn, N., Herzog, D., Schneiderman, N. Cander Res. 1983, 43, 763-769.
30. Harrison, J.J., Anisowicz, A., Gadi, I.K., Raffeld, M., Sager, R. Proc. Natl. Acad. Sci. 1983, 80, 6606-66.0.
31. Gautsch, J.W., Wilson, M.C. Nature 1983, 301, 32-37.
32. Graessman, M., Graessmann, A., Wagner, H., Werner, E., Simon, D.
Proc. Natl. Acad. Sci. 1983, 80, 6470-6474.

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VITAMIN B_{12} AS A POSSIBLE ADJUNCT IN PREVENTION OF METHOTREXATE HEPATOTOXICITY

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Abstract

It has been established by several investigators that hepatic betaine levels reflect whether or not a lipotrope deficiency state exists in the experimental animal.

Since methotrexate through its antagonism of folate metabolism in the organism may in turn interfere with one carbon metabolism, the possibility exists that methotrexate hepatotoxicity may be due to a drug-imposed lipotrope deficiency.

Several studies are reviewed in which the effects of methotrexate administration on levels of hepatic betaine were measured in rats. It was observed that animals receiving dietary vitamin B_{12} were protected against a methotrexate-induced lowering of hepatic betaine. The livers of those animals on a vitamin B_{12} deficient diet, however, showed a marked reduction in this important methylating agent when administered methotrexate. Since betaine is a reflection of lipotrope deficiency, the use of vitamin B_{12} as a means of protecting against methotrexate hepatotoxicity is considered.

It is well known that methotrexate (MTX), a folate antagonist used in cancer chemotherapy and psoriasis, inhibits the enzyme dihydrofolate reductase. This inhibition reduces the amount of tetrahydrofolate coenzymes (1) available for purine synthesis, and in turn, reduces nucleic acid formation in the body hence restricting rapidly growing tissue. A common practice in treating neoplastic disease has been to administer citrovorum-factor along with the MTX in order to "rescue" the normal cells from the deleterious effects of inhibited folate metabolism. Since tumor cells are more vulnerable than normal cells to the lack of nucleic acid synthesis, the citrovorum factor furnishes needed folate coenzymes and helps rescue normal cells in the wake of the methotrexate effects.

If the tetrahydrofolate coenzyme pool of the body is depleted by the action of MTX, it is entirely feasible that this may impair certain vital methyl transfer reactions in the liver. For example, this may inhibit the ^{N5}-methyltetrahydrofolate-homocysteine methyltransferase involved in the

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formation of lipotropic substances such as methionine, S-adenosylmethionine (SAM) and choline. Since these substances are the major lipotropic compounds in the body, a serious deficiency could lead to fatty metamorphosis in the liver and the liver damage seen with the use of MTX.

Several workers have attempted to approach this question by conducting "protective" experiments with choline and other lipotropes when administering MTX to humans and animals. Weinstein and Frei (2) have suggested that concomitant administration of choline with methotrexate may prevent MTX-related hepatotoxicity in humans. Freeman-Narrod et al. (3) administered MTX alone and in combination with varying amounts of choline to rats. The rats receiving the combined treatment showed significantly lower levels of triglyceride in their livers than did those given MTX alone and the protective effect of the choline on the liver appeared to be dose related.

A past study in this laboratory (4), using the rat as the experimental model, has lent support to the findings of the above authors. This work tested the effect of MTX on hepatic fatty infiltration (a measure of liver injury) in rats fed a lipotrope-deficient diet and the effect of MTX on similarly fed animals when the diet was individually supplemented with the lipotropes methionine and choline. It was found that triglyceride and cholesterol esters were elevated in the rats fed the basal diet and that this accumulation was potentiated in animals receiving MTX. In animals whose diets were supplemented with choline or methionine, however, the drug did not affect the hepatic levels of triglyceride and cholesterol esters. These results indicated that MTX may interfere with the formation of choline and methionine but does not interfere with the lipotropic action of preformed choline and methionine. Since choline and methionine are products of one-carbon metabolism beyond the involvment of MTX with folate, these results support the case for supplementation with lipotropic agents during MTX administration.

Both choline and methionine are lipotropic substances by virtue of their contribution to phosphatidylcholine synthesis which is involved in proper cellular membrane function and structure (5). Methionine makes its contribution in this respect by its conversion to SAM which plays a vital role in sequential phospholipid methylation to produce essential phosphatidylcholine. The contribution of choline takes place in two different ways. One, through conversion to phosphatidylcholine in the Kennedy pathway (6) and secondly, via the oxidative pathway for choline which forms betaine. Betaine is then utilized by betaine-homocysteine methyltransferase to methylate homocysteine in an alternate pathway to form methionine and hence SAM.

According to Finkelstein and Martin (7), the reaction mediated by N^5-methyltetrahydrofolate-homocysteine methyltransferase and the reaction utilizing betaine through the medium of betaine-homocysteine methyltransferase are of equal importance in methionine synthesis and in promoting methylation in the liver. Once thought to fulfill a very minor function in the organism, betaine is now felt to play a very active and important role as a methylating substance and therapeutic agent (8,9). Some workers feel that the hepatic betaine level is a good index of a choline deficiency in the liver. Wong and Thompson (10), Barak and Tuma (11), and Martin and Finkelstein (12) have all shown a marked reduction in hepatic betaine levels in animals fed diets deficient in choline.

Since it was felt that hepatic betaine levels reflected the choline deficiency state of the animal, several studies (13-15) were conducted in this laboratory to determine the effect of MTX administration on hepatic betaine

levels in the rat. In this work, rats were divided into pairs and pair-fed with each of three different kinds of widely used control diets. These were the control diet of Iseri et al. (16), the control diet of French (17) and Purina rat chow, respectively. The experimental animals in each pair were injected daily and intraperitoneally with 0.1mg MTX/kgm body weight. Control animals were injected daily with saline. Rats fed the Iseri control diet showed no changes in hepatic betaine when treated with MTX for 10 days. Rats fed the Purina rat chow showed no changes in liver betaine in 30 days on the drug. However, rats fed the French control diet demonstrated a highly significant lowering of betaine in their livers with MTX treatment for 10 days.

The results summarized above posed the strong question of why MTX should produce a choline (betaine) deficiency state (10-12) when rats were fed the French control diet but not when fed the Iseri control diet or the Purina rat chow in the times described. On close study, it was determined that the major difference between the three diets used, other than the fat, was the vitamin B_{12} content. The French control diet, either as originally described or as supplied commercially, is devoid of vitamin B_{12}.

To determine whether the absence of dietary vitamin B_{12} was responsible for the lowering of hepatic betaine in the livers of rats receiving MTX, a further study (18) was conducted in this laboratory. Rats were divided into pairs. One group of paired rats was fed on the French choline deficient diet to which had been added choline at the level of 0.4 g per 100g of diet and vitamin B_{12} at the level of 2.2 µg per 100g of diet. A second group of paired rats was fed the same diet from which the B_{12} had been eliminated. Control animals of each pair in both groups were pair-fed to the consumption of the experimental animals in each group which were treated with MTX for 10 days. On sacrifice, it was shown that hepatic betaine was not reduced by the MTX in animals receiving the dietary B_{12}, however, the hepatic betaine was markedly lowered in those animals receiving MTX with no B_{12}.

At present the reason for the sparing effect of vitamin B_{12} for hepatic betaine is not clear. One would expect that hepatic betaine would be depleted by MTX even when dietary B_{12} is furnished. This should occur through the inhibition of the folate contribution to methionine biosynthesis with the pool size of betaine becoming lowered through the action of betaine-homocysteine methyltransferase as a compensatory mechanism to maintain essential methionine. This did not happen, however, and only with the lack of B_{12}, a coenzyme for N-methyltetrahydrofolate-homocysteine methyltransferase, was the betaine depleted.

It is feasible that the dietary presence of B_{12} may maintain or stimulate the action of N^5-methyltetrahydrofolate-homocysteine methyltransferase in the face of the MTX to preserve hepatic betaine or act in some unknown manner to increase betaine production through choline oxidase. Whatever the function of the B_{12}, the fact that this vitamin prevented a condition of hypomethylation from occurring in the experimental animal may prove to have practical application in the prevention of liver injury during MTX chemotherapy in humans.

REFERENCES

1. A.V. HOFFBRAND and F. TRIPP. Br. Med. J. 2:140-142, 1972.
2. G. WEINSTEIN and E. FREI. Abstracts of Second International Symposium on Psoriasis, Stanford University, Stanford, California, 1976.
3. M. FREEMAN-NARROD, S.A. NARROD and R.P. CUSTER. J. Nat'l Cancer Inst.

59:1013-1017, 1977.
4. D.J. TUMA, A.J. BARAK and M.F. SORRELL. Biochem. Pharmacol. 24:1327-1331, 1975.
5. S. HIRATA and J. AXELROD. Science 209:1082-1090, 1980.
6. E.P. KENNEDY and S.B. WEISS. J. Biol. Chem. 222:193-214, 1956.
7. J.D. FINKELSTEIN and J.J. MARTIN. J. Biol. Chem. 259:9508-9513, 1984.
8. A.J. BARAK and D.J. TUMA. Life Sci. 32:771-774, 1983.
9. Nutr. Revs. 42:180-182, 1984.
10. E.R. WONG and W. THOMPSON. Biochem. Biophys. Acta 260:259-271, 1972.
11. A.J. BARAK and D.J. TUMA. Lipids 14:860-863, 1979.
12. J.J. MARTIN and J.D. FINKELSTEIN. Anal. Biochem. 111:72-76, 1981.
13. A.J. BARAK and R.J. KEMMY. Drug-Nutr. Interact. 1:275-278, 1982.
14. A.J. BARAK, R.J. KEMMY and D.J. TUMA. Drug-Nutr. Interact. 1:303-306, 1982.
15. A.J. BARAK. Unpublished Data, 1984.
16. O.A. ISERI, L.S. GOTTLIEB, M. HEGSTED, and J.J. VITALI. Lab. Invest. 27:226-235, 1972.
17. S.W. FRENCH. J. Nutr. 88:291-302, 1966.
18. A.J. BARAK and H.C. BECKENHAUER. Biochem. Arch. 1:61-65, 1985.

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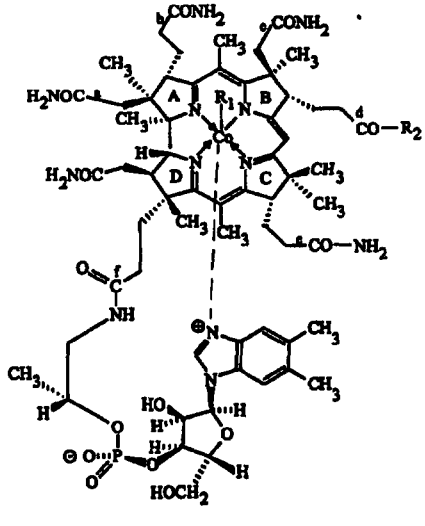
(54) Title: RECEPTOR MODULATING AGENTS AND METHODS RELATING THERETO

(57) Abstract

Receptor modulating agents capable of modulating cell surface receptors by affecting the cell surface receptor trafficking pathway. The receptor modulating agents are comprised of a covalently bound rerouting moiety and targeting moiety.



R_1 = CN ; R_2 = NH_2 (Cyanocobalamin)
R_1 = CN ; R_2 = OH (Cyanocobalamin -(3)-free acid)
R_1 = CN ; R_2 = HN-CH_2-CH_2-CH_2-CO_2H (GABA adduct)
R_1 = CN ; R_2 = GABA - Peptide (where GABA = linker)
R_1 = CN ; R_2 = Peptide
R_1 = CN ; R_2 = HN-(linker)-tyramine-^{125}I
R_1 = CN ; R_2 = HN-(linker)-lysosomotropic agent
R_1 = CN ; R_2 = HN-(linker)-X-linking agent
R_1 = CN ; R_2 = HN-(linker)-biotin
R_1 = CN ; R_2 = NH-$(CH_2)_{12}NH_2$

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Description

RECEPTOR MODULATING AGENTS AND METHODS RELATING THERETO

Technical Field

The present invention is generally directed to receptor modulating agents which modulate cell surface receptors and, more specifically, to receptor modulating agents which bind to cell surface receptors and affect the receptor trafficking pathway and methods related thereto.

Background of the Invention

Cell surface receptors constitute a class of proteins which are responsible for receptor-mediated endocytosis of specific ligands. Basically, the receptors serve as escorts for ligand delivery to intracellular destinations.

Ligand delivery is generally achieved through coated regions on the plasma membrane called "coated pits." These pits continually invaginate and pinch off, forming "coated vesicles" in the cytoplasm. Coated pits and vesicles provide a pathway for receptor mediated endocytosis of specific ligands. The ligands that bind to specific cell surface receptors are internalized via coated pits, enabling cells to ingest large numbers of specific ligands without taking in correspondingly large volume of extracellular fluid. The internalized coated vesicles may or may not lose their coats and bind with other vesicles to form larger vesicles called "endosomes." In the endosome the ligand and the receptor are separated or "sorted." Endosomes which sort ligands and receptors are known as "compartment of uncoupling of receptor and ligand" or "CURL."

Endosomes may fuse with primary lysosomes, where their contents are digested, or they may be delivered to other intracellular destinations. The receptor proteins are generally not digested, but are rather recycled to the cell membrane surface through a process called "exocytosis," or transferred to early or late endosomes via multivesicular bodies. The entire pathway is referred to as the "receptor trafficking pathway."

Some receptors deliver their ligand directly to the cytoplasm or other specific intracellular locations. Perhaps one of the most studied receptor trafficking pathways is that of iron transport. In this pathway, a serum carrier protein, transferrin, binds iron and transports it to transferrin receptors on the plasma membrane surface.

After binding and internalization, via coated pits, the resulting vesicle combines first with early endosomes and then with late endosomes. This process results in the gradual drop in pH in the vesicle. The drop in pH causes the transferrin carrier protein to lose its affinity to iron. When this occurs, the iron translocates through the membrane of the vesicle and joins the intracellular pool of enzymes. The transferrin receptor may then recycle to the cell surface where it may repeat the process.

Other receptors may deliver their ligand directly to the lysosomes for digestion. For example, the epidermal growth factor ("EGF") receptor delivers its ligand directly to a lysosome for degradation (Prog. Histochem. Cytochem. 26:39-48,1992). The EGF receptor may recycle to the cell surface depending on its state of phosphorylation (Cancer Treat. Rep. 61:139-160, 1992; J. Cell. Biol. 116:321-330, 1992).

A single receptor may utilize more than one receptor trafficking pathway within the same cell. For example in polarized cells, such as specialized transport epithelia cells, membrane trafficking is distinct between apical and basal sides of the cell (Sem. Cell. Biol. 2:387-396, 1991). Moreover, non-polarized epithelia cells may simultaneously follow two separate sorting pathways.

The control or regulation of cell surface receptors may be achieved by a variety of techniques. Regulation of cell surface receptors may be accomplished, at a very basic level, by the binding of naturally occurring ligands. As discussed above, receptor binding of a ligand will generally trigger the internalization of the ligand-receptor complex. Such internalization may desensitize the cell to further ligand binding. (J. Immunol. 150:3161-9, 1993; Mol. Endocrinol. 6:2090-102, 1992; J. Cell. Physiol. 154:281-8, 1993; Receptor 1:13-32, 1990-91; Biochem. J. 288:55-61, 1992; J. Immunol. 148:2709-11, 1992; J. Cell. Physiol. 148:24-34, 1991). This type of regulation, however, is transient in nature and does not result in diminution of biologic response.

Regulation of cell surface receptors may also be accomplished by administration of receptor antagonists or agonists. Receptor antagonists are organic protein or peptide ligands generally derived through empirical structure-function studies, or through the use of detailed knowledge of ligand and receptor interaction. Essentially, an antagonist may constitute any molecule with similar binding activity to a natural ligand, but incapable of producing the biological response normally induced by the natural ligand. Thus, the antagonist competitively blocks receptor activity. With a competitive antagonist, the regulation of receptor activity is dependent upon both the antagonist's affinity for the receptor, as well as its extracellular concentration over time.

Receptor agonists are protein or peptide ligands derived in a similar manner as antagonists. Essentially, an agonist may constitute any molecule which binds to the receptor in a manner superior to that of the natural ligand.

One receptor of particular interest is the vitamin B_{12} receptor. As has been demonstrated in experimental in vitro data, pre-clinical animal models, and patient studies, vitamin B_{12} is a co-enzyme necessary in cell division, as well as cellular metabolism, in proliferating normal and neoplastic cells. Insufficient vitamin B_{12} causes cellular division to be held in abeyance and ultimately may result in apoptosis. The nutrient is generally derived from dietary intake and is transported throughout the body complexed to transport proteins. The complex of transport protein and vitamin B_{12} is recognized by a cellular receptor which internalizes the complex and releases the vitamin intracellularly. The overall process has been reviewed in GUT 31:59, 1991. Vitamin B_{12} is taken in through the diet. Binding proteins in the saliva (R-binder) and gut (intrinsic factor-(IF)) complex vitamin B_{12} after release from endogenous binding proteins by action of enzymes and low pH in the stomach. Vitamin B_{12} is transferred across the intestinal epithelium in a receptor specific fashion to transcobalamin II (TcII). The vitamin B_{12}/transcobalamin II complex is then transported throughout the body and recognized by receptors present on dividing cells, internalized and released within the cell where it is utilized by certain enzymes as a co-factor.

The high affinity receptor in dividing tissues or cells responsible for internalization of vitamin B_{12} recognizes transcobalamin II complexed with vitamin B_{12}. The vitamin B_{12}/TcII receptor recognizes only the vitamin B_{12}/TcII complex and not the serum transport protein or the vitamin alone. The receptor is undetectable on non-dividing cells; the mechanism for supplying non-dividing cells with vitamin B_{12} is poorly understood. However, it is known that more vitamin B_{12} is required during cell division than during metabolism, and that the vitamin B_{12}/TcII receptor is the only high affinity means for cellular uptake of vitamin B_{12} during cell division. When stimulated to divide, cells demonstrate transient expression of this receptor leading to vitamin B_{12} uptake which precedes actual DNA synthesis (J. Lab. Clin. Med. 103:70, 1984). Vitamin B_{12} receptor levels may be measured by binding of ^{57}Co-vitamin B_{12} complexed to transcobalamin II (present in serum) on replicate cultures grown in chemically defined medium without serum. No receptor mediated uptake occurs in the absence of carrier protein.

Dividing cells, induced to differentiate, lose receptor expression and no longer take up vitamin B_{12}. More importantly, leukemic cells, deprived of vitamin B_{12}, will stop dividing and die (Acta Haemat. 81:61, 1989). In a typical experiment,

leukemic cell cultures were deprived of serum for 3 days, and then supplemented either with serum (a source of vitamin B_{12}) or a non-metabolizable analogue of vitamin B_{12} and cultured up to five days. Cell cultures supplemented with vitamin B_{12} continued to grow, whereas those deprived of the active nutrient stopped growing and die.

Based on these observations, it has been suggested that whole body deprivation of vitamin B_{12} may be useful in the treatment of cancer or other disorders characterized by uncontrolled growth of cells. Moreover, because of the critical role played by vitamin B_{12}-containing enzymes in cell division, it is believed that vitamin B_{12} deprivation may be used in combination with chemotherapeutic drugs which inhibit cellular replication. For example, when vitamin B_{12} depletion was combined with methotrexate, the two modalities together were more efficient in depleting folate levels in leukemic cells than either alone (FASEB J. 4:1450, 1990; Arch. Biochem. Biophys. 270:729, 1989; Leukemia Research 15:165, 1991). Folates are precursors in the production of DNA and proteins. In typical experiments, cultures of leukemic cells were exposed to nitrous oxide for several hours to convert the active form of endogenous vitamin B_{12} to an inactive form. Replicate cultures were then left without further treatment, or additionally treated with methotrexate. Cellular folate levels were measured three days later. Cells treated with the combination (*i.e.*, both methotrexate and inactive vitamin B_{12}) showed a more striking decrease in cellular folate levels than with either of the two approaches alone. This combination also results in a higher cell kill in vitro. When this approach was applied to the treatment of highly aggressive leukemia/lymphoma in animal models (Am. J. Haematol. 34:128,1990; Anticancer Res. 6:737, 1986; Cancer Chemother. Pharmacol. 17:114, 1986; Br. J. Cancer 50:793, 1984), additive or synergy of anti-tumor action was observed, resulting in prolonged remissions and cures.

A key finding in the experiments described above was that short-term (hours to days), whole body depletion of vitamin B_{12} can act synergistically with chemotherapeutic drugs (such as methotrexate and 5-FU) to inhibit tumor growth and treat animals with leukemia/lymphoma. Despite synergistic anti-tumor activity, there was no toxicity attributable to the short-term vitamin B_{12} depletion for proliferating normal cells. This combination therapy was demonstrated in multiple animal models. Observations in patients have indicated that long-term (months to years) vitamin B_{12} depletion is required to produce significant normal tissue toxicity. Even in those cases, subsequent infusion of vitamin B_{12} can readily reverse symptomology (Br. J. Cancer 5:810, 1989).

Because of the promise of this therapeutic approach, various methods have been sought to efficiently and controllably perform a temporary depletion of vitamin B_{12}. Such methods, however, affect all of the body's stores of vitamin B_{12}. They include dietary restriction, high doses of vitamin B_{12} analogues (non-metabolizable-competitive antagonists which act as enzyme inhibitors), and nitrous oxide (transformation of vitamin B_{12} to inactivate form). These different methods have been used in culture systems and in animals to deplete vitamin B_{12}. The most efficient and the most utilized method has been the inhalation of nitrous oxide (laughing gas). Animals are maintained typically under an atmosphere of 50% to 70% of nitrous oxide for periods from a few hours to a few days, causing the conversion of endogenous vitamin B_{12} into an inactive form. This methodology has been utilized in combination with drugs for therapy of leukemia/lymphoma. A further method for vitamin B_{12} depletion involves infusion of a non-metabolizable analogue of vitamin B_{12} which essentially dilutes out the active form. This form of therapy is not specific for dividing cells but affects liver dependent metabolic processes. Another approach includes restricting the dietary intake of vitamin B_{12}. This method, however, requires very long periods of dietary restriction and is offset by hepatic storage of vitamin B_{12}. All of these methods suffer from problems of specificity, since they affect both vitamin B_{12}-dependent growth as well as basal metabolism, and therefore are not particularly suited to the development of anti-proliferative pharmaceutical products.

In view of the biological importance of cell surface receptors, receptor-controlling agents have emerged as a class of pharmaceutical drugs. Moreover, with the advent of genetic engineering for the isolation and amplification of genes for cell surface receptors, as well as computer programs to model the interactions between ligands and receptors (*i.e.*, "rational" drug design), the production of receptor-controlling drugs has been significantly enhanced.

To date, many months or even years of scientific research, as well as significant financial resources, are required to produce new receptor antagonists or agonists. To speed up this process, new screening technologies have been developed which utilize peptide or antibody recombinant libraries (*see, e.g.*, Gene 73:305, 1988; Proc. Nat. Acad. Sci. (USA) 87:6378, 1990; Biochromatography 5:22, 1990; Protein Engineering 3:641, 1989). While library screening does not require the same degree of knowledge of a specific receptor/ligand system, it does involve an intensive screening effort utilizing functional receptor-specific assays. Moreover, the initial compounds identified by such screening programs are generally only precursors to the development of therapeutic products through more typical structure-functional assessments.

While antagonists and agonists are generally capable of regulating a biological response, the surface receptors which bind such ligands are continually being re-expressed on the cell surface. Thus, effective regulation by antagonists or agonists must rely on a relatively high and sustained serum concentration in order to bind the new surface receptors continually being expressed on the cell surface.

Accordingly, there is a need in the art for agents which bind cell surface receptors and thus regulate biological responses associated therewith, and which further effect normal cellular trafficking of the bound receptor. There is also a need in the art for agents which, when bound by a cell surface receptor and internalized, promote retention of the receptor within the cell. Moreover, there exists a need for methods relating to the administration of such agents to regulate a biological response. The present invention fulfills these needs and provides further related advantages.

Summary of the Invention

Briefly stated, the present invention provides receptor modulating agents which are capable of affecting a receptor trafficking pathway of the cell. Receptor modulating agents of the present invention are comprised of a rerouting moiety coupled to a targeting moiety.

Suitable targeting moieties include, by way of example, a vitamin B_{12} molecule or any one of several proteins and peptides.

Suitable rerouting moieties include, by way of example, lysosomotropic moieties, such as gentamycin, kanamycin, neomycin, and streptomycin; intracellular polymerizing moieties, such as dipeptide esters and leucine zippers; peptide sorting sequences, such as endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides; conditional membrane binding peptides, such as charged glutamate, aspartate, and histidine; and bi- or multi-valent receptor cross-linking moieties.

In a preferred embodiment of the present invention, a receptor modulating agent, is comprised of a vitamin B_{12} molecule coupled to a rerouting moiety by a linker. Generally, the linker is at least 4 atoms in length, typically, the linker is about 6 to 20 atoms in length and preferably, the linker is 12 atoms in length. Suitable linkers include linkers which include an amino group, such as diaminoalkyl, diaminoalkylaryl, diaminoheteroalkyl, diaminoheteroalkylaryl and diaminoalkanes. Preferably, the linker is $-NH(CH_2)_xNH-$ wherein x = 2-20 or $-NH(CH_2)_yCO-$, wherein y = 3-12. In one embodiment the linker is a trifunctional linker.

In a preferred embodiment of this aspect of the present invention, a B_{12} molecule is coupled to a rerouting moiety at a *b*-, *d*- or *e*- coupling site. In a particularly preferred embodiment of the present invention, a B_{12} molecule is coupled to a rerouting moiety at a *d*- or *e*- coupling site. In another embodiment, the B_{12} molecule is coupled to a rerouting moiety at a ribose coupling site. In yet another embodiment, the receptor modulating agent is bound to transcobalamin.

Receptor modulating agents of the present invention may act by affecting a receptor trafficking pathway in any one of several ways, including, by redirecting an agent/receptor complex; by cross-linking one or more cell surface receptors; by anchoring a cell surface receptor in the membrane; and by retaining a receptor in an endosome.

Another aspect of the present invention includes a vitamin B_{12} dimer comprising a first and a second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling sites *h*, and coupling sites *i*. In a preferred embodiment, the B_{12} molecule coupled through an *e*- or *d*- coupling site.

In another embodiment, B_{12} molecules are coupled by a linker. Generally, the linker is at least 4 atoms in length, typically, the linker is about 10 to 55 atoms in length and preferably, the linker is 35 to 45 atoms in length. In a preferred embodiment, the linker is a trifunctional linker. Suitable linkers include linkers which include an amino group, such as diaminoalkyl, diaminoalkylaryl, diaminoheteroalkyl, diaminoheteroalkylaryl and diaminoalkanes. Preferably, the linker is $-NH(CH_2)_xNH-$ wherein x = 2-20 or $-NH(CH_2)_yCO-$, wherein y = 3-12.

In another aspect of this embodiment, a vitamin B_{12} dimer is coupled to at least one transcobalamin II molecule. In yet another aspect of this embodiment, at least one of said first and said second vitamin B_{12} molecules of the dimer is a vitamin B_{12} derivative.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references set forth below which describe certain procedures or compositions in more detail are incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a schematic illustrating a mechanism of action of a receptor modulating agent of the present invention. A healthy receptor will internalize when bound by the appropriate ligand, release the ligand within the cell and then recycle to

the cell surface. Receptor modulating agents of the present invention impede the receptor trafficking pathway by inhibiting the recycling of receptors to the cell surface. Essentially, the targeting moiety on receptor modulating agents bind the receptor and the rerouting moiety redirects the receptor/receptor modulating agent complex to other points within the cell, where it may be retained or degraded. (Not shown in this schematic are receptors synthesized de novo).

Figures 2-5 are formulae representing families of antibiotics which act as rerouting moieties. The preferred reactive groups for coupling with a targeting moiety are indicated. These rerouting moieties facilitate retention of the receptor/receptor modulating agent complex through protonation of the complex, eventually delivering it to lysosomes for degradation.

Figure 2 illustrates formulae representing the gentamycin, sisomicin, and netilmicin families of antibiotics.

Figure 3 illustrates formulae representing the kanomycin, tobramycin, and amikacin families of antibiotics.

Figure 4 illustrates formulae representing the neomycin, paromomycin, ribostamycin, and butirosin families of antibiotics.

Figure 5 illustrates formulae representing the streptomycin family of antibiotics.

Figure 6 illustrates formulae representing substituted aminoquinolines (*e.g.*, chloroquine) substituted aminoacridines (*e.g.*, quinacrine), and substituted aminonapthalines (*e.g.*, dansyl cadaverine), all of which are representative rerouting moieties of the present invention. These rerouting moieties impede the receptor trafficking pathway through protonation and intracellular retention.

Figure 7 illustrates formulae representing glycosylation inhibitors, all of which are representative rerouting moieties of the present invention. These sugars may be conjugated to targeting moieties using linkages typical of oligomeric carbohydrate chains. The resulting receptor modulating agent is recognized by internal glycosyl transferases, subject to intracellular retention, and, ultimately, degradation in the lysosomes.

Figure 8 illustrates a formula representing a vitamin B_{12} (cyanocobalamin) molecule and identifies a preferred coupling site suitable for use in the present invention for derivatization and conjugation.

Figure 9 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12}-GABA adduct.

Figure 10a is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} derivative comprising a vitamin B_{12} molecule with a diaminododecane linker arm coupled to any one of coupling sites *d*-, *e*-, or *b*-.

Figure 10b is a schematic depicting a representative reaction scheme for coupling a succinic anhydride to a vitamin B_{12} diaminododecane adduct in preparation for coupling the adduct to a rerouting moiety, or other molecule, with an amino reaction site.

Figure 11 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} derivative comprising a vitamin B_{12} molecule and a diaminododecane linker arm coupled to a ribose coupling site.

Figure 12 is a schematic depicting a representative reaction scheme for coupling vitamin B_{12} or a vitamin B_{12}-GABA adduct to amikacin.

Figure 13 is a schematic depicting a representative reaction scheme for coupling vitamin B_{12} or a vitamin B_{12}-GABA adduct to streptomycin.

Figure 14 is a schematic depicting a representative reaction scheme for coupling a vitamin B_{12} carboxylate derivative or a vitamin B_{12}-GABA adduct to acridine.

Figure 15 is a schematic depicting a representative reaction scheme for the synthesis of a bivalent receptor modulating agent, a vitamin B_{12} dimer, using a trifunctional linker. The trifunctional linker allows for coupling with additional compounds (*e.g.*, R-NH_2) such as, by way of example, aminoglucosides (Figures 2-5), aminoacridines (Figure 6), glycosylation inhibitors (Figure 7), and biotin.

Figure 16 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} dimer using a homobifunctional or homotrifunctional cross-linking reagent.

Figure 17 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} dimer using a heterobifunctional cross-linker.

Figures 18-21 are schematics depicting representative reaction schemes for the synthesis of various receptor modulating agents generally comprised of a rerouting moiety, designated by the reactive group and R, selected from those represented in Figures 2-7, and a vitamin B_{12} molecule or derivative thereof as a targeting moiety.

Figure 22 is a graph illustrating the binding curve of Transcobalamin II to the cyanocobalamin monocarboxylic acids produced in Example 1. AD = Cyanocobalamin **(1)**; AL = Cyanocobalamin *b*-monocarboxylic acid **(2)**; AM =

Cyanocobalamin *e*-monocarboxylic acid (3); and AN= Cyanocobalamin *d*-monocarboxylic acid (4).

Figure 23 is a graph illustrating the binding curve of Transcobalamin II to the cyanocobalamin diaminododecane adducts produced in Example 3 and 4. AH = Cyanocobalamin *b*-monocarboxylic acid conjugate diaminododecane (7); AI = Cyanocobalamin *e*-monocarboxylic acid conjugate diaminododecane (8); AJ = Cyanocobalamin *d*-monocarboxylic acid conjugate diaminododecane (9); AK = Cobalamin *e*-monocarboxylic acid conjugate diaminododecane, and AE = Cyanocobalamin ribose-succinate (11).

Figure 24 is a graph illustrating the binding curve of Transcobalamin II to a series of vitamin B_{12} dimers. Dimer X = *b*-acid dimer with isophthaloyl dichloride (36); Dimer Y = *e*-acid dimer with isophthaloyl dichloride (37); dimer Z = *d*-acid dimer with isophthaloyl dichloride (38); Dimer A= *b*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride (58); Dimer B = *e*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride (59); and Dimer C = *d*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride (60). These dimers were prepared as set forth in the Examples below. (*see* Examples 13 and 16.)

Figure 25 is a graph illustrating the binding curve of Transcobalamin II to a series of biotinylated vitamin B_{12} molecules. AA = Cyanocobalamin *b*-monocarboxylic acid conjugate diaminododecane and biotin (17); AB = Cyanocobalamin *e*-monocarboxylic acid conjugate diaminododecane and biotin (18); AC = Cyanocobalamin *d*-monocarboxylic acid conjugate diaminododecane and biotin (19); AF = Cyanocobalamin ribose-succinate conjugate diaminododecane (13); and AG = Cyanocobalamin ribose-succinate conjugate diaminododecane and biotin (20). These biotinylated molecules were prepared as set forth in Examples below. (*see* Example 8.)

Detailed Description of the Invention

The present invention is generally directed to a receptor modulating agent which is capable of binding to a cell surface receptor to form a receptor modulating agent/receptor complex ("agent/receptor complex"). The binding of a suitable receptor modulating agent to a cell surface receptor generally results in invagination of the agent/receptor complex into the cell into the vesicular system in the same manner as the natural ligand. However, once internalized, or as part of the internalization process, a receptor modulating agent of the present invention affects the receptor trafficking pathway by effectively impeding, preventing, or delaying the

receptor from recycling to the surface, thus depriving the cell of receptors able to engage in binding its natural ligand and triggering related biological responses.

Within the context of the present invention, "affecting the receptor trafficking pathway" refers to impeding the receptor trafficking pathway in such a manner so as to affect biological response. This would include trapping, delaying, retaining, re-directing, or degrading the cell surface receptor. A "receptor modulating agent" is comprised of at least one targeting moiety covalently attached to at least one rerouting moiety. A "targeting moiety," as described in detail below, is a moiety capable of specifically binding to a cell surface receptor to yield an agent/receptor complex and, in a preferred embodiment, has an affinity for the cell surface receptor of within 100-fold, and more preferably, within 10-fold, of the affinity of the natural ligand for the receptor. A preferred targeting moiety is a vitamin B_{12} molecule. In contrast, a "rerouting moiety" is a moiety which redirects an agent/receptor complex, resulting in prolonged retention, degradation, and/or modulation of the receptor within the interior of a cell or on the cell surface, including, by way of example, retaining the receptor in the cell membrane or directing the receptor to a lysosome within the cell. Suitable rerouting moieties are described in detail below.

A targeting moiety is coupled to a rerouting moiety to yield the receptor modulating agent by any suitable means known in the art, including direct covalent linkage of an appropriate chemical linker or through a very tight association in non-covalent attachment. By way of example for the latter, in one embodiment, coupling is accomplished through the combination of an avidin or streptavidin conjugate with a vitamin B_{12}/biotin conjugate. Coupling of the targeting moiety and the rerouting moiety should be of a nature which resists cleavage by the enzymatic and low pH conditions normally encountered within the internal portion of the cell, including endosomes and lysosomes. Suitable linkers are noted below. The ability to resist cleavage may be detected by any means known in the art, including exposing the receptor modulating agent to enzymes at low pH and measuring release of the targeting or rerouting moiety using techniques known in the art.

Coupling of a targeting moiety and a rerouting moiety should not significantly hinder the ability of the targeting moiety to specifically bind the cell surface receptor. The receptor modulating agent may also include additional moieties, so long as they do not interfere with either the targeting or the rerouting moieties. For example, such moieties may be coupled to the receptor modulating agent through the use of a trifunctional linker or they may be coupled to a rerouting or targeting moiety. Optimal attachment of the two moieties may be determined by comparing the affinity of

binding of the receptor modulating agent with free targeting moiety in assays of inhibition of binding.

These, and other suitable techniques, are described in detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989.

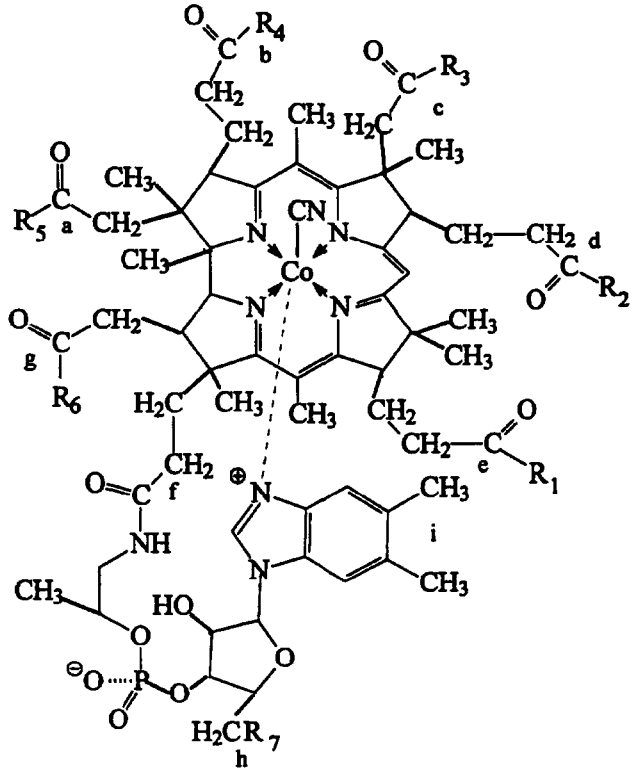
Coupling of a targeting moiety and a rerouting moiety should also not significantly affect the ability of the rerouting moiety to retain or delay the agent/receptor complex within the cell. This may be empirically determined by any one of several methods known in the art, including using labeling techniques to compare intracellular retention of the targeting moiety versus that of the receptor modulating agent as exemplified below.

As noted above, targeting moieties of a receptor modulating agent include any moiety which specifically binds to a cell surface receptor. Suitable targeting moieties include proteins and peptides. Representative examples of suitable targeting moieties include peptides such as bombesin, gastrin-releasing peptide, cell adhesion peptides, substance P, neuromedin-B, neuromedin-C and metenkephalin; hormones, including EGF, alpha- and beta-TGF, estradiol, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, and human growth hormone; proteins corresponding to ligands for known cell surface receptors, including low density lipoproteins, transferrin and insulin; fibrinolytic enzymes; and biological response modifiers, including interleukin, interferon, erythropoietin and colony stimulating factor also constitute targeting moieties of this invention. Moreover, analogs of the above targeting moieties that retain the ability to specifically bind to a cell surface receptor are suitable targeting moieties. Essentially, any analog having about the same affinity as a targeting moiety, herein specified, could be used in synthesis of receptor modulating agents.

In a preferred embodiment, a targeting moiety is a vitamin B_{12} molecule. Vitamin B_{12} is an essential nutrient for dividing cells. By inhibiting its uptake, the growth of dividing cells can be halted. The cell surface receptor for vitamin B_{12} is the transcobalamin II/vitamin B_{12} ("TcII/B_{12}") receptor, which is characterized by a high affinity for the carrier protein, transcobalamin II (TcII), when complexed with vitamin B_{12} ("TcII/B_{12} complex"). The TcII/B_{12} receptor does not recognize vitamin B_{12} alone, but does recognize the carrier protein TcII with reduced affinity when not complexed with vitamin B_{12}. In many respects, this receptor system is similar to that for transferrin/iron in that the goal of the receptor system is to deliver vitamin B_{12} into cells such that it can be utilized by enzymes involved in DNA synthesis. Within the context of the present invention, the term "vitamin B_{12}" refers to the class of

compounds known as cobalamins and derivatives thereof, including, by way of example, cyanocobalamin. The term "vitamin B_{12}" is used interchangeably with the term cyanocobalamin.

Suitable vitamin B_{12} molecules includes any vitamin B_{12} capable of coupling to another molecule while maintaining its ability to form a TcII/B_{12} complex. A preferred vitamin B_{12} targeting moiety is generally comprised of a vitamin B_{12} molecule, such as a cyanocobalamin, and a linker, described in detail below. The linker may be coupled to any one of several sites on a vitamin B_{12} molecule, including potential carboxyl coupling sites *a*- through *g*-, an alcohol (ribose) coupling site ("coupling site *h*") or a benzimidazole coupling site ("coupling site *i*.") (*See* structure I below.) Preferably, a linker is coupled to coupling sites *b*-, *d*- or *e*- on a vitamin B_{12} molecule. Even more preferably, a linker is coupled to coupling site *d*- or *e*-. This embodiment of the present invention includes compounds represented by the following formula:



STRUCTURE I

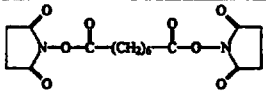
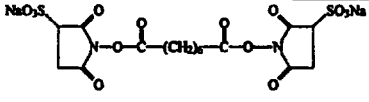
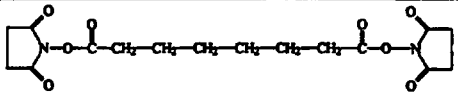
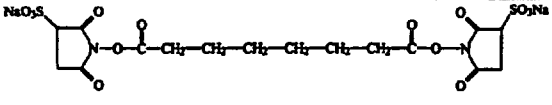
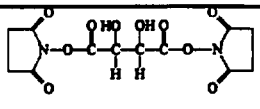
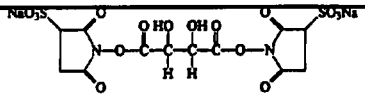
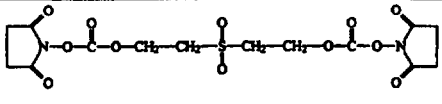
wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker. One of ordinary skill in the art will appreciate that a number of other coupling sites on the vitamin B_{12}

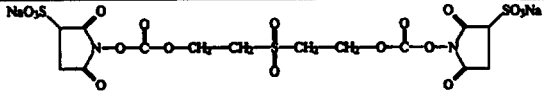
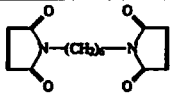
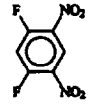
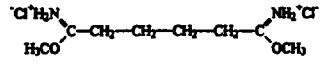
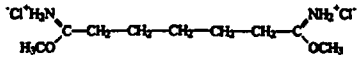
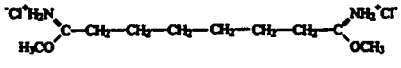
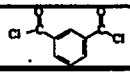
molecule may be chemically altered without affecting coupling of the molecule with a linker or TcII. Coupling sites which are not occupied by a linker may have a variety of chemical moieties attached thereto, including an amino, secondary amino, tertiary amino, hydroxy, lower alkyl, lower alkoxy, alkoxyalkyl, alkoxyalkoxy, cycloalkylalkoxy, and thioalkyl groups.

In a preferred embodiment, R_1, R_2 or R_4 is a linker and the remaining R groups are $-NH_2$, with the exception of R_7, which is preferably -OH. In an especially preferred embodiment, R_2 is a linker, R_1, R_3-R_6 are $-NH_2$ and R_7 is -OH.

In another preferred embodiment, R_7 is a linker and R_1-R_6 are $-NH_2$.

TABLE 1
HOMOBIFUNCTIONAL LINKERS

	disuccinimidyl suberate (DSS)*
	bis(sulfosuccinimidyl) suberate (BS^3)*
	disuccinimidyl suberate (DSS)*
	bis(sulfosuccinimidyl) suberate (BS^3)*
	disuccinimidyl tartarate (DST)*
	disulfosuccinimidyl tartarate (Sulfo-DST)*
	bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone BSOCOES)*

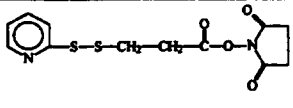
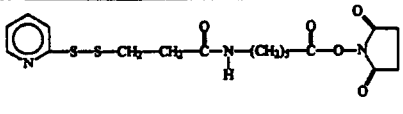
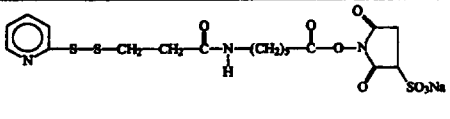
	bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone (Sulfo-BSOCOES)*
	bismaleimidohexane (BMH)*
	1,5-Difluoro-2,4-dinitrobenzene (DFDNB)*
	dimethyl adipimidate-2 HCl (DMA)*
	dimethyl pimelimidate-2 HCl (DMP)*
	dimethyl subevimidate-2 HCl (DMS)*
	isophthaloyl dichloride**

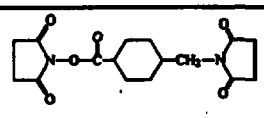
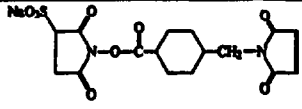
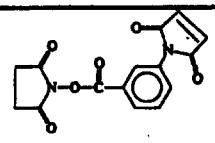
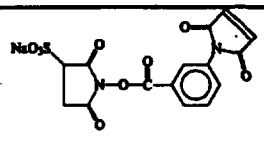
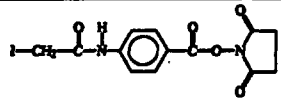
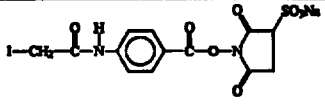
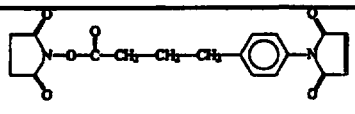
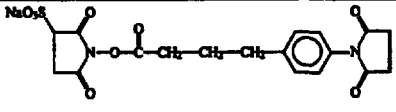
*Pierce Chemical, Co., Rockford, Illinois

**Aldrich Chemical Co., Milwaukee, Wisconsin

TABLE 2

HETEROBIFUNCTIONAL LINKERS

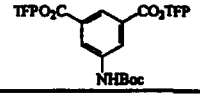
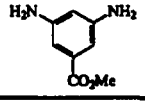
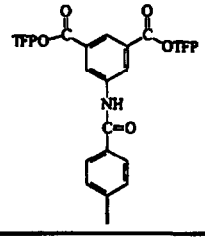
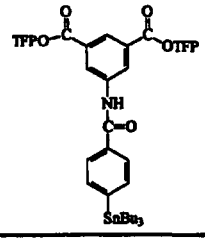
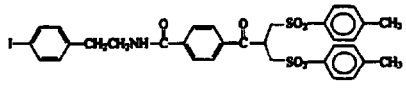
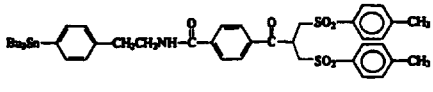
	N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)*
	succinimidyl 6[3(2-pyridyldithio) propionamido] hexanoate (LC-SPDP)*
	sulfosuccinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (Sulfo-LC-SPDP)*

	succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)*
	sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC)*
	m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)*
	m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Sulfo-MBS)*
	N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB)*
	sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (Sulfo-SIAB)*
	succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB)*
	sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate (Sulfo-SMPB)*

*Pierce Chemical, Co., Rockford, Illinois

TABLE 3

TRIFUNCTIONAL LINKERS

	Derived from 5-amino isophthalic* acid - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	Derived from 3,5-diaminovbenzoic acid* - unreported synthesis
	5-(*p*-iodobenzoyl)amino-1,3-isophthaloyl ditetra-fluorophenyl ester - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	5(*p*-tri-N-butylisomylbenzoyl)-amino-1,3-isophthaloyl ditchtrafluorophenyl ester - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	D.S. Wilbur et al., Bioconjugate Chem. 5(3):220-235, 1994.
	D.S. Wilbur et al., Bioconjugate Chem. 5(3):220-235, 1994.

*Aldrich Chemical Co., Milwaukee, Wisconsin

Suitable linkers include any one of several linkers, preferably containing at least two coupling or reactive groups, allowing the linker to bind to both vitamin B_{12} and a rerouting moiety. In the context of the present invention, the terms "coupling group" and "reactive group" are used interchangeably. By way of example, a linker may be homobifunctional, heterobifunctional, homotrifunctional, or heterotrifunctional. Homobifunctional agents may facilitate cross-linking, or dimerization of vitamin B_{12}

molecules in a single step, hence a coupling reaction using these agents should be performed with an excess of homobifunctional agents, unless dimerization is the desired result, as in the synthesis of dimers described in detail below.

Suitable homobifunctional agents include those listed in Table 1, as well as those described in detail below. Heterobifunctional agents facilitate cross-linking in a stepwise method, allowing more than one linker to be incorporated and a variety of targeting agents such as vitamin B_{12} molecules to be linked. Suitable heterobifunctional agents include those listed in Table 2 as well as those described in detail below. Homo- and hetero- trifunctional linkers are coupled to a rerouting moiety and a vitamin B_{12} molecule as described above, with the additional advantage of a third coupling site on the linker. One of ordinary skill in the art will appreciate that this allows for any number of different molecules to couple with the rerouting moiety, including, by way of example, markers, such as radiolabeled and fluorescent molecules; proteins and peptides, such as antibodies; and conjugating molecules, such as biotin. Suitable trifunctional linkers are listed in Table 3. Homobifunctional, heterobifunctional, homotrifunctional, and heterotrifunctional linkers are commercially available.

Suitable linkers are generally relatively linear molecules greater than 4 atoms in length, typically between 6 and 30 atoms in length, and preferably are 8 to 20 atoms in length. In a particularly preferred embodiment, the linker is a linear molecule of 12 atoms in length. In the context of the present invention, the term "atom" refers to a chemical element such as, by way of example, C, N, O, or S. The ranges provided above are based on the relatively linear accounting of the linker. One of ordinary skill in the art will appreciate that a linker may be linear, branched, and even contain cyclical elements.

Coupling or reactive groups include any functional group capable of coupling a linker to a vitamin B_{12} molecule. Suitable coupling groups include, nucleophilic and electrophilic functional groups. Suitable nucleophilic groups include hydroxy groups, amino groups, and thio groups. Suitable electrophilic groups include carboxylic acid groups and carboxylic acid derivatives including acid halides, acid anhydrides, and active esters such as NHS esters.

Suitable homobifunctional linkers include, by way of example, diaminoalkanes, such as those represented by the formula $NH_2(CH_2)_xNH_2$, wherein x = 2-20. A preferred linker is a diaminododecane. Suitable heterobifunctional linkers include those represented by the formula $NH_2(CH_2)_yCOOH$, wherein y = 3-12. Those

of ordinary skill in the art will appreciate that a protecting group may be necessary when utilizing a heterobifunctional group.

A linker may be coupled to the preferred *b*-, *d*- or *e*- coupling sites (*see* Structure I above) by any one of several suitable means, including, by way of example, activating a vitamin B_{12} molecule by hydrolyzing its propionamide groups to produce monocarboxylates, purifying the resulting monocarboxylates, and coupling a linker to a selected coupling site. Hydrolysis of the coupling sites may be accomplished by exposing vitamin B_{12} to aqueous acid for a period of time and under suitable conditions to hydrolyze the desired propionamide groups. Preferably, hydrolysis is performed by exposure of the amide to dilute aqueous acid for a period of about 6 to 12 days, typically about 9 to 11 days, and most preferably about 10 days at room temperature. Suitable aqueous acids include, by way of example, 0.1N hydrochloric acid, 0.5N phosphoric acid or 0.5N sulfuric acid.

Purification of *b*-, *d*- and *e*- monocarboxylates can be accomplished by any one of several means, including column chromatography, such as gel permeation chromatography, adsorption chromatography, partition chromatography, ion exchange chromatography, and reverse phase chromatography. Preferably, column chromatography is preparative reverse phase liquid chromatography. These techniques are described in detail in Lim, HPLC of Small Molecules, IRL Press, Washington, D.C., 1986. Purification of monocarboxylates by preparative liquid chromatography (LC) should be accomplished at a very slow flow rate. For example, LC purification may be conducted at a flow rate of 0.15 mL/min. on a 5 μm, 4.6 X 250 mm propylamine column (RAININ microsorb-MV amino column) eluting with 58 μM pyridine acetate, pH 4.4 in H_2O : THF (96 : 4) solution. Even more preferably, the coupling reaction is monitored using analytical high pressure liquid chromatography (HPLC). Reverse-phase HPLC chromatography is preferably carried out using an analytical version of above-noted propylamine column using a gradient solvent system at a flow rate of 1 mL/min. Within the context of the present invention, the *d*- isomer is identified as the longest retained peak (third), the *e*- isomer is identified as the second retained peak, and the *b*- isomer is identified as the shortest retained peak (first) eluted from the LC column. The *d*- isomer may also be identified as that vitamin B_{12} derivative demonstrating the greatest biological activity as noted below.

A ribose coupling site (coupling site *h*, *see* structure I) may be activated by any one of several suitable means including, activating a hydroxyl group at coupling site *h* by reaction with a suitable reagent (*e.g.*, succinic anhydride), to yield a ribose derivative which bears a reactive group (*e.g.*, a carboxylate group). This technique is

described in detail in Toraya, Bioinorg. Chem. 4:245-255, 1975. Separation and purification of the activated molecule may be accomplished on a C18 column as noted below. Once coupling site *h* has been activated, a linker may be coupled to this site in the same manner as described below.

After activating the vitamin B_{12} molecule at a selected coupling site, linkers may be coupled to a vitamin B_{12} molecule to form a vitamin B_{12} linker adduct using any one of several means, including, by way of example, an amide forming reaction, employing an amine group on the linker and a carboxylate coupling site on a vitamin B_{12} molecule. Alternatively, a linker may be coupled to a vitamin B_{12} molecule through an amide forming reaction, employing a carboxylate group on the linker and an amino group on a B_{12} molecule. The amide forming reaction may include the use of a coupling agent. Suitable coupling agents include carbodiimide coupling agents, such as, by way of example, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1-benzyl-3-(3-dimethylaminopropyl), carbodiimide (BDC), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC), and 1,3-dicyclohexylcarbodiimide (DCC). Preferably, the coupling agent is water soluble. Even more preferably, the coupling agent is EDC.

Alternatively, the amide forming reaction coupling the linker to a B_{12} molecule may employ a reactive carboxylic acid group and an amine. Suitable reactive carboxylic acid groups include carboxylic acid derivatives which yield an amide upon reaction with an amine. Such reactive groups include, by way of example, any reactive carboxylic acid derivative, including, by way of example, carboxylic acid halides, such as acid chlorides and bromides; carboxylic acid anhydrides, such as acetic anhydrides and trifluoroacetic anhydrides; esters, such as p-nitrophenyl esters and N-hydroxysuccinimide esters. Such techniques are described in detail in Bodanszky, Principles of Peptide Synthesis, Springer Verlag, Berlin, 1984.

Although coupling of a linker through a cyano coupling site is possible it is not preferred, due to the instability of linkers coupled to this site. Dolphin, D., [205] Methods Enzymol. 18C:34-52, 1971. Additionally, a linker may be coupled to a benzimidazole (coupling site *i*, *see* Structure I) using techniques described in detail in Jacobsen, Anal. Biochem. 113:164-171, 1981.

Vitamin B_{12} linker adducts may be separated and purified using any suitable means, including column chromatography, such as gel permeation chromatography, adsorption chromatography, partition chromatography, ion exchange chromatography, and reverse phase chromatography. Preferably, column

chromatography is preparative LC. These techniques are described in detail in Lim, HPLC of Small Molecules, IRL Press, Washington, D.C., 1986.

As noted above, the vitamin B_{12} receptor modulating agents of the present invention must be capable of binding transcobalamin II. The ability of a receptor modulating agent to bind TcII may be ascertained using any one of several means known in the art, including competitive binding assays with the receptor modulating agent competing with native vitamin B_{12}.

Rerouting moieties of the present invention include any moiety which is capable of affecting the receptor trafficking pathway. This characteristic can be assessed by employing a receptor modulating agent having a radiolabeled targeting moiety and following its path through the cell. This is accomplished using techniques known in the art, including using radiolabeled, biotinylated, or FITC labeled targeting moiety followed by binding assays, ELISA, or flow cytometry. A preferred receptor modulating agent is one which results in the removal of the highest percent of receptor for the longest period of time.

Suitable rerouting moieties of this invention do not significantly detract from the selectivity of the targeting moiety. Whether a rerouting moiety detracts from the selectivity of a targeting moiety may be determined by any one of several methods known in the art, including comparing binding of the receptor modulating agent on receptor positive and receptor negative cells, as assessed by ELISA, flow cytometry, or other binding assays.

Rerouting moieties cause the retention/degradation of an agent/receptor complex within at least one cell type, but not necessarily in all cells. In like fashion, a rerouting moiety causes retention of an agent/receptor complex in some cells, but not necessarily other agent/receptor complexes in other cells. Different rerouting moieties may also distinguish between receptor species, for example, as in polarized epithelium where the same receptor may independently traffic on the apical, basal, or basolateral sides of the cell. To determine if a particular rerouting moiety is suitable, a rerouting moiety is covalently attached to the targeting moiety, and the resulting receptor modulating agent is compared for receptor modulation on different receptor-bearing cells using binding or functional assays known in the art.

Suitable rerouting moieties of this invention may be categorized into five different functional classes: (1) lysosmotropic moieties; (2) intracellular polymerizing moieties; (3) protein sorting signals or sequences; (4) conditional membrane binding peptides; and (5) bi- or multi-valent receptor cross linking moieties. While such rerouting moieties may have different functional mechanisms of action, all promote

retention of the agent/receptor complex within the intracellular vesicular system. All of these classes of rerouting moieties will impart the ability to affect the receptor trafficking pathway.

In one aspect of the present invention, a first functional class of rerouting moieties, lysosomotropic moieties, are disclosed. Within the context of the present invention, the term "lysosomotropic moieties" refers to moieties which route the agent/receptor complex to the lysosomes. Numerous suitable lysosomotropic moieties are known, and are reviewed in Biochem. Pharmacol. 23:2495-2531, 1974.

A preferred lysosomotropic moiety includes an aminoglycoside antibiotic marked by the characteristic ability to accumulate in lysosomes after intracellular protonation. Intracellular protonation occurs in the increasingly acidic conditions which occur during the transfer from early to late endosomes and, finally, to the lysosome. Strong positive charges prohibit the lysosomotropic moiety from leaving the membrane-enclosed vesicles, thus trapping the agent/receptor complex in the vessel.

Aminoglycoside antibiotics are similar in structure, but are divided into structurally related families of compounds based upon the sugar units. Each of the families of aminoglycoside antibiotics, as well as representative members thereof, are set forth in Figures 2-5. These families include gentamycin, kanamycin, neomycin and streptomycin. The gentamycin family includes gentamycin C_1, gentamycin C_2, gentamycin C_{1a}, sisomicin and netilmicin; the kanamycin family includes kanamycin A, tobramycin and amikacin; the neomycin family includes neomycin B, paromomycin, ribostamycin and bytirosin B; and the streptomycin family includes streptomycin A and streptomycin B.

In a particularly preferred embodiment of the present invention, the rerouting moiety is gentamycin, which accumulates in lysosomes in concentration as much as 300 fold that of the extracellular concentration (J. Pharmacol. Exp. Ther. 255:867-74, 1990; Ren. Fail. 14:351-7, 1992).

Suitable aminoglycosides have reactive amine groups capable of being coupled through peptide or other chemical linkers. Thus, a targeting moiety may be readily attached via covalent linkage to these rerouting moieties using any one of several techniques known in the art to form covalent bonds, for example, using thioether, disulfide, ether, ester and peptide bonds. Since many of the aminoglycoside antibiotics have several amines which could be derivatized in a conjugation procedure, a primary amine contained in these compounds can be selectively reacted to favor covalently attachment to the targeting moiety through this amine (*see* amine indicated with arrow in Figures 2-4). With regard to streptomycin, covalent attachment to the

targeting moiety may be accomplished by converting the aldehyde moiety indicated in Figure 5 to an amine, and attaching to the targeting moiety using carbodiimide or other suitable activated carboxylic acid. Aminoglycosides are water soluble and do not readily bind to other proteins, and thus do not impart non-specific binding to a receptor modulating agent.

Particularly preferred aminoglycosides include those which allow for preferential derivation of a selected amine. Specifically, preferred aminoglycosides include those compounds to which protective groups can be added to various nitrogen atoms thereof and, subsequently, selectively deprotected to yield a single free amine. The free amine can be further derivatized, for example, by addition of a peptide linker or covalently attached directly to the targeting moiety. These rerouting moieties include ribostamycin (*see* Figure 4), kanamycin (*see* Figure 3), amikacin, and streptomycin. Ribostamycin is particularly preferred, due to its relative low toxicity and its derivatization chemistry, allowing an acyl migration reaction to be effected on a hydroxyl protected ribostamycin to yield a single amine adduct. Kanamycin may also be used in a selective protection/acylation reaction; Amikacin is commercially available in a form which allows attachment without deprotecting its amines or alcohol groups; and streptomycin can also be readily derivatized by protonating guanidinium groups under physiologic conditions to provide the polycations necessary for cellular or lysosomal retention.

In another aspect of the present invention, non-aminoglycoside lysosomotropic compounds which may accumulate after intracellular protonation are also suitable rerouting moieties (*see* Figure 6). Suitable non-aminoglycoside compounds exhibiting this characteristic are known in the art, a series of aminoacridine and amino quinoline dyes, typified by cholquinine and quinacrine; a group of amino naphthalenes, typified by dansyl cadaverine; and derivatives thereof. Such dyes are characterized by cellular retention and low toxicity. All of these compounds have characteristic sites for covalent attachment to a targeting moiety via the nitrogen indicated in Figure 6 and may be attached thereto as described above.

Another aspect of the present invention utilizes a lysosomotropic peptide subject to charge modification under intracellular conditions is employed as a rerouting moiety. Once charge-modified, the rerouting peptide acts to retain an agent/receptor complex in the intracellular vesicular system until membrane flow delivers it to the lysosome for degradation. Preferably, these peptides are capable of being phosphorylated by intracellular protein kinases. When phosphorylated by the intracellular enzymes, such peptides would be highly anionic.

Charge-based retention can be an inherent property of the rerouting peptide or can be imparted by intracellular modification. Intracellular modification may be accomplished by any of several means known in the art, including phosphorylation of certain residues of some receptors (*e.g.*, the EGF receptor) may cause intracellular rerouting (Cancer Treat. Res. 61:139-160, 1992; J. Cell. Biol. 116:321-30, 1992).

The rerouting peptides may be covalently attached to a targeting moiety by any means, including, for example, covalently linking the peptide directly to the targeting moiety, or by use of an appropriate linker moiety, such as G-G-G, which may be derivatized and covalently attached to the targeting moiety.

Preferred rerouting peptides include protein kinase-substrate peptides that incorporate serine. These peptides are particularly preferred for enhancement of receptor rerouting in tumor target cells, which have increased levels of protein kinase activity for serines or tyrosines. Increased levels of kinase activity within tumor cells may be attributed to the presence of oncogene products, such as H-ras, on the cytoplasmic side of tumor cell plasma membranes (C.I.B.A. Found. Symp. 164:208-18, 1992).

Suitable rerouting peptides also include protein kinase substrates and peptides that possess a single positive charge. The latter type of rerouting peptide may form an ion pair with a "glutamate-like" residue of an attached or closely associated residue(s) of the receptor. Particularly preferred rerouting peptides may be derived, using technologies known in the art, from the proteins and the amino acid sequences identified in Table 4.

TABLE 4
REROUTING PEPTIDES

PEPTIDE SOURCE	AMINO ACID SEQUENCE
EGF receptor	DVVDADEYLIPQ
EGF fragment	CMHIESLDSYTC
Phosphorylase kinase	RTKRSGSVYEPLKI
Protein kinase C pseudosubstrate	RFARK-GALRQKNV
Myelin basic protein	S/T-XAA-K/R (where XAA is an uncharged residue)
Kemptide	RGYALG or RGYSLG
Glycogen synthetase	PLSRTLSVAA

Transferrin receptor	FSLAR
III histone	ASGSFKL
Casein kinase II substrate	AAAAAASEEE or AAAAAASDDD
Insulin receptor auto-phosphorylation substrate	DIYETDYYR
calmodulin-dependent protein kinase II	Waxman and Arenowski Biochem. 32(11):2923-30, 1993
Neurogranin	Chen et al., Biochem. 32(4):1032-9, 1993
MARCKS	Heemskerk et al., Biochem. Biophys. Res. Commun. 190(1):236-41, 1993
Glycogen synthase	Marais et al., FEBS Letters 277:151-5, 1990
Ribosomal protein S6	Munro et al., Biochem. Biophys. Acta 1054:225-30, 1990
Co-polymers which serve as substrates for protein kinase A, C, P	Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 86:1761-5, 1989; Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 85:1408-11, 1988
Serine-threonine kinases	Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 86:1761-5, 1989; Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 85:1408-11, 1988

In another aspect of the present invention, the rerouting moiety is a lysosomotropic amino acid ester which, in high concentration, can cause the lysis of granule containing cells, such as NK cells, cytolytic T cells and monocytes. The concentration must generally be maintained below 100 mM to avoid lysis. Suitable lysosomotropic amino acid esters and their sources are presented in Table 5.

TABLE 5
LYSOSOMOTROPIC AMINO ACID ESTERS

Leu-O-Me	Res. Immunol. 143:893-901, 1992 Eur. J. Immunol. 23:562-5, 1993 Intl. Arch. Aller. & Immunol. 100:56-59, 1993 Cell. Immunol. 139:281-91, 1992 Exp. Pathol. 42:121-7, 1991

Iso-leu-O-Me	Res. Immunol. 143:893-901, 1992
L-Val-O-Me	J. Immunol. 134:786-93, 1985
Phe-O-Me	J. Immunol. 148:3950-7, 1992 Blood 79:964-71, 1992
Phe-, Ala-, Met-, Trp-, Cys-, Try-, Asp-, & Glu-O-Me	Int. J. Immunopharmacol. 13:401-9, 1991

The lysosomotropic amino acid esters identified in Table 5 can be used to retain the agent/receptor complex in lysosomes after intracellular cleavage of the ester. In one embodiment, such amino acid esters may be utilized as the C-terminal portion of a larger peptide containing a linker sequence and/or a phosphorylation substrate sequence, and with suitable residues, such as cysteine, for covalent attachment to a targeting moiety, such as a sequence encoding a peptide or protein ligand for a given cell surface receptor.

In another embodiment of the present invention, a second functional class of rerouting moieties is disclosed. This class includes peptides which undergo polymerization within endosomes or lysosomes, inhibiting their passage through intracellular membranes.

Intracellular polymerizing compounds can be incorporated into a larger peptide containing the targeting moiety and a linker. Suitable peptides include the dipeptide ester referenced in Table 5 (*i.e.*, L-Leucyl-L-Leucine-O-Me). When transported into cells, these dipeptide esters preferentially accumulate in lysosomes and secondary granules of cytotoxic cells. These dipeptides also undergo self-association and polymerization, which results in trapping at low concentrations, and membrane rupture at higher concentrations.

TABLE 6

POLYMERIZING DI-PEPTIDE ESTER: L-LEUCYL-L-LEUCINE-O-ME

J. Invest. Dermat. 99:805-825, 1992
J. Clin. Invest. 84:1947-56, 1989
Transpl. 53:1334-40, 1992
J. Immunol. 138:51-7, 1987
J. Immunol. 148:3950-7, 1992

J. Immunol. 136:1038-48, 1986
Cryobiology 29:165-74, 1992
Acta. Biochem Biophys. Hung 24:299-311,1989
Blood 79:964-71, 1992
Blood 78:2131-8, 1991
J. Immunol. 139:2137-42, 1987
J. Exp. Med. 172:183-194, 1990
J. Clin. Invest. 78:1415-20, 1986
PNAS 87:83-7, 1990
J. Immunol. 137:1399-406, 1986
PNAS 82:2468-72, 1985

Suitable intracellular polymerizing compounds also include peptides that can self-associate into alpha-helical structures termed "leucine zippers". In the context of this invention, such structures may be used to form intracellular polymers that are incapable of exiting intracellular vesicles. Such sequences can be selected by observing self association of the compounds in solution, and the formation of polymers capable of binding to DNA. Suitable peptide sequences that can self-associate into alpha helical structures are presented in Table 7.

TABLE 7
LEUCINE ZIPPERS

Boc(t-butoxycarbonyl)-Aib(alpha-aminoisobutyryl) Glu(OB_nl)-(benzoyl ester)-Leu-Aib-Ala-Leu-Aib-Ala-
Boc-Aib-Leu-Aib-Aib-Leu-Leu-Aib-Leu-Aib-O-Me Proteins 12:324-30, 1992 Lys(Z)(benzyloxy-carbonyl)-Aib-O-Me PNAS 87:7921-5, 1990
GELEELLKHLKELLKGER Biochem. 31:1579-84, 1992

In another embodiment of the present invention, a third functional class of rerouting moieties is disclosed. This class includes moieties that can be recognized by intracellular receptors. Such sequences are identified by their ability to stop movement of endogenously synthesized proteins to the cell surface. Suitable peptides include certain peptide sequences (such as sorting or signal sequences) associated with the trafficking of endogenously synthesized proteins (Cur. Opin. Cell. Biol. 3:634-41, 1991). Such peptide sequences, when covalently attached to the C-terminus of an exogenously added targeting moiety, result in the retention of the agent/receptor complexes in the endoplasmic reticulum ("ER"), Golgi apparatus, or lysosomes.

Such peptide sequences are recognized by intracellular receptors, examples of which include both mammalian and bacterial versions of ER receptors described in detail in J. Cell. Biol. 120:325-8, 1993; Embo. J. 11:4187-95, 1992; Nature 348:162-3, 1990. Further exemplary peptide sequences and variants thereof (shown in parentheses) that can be recognized by intracellular receptors are set forth in Table 8, Sections A and B.

Certain signal sequences may be preferred for retention by one type of organism versus another type. For example, REDLK is a preferred sequence recognized by prokaryotic cells and to a lesser degree by eukaryotic cells (*see* Table 8, section C). Thus, employing this sequence as the rerouting moiety, receptor modulating agents can be constructed to selectively inhibit a receptor-mediated process in bacteria, while having little effect on mammalian cells.

TABLE 8

PEPTIDE SEQUENCES WHICH BIND INTRACELLULAR RECEPTORS

A. Endoplasmic Reticulum or Golgi Retention Peptides	
1. KDEL (DKEL, RDEL, KNEL, SDEL, KEEL, QDEL, KEDL, KDEL)	J. Biol. Chem. 265:5952-5, 1990 Biochem. Biophys. Res. Commun. 172:1384-91, 1990 J. Virol. 65:3938-42, 1991 Exp. Cell Res. 197:119-24, 1991 Growth Factors 5:243-53, 1991 J. Biol. Chem. 267(10):7022-6, 1992 J. Biol. Chem. 267:10631-7, 1992 J. Cell. Biol. 118:795-811, 1992 J. Cell. Biol. 119:85-97, 1992 Exp. Cell. Res. 203:1-4, 1992 P.N.A.S. 90:2695-9, 1993 Mol. Biochem Parasitol 48:47-58, 1991 Embo J. 4:2345-55, 1992 J. Biol. Chem. 266:14277-82, 1991 Mol. Cell Biol. 11:4036-44, 1991
2. HDEL (HVEL, HNEL, HTEL, TEHT, DDEL, HIEL)	J. Biol. Chem. 268:7728-32, 1993 Mol. Biochem Parasitol 57:193-202, 1993 J. Cell SCI 102:261-71, 1992 Eur J. Biochem. 206:801-6, 1992 J. Biol. Chem. 266:20498-503, 1991
3. ADEL	Embo J. 11:1583-91, 1992
4. REDLK	J. Biol. Chem. 266:17376-81, 1991
5. SEKDEL	Growth Factors 5:243-53, 1991
6. KTEL	J. Virol. 66:4951-6, 1992
B. Lysosomal Retention Peptides	
1. KFERQ	Trends Biochem SCI 15:305-9, 1990
2. Tyrosine-containing polypeptides	J. Cell Biol. 111:955-66, 1990
C. ORGANISM-SPECIFIC RETENTION PEPTIDES	
1. REDLK	J. Biol. Chem. 266:17376-17381, 1991

D. CLATHRIN-BINDING PEPTIDES (INTERNALIZATION SIGNALS)		
1.	LLAV	J. Cell. Biol. 199:249-57, 1992
2.	YKYSKV	J. Cell. Biol. 199:249-57, 1992 Embo. J. 7:3331-6, 1988
3.	PPGYE	Cell 67:1203-9, 1991 Curr. Opin. Cell Biol. 3:1062, 1991

A further class of peptide sequences of this invention, termed "internalization signals," function by binding to clathrin, both in the coated pits, as well as those intracellular vesicles which maintain a clathrin coat. Representative examples of such clathrin-binding peptides (CBP) are disclosed in Table 8, section D. The CBP binds clathrin in the coated pits initially located on the cell surface causing retention of the targeting moiety to which it is conjugated.

A further class of moieties capable of recognizing intracellular receptors includes carbohydrates. Suitable carbohydrates include any carbohydrate which is capable of binding to intracellular carbohydrate (CHO) receptors but not cell surface CHO receptors. Such carbohydrates include: mannose-6-phosphate and glucose-6-phosphate. Suitable carbohydrate moieties include those which bind to the insulin-like growth factor II/mannose-6-phosphate (IGF II/M6P) receptor, include analogs of mannose-6-phosphate, as well as other phosphorylated saccharides (Carbohydrate Res. 213:37-46, 1991; FEBS Lett. 262:142-4, 1990).

The affinity of the rerouting moiety can be varied by changes in the chemical nature of the phosphorylated saccharides (J. Biol. Chem. 264:7970-5, 1989; J. Biol. Chem. 264:7962-9, 1989) (monosaccharides bind with the lowest affinity, while di- or tri-saccharides bind with increasingly higher affinity). Clustering of phosphorylated saccharides on protein carriers can dramatically increase affinity to the intracellular receptor.

Synthesis of various oligosaccharides are reviewed in Sem. Cell. Biol. 2:319-326, 1991. Although, mannose-6-phosphate receptor expression is primarily intracellular, expression also occurs on cell surfaces. Thus, in the context of the present invention, covalent attachment of a targeting moiety with a carbohydrate which binds the mannose-6-phosphate receptor should be constructed so as to give at least 100-fold difference in binding affinity between the targeting moiety and the rerouting moiety. For example, a vitamin B_{12}/transcobalamin II receptor targeting moiety, in this case vitamin B_{12}, would have a binding affinity for the carrier protein, transcobalamin II

(TcII), of $\geq 10^{-10}$ M and an affinity for the IGF II/M-6-P receptor of 10^{-8} M or less. This will maintain the specificity of the vitamin B_{12} binding (via TcII), while allowing transfer of the receptor modulating agent from serum M-6-P soluble receptor to cell surface receptor.

In addition to IGF II/M-6-P receptor moieties, other carbohydrate-based rerouting moieties also promote retention of the modulating agent/receptor complex in the ER or Golgi complex. Such moieties are based on the recognition by various glycosyl transferases of carbohydrate moieties, either as a natural substrate or as an inhibitor. Such moieties are reviewed in Sem. Cell. Biol. 2:289-308, 1991. For example, saccharide recognition moieties include penultimate sugars, such as glucose and N-acetyl glucosamine (which are natural substrates). More preferred, however, are glycosylation inhibitors which are recognized by glycosyl transferases, but cannot serve to append further carbohydrate residues on growing chains (Sem. Cell. Biol. 2:309-318, 1991) (*see* Figure 7).

In yet another embodiment of the present invention, a fourth functional class of rerouting moieties is disclosed. This class is generally comprised of rerouting moieties which anchor the receptor to the cell membrane. By way of example, this class includes membrane-binding peptides that exhibit conditional pH-dependent membrane binding. Such peptides exhibit α-helical character in acid but not neutral pH solutions. When a conditional membrane-binding peptide assumes a helical conformation at an acidic pH, it acquires the property of amphiphilicity, (*e.g.*, it has both hydrophobic and hydrophilic interfaces). More specifically, within a pH range of approximately 5.0-5.5, such a peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the peptide into a target membrane. An alpha helix-induced acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes or lysosomes. In aqueous solution at physiological pH, a conditional, membrane-binding peptide is unfolded (due to strong charge repulsion among charged amino acid side chains) and is unable to interact with membranes.

Suitable conditional membrane-binding peptide sequences include the charged amino acids glutamate, aspartate, and histidine. A preferred conditional membrane-binding peptide includes those with a high percentage of helix-forming residues, such as glutamate, methionine, alanine, and leucine. Further, conditional membrane-binding peptide sequences include ionizable residues having pKas within the range of pH 5-7, so that a sufficiently uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane. Conditional membrane-binding peptides can be incorporated through covalent bonds to

a chemical or peptide targeting moiety or synthesized as an entire peptide sequence including a linker and peptide targeting moiety.

A particularly preferred conditional membrane-binding peptide is aa1-aa2-aa3-EAALA(EALA)$_4$-EALEALAA-amide, which represents a modification of a published peptide sequence (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue such as cysteine or lysine, that facilitates chemical conjugation of the conditional membrane-binding peptide to a targeting protein. The peptide can also be incorporated into a fusion protein with a protein or peptide targeting moiety (*see* Example 7). Amino acid residues 2-3 (*i.e.*, aa2-aa3) may be selected to modulate the affinity of the translocating peptide for different membranes. For instance, if both residues 2 and 3 are lysine or arginine, the peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the peptide will insert into neutral membranes.

Yet another preferred conditional membrane-binding peptide can be derived from sequences of apo-lipoprotein A-1 and B; peptide toxins such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, and pancreatic polypeptide. Such peptides normally bind membranes at physiologic pH but through attachment of substituents the peptides can be enhanced in their ability to form alpha-helices at acidic pH and reduced in their membrane-binding at physiologic pH. An example of such a modified peptide having pH-dependent membrane binding at acidic pH is fully succinylated melittin. In this example, a peptide (melittin) that normally binds to membranes at physiological pH is converted to a pH-dependent peptide through succinylation of lysines. Upon succinylation, the peptide displays an amphipathic character only at acidic pHs.

Insertion of a conditional membrane-binding peptide into a target cell membrane is enhanced through stabilization of the amphiphilic alpha helix. Helix stabilization may be achieved: (1) by adding repeating "EALA" units to form a longer peptide; (2) by placing an amide at the C-terminus of the peptide, in order to counteract the helical dipole; (3) by polymerizing the peptide; (4) by substituting a natural helix-former for one or more of the stacked glutamates; or (5) by attaching the peptide to a targeting moiety through use of a longer linker, in order to provide sufficient distance between the membrane binding peptide and the targeting moiety for the peptide to contact and interact with the target cell intracellular membranes.

In yet another embodiment of the present invention, a fifth functional class of rerouting moieties is disclosed. In this context, the rerouting moiety merely functions as a modulating agent in that the moiety disables the receptors by crosslinking the same. This class includes bi- or multi-valent receptor crosslinking moieties formed from monovalent binding targeting moieties. Cross-linking of receptors in some receptor systems is sufficient to cause a rerouting of cell surface receptors to lysosomes for degradation, rather than their normal pathway of receptor recycling. The synthesis of a bivalent receptor modulating agent is exemplified in greater detail in the examples below.

A preferred cross-linking receptor modulating agent is a vitamin B_{12} dimer. In this embodiment, each vitamin B_{12} molecule acts as a targeting agent and a rerouting agent; cross-linking the B_{12} dimer will cross-link the vitamin B_{12} receptors, thus impeding the receptor trafficking pathway. A preferred vitamin B_{12} dimer is generally comprised of two vitamin B_{12} molecules, such as cyanocobalamin, coupled by one or more linkers through coupling sites independently selected from *a-g*, *h* (ribose), and *i* (benzimidazole). Preferably, cross-linking occurs between *d-* or *e-* coupling sites on both molecules. The dimer must be capable of forming a B_{12}/TcII complex. As noted above, this characteristic may be assayed using any one of several techniques known in the art, including competitive binding assays.

A vitamin B_{12} may be coupled to a second vitamin B_{12} molecule in the same manner as described in detail for conjugation of rerouting moieties to vitamin B_{12} targeting moieties. As noted above, dimers may be synthesized using one or more linkers of various lengths and any combination of homobifunctional, heterobifunctional, homotrifunctional, or heterotrifunctional linkers. As noted above, the use of a trifunctional linker allows for coupling with any number of additional moieties.

In selecting a linker for dimer synthesis, it should be noted that the total number of atoms comprising the linker between the vitamin B_{12} molecules should generally be greater than 10 atoms, typically be in the range of 30 to 55 atoms and, preferably be 45. As noted above, one of ordinary skill in the art will appreciate that although the number of atoms is calculated relative to a linear chain of atoms, linear chain, branched chain, and cyclical chain linkers or combinations thereof would be suitable. Hence, the structure of the atom chain in a linker would include, by way of example, alkyl, heteroalky, alkylaryl, and heteroalkyl aryl.

By way of example, a dimer may be synthesized by combining two different vitamin B_{12} linker adducts in the presence of a coupling agent. The linkers

couple and dimers may then be separated and purified using the same methods outlined above.

Alternatively, activated vitamin B_{12} may simply be combined with a homobifunctional or homotrifunctional linker (Tables 1 and 3). Preferably, in this embodiment, the ratio of vitamin B_{12} to linker should be in the range of 2:1. Preferably, a 1:1 ratio is used in preparation of mixed dimers (*e.g.*, *b*- and *e*-acid derivatives) or mixed ligands (*e.g.*, B_{12} and hormone). Dimers may be separated and purified as noted above.

In still another alternative, vitamin B_{12} linker adducts, synthesized as described, above may be coupled by a third linker. The third linker, a "cross-linker," serves to bridge the linkers on the vitamin B_{12} linker adducts. Suitable cross-linkers include those noted in Tables 1, 2, and 3.

Polymerization of peptides may be accomplished by placing a cysteine residue at each end of a peptide, followed by oxidation using dissolved oxygen or other mild oxidizing agent, such as oxidized glutathione. The average length of a polymerized peptide may be controlled by varying the polymerization reaction conditions.

The amino acid sequence of any of the peptides of this invention may be selected to include all L-amino acids or all D-amino acids having a side chain pK_a from 5.0 to 9.0. D-amino acids may be advantageously used to form non-proteolyzable peptides, since the D-amino acids are not metabolized within the cell. Further, the peptides of the present invention may include a combination of L- and D-amino acids, wherein D-amino acids are substituted for L-amino acids on either side of a proteolytic cleavage site. Yet another preferred noncleavable peptide incorporates peptide bond analogs that are not susceptible to proteolytic cleavage by cellular enzymes.

As discussed above, the receptor modulating agents of this invention comprise a targeting moiety coupled to the rerouting moiety. The rerouting moieties identified above may be covalently attached to the targeting moiety by any one of several techniques known in the art, including (a) by chemical modifications such as a disulfide formation, thioether formation, amide formation or a reduced or non-reduced Schiff's base, (b) by direct peptide bond formation as in a fusion protein, or (c) by use of a chemical and peptide linker. Suitable peptide linkers in this regard correspond to two or more amino acid residues that allow the rerouting peptide to assume its active conformation independent of its interaction with the targeting moiety, and which allows sufficient distance for rerouting moiety access to, for example, intracellular membranes from the peptide attachment site on the targeting moiety.

In one embodiment, a rerouting moiety may be conjugated to a vitamin B_{12} targeting moiety by any one of several means, including, by way of example, coupling a rerouting moiety to a reactive group on a vitamin B_{12} linker adduct; coupling a vitamin B_{12} to a reactive group on a rerouting moiety linker adduct or an appropriate side chain thereof; coupling a vitamin B_{12} linker adduct to a rerouting moiety linker adduct or an appropriate side chain thereof; coupling a rerouting moiety/biotin binding protein conjugate to a vitamin B_{12}/biotin conjugate; or coupling a rerouting moiety biotin conjugate to a vitamin B_{12}/biotin binding protein conjugate.

Coupling of a rerouting moiety to a vitamin B_{12} linker adduct, or a vitamin B_{12} to a rerouting moiety linker adduct, may be accomplished using the same techniques noted above for coupling a vitamin B_{12} molecule with a linker. The only critical consideration of this aspect of the invention is that the total linker length must be sufficient to avoid steric hindrance. Preferably, the total linker length is at least 6 atoms.

Coupling of a rerouting moiety/biotin binding protein conjugate to a vitamin B_{12}/biotin conjugate may be accomplished using any one of several means described in detail in Avidin-Biotin Chemistry: A Handbook, ed. D. Savage, Pierce Chemical Co., 1992. Briefly, a biotin binding protein conjugate is prepared using a rerouting moiety or, as in a second embodiment, a vitamin B_{12} molecule. Suitable biotin binding proteins include avidin or streptavidin. In some circumstances, a linker may be utilized to distance the molecules. For example, when coupling a vitamin B_{12} to an avidin, a linker of at least 6 atoms is preferred.

A biotin conjugate is prepared using a vitamin B_{12} molecule or, as in a second embodiment, a rerouting moiety. By way of example, a vitamin B_{12} molecule is combined with an NHS ester of biotin. Preferably, the vitamin B_{12} molecule is a vitamin B_{12} linker adduct as described above. Even more preferably, the vitamin B_{12} molecule is a vitamin B_{12} linker adduct characterized by a 12 atom linear linker coupled to the *d*- or *e*- coupling site.

Once formulated, coupling between the biotin conjugates and biotin binding protein conjugates is easily accomplished by combining the complementing conjugates, *i.e.*, a vitamin B_{12}/biotin conjugate with a rerouting moiety/avidin conjugate.

In another aspect of the present invention, a B_{12}/biotin conjugate is utilized to couple a vitamin B_{12} to any number of compounds through biotin binding protein conjugates. Using a vitamin B_{12}/biotin conjugate, any compound which is capable of coupling a biotin binding protein may be coupled to a vitamin B_{12} and

thereby internalized into cells expressing the vitamin B_{12} receptor. Such compounds include, in addition to the rerouting moieties described in detail below, hormones, enzymes, antibodies or fragments thereof, markers, or therapeutics. Coupling any of these compounds to a biotin binding protein, such as avidin or streptavidin, may be accomplished using techniques described in detail in Avidin-Biotin Chemistry: A Handbook, ed. D. Savage, Pierce Chemical Co., 1992.

In one aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a therapeutic/avidin conjugate directed at neoplastic disorders. Neoplastic disorder therapeutics which may be coupled to a vitamin B_{12}/biotin conjugate through avidin include doxorubicin, daunorubicin, etoposide, teniposide, vinblastine, vincristin, cyclophophamide, cisplatin and nucleoside antimetabolites such as arabinosylcytosine, arabinosyladenine and fludarabine.

In another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a marker conjugated with a biotin binding protein. Suitable markers include, by way of example, fluorescent molecules or radiolabeled molecules. This combination may be utilized as a detection system incorporated into a screening device to identify patients with low receptor bearing cells or in the evaluation of receptor up-regulation, for example, following treatment of patients for any one of a wide variety of receptor modulation disorders.

In another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a radioisotope conjugated to a biotin binding protein. Suitable radioisotopes include, any high energy emitting radioisotopes capable of conjugating a biotin binding protein. This combination may be utilized as a targeted radiodiagnostic or radiotherapeutic.

In yet another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is used to immobilize vitamin B_{12} to a solid matrix or avidin-coated substrate. By way of example, this would enable one to isolate TcII, TcII receptors, and evaluate coupling sites on the Vitamin B_{12}.

The receptor modulating agents of this invention regulate receptor-dependent biological responses through alterations in the receptor trafficking pathway. As illustrated in Figure 1, with specific reference to the receptor for vitamin B_{12}, cell surface receptors are often associated with clathrin-coated pits. When bound by the receptor modulating agent of the present invention, the coated pits invaginate to form vesicles. The vesicles are then directed by the rerouting agent to lysosomes for receptor degradation or delivered to endosomes where the rerouting agent securely binds or

delays the agent/receptor complex. Thus, the receptor modulating agents can incapacitate the receptors normally undergoing recycling.

Newly synthesized receptors will eventually replace the internalized receptor on the cell surface. However, this process is far more time consuming than recycling--many cells require hours or days to achieve maximal receptor re-expression. Continued exposure of the cell to the receptor modulating agents will exhaust the intracellular receptor pools. Thus, by modulating a plasma membrane receptor, re-expression of the receptor can be substantially delayed, thereby regulating a biological response associated with that receptor for a prolonged period of time.

Biological activity of receptor modulating agents of the present invention may be ascertained in vitro by any one of several means known in the art including, competition binding assays or cell proliferation studies. These techniques are described in detail in Laboratory Techniques in Biochemistry and Molecular Biology: An Introduction to Radioimmunoassay and Related Techniques, 3rd Edition, ed. Burdon and van Knippenberg, Elsevier, 1987. By way of example, a receptor modulating agent may be cultured with a suitable cell line, such as K562 cells (ATCC CCL 243), under conditions representing in vivo conditions. Such conditions would include the provision of a human source of TcII (such as human serum), vitamin B_{12}, and, preferably by careful removal by chromatography, of all TcII from other medium supplements such that proliferation is solely dependent on a known amount of exogenous TcII. Cell cultures deprived of vitamin B_{12} gradually lose their proliferative capacity, eventually resulting in cell death. Biological activity may be evaluated in vivo using techniques described in detail in Shieh et al., J. Immunol. 152(2):859-866, 1994 in which human tumor cell lines are injected into nude mice, followed by therapy with receptor modulating agents. Next, tumor cells are removed, single cell suspensions prepared and TcII cell surface receptor density may be evaluated by flow cytometry and biotinylated vitamin B_{12} and avidin FITC.

The receptor modulating agent of the present invention may be administered in a therapeutically effective amount to treat a variety of disorders characterized in which control of the disease process or symptoms can be achieved by modulation of one or more receptor systems and the associated biological responses. Such disorders include neoplastic disorders, autoimmune diseases, rheumatic arthritis, cardiovascular disease, and neurodegenerative diseases.

Common to many non-neoplastic disease processes is a stage in which the disease process itself, or its symptoms, can be halted or ameliorated by the use of an anti-proliferative agent such as vitamin B_{12}/TcII receptor modulating agents. These

commonly recognized stages include a sensitization or elicitation phase in which immune cells responsible for the disease become turned on by antigen specific or non-specific means, followed by a proliferative phase in which the immune cells expand in number, and finally a symptomatic phase in which the expanded immune cells create tissue damage directly or indirectly. Neoplastic disorders include, by way of example, leukemia, sarcoma, myeloma, carcinoma, neuroma, melanoma, cancers of the breast, lung, liver, brain, colon, cervix, prostrate, Hodgkin's disease, and non-Hodgkin's lymphoma. Because of this, anti-proliferative chemotherapeutic drugs are commonly utilized in the treatment of many diseases other than cancer, but are limited in use to life threatening situations due to their associated toxicity. Anti-proliferative agents, such as the ones of the present invention (with little of the direct toxicity of chemotherapeutic drugs), may be used more widely. More specifically, the vitamin B_{12} receptor modulating agents of the present invention are not destructive to plasma membrane processes (*e.g.*, ion transport). In addition, the anti-proliferative activity is reversible by administration of vitamin B_{12}. Furthermore, the agents of this invention may not be mutagenic, teratogenic, or carcinogenic since they act at the level of the plasma membrane, and not at the level of the nucleus, and DNA by intercalation or cross-linking (as many chemotherapeutic drugs act).

An understanding of the pharmaceutical applications for B_{12}/TcII receptor modulating agents requires a knowledge of the cell types targeted by such therapy. To this end, various pharmaceutical applications are disclosed in Table 9 below.

TABLE 9

TARGET CELLS FOR VITAMIN B_{12} RECEPTOR MODULATING AGENTS

TARGET CELL	OTHER PROLIFERATION ASSOCIATED MARKERS	POTENTIAL PHARMACEUTICAL APPLICATIONS
Activated T-Cell	IL-2 receptor Transferrin Receptor Insulin Receptor Class II Histocompatibility Antigens	Graft versus Host Disease Organ Transplants Auto-Immune Diseases Asthma Crohn's Disease
Tumor Cells	Tumor Assoc. Ags. Ki67 Transferrin Receptor	Tumor Therapy (alone and in combination with chemotherapeutic drugs)

Bone Marrow Stem Cells	CD-34 Transferrin Receptor Class II Histocompatibility Antigens IL-1, IL-3 Receptors	Allogeneic Bone Marrow Transplants Reduction in Toxicity of Chemotherapy
Proliferating Fibroblasts	Thy 1.1 Transferrin Receptor Insulin & Insulin-like Growth-Factor Receptors Fibroblast Growth-Factor Receptor	Inhibition of Adhesions, Scarring Scleroderma
Proliferating Epithelium or Epidermal (Keratinocytes)	EGF Receptor Proto-Oncogenes	Psoriasis

Proliferating and activated T-cells can cause a wide variety of diseases ranging from the chronic inflammation of Crohn's disease to more acute organ graft rejection. In all of these diseases, the T-cell may serve a central pathogenic role or a more accessory role. Anti-proliferative chemotherapeutic drugs serve to reduce symptomotology and in some cases lead to long-term remission. Similarly, proliferating fibroblasts and epithelial cells may give rise to diseases characterized by cell overgrowth. Vitamin B_{12} receptor modulating agents may be used to replace or used in combination with existing chemotherapeutic regimens in these diseases. An important aspect of the use of anti-proliferative vitamin B_{12} receptor modulating agents in these diseases is not to apply it so aggressively or with improper timing such that normal healing (adhesions, scarring) or cell renewal (psoriasis) processes are also inhibited. As such, low doses of receptor modulating agents may be used during healing and higher doses once healing is completed. Alternatively, receptor modulating agents may not be administered at all until after healing is completed.

As previously mentioned, B_{12}/TcII receptor modulating agents can be used to deprive neoplastic cells of vitamin B_{12}. It has already been shown that sufficient deprivation leads to the death of rapidly proliferating lymphoid neoplasms such as leukemia and lymphoma. Moreover, short term treatment to reduce cellular availability of this nutrient, combined with existing chemotherapeutic agents, markedly improves therapeutic efficacy.

For solid tumors, vitamin B_{12} depletion may induce cytostasis and differentiation as well as cell death. Thus, B_{12}/TcII receptor modulating agents may be used to induce differentiation in hormonally responsive solid tumors. An increase in the number of cells expressing a differentiated phenotype should translate into an increase in expression of hormone receptors. The hormone receptor status of tumors, such as breast and prostrate cancer, are directly correlated with their response to hormonal therapy. Accordingly, B_{12}/TcII receptor modulating agents can be used to increase the number of receptor positive tumor cells or increase receptor density in order to enhance efficacy of subsequent hormonal therapy.

Vitamin B_{12} receptor modulating agents may affect both replicating neoplastic and normal cells. However, bone marrow progenitors demonstrate differential sensitivity or response. Thus, B_{12} receptor modulating agents can be used to modulate sensitivity of bone marrow progenitors so as to enhance their resistance to the toxic effects of chemotherapeutic agents. Such chemotherapeutic drugs act primarily on replicating cells, with non-replicating cells being much less sensitive. Decreasing the sensitivity of progenitors to toxic drugs would increase the bone marrow reserves and enhance subsequent response to colony stimulating factors, and enable higher doses of chemotherapy or reduce the interval to reconstitution. It should also be recognized that such positive effects on bone marrow progenitors, as a natural consequence of B_{12} receptor therapy for cancer, is an additional mechanism by which the therapeutic index of chemotherapeutic drugs other than 5-FU and methotrexate can be improved.

In a variety of autoimmune diseases, graft versus host disease, ectopic allergy, and organ transplantation, an initial 'induction' phase, in which the patient becomes sensitized to self or allo-antigens, is followed by a "proliferative" phase in which forbidden or unregulated clones of B- or T-cells are expanded. It has long been known that treatment with anti-proliferative, chemotherapeutic drugs following induction can inhibit expansion of forbidden clones, inhibit progression of disease, and restore a stable state of tolerance.

Inflammation is an application for which antibodies are already being utilized in clinical trials. The primary emphasis has been on inhibiting the early manifestations of inflammation by inhibiting recruitment or binding of inflammatory cells to vascular endothelium of injured tissue. It also well recognized that proliferation of cells at the site of inflammation contributes to the pathology and tissue destruction of both acute as well as chronic inflammation. To this end, anti-proliferative, chemotherapeutic drugs have been widely used to inhibit sequelae of inflammation.

Methotrexate is one such drug commonly used to treat symptoms associated with rheumatoid arthritis. The drug acts to reduce both localized (*e.g.*, synovium) and generalized inflammation associated with disease progression. Methotrexate acts synergistically with vitamin B_{12} depletion in therapy of leukemia. B_{12} receptor modulating agents can therefore be combined with methotrexate to enhance efficacy in rheumatoid arthritis. Other methotrexate applications include treating destructive inflammation associated with chronic heart disease and colitis.

Surgery, radiation or chemotherapy to the abdomen is often complicated by the development of tissue adhesions. These represent a considerable clinical problem because they lead to bowel blockage and require surgical intervention. Peritoneal adhesions arise as a result of proliferation of the cells of the peritoneal membrane lining the abdomen. A non-toxic means of interfering with such proliferation could lead to restoration of these normal cells to homeostatic control mechanisms and thereby inhibition of adhesion formation. A similar process of benign proliferation and subsequent scarring is a complication of retinal surgery. Direct instillation of a small molecule analog of an antibody receptor antagonist could prevent such disabling complications.

The term "treatment" as used within the context of the present invention, refers to reducing or alleviating symptoms in a subject, preventing symptoms from worsening or progressing, inhibition or elimination of the causative agent, or prevention of the infection or disorder in a subject who is free therefrom. Thus, for example, treatment of infection includes destruction of the infecting agent, inhibition of or interference with its growth or maturation, neutralization of its pathological effects and the like. A disorder is "treated" by partially or wholly remedying the deficiency which causes the deficiency or which makes it more severe.

The receptor modulating agents of the present invention are administered in a therapeutically effective dose. A therapeutically effective dose may be determined by in vitro experiment followed by in vivo studies.

Pharmaceutical compositions containing the receptor modulating agents in an admixture with a pharmaceutical carrier or diluent can be prepared according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration (*e.g.*, intravenous, oral topical, aerosol, suppository, parenteral or spinal injection). Preferably, administration is via stereotactical injection.

The following examples are offered by way of illustration, not limitation.

EXAMPLES

In summary, the examples which follow disclose the synthesis of several receptor modulating agents of this invention utilizing different functional classes of rerouting moieties. More specifically, a series of examples are presented which employ vitamin B_{12} as a targeting moiety in a receptor modulating agent.

All chemicals purchased from commercial sources were analytical grade or better and were used without further purification unless noted. Isophthaloyl dichloride was purchased from Lancaster Synthesis Inc. (Windham, NH). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Ion exchange chromatography was conducted with 200-400 mesh strongly basic anion 2% cross-linking Dowex-1-chloride (Aldrich Chemical Co). Amberlite XAD-2 nonionic polymeric adsorbent and octadecyl functionalized silica gel for column chromatography were obtained from Aldrich Chemical Co.

^{1}H NMR were obtained on Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as ppm (δ) using tetramethylsilane as internal reference. IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. UV data were obtained on a Perkin-Elmer Lambda 2 UV/V is spectrophotometer. Mass spectral data were obtained on a VG 7070H mass spectrometer using fast atom bombardment (FAB).

HPLC separations of compounds were obtained on Hewlett-Packard quaternary 1050 gradient pumping system with a UV detector. Analysis of the HPLC data were obtained on a Hewlett-Packard HPLC Chemstation software.

HPLC for Monomers: HPLC separations were conducted at a flow rate of 1 mL/min. on a 5 mm, 4.6 250 mm NH_2 column (RAININ microsorb-MV amino column) eluting with 58 mM pyridine acetate, pH 4.4 in H_2O : THF (96 : 4) solution. Retention times were: **1**= 4.3 min; **2** = 6.5 min; **3** = 8.0 min; **4** = 8.8 min; **5** = 10.9 min; **6** = 2.3 min; **7** = 2.3 min; **8** = 3.0 min; **9** = 2.9 min; **10** = 2.9 min; **13** = 3.4 min. Reverse-phase HPLC chromatography was carried out using a Hewlett-Packard Lichrospher 100 RP-18 (5 mm, 125 X 4 mm) C-18 column using a gradient solvent system at a flow rate of 1 mL/min. Solvent A in the gradient was methanol. Solvent B was H_2O. Starting from an 40% A, the gradient was increased to 100% A over 10 min. The gradient was then brought back to 40% A over a 5 min period. Retention times under these conditions for biotin conjugates were: **17** = 7.1 min; **18** = 7.2 min; **19** = 6.9 min; **20** = 6.4 min.

Preparative LC was conducted to separate the mixture of monocarboxylic acids using RAININ Rabbit-plus peristaltic pumping system with a DYNAMAX (model UV-1) UV-visible absorbance detector at a flow rate of 0.15 mL/min. ID column (Alltech, 150 psi), (1000 mm X 25 mm) packed with aminopropyl silica (40-63 mm) was used.

HPLC for Dimers: For dimers **36, 37,** and **38** solvent A in the gradient was methanol. Solvent B was H_2O. The gradient was held at the starting mixture of 70% A for 2 min, then the percentage of A was linearly increased to 100% over the next 10 min. The gradient was held at 100% A for 20 min. Retention times under these conditions for dimers were: **36** = 8.7 min; **37** = 9.0 min; **38** = 8.9 min. For dimers **58-60** and **64-66** Solvent A in the gradient was methanol. Solvent B was aqueous 1% acetic acid. The gradient was begun at 40% A and was held at that composition for 2 min, then the percentage of A was linearly increased to 100% over the next 10 min. Retention times for the compounds examined under these conditions were: **58** = 14.0 min; **59** = 14.1 min; **60** = 13.9 min; **64** = 8.7 min; **65** = 8.6 min; **66** = 9.0 min.

EXAMPLE 1

PREPARATION AND PURIFICATION OF CYANOCOBALAMIN MONOCARBOXYLATES: MODIFICATION ON THE CORRIN RING

This example serves to demonstrate the hydrolysis of *b*-, *d*- and *e*-propionamide sites on a vitamin B_{12} molecule using dilute acid in preparation for coupling of a linker to the sites. Importantly, the hydrolysis of the *b*-, *d*- and *e*-propionamides is selective over the hydrolysis of *a*-, *c*- and *g*-acetamides, or the *f*-amide in the heterocyclic chain connecting the benzimidazole. An optimal yield of monocarboxylate to di- and tri-carboxylate derivatives was obtained at room temperature in 0.1 N HCl over a 10 day period. The non-hydrolyzed vitamin B_{12} and the di- and tri-carboxylates produced were readily isolated from the desired monocarboxylates by preparative liquid chromatography.

Specifically, cyanocobalamin (**1**) (3.7 mmol, 5 g) was dissolved in 500 mL of 0.1 N HCl and stirred at room temperature for 10 days under argon atmosphere. The solution was then neutralized with 6 N NaOH and the cobamides were desalted by extraction into phenol and applied to a 200 g (60 x 4 cm, 200-400 mesh) Dowex Cl^- x 2 column (acetate form; prepared by washing with saturated sodium acetate until it was free from Cl^-, then washing with 200 mL water). The column was eluted with water to

remove unreacted cyanocobalamin and then eluted with 0.04 M sodium acetate (pH 4.67).

The first fraction of the elution contained three monocarboxylic acids. These were desalted by extraction into 100 mL of 90% (w/w) phenol, twice with 25 mL and once with 10 mL of phenol. Three volumes of ethyl ether (3 x 160 mL) and 1 volume of acetone (160 mL) were added to the combined phenol extracts. Monocarboxylic acids were removed from the organic phase by extraction with water (2 x 100 mL). The combined aqueous phases were extracted twice with 20 mL of ether to remove residual phenol. The aqueous solution of monocarboxylic acids was evaporated to dryness. Yield: 2.5 g (50%).

The mixture of three acids (0.350 g) was then applied to a 200 g (1000 mm x 25 mm) column of aminopropyl coated silica (40-63 mm) and was eluted with 58 mM pyridine acetate pH 4.4 in H_2O : THF (96 : 4); the elute was collected with an automatic fraction collector. The first eluted acid was found to be *b*-monocarboxylic acid **(2)**, the second eluted acid was *e*-monocarboxylic acid **(3)** and the third eluted acid was *d*-monocarboxylic acid **(4)**. The acid fractions were desalted by phenol extraction. The solids obtained were crystallized from aqueous acetone.

b-acid **(2)**: yield 0.122 g (35%), mp 267-270°C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.00 (m, 2H); 1.18 (s, 3H, C-46 CH_3); 1.24 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.9 (d, 7H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.26 (d, 6H, B10 & B11, CH_3); 2.36 (d, 2H, C-26 CH_2); 2.57 (s, 10H, C-35 CH_3, C-31 CH_2, C-37 CH_2, C-53 CH_3); 2.8 (m, 2H, C-60 CH_2); 3.3 (m, 3H, C-8H, C-13H); 3.6 (m, 2H, Pr_1 CH_2); 3.7 (d, 1H, R_5); 3.9 (d, 1H, R_5); 4.0 (m, 1H, R_4); 4.12 (d, 1H, C-19); 4.17 (s, 1H, C-3); 4.3 (m, 1H, R_2); 4.5 (m, 1H); 4.7 (m, 1H, R_3); 6.0 (s, 1H, C-10); 6.2 (s,1H, R_1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε23441)

e-acid **(3)**: yield 0.168 g (48%), mp 245-250° C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.01 (m, 2H); 1.15 (s, 3H, C-46 CH_3); 1.23 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.83 (s, 4H, C-55 CH_2); 1.93 (m, 6H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.22 (d, 6H, B10 & B11 CH_3); 2.35 (s, 3H,C-26 CH_2); 2.5 (d, 13H, C-35 CH_3, C-31 CH_2, C-37 CH_2, C-53 CH_3); 2.9 (m, 1H, C-60 H); 3.2 (m, 1H, C-13H); 3.4 (m, 1H, C-8 H); 3.6 (d, 1H, Pr1 CH); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 2H); 4.1 (d, 1H); 4.2 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s,

1H, B7). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε21 842)]

d-acid (4): yield 0.060 g (17%), mp > 300° C, ^1H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.04 (m, 2H); 1.15 (s, 3H, C-46 CH_3); 1.25 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.85 (s, 4H); 2.01 (s, 6H); 2.23 (d, 8H, B10 & B11 CH_3); 2.38 (d, 3H, C-26 CH_2); 2.53 (d, 13H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.6 (m, 5H); 2.9 (m, 1H, C-60 H); 3.3 (d, 1H, C-13H); 3.4 (m, 1H, C-8 H); 3.6 (d, 1H, Pr_1 CH); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 2H); 4.1 (d, 1H); 4.3 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); UV (MeOH): λ360 (ε22 127). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 2

CYANOCOBALAMIN MODIFIED ON RIBOSE: SUCCINATE CONJUGATE (5)

This example serves to demonstrate the activation of the ribose coupling site coupling site *h* (*see* structure I) with succinic anhydide. Cyanocobalamin (1) (0.15 mmoL, 200 mg) was dissolved in 40 mL of dimethylsulfoxide (DMSO) containing 8 g (80 mmoL) of succinic anhydride and 6.4 mL of pyridine. After 14-16 h at room temperature, the excess of succinic anhydride was destroyed by adding 500 mL of water and keeping the pH of the reaction mixture at 6 with 10% KOH. KCN was then added at a final concentration of 0.01 M and the pH of the solution was readjusted to 6 with 3 N HCl. After 1 h the cyanocobalamin components were desalted by phenol extraction and applied to a 100 g of Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The cyanocobalamin was eluted with water. Succinate conjugate (5) was eluted with NaOAc (0.04 M, pH 4.67) which yielded 180 mg (85 %) after isolation. The O2',O5'-disuccinyl derivative remained absorbed on the column under these conditions. mp 208-210° C with decomposition.

^1H NMR (D_2O-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 0.95 (m, 2H); 1.15 (s, 3H); 1.2 (d, 3H); 1.35 (d, 7H); 1.4 (s, 3H); 1.8 (s, 3H); 1.9 (s, 12H); 2.2 (d, 6H); 2.36 (d, 2H); 2.5 (d, 10H); 2.6-2.7 (m, 7H); 3.0 (m, 1H); 3.3 (d, 1H); 3.37 (m, 1H); 3.5 (d, 1H); 4.0 (d. 1H); 4.18 (m, 2H); 4.25 (m, 3H); 4.54 (d, 1H); 6.0 (s, 1H); 6.3 (d, 1H); 6.4 (s, 1H); 7.0 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1455 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε 26041).

EXAMPLE 3

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH 1,12-DIAMINODODECANE: REACTION WITHOUT SODIUM CYANIDE

This example serves to demonstrate the coupling of a linker to a cyanocobalamin monocarboxylate. Coupling of the monocarboxylates (**2, 3, 4**) with diaminododecane was first attempted using N-ethyl-N'-dimethylamino-propyl-carbodiimide hydrochloride (EDC) in H_2O according to Yamada and Hogenkamp, J. Biol. Chem. 247, 6266-6270, 1972. However, the products obtained did not have a reactive amino group. Alteration of the reaction conditions by changing the reaction mixture to DMF/H_2O and adding NaCN/N-hydroxysuccinimide (*see* Example 4) to the reaction mixture gave the desired diaminododecane adducts.

A mixture of cyanocobalamin monocarboxylic acid (0.370 mmoL, 500 mg) and 1,12-diaminododecane (3.6 g) in 100 mL H_2O was adjusted to pH 6 with 1 N HCl. The solution was then treated with N-ethyl-N'-dimethylamino-propyl-carbodiimide-hydrochloride (EDC) (726 mg) and stirred at room temperature for 22 h. In 5 intervals of 6 to 14 h, 650 mg of EDC was added to the reaction mixture. After a total reaction time of 4 days (HPLC monitoring) the solution was evaporated to dryness, the residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of water and applied to an 175 g Amberlite XAD-2 (60 x 4 cm) column. Contaminates were washed from the column with 1L water, then the crude product was eluted with 500 mL of methanol. The solution was evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The final product was eluted using 250 mL of water, thereby leaving non-converted acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer pH 4.67. The fraction containing the final product was evaporated to dryness.

The mass spectral value obtained indicated that HCN was lost from the desired product. Further, ^1H NMR data suggested that some protons were being affected by the cobalt. Thus, this reaction was conducted with NaCN (Example 4) to drive the equilibrium towards retention of Co-CN. N-hydroxy succinimide was also added to facilitate the coupling reaction.

e-acid adduct (**6**): Yield: 222 mg (40%). mp 172-174° C with decomposition. ^1H NMR (MeOH-d_4, δ): 0.43 (m, 3H, C-20 CH_3); 1.06 (t, 4H, C-46 CH_3); 1.16 (m, 5H); 1.2 (m, 5H); 1.33 (m, 7H); 1.43 (s, 3H); 1.68 (m, 4H); 1.86 (m,

5H); 2.2 (m, 8H); 2.3 (m, 6H); 2.4 (m, 10H); 2.55 (m, 10H); 2.8 (m, 4H); 3.1 (m, 6H); 3.3 (m, 5H); 3.6 (m, 2H); 3.7 (m, 2H); 3.8 (m, 1H); 4.0 (m, 1H); 4.1 (m, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 1H); 6.0 (d 1H, C-10); 6.2 (m, 1H, R1); 6.5 (m, 1H, B4); 7.1 (m, 1H, B2); 7.2 (m, 1H, B7). MS (FAB^+): m/e 1512. IR (KBr): 3400, 3200, 2950, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε21 877).

d-acid adduct (7): yield: 225 mg (45%), mp 195-198° C with decomposition. ^1H NMR (MeOH-d_4, δ): 0.43 (m, 3H, C-20 CH_3); 1.09 (m, 7H); 1.14 (m, 6H); 1.2 (m, 10H); 1.27 (m, 10H); 1.33 (m, 6H); 1.5 (m, 3H); 1.77 (s, 3H); 2.2 (m, 8H); 2.26 (s, 2H); 2.5 (m, 10H); 2.7 (m, 5H); 3.0 (m, 2H); 3.1 (m, 2H); 3.2 (m, 3H); 3.5 (m, 2H); 3.6 (m, 1H); 3.8 (m, 1H); 3.9 (m, 1H); 4.0 (m, 1H); 4.1 (m, 1H); 4.2 (m, 1H); 4.4 (m, 1H); 4.6 (m, 1H); 6.0 (d 1H, C-10); 6.1 (m, 1H, R_1); 6.4 (m, 1H, B4); 7.0 (m, 1H, B2); 7.1 (m, 1H, B7); MS (FAB^+): m/e 1512, IR (KBr): 3400, 3200, 2950, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε22 680).

EXAMPLE 4

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH 1,12-DIAMINODODECANE: REACTION CONTAINING SODIUM CYANIDE

Cyanocobalamin monocarboxylic acid **(2, 3, 4)** (0.370 mmoL, 500 mg) and N-hydroxysuccinimide (1.48 mmoL, 170 mg) were dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and 363 mg of NaCN was added. 1,12-Diaminododecane was dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and the pH was adjusted to 6 with 1 N HCl. The diaminododecane solution was then added in one portion to the cyanocobalamin solution. EDC (285 mg) was added and the pH of the solution was readjusted to 5.5. The reaction mixture was then stirred overnight in the dark at room temperature. In 5 intervals of 6-14 h, 170 mg of N-hydroxysuccinimide and 285 mg of EDC were added to the solution, readjusting the pH value 5.5 each time. After a total reaction time of 4 days (reaction followed by HPLC), the solution was evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of H_2O and applied to an 200 g Amberlite XAD-2 (60 x 4 cm) column. The column was eluted with 1 L water to remove undesired materials, then the desired product was eluted with 500 mL methanol. The solution was evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100 g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The desired product was eluted from the column with 250 mL water, leaving any non-reacted acid bound to the column. This was followed by elution with 0.04

mol/L sodium acetate buffer pH 4.7. The fractions containing the final product were evaporated to dryness.

b-isomer (**8**): yield 410 mg (82%), mp 172-174° C with decomposition. ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.18 (s, 4H); 1.3 (m, 13H); 1.39 (m, 13H); 1.45 (s, 5H); 1.6 (m, 4H); 1.72 (m, 2H); 1.9 (s, 6H); 2.25 (d, 6H, B10 & B11 CH_3); 2.35 (m, 5H); 2.56 (m, 5H); 2.8-3.0 (m, 8H); 3.15 (m, 4H); 3.3 (m, 2H); 3.4 (m, 2H); 3.6 (m, 1H); 3.68 (m, 1H); 3.75 (m, 1H); 3.9 (d, 1H); 4.07 (m, 1H); 4.12 (d, 1H); 4.2 (br s, 1H); 4.3 (m, 1H); 4.47 (m, 1H); 4.7 (m, 1H); 6.0 (s, 1H, C-10); 6.2 (d,1H, R_1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB$^+$): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε15409).

e-isomer (**9**): yield: 430 mg (86%), mp 175-180° C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.22 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.6 (m, 3H); 1.87 (s, 8H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.36 (m, 3H); 2.55 (d, 10H); 2.8 (s, 4H); 3.06 (t, 2H); 3.1 (m, 3H); 3.3 (s, 1H); 3.34 (m, 1H); 3.4 (m, 1H); 3.58 (m, 1H); 3.65 (m, 1H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 2H); 4.48 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB$^+$): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε16 720)

d-isomer (**10**): yield: 400 mg (80%), mp 174-178° C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.07 (m, 3H, C-46 CH_3); 1.2 (d, 4H, Pr_3 CH_3); 1.27 (m, 15H); 1.35 (br s, 9H); 1.42 (s, 3H); 1.53 (m, 2H); 1.6 (m, 4H); 1.86 (s, 4H); 2.25 (d, 6H, B10 & B11 CH_3); 2.5 (d, 10H); 2.8 (s, 3H); 2.9 (m, 6H); 3.15 (m, 3H); 3.2 (m, 4H); 3.4 (m, 3H); 3.6 (d, 1H); 3.75 (d, 1H); 3.96 (d, 1H); 4.08 (m, 2H); 4.19 (m, 1H); 4.3 (m, 2H); 4.65 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); UV (MeOH): λ360 (ε17 665). MS (FAB$^+$): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 5

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH GAMMA-AMINOBUTYRIC ACID (GABA)

This example serves to demonstrate the coupling of a gamma-aminobutyric acid (GABA) linker to a vitamin B_{12} molecule. This reaction scheme is represented in Figure 9.

Gamma-aminobutyric acid (GABA) *tert*-butyl ester **(11)** (1 mmol) and cyanocobalamin monocarboxylates **(2, 3, 4)** (0.1 mmol.) are mixed in 20 mL H_2O and sufficient 0.1 N HCl is added to adjust to pH to 6.0. N-ethyl-N^1-dimethylaminopropylcarbodiimide hydrochloride (EDC) (0.5 mmol) is added to the solution. The reaction mixture is stirred at room temperature for 24 hours and then the mixture is dried under vacuum. This reaction mixture is treated with TFA to remove the *tert*-butyl ester. A cyanocobalamin-GABA adduct **(12)** was purified. Reverse-phase HPLC chromatography is carried out as described above. A cyanocobalamin-GABA adduct **(12)** can be further activated with a carbodiimide and coupled to a moiety as described below.

EXAMPLE 6

CYANOCOBALAMIN MODIFIED ON RIBOSE: SUCCINATE-DIAMINODODECANE CONJUGATE (13)

Cyanocobalamin-Ribose-Succinate **(5)** (0.370 mmoL, 538 mg) and N-hydroxylsuccinimide (1.48 mmoL, 170 mg) were dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and 363 mg of NaCN was added. This reaction scheme is represented in Figure 11. 1,12-Diaminododecane was taken in a mixture of DMF : H_2O (1:1) (18.4 mL), pH was adjusted to 6 with 1N HCl. The diaminododecane solution was then added in a portion to the cyanocobalamin solution. EDC (285 mg) was added, the pH of the solution was readjusted to 5.5 and the reaction mix. was stirred overnight in the dark at room temperature. In 5 intervals of 6 to 14 h 170 mg of N-hydroxysuccinimide and 285 mg of EDC was added to the solution, readjusting the pH 5.5 each time. After a total reaction time of 4 days (HPLC monitored) the solution was evaporated to dryness, the residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of H_2O and applied to an 200 g Amberlite XAD-2 (60 x 4 cm) column. Contaminates were washed from the column with 1 L water and then the crude product was eluted with 500 mL methanol. The solution was

evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100 g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The final product was eluted using 250 mL water, thereby leaving non-converted acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer pH 4.7. The fraction containing the final product **(13)** was evaporated to dryness. Yield : 425 mg (70%), mp 185-187° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.15 (s, 3H); 1.2 (d, 3H); 1.3 (s, 27H); 1.4 (m, 3H); 1.55 (m, 6H); 1.85 (m, 12H); 2.2 (d, 6H); 2.3 (d, 6H); 2.5 (d, 10H); 2.8 (m, 10H); 3.0 (t, 3H); 3.1 (t, 3H); 3.2 (s, 6H); 3.3 (m, 4H); 3.58 (m, 2H); 3.6 (d, 1H); 4.1 (d. 1H); 4.2 (m, 2H); 4.3 (m, 1H); 4.4 (d, 1H); 6.0 (s, 1H); 6.2 (d, 1H); 6.5 (s, 1H); 7.1 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1638 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360.

EXAMPLE 7

MODIFICATION OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS CONJUGATED WITH 1,12-DIAMINODODECANE: REACTION WITH SUCCINIC ANHYDRIDE

This example serves to demonstrate modification of an amino terminus linking moiety to a carboxylate terminus. Such a modification may be necessary for conjugating amino containing rerouting agents (*e.g.*, aminosugars) to cyanocobalamin derivatives containing a linker.

Cyanocobalamin carboxylic acid diaminododecane conjugate **(8, 9, 10)** (0.138 mmoL, 200 mg) was dissolved in 40 mL of dimethylsulfoxide (DMSO) containing 8 g (80 mmoL) of succinic anhydride and 6.4 mL of pyridine. After 14-16 h at room temperature, the excess of succinic anhydride was destroyed by adding 500 mL of water and keeping the pH of the reaction mixture at 6 with 10% KOH. KCN was then added at a final concentration of 0.01 M and the pH of the solution was readjusted to 6 with 3 N HCl. After 1 h the cyanocobalamin components were desalted by phenol extraction. The residue was digested with 100 mL of acetone and the solvent was decanted. It was dissolved in 40 mL of H_2O. 1N NaOH (2 mL) was added to it and the reaction was stirred at room temperature for 15-20 min. It was then neutralized with 1N HCl and the cyanocobalamin components **(14, 15, 16)** were desalted by phenol extraction. Yield: 80 mg (40%); mp 190-198° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.23 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.87 (s, 4H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.35 (m, 3H); 2.4 (m, 5H); 2.55 (d,

10H); 2.7 (s, 5H); 2.8 (m, 2H); 3.1 (m, 6H); 3.3 (s, 6H); 3.4 (m, 1H); 3.65 (m, 2H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7). MS (FAB^+): m/e 1639 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε 22 564).

EXAMPLE 8

CYANOCOBALAMIN MODIFIED ON MONOCARBOXYLIC ACID: DIAMINODODECANE-BIOTIN CONJUGATES

This example serves to demonstrate coupling a vitamin B_{12} derivative and biotin. Biotin conjugates **(17, 18, 19)** were obtained by reaction of activated cyanocobalamin monocarboxylic acid diaminododecane **(14), (15),** and **(16)** with the NHS ester of biotin (Sigma Chemical Co.).

To a solution of cyanocobalamin monocarboxylic acid diaminododecane conjugate **(14, 15, 16)** (300 mg, 0.195 mmoL) in DMF (35 mL), was added triethylamine (0.027 mL, 0.195 mmoL). N-Hydroxysuccinimidobiotin (100 mg, 0.295 mmoL) was then added over a period of 10-15 min and evaporated to dryness. The solid residue was dissolved in 20 mL of water and applied to an 75 g of Dowex Cl^- (40 x 2 cm) (acetate form, 200-400 mesh) column. The product was eluted using 250 mL of water. It was then evaporated to dryness, the residue was dissolved in a 10 mL of methanol - water (7:3 v/v) and the solution was applied to a reverse phase C-18 column (500 mm x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector. The eluate was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-isomer **(17)**: yield 159 mg (53%), mp 210-212° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.18 (s, 4H); 1.3 (m, 13H); 1.39 (m, 13H); 1.45 (s, 5H); 1.6 (m, 4H); 1.72 (m, 2H); 1.9 (s, 6H); 2.2 (d, 8H, B10 & B11 CH_3); 2.6 (d, 12H); 2.7 (m, 3H); 2.8-3.0 (m, 8H); 3.1 (m, 3H); 3.2 (m, 2H); 3.4 (s, 1H); 3.6 (m, 2H); 3.68 (d, 1H); 3.75 (m, 1H); 3.9 (d, 1H); 4.07 (m, 1H); 4.12 (d, 1H); 4.2 (s, 1H); 4.3 (m, 1H); 4.47 (m, 1H); 4.7 (m, 1H); 6.0 (s, 1H, C-10); 6.2 (d,1H, R1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε23 746).

Anal. Calcd. for $C_{85}H_{127}N_{17}O_{16}CoPS \cdot 11H_2O$: C, 51.98; H, 7.59; N, 12.13. Found: C, 51.91; H, 7.81; N, 12.31.

e-isomer **(18)**: yield 174 mg (58%), mp 222-224° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.22 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.6 (m, 4H); 1.72 (m, 2H); 1.87 (s, 4H); 2.17 (m, 3H); 2.25 (s, 6H, B10 & B11 CH_3); 2.36 (m, 3H); 2.55 (d, 10H); 2.64 (m, 2H); 2.8 (s, 4H); 2.97 (s, 4H); 3.1 (m, 3H); 3.3 (m, 1H); 3.4 (m, 1H); 3.58 (m, 1H); 3.65 (m, 1H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 2H); 4.48 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε24 441).

Anal. Calcd. for $C_{85}H_{127}N_{17}O_{16}CoPS \cdot 9H_2O$ (13): C, 52.96; H, 7.53; N, 12.35. Found: C, 52.85; H, 7.55; N, 12.30.

d-isomer **(19)**: yield 165 mg (55%), mp 216-218° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.16 (s, 3H, C-46 CH_3); 1.2 (d, 4H, Pr_3 CH_3); 1.28 (s, 15H); 1.35 (br s, 9H); 1.42 (s, 3H); 1.53 (m, 2H); 1.6 (m, 4H); 1.72 (m, 2H); 1.86 (s, 6H); 2.16 (m, 3H); 2.02 (m, 4H); 2.25 (d, 6H, B10 & B11 CH_3); 2.5 (d, 10H); 2.7 (d, 1H); 2.8 (m, 5H); 3.1 (m, 6H); 3.2 (m, 3H); 3.4 (m, 1H); 3.57 (m, 1H); 3.6 (d, 1H); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.11 (d, 1H); 4.17 (m, 1H); 4.3 (m, 2H); 4.4 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε29 824).

Anal. Calcd for $C_{85}H_{127}N_{17}O_{16}CoPS \cdot 10H_2O$: C, 52.46; H, 7.56; N, 12.24. Found: C, 52.27; H, 7.56; N, 12.34.

EXAMPLE 9

CYANOCOBALAMIN MODIFIED ON RIBOSE: SUCCINATE-DIAMINODODECANE-BIOTIN CONJUGATE (20)

This example serves to demonstrate the conjugation of the ribose-linked diaminododecane adduct **(13)** with biotin to produce a cyanocobalamin biotin conjugate **(20)**.

To a solution of **(11)** (300 mg, 0.183 mmoL) in DMF (35 mL), triethylamine (0.025 mL, 0.183 mmoL) was added. N-hydroxysuccinimidobiotin (100

mg, 0.295 mmoL) was added over a period of 10-15 min. and then evaporated to dryness. The solid residue was dissolved in 20 mL of water and adjusted to pH 10 with 1N NaOH and applied to an 75 g Dowex Cl^- (40 x 2 cm) (200-400 mesh) column. The water fraction was discarded. The product was then eluted with 0.1N NH_4OAc and was desalted by phenol extraction. The residue was dissolved in a 10 mL of methanol - water (7:3 v/v) and the solution was applied to a reverse phase column (octadecyl) which was developed with the same solvent. The fractions containing the final product **(20)** (HPLC monitored) were evaporated to dryness. Yield 135 mg (45 %), mp 198-205 ° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.15 (s, 3H); 1.2 (d, 3H); 1.3 (s, 27H); 1.36 (m, 6H); 1.4 (m, 3H); 1.6 (m, 4H); 1.7 (m, 2H); 1.85 (m, 12H); 2.0 (d, 3H); 2.17 (m, 3H); 2.2 (d, 6H); 2.3 (d, 6H); 2.5 (d, 10H); 2.64 (m, 2H); 2.8 (m, 10H); 3.1 (m, 6H); 3.25 (m, 6H); 3.58 (m, 2H); 4.0 (m, 1H); 4.1 (m, 1H); 4.16 (m, 1H); 4.4 (m, 1H); 4.6 (s, 2H); 4.7 (m, 1H); 6.0 (s, 1H); 6.2 (d, 1H); 6.5 (s, 1H); 7.1 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1866 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε28 434).

EXAMPLE 10

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (STREPTOMYCIN) RECEPTOR MODULATING AGENT

This example demonstrates coupling of streptomycin to a cyanocobalamin or cobalamin derivative. Streptomycin **(21)** is conjugated with cyanocobalamin monocarboxylate **(2, 3, 4)** or a diaminoalkylsuccinate derivative **(14, 15, 16)** through the use of an oxime coupled linking moiety (Figure 13). The linking group, ((3-aminopropyl)aminoxy)acetamide **(22)** is prepared by reaction of the N-hydroxysuccinimidyl ester of 1,1-dimethylethoxycarbonyl-aminooxyacetic acid **(23)** (J. Med. Chem. 36:1255-126, 1993) with an excess of diaminopropane in anhydrous THF. The linking group is separated from other compounds in the reaction mixture by preparative chromatography. The linker (1 g) is then mixed with streptomycin (0.5g) in 10 mL of H_2O containing sodium acetate. The aqueous solution is warmed in a H_2O bath for 10 minutes to yield a crude streptomycin-linker adduct **(25)** which may be purified by chromatography on acid washed alumina (J. Am. Chem. Soc. 68:1460, 1946). The aqueous solution containing the streptomycin linker adduct (0.15 mmol) is mixed with an aqueous solution of activated cyanocobalamin **(2, 3, 4)** (01. mmol) and EDC (0.5 mmol) is added. The reaction mixture is stirred at room temperature for 24

hours, then run over a reversed-phase preparative chromatography column for purification of the cyanocobalamin-streptomycin receptor modulating agent **(26)**.

EXAMPLE 11

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (ACRIDINE) RECEPTOR MODULATING AGENT

This example demonstrates the coupling of the vitamin B_{12} to acridine. Chloroquine, quinacrine and acridine are lysosomotropic dyes which are relatively non-toxic and concentrated as much as several hundred fold in lysosomes. Acridine derivatives may be covalently attached to a targeting moiety (such as cyanocobalamin) by the reaction scheme illustrated in Figure 14, method A, or similarly as described in method B. Both reaction schemes produce a cyanocobalamin-acridine conjugate.

Method A: A diamine side chain is first synthesized in a manner analogous to the side chain of quinacrine. Specifically, mono-phthaloyl protected 1,4-diaminobutane **(27)** is reacted with 6,9-dichloro-2-methoxyacridine **(28)** in phenol (J. Am. Chem. Soc. 66:1921-1924, 1944). The reaction mixture is then poured into an excess of 2 N NaOH and extracted with ether. The ether extract is washed with 1 M $NaHCO_3$, then H_2O, and dried over $MgSO_4$. The crude product is recrystallized from H_2O-alcohol. The phthaloyl protecting group is removed using anhydrous hydrazine in MeOH (Bioconjugate Chem. 2:435-440, 1991) to yield the aminoacridine, **(29)**. Aminoacridine **(29)** is then conjugated with vitamin B_{12} monocarboxylic acid **(2, 3, 4)** to yield a cyanocobalamin-acridine conjugate **(30)**.

Method B: Acridine derivative **(31)** (0.098 mmol, 0.045 g) was dissolved in 0.5 mL of trifluoroacetic acid. This solution was stirred at room temperature for 0.5 h. TFA was removed by aspirator vacuum. The residue was dissolved in 5 mL of acetonitrile and was neutralized by few drops of triethylamine. Acetonitrile was then removed by aspirator vacuum. The residue was dissolved in DMSO (10 mL) and cyanocobalamin carboxylic acid-diaminododecane-succinyl derivative **(15, 16, 17)** (0.098 mmol, 134 mg) was added followed by triethylamine (12 µL). The reaction mixture was then stirred at room temperature for 24 h. (HPLC monitored), and evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted yielding a cyanocobalamin-acridine conjugate **(32)**. Yield: 120 mg (62%). mp 182-188 °C.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.23 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.65 (m,

2H); 1.87 (s, 4H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.35 (m, 3H); 2.4 (d, 5H); 2.44 (d, 2H); 2.55 (d, 10H); 2.64 (s, 5H); 2.8-2.9 (m, 8H); 3.1-3.15 (m, 6H); 3.3 (s, 6H); 3.4 (m, 1H); 3.65 (m, 2H); 3.75 (d, 1H); 3.9 (d, 1H); 3.98 (s, 2H); 4.0 (m, 2H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); 7.3 (t, 1H); 7.4 (dd, 1H); 7.6 (dd, 1H); 7.7 (2dd, 2H); 7.8 (d, 1H); 7.9 (d, 1H); 8.4 (d, 1H).

EXAMPLE 12

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (AMIKACIN) RECEPTOR MODULATING AGENT

This example demonstrates conjugation of amikacin to a cyanocobalamin molecule to form a cyanocobalamin-amikacin conjugate. A reaction scheme for the conjugation is depicted in Figure 12. As noted above, chemical moieties that are retained subcellularly within lysosomes are termed lysosomotropic. Aminoglycosides are lysosomotropic compounds, and thus may be used as rerouting moieties of this invention. The primary long chain amine on the hydroxyaminobutyric acid side chain of the aminoglycoside, amikacin (*see* Figure 3), is preferentially reactive. Specifically, amikacin **(33)** (Sigma Chemical Co., St. Louis), is reacted with a vitamin B_{12} monocarboxylate **(2, 3, 4)** in the presence of EDC. A cyanocobalamin-amikacin conjugate **(34)** is then separated and purified by reverse-phase LC chromatography under conditions noted above.

EXAMPLE 13

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHALOYL DICHLORIDE CROSS-LINKING

This example demonstrates the production of a cyanocobalamin dimer suitable for use as a cross-linking receptor modulating agent. Cross-linking of receptors in some receptor systems is sufficient to cause a rerouting of cell surface receptors to lysosomes for degradation, rather than their normal pathway of receptor recycling.

To a solution of cyanocobalamin monocarboxylic acid diaminododecane conjugate **(8, 9, 10)** (0.192 mmol, 0.300 g) in DMF (30 mL), was added triethylamine (18 µL). Isophthaloyl dichloride **(35)** (0.096 mmol, 0.0195 g) was added over a period of 10-15 min. The reaction mixture was stirred at 55-60°C for 48 h (HPLC monitored) and evaporated to dryness. The solid residue was dissolved in 20 mL of methanol :

H_2O (7:3) and applied to a reverse phase C-18 column (500 mm x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector; the elute was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-acid dimer (**36**): yield 96 mg (30%), mp 217-220° C with decomposition, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.18 (s, 8H); 1.3 (m, 36H); 1.37 (m, 12H); 1.46 (s, 10H); 1.6 (m, 8H); 1.9 (d, 12H); 2.05 (m, 10H); 2.2 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8-3.0 (m, 16H); 3.15 (m, 6H); 3.3 (s, 8H); 3.37 (m, 14H); 3.6 (m, 4H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (s, 2H); 4.68 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.95 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV: λ360 (ε42 380).

e-acid dimer (**37**): yield 121 mg (38%), mp 220-222° C with decomposition, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.17 (s, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.87 (s, 8H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.8 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 14H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (s, 2H); 4.66 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.93 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε 33 854)

d-acid dimer (**38**): yield 96 mg (30%), mp 225-228° C with decomposition, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.16 (s, 8H); 1.29 (m, 36H); 1.35 (d, 12H); 1.44 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.85 (s, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (m, 8H); 3.13 (m, 8H); 3.28 (s, 12H); 3.35 (m, 12H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.64 (m, 2H); 4.7 (s, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.93 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} UV (MeOH): λ360 (ε 31 747).

EXAMPLE 14

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ETAC CROSS-LINKING

This example serves to illustrate synthesis of a bivalent receptor modulating agent using a heterotrifunctional cross-linker. The reaction scheme for this synthesis is depicted in Figure 15. The heterotrifunctional cross-linker is formed an ETAC reagent (Bioconjugate Chem. 1:36-50, 1990; Bioconjugate Chem. 1:51-59, 1990; J. Am. Chem. Soc. 101:3097-3110, 1979). Bivalency, in addition to enhancing affinity of binding, also imparts the ability to cross-link neighboring receptors and trigger endocytosis. The bivalent "arms" of the agent may be lengthened with peptide or other linking molecules to enable simultaneous binding of both "arms". In the case of vitamin B_{12} this may be assessed by gel filtration. If the linkers allow simultaneous interaction, there will be 2 moles of TcII for every mole of ETAC dimer present in a single peak of 80,000 m.w. (versus 40,000 m.w. of monomeric TcII). Simultaneous binding of 2 moles of TcII will then have the potential for bivalent binding to cell surface receptor. This can be tested by comparing the affinity of monomer and dimer binding to receptor. While the bivalent agent can be synthesized to include any rerouting moiety of this invention which enhances lysosomal targeting and retention, the compound tyramine, useful for radio-labeling is disclosed for the purpose of illustration.

Referring to Figure 15, carboxy-ETAC **(39)** is prepared by the method of Liberatore et al. (Bioconjugate Chem. 1:1990). The carboxy-ETAC is converted to its acid chloride by reaction in thionyl chloride. Addition of amine **(40)** gives the amine-ETAC adduct **(41)**. Reaction of amine-ETAC (1 mmol) in CH_3CN with 1 M aqueous cysteamine (10 mmol) is conducted by stirring at room temperature for 24 h. This compound is reduced with $NaCNBH_3$ under acidic conditions. The crude amine-ETAC-cysteamine adduct **(42)** is purified by reverse-phase LC, using conditions noted above. A vitamin B_{12} monocarboxylate **(2, 3, 4)** is conjugated with tyramine-ETAC-cysteamine compound by reaction with EDC in H_2O. The resultant vitamin B_{12}-ETAC-tyramine dimer **(43)** is purified by reverse phase LC, using conditions described above.

EXAMPLE 15

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHLATE CROSS-LINKING WITH BIOTIN MOIETY

This example illustrates the synthesis of a bivalent receptor modulating agent which is additionally coupled to a biotin moiety **(44)**. Further modification can be obtained by coupling of this molecule with an avidin or streptavidin moiety.

Reaction Step A: Biotin (12.3 mmol, 3 g) was dissolved in warm (bath temperature 70°C) DMF (60 mL) under argon atmosphere. It was then cool to ambient temperature and DCC (13.5 mmol, 2.79 g) was added, followed by tetrafluorophenol (24.6 mmol, 4.08g). The reaction mixture was then cooled to 0°C and stirred for 0.5 h. It was then brought back to ambient temperature and stirred for another 4-5 h. The reaction mixture was filtered and the filtrate was evaporated to dryness. The precipitate was washed with acetonitrile (50 mL) and was filtered to yield 5 g (98%) of white solid **(45)**.

^{1}H NMR (DMSO, δ): 1.4 (m, 2H); 1.7 (m, 2H); 2.5 (t, 2H); 2.8 (t, 2H); 3.1 (m, 1H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.9 (m, 1H).

Reaction Step B: 6-Aminocaproic acid **(46)** (7.5 mmol, 0.99g) was dissolved in H_2O (75 mL). Triethylamine (0.5 mL) was added followed by a solution of TFP ester of Biotin (5 mmol, 1.96 g) in warm acetonitrile (300 mL). The reaction was stirred overnight at room temperature. It was then filtered, washed with H_2O (50 mL) and dried on high vacuum. Yield: 0.870 g (47%). The filtrate was evaporated to dryness. The residue was taken in boiling acetonitrile (75 mL) and was filtered, washed with hot acetonitrile. The solid **(47)** was dried on high vacuum to give 0.6 g, for a total yield of 1.47 g (79%).

^{1}H NMR (DMSO-d_6, δ): 1.2-1.6 (m, 8H); 2.0 (t, 2H); 2.2 (t, 2H); 2.5 (dd, 2H); 2.8 (dd, 2H); 3.1 (m, 3H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.7 (m, 1H).

Reaction Step C: Biotin conjugated caproic acid **(47)** (2.68 mmol, 1 g) was dissolved in DMSO (50 mL). Triethylamine (0.4 mL) was added followed by TFP acetate (4.02 mmol, 1.05 g). The reaction mixture was then stirred at room temperature for 15-20 min (HPLC monitored). It was then evaporated to dryness. The residue was washed with ether and dichloromethane and dried on high vacuum **(48)**. Yield: 1.24 g (89%).

^{1}H NMR (DMSO-d_6, δ): 1.2 (t, 2H); 1.3-1.7 (m, 5H); 2.1 (t, 2H); 2.6 (dd, 2H); 2.8 (m, 4H); 3.1 (m, 4H); 4.2 (m, 1H); 4.4 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.0 (m, 1H).

Reaction Step D: TFP ester of Biotin-caproic acid **(48)** (0.67 mmol, 0.35 g) was dissolved in DMF (40 mL). Triethylamine (80 μL) was added followed by aminoisophthalic acid (1.005 mmol, 0.182 g). The reaction was stirred at room temp. for 8 days (HPLC monitored) while adding triethylamine (80 μL) every after 24 h. It was then evaporated to dryness. The residue was then applied to a column of silica and was initially eluted with acetonitrile (450 mL). It was then eluted with methanol, 20 mL of fractions were collected, at the fraction 2 the solvent was changed to DMF. The fractions containing the final product (HPLC monitored) were evaporated to dryness **(49)** to yield 230 mg (65%).

^{1}H NMR (DMSO-d_6, δ): 1.3-1.7 (m, 8H); 2.1 (t, 2H); 2.3 (t, 2H); 2.6 (m, 2H); 2.8 (m, 2H); 3.1 (m, 3H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.1 (m, 1H); 8.46 (s, 2H).

Reaction Step E: Biotin-caproic acid-isophthalic acid **(49)** (0.376 mmol, 200 mg) was dissolved in DMF (30 mL) under argon atmosphere. TFP acetate (0.94 mmol, 241 mg) was added by double ended needle, followed by triethylamine (112 μL). The reaction was then stirred at room temp. for 24 h (HPLC monitored). It was then evaporated to dryness. The light brownish oil was taken in ether, solid was filtered and was washed with ether (50 mL) **(50)** to yield 250 mg (86%).

^{1}H NMR (DMSO-d_6, δ): 1.3-1.7 (m, 8H); 2.1 (t, 2H); 2.3 (t, 2H); 2.6 (m, 2H); 2.8 (m, 2H); 3.1 (m, 3H); 4.2 (m, 1H); 4.4 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.1 (m, 2H); 8.57 (s, 1H); 8.9 (s, 2H).

Reaction Step F: In a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(8, 9, 10)** (0.130 mmol, 0.2 g) in a mixture of DMF : H_2O (3:1) (40 mL) triethylamine (12 μL) was added. DiTFP ester of biotin-caproic acid-isophthalic acid **(50)** (0.065 mmol, 0.050 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 3 h (HPLC monitored). It was then evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted to yield 230 mg (62%) **(51)**. mp 195-198°C with decomposition.

EXAMPLE 16

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHALATE CROSS-LINKING WITH PARA-IODOBENZOYL MOIETY

This is an example of a bivalent receptor modulating agent which is also conjugated to a *para*-iodobenzoyl moiety.

Reaction Step A: A 5g (28 mmol) quantity of 5-aminoisophthalic acid **(52)** was dissolved in 30 mL 1N NaOH and placed in an ice/water bath. To the cold solution was added 7.5g (28 mmol) 4-iodobenzoyl chloride **(52)** in 60 mL of acetonitrile, dropwise. The thick white precipitate was then stirred for 10 minutes before removing the ice/water bath and allowing the mixture to stir an additional 10 minutes. The reaction mixture was adjusted to pH 4 with acetic acid and the resulting solid collected. This solid was then dissolved in 30 mL 1N NaOH and washed with ether (2 x 50 mL). The resulting aqueous solution was filtered and acidified to pH 4 with acetic acid. The white precipitate was the collected and dried on high vacuum to yield .6 g (99+%) of **(54)**. mp >300 °C; IR (Nujol, cm^{-1}) 3570(m), 3300(m), 1645, 1580(m), 1525(m), 760(m); ^1H NMR (DMSO-d_6, δ), 8.51 (2H, d, J = 0.7 Hz), 8.27 (1H, s), 7.94 (2H, d, J = 4.2 Hz), 7.84 (2H, d, J = 4.1 Hz).

Reaction Step B: A 5g (12.2 mmol) quantity of 5-[N-iodobenzoyl)amino]-isophthalic acid **(54)** was suspended in 100 mL anhydrous ethyl acetate. To this was added 12.5g (73 mmol) 2,3,5,6-tetrafluorophenol **(55)** followed by 5g (24.2 mmol) 1,3-dicyclohexylcarbodiimide. This suspension was then stirred at room temperature for 3 days before filtering off the solid and washing with an additional 20 mL of ethyl acetate. The filtrate was then evaporated to dryness. The resulting sticky white solid was suspended in 50 mL acetonitrile and stirred for 30 minutes. Filtering yielded 3.75g of white solid (43%) **(56)**. mp 250-251 °C; IR (Nujol, cm-1) 3220(m), 3060(m), 1750, 1655, 1520, 1485, 1330, 1195, 1110, 1085, 955(m), 945(m); ^1H NMR (DMSO-d_6, δ), 9.06 (2H, d, J = 0.7 Hz), 8.57 (1H, t, J = 1.4 Hz), 8.04 (2H, m), 7.94 (2H, d, J = 4.2 Hz), 7.81 (2H, d, J = 4.3 Hz).

Reaction Step C: To a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(56)** (0.192 mmol, 0.3 g) in a mixture of DMF : H_2O (3:1) (40 mL) was added triethylamine (0.018 mL). To this solution, DiTFP ester of 5-[N-(p-Iodobenzoyl)amino]-Isophthalic acid **(57)**(0.096 mmol, 0.068 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 4-5 h (HPLC monitored). It was then evaporated to dryness. The solid residue was dissolved in 20 mL of methanol : H_2O (8:2) and applied to a reverse phase C-18 column (500 mm

x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector; the elute was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-acid dimer (**58**): yield: 280 mg (76%), mp 230-233 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.19 (s, 8H); 1.3 (m, 36H); 1.37 (d, 12H); 1.46 (s, 10H); 1.63 (m, 8H); 1.87 (s, 12H); 2.05 (m, 10H); 2.27 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8 (s, 8H); 3.0 (s, 10H); 3.15 (m, 8H); 3.3 (d, 8H); 3.37 (m, 14H); 3.6 (m, 2H); 3.68 (d, 2H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.64 (m, 4H); 6.0 (s, 2H, 2C-10); 6.26 (d, 2H, $2R_1$); 6.6 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.9 (d, 2H); 7.99 (d, 1H); 8.28 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360.6 (ε48 871)

e-acid dimer (**59**): yield: 258 mg (70%), mp 285-290 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.17 (s, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.86 (s, 12H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.83 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 8H); 3.58 (m, 2H); 3.65 (m, 2H); 3.75 (m, 2H); 3.9 (d, 2H); 4.06 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.57 (s, 2H); 4.65 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d, 2H, 2R1); 6.5 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.89 (d, 2H); 7.98 (s, 1H); 8.26 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε41 481).

d-acid dimer (**60**): yield 265 mg (72%), mp 253-255 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.16 (s, 8H); 1.22 (d, 12H); 1.33 (m, 36H); 1.43 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.86 (s, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (s, 4H); 3.0 (s, 4H); 3.28 (s, 10H); 3.35 (m, 8H); 3.58 (m, 2H); 3.65 (m, 2H); 3.73 (m, 2H); 3.88 (d, 2H); 4.05 (m, 2H); 4.1 (m, 2H); 4.17 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.57 (s, 2H); 4.63 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.5 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.89 (d, 2H); 7.98 (s, 1H); 8.26 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε48 245).

EXAMPLE 17

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTAHATE CROSS-LINKING WITH PARA-(TRI-BUTYLSTANNYL)BENZOYL MOIETY

This is an example of a bivalent receptor modulating agent coupled to a *para*-tri-N-butyl stannyl moiety.

Reaction Step A: A 2 g (2.8 mmol) quantity of the diTFP ester of 5-[N-(p-Iodobenzoyl)amino]-Isophthalic acid **(57)** (as prepared above) was dissolved in 20 mL dry toluene under argon. To this was added 2.8 mL (5.5 mmol) of *bis*(tributyltin) **(61)** followed by 40 mg (0.04 mmol) tetrakis(triphenylphosphine)palladium **(62)**. The mixture was stirred at room temperature for 15 minutes before heating to 80°C for 2 h. Since the mixture only darkened slightly over the 2 h period, an additional 40 mg of palladium catalyst was added. Within 1 hour the mixture had turned black. After cooling to room temperature, the toluene was removed by rotary evaporation. The resulting black oil (containing solids), was then taken into 20 mL ethyl acetate and dried onto 10 g silica gel (via rotoevaporation). This solid was then added to a 250 g (40 x 3.5 cm) silica gel column and eluted initially with hexanes containing 5% acetic acid. After 600 mL, the solvent was changed to 90/10 hexanes/ethyl acetate (containing 5% acetic acid). Fractions 14-16 were combined and dried to yield 1.5 g (62%) of white solid **(62)**. mp 120-123 °C;

^{1}H NMR ($CDCl_3$, δ), 8.87 (2H, d, J = 0.7 Hz), 8.76 (1H, t, J = 1.6 Hz), 8.38 (1H, s), 7.84 (2H, d, J = 4.1 Hz), 7.62 (2H, d, J = 4.1 Hz), 7.07 (2H, m), 1.55 (6H, m), 1.36 (15H,m), 1.11 (6H,m), 0.89 (9H, t, J = 7.3 Hz); MS (FAB^+) M+H patterns calculated 870 (75.1%), 871 (52.9%), 872 (100%), 873 (41.0%), 874 (21.4%), found 870 (82.1%), 871 (55.1%), 872 (100%), 873 (42.1%), 874 (25.2%).

IR (Nujol, cm^{-1}) 1750, 1645, 1520, 1480(m), 1185, 1100, 1085.

Reaction Step B: In a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(8, 9, 10)** (0.065 mmol, 0.1 g) in a mixture of DMF : H_2O (3:1) (40 mL) triethylamine (0.006 mL) was added. DiTFP ester of 5-[N-(p-tributyltin benzoyl) amino]-Isophthalic acid **(63)**(0.0325 mmol, 0.028 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 12-14 h (HPLC monitored). It was then evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted.

b-acid dimer **(64)**: yield: 90 mg (70%), mp 208-212 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.15 (t,

12H); 1.19 (s, 8H); 1.3 (m, 36H); 1.37 (d, 12H); 1.46 (s, 10H); 1.6 (m, 8H); 1.9 (s, 12H); 2.05 (m, 10H); 2.28 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8-2.9 (m, 16H); 3.15 (m, 8H); 3.3 (s, 8H); 3.37 (m, 14H); 3.6 (m, 4H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.68 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.6 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (d, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.99 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

e-acid dimer (**65**): yield: 93 mg (72%), mp >300 °C, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.12 (t, 12H); 1.17 (d, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.87 (d, 12H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.8 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 14H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.66 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.98 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

d-acid dimer (**66**): yield: 100 mg (78%), mp 202-205 °C with decomposition, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.12 (t, 12H); 1.15 (s, 8H); 1.29 (m, 36H); 1.35 (d, 12H); 1.44 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.86 (d, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (m, 8H); 3.13 (m, 8H); 3.28 (s, 10H); 3.35 (m, 10H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.05 (m, 2H); 4.1 (m, 2H); 4.17 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.98 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 18

EVALUATION OF THE ABILITY OF VITAMIN B_{12} RECEPTOR MODULATING AGENTS TO BIND TO TCII

This example serves to demonstrate a competitive binding assay suitable for evaluating the ability of vitamin B_{12} receptor modulating agents to bind TcII. Binding of the vitamin B_{12} derivatives to recombinant transcobalamin II was conducted in picomolar concentrations and the percent bound ascertained.

In this competitive binding assay, various B_{12} derivatives, including vitamin B_{12} receptor modulating agents, were evaluated for their ability to bind to TcII

relative to radiolabeled B_{12}. Varying concentrations of each derivative were incubated with immobilized TcII in the presence of a constant amount of radiolabeled B_{12}. After incubation for 20 minutes at 37° C, the free radiolabeled B_{12} was separated from the TcII bound tracer by removal of the supernatant. The radioactivity of the supernatant solution was then measured to determine the amount of free radiolabeled B_{12} present at the end of each competition. By measuring the amount of free radiolabeled B_{12} for each competition, the ability of each derivative to inhibit radiolabeled B_{12} binding was determined. A binding curve was then be constructed for each B_{12} derivative where the amount of radiolabeled B_{12} bound (% radiolabel bound) was correlated with the concentration of derivative present in the original mixture. The more effective the derivative is in binding to TcII, the lower the percent bound radiolabeled vitamin B_{12}.

Figure 22 illustrates the binding curve of Transcobalamin II to the cyanocobalamin monocarboxylic acids produced in Example 1. AD = Cyanocobalamin **(1)**; AL = Cyanocobalamin *b*-monocarboxylic acid **(2)**; AM = Cyanocobalamin *e*-monocarboxylic acid **(3)**; and AN= Cyanocobalamin *d*-monocarboxylic acid **(4)**. The *d*-carboxylate **(3)** appears to bind nearly as well as cyanocobalamin. Two samples of vitamin B_{12} were used, one as a known standard and the other as an unknown.

Figure 23 illustrates the binding curve of Transcobalamin II to the cyanocobalamin diaminododecane adducts **(8, 9, 10)** and succinate adduct **(13)** produced in Example 3 and 4 above. AH = Cyanocobalamin *b*-monocarboxylic acid conj Diaminododecane **(7)**; AI = Cyanocobalamin *e*-monocarboxylic acid conj Diaminododecane **(8)**; AJ = Cyanocobalamin *d*-monocarboxylic acid conj Diaminododecane **(9)**; AK = Cobalamin *e*-monocarboxylic acid conj Diaminododecane, and AE = Cyanocobalamin Ribose-Succinate **(11)**. The *b*-conjugate **(17)** has the least binding, whereas the *e*-conjugate **(18)** has intermediate binding, and the *d*-conjugate **(19)** binds quite well. The biotin conjugate attached to the ribose site **(13)** appears to bind very well, as does its precursor amino derivative **(12)**. The additional compound studied is of unknown structure, but may have the amine group coordinated with the cobalt atom as the mass spectrum indicates that it has the appropriate mass for **(7)** minus HCN. It is clear that this unknown compound is not likely to bind TcII.

Figure 24 illustrates the binding curve of Transcobalamin II to a series of vitamin B_{12} dimers. Dimer X = *b*-acid dimer with Isophthaloyl dichloride **(36)**; Dimer Y = *e*-acid dimer with Isophthaloyl dichloride **(37)**; dimer Z = *d*-acid dimer with Isophthaloyl dichloride **(38)**; Dimer A= *b*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(58)**; Dimer B = *e*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(59)**; and Dimer C = *d*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(60)**.

Figure 25 illustrates the binding curve of Transcobalamin II to a series of biotinylated vitamin B_{12} molecules. AA = Cyanocobalamin *b*-monocarboxylic acid conj Diaminododecane and Biotin (17); AB = Cyanocobalamin *e*-monocarboxylic acid conj Diaminododecane and Biotin (18); AC = Cyanocobalamin *d*-monocarboxylic acid conj Diaminododecane and Biotin (19); AF = Cyanocobalamin Ribose-Succinate conj Diaminododecane (13); and AG = Cyanocobalamin Ribose-Succinate conj. Diaminododecane and Biotin (20).

EXAMPLE 19

ASSAY FOR BIOLOGICAL ACTIVITY OF VITAMIN B_{12} RECEPTOR MODULATING AGENTS

This example serves to demonstrate the use of an assay to ascertain biological activity of the receptor modulating agents of the present invention.

Receptor down-modulation involves a comparison of treatment of a target cell line such as K562, each sample is treated with vitamin B_{12} or a vitamin B_{12} receptor modulating agent at 4°C for 24 hours. Following this period, cells of each sample are separated from a vitamin B_{12} or a vitamin B_{12} receptor modulating agent by centrifugation. The cells are then washed and resuspended in phosphate buffered saline containing 2 mM EDTA for a brief period of time not to exceed 15 minutes at 4°C. Then, the cells are washed again and returned to a tissue culture medium at 4°C. The tissue culture medium containing TcII and a radiolabeled TcII/B_{12} complex. The time course of TcII/B_{12} binding to the cell receptor is determined by measuring the percent radiolabel bound to the cell at 0, 15, 30, 60, 120, and 240 minutes. Those samples exposed to the vitamin B_{12} receptor modulating agents of the present invention show significantly reduced TcII/B_{12} complex binding compared to cells cultured in vitamin B_{12}. Trypsin treated cells reveal any nonspecific binding or uptake of the labeled vitamin B_{12} on or within the cell.

EXAMPLE 20

METHOD FOR ASSESSING BIOLOGICAL ACTIVITY OF A RECEPTOR MODULATING AGENT

This example serves to demonstrate a method suitable for assessing the biological activity of a receptor modulating agent of the present invention.

$0.2x10^6$ cells/ml K562 cells were cultured in RPMI medium modified by addition of 10 μM MeTHF, 2.7 nM vitamin B_{12} and 1% human serum. No folate was added. 10 μM *d*-diamimododecane adduct (7) was added and cultured over 9 days at 37°C. 10 μM vitamin B_{12} cultured under identical conditions as (7) was utilized as a control. The cultures were then independently assessed for proliferation and cell death by Trypan blue exclusion. The results are described in Table 10, below, in terms of the percent cell death.

Table 10

	Control	*d*-diaminododecane adduct (7)
Proliferation	98%	9 %
Cell Death	8 %	85 %

The receptor modulating agent, in this case *d*-diaminododecane adduct (7), clearly demonstrates the marked biological activity of the receptor modulating agent.

EXAMPLE 21

SYNTHESIS OF AN ANTI-INFLAMMATORY RECEPTOR MODULATING AGENT

The synthetic peptide f-met-leu-phe is equivalent to a bacterial cell wall constituent (Biochem. Soc. Trans. 19:1127-9, 1991; Agents Actions Suppl. 35:3-8, 1991; Agents Actions Suppl. 35:11-6, 1991; J Immunol. 146:975-80, 1991). This peptide is recognized by receptors on PMN which can respond by chemotaxis to sites of local inflammation along a gradient of the peptide. During inflammation, receptor expression can be dramatically increased by mobilizing receptor from intracellular pools. Non-specific methods used to abrogate this up-regulation also inhibit chemotaxis and presumably the anti-inflammatory reaction associated with local inflammation (J. Immunol. 145:2633-8, 1990). The synthesis of a receptor modulation agent useful as an inhibitor of early inflammation is described below.

The peptide f-met-leu-phe-$(gly)_3$-leu-O-Me is synthesized using tea-bag methodology or solid phase peptide synthesis procedures described by Merrifield et al. (Biochemistry 21:5020-31, 1982) and Houghten (Proc. Nat'l. Acad. Sci. (USA) 82:5131-35, 1985), or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A peptide synthesizer. The peptide-amide is deprotected

in 45% trifluoroacetic acid-51% methylene chloride-2% ethanedithiol-2% anisole for 20 minutes, and cleaved from the 4-methylbenzhydrylamine resin using the Tam-Merrifield low-high HF procedure (J. P. Tam et al., J. Am. Chem. Soc. 105:6442-55, 1983). The peptide is then extracted from the resin using 0.1 M ammonium acetate buffer, pH 8, and is lyophilized. The crude peptide is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separations Group, Hesperia, Calif.), and a linear gradient of 0.5-1.0%/min. from 100% acetonitrile + 0.1%v/v trifluoroacetate to 100% acetonitrile + 0.1% trifluoroacetate. The HPLC-purified peptide is analyzed by amino acid analysis (R. L. Heinriksen and S. C. Meredith, Anal. Biochem. 160:65-74, 1984) after gas phase hydrolysis (N. M. Meltzer et al., Anal. Biochem. 160:356-61, 1987). The sequence of the purified peptide may be confirmed by Edman degradation on a commercially available sequencer (R. M. Hewick et al., J. Biol. Chem. 15:7990-8005, 1981). The peptide amide is converted to an O-methyl ester (*i.e.*, f-met-leu-phe-$(gly)_3$-leu-O-Me) by treatment with dimethylformamide (5g/60 mL with 1.3 equivalents of $NaHCO_3$ in excess methyl iodide (4 equivalents). The mixture is stirred under argon gas at room temperature for 40 hours. If required, the peptide is extracted to dryness with 150 mL of ethyl acetate. The receptor for modulating agent is used to treat PMN, activated with GM-CSF (to increase expression of fMLP receptors). Loss of binding of biotinylated fMLP is compared on fMLP versus f-MLP receptor modulating agent treated cells.

EXAMPLE 22

SYNTHESIS OF A FUSION PROTEIN RECEPTOR MODULATING AGENT

An EGF receptor modulating agent containing a genetically engineered fusion protein is hereby described. Briefly, the C-terminus of a DNA sequence encoding EGF, or its receptor binding domain, is ligated by conventional procedures (*e.g.*, using T_4DNA ligase) to a DNA sequence corresponding to a GGG spacer. The C-terminus of the EGF-GGG DNA sequence is then fused to the N-terminus of a DNA sequence encoding the conditional, membrane binding peptide KGEAALA$(EALA)_4$-EALEALAA. Alternately, peptide-spacer DNA sequences may be synthesized *in vitro* using standard oligonucleotide synthesis procedures (*see, e.g.*, U.S. Pat. Nos. 4,500,707 and 4, 668,777). The recombinant EGF peptide DNA sequence is cloned in an *E. coli* expression vector using conventional procedures. *E. coli* strain HB101 is transformed with the fused recombinant DNA sequence and cultured to produce the EGF peptide. The fusion protein is purified form the transformed *E. coli* culture by standard methods,

including anti-EGF affinity chromatography. The fusion protein may be eluted from the affinity matrix using standard techniques, such as high salt, chaotropic agents, or high or low pH. Loss of EGF receptor is measured by flow cytometry and mouse monoclonal antibody to EGF receptor.

From the foregoing, it will be appreciated that, although specific embodiments of this invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except by the appended claims.

Claims

1. A receptor modulating agent, comprising a vitamin B_{12} molecule coupled to a rerouting moiety.

2. The receptor modulating agent of claim 1 wherein said B_{12} molecule is coupled to said rerouting moiety by a linker.

3. The receptor modulating agent of claim 2 wherein said linker is at least 4 atoms in length.

4. The receptor modulating agent of claim 3 wherein said linker is 6 to 20 atoms in length.

5. The receptor modulating agent of claim 4 wherein said linker is 12 atoms in length.

6. The receptor modulating agent of claim 2 wherein said linker includes at least one amino group.

7. The receptor modulating agent of claim 6 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

8. The receptor modulating agent of claim 6 wherein said linker is selected from the group consisting of a diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

9. The receptor modulating agent of claim 6 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x = 2-20.

10. The receptor modulating agent of claim 6 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y = 3-12.

11. The receptor modulating agent of claim 2 wherein said linker is coupled to said rerouting moiety through a coupling site on said vitamin B_{12} derivative selected from the group consisting of *b-*, *d-* and *e-*.

12. The receptor modulating agent of claim 11 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites.

13. The receptor modulating agent of claim 2 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

14. The receptor modulating agent of claim 2 wherein said linker is a trifunctional linker.

15. The receptor modulating agent of claim 14 wherein a biotin molecule is coupled through a reactive site on said trifunctional linker.

16. The receptor modulating agent of claim 1 wherein said rerouting moiety is selected from the group consisting of lysosomotropic moieties, intracellular polymerizing moieties, peptide sorting sequences, conditional membrane binding peptides and bi- or multi-valent receptor cross-linking moieties membrane anchors.

17. The receptor modulating agent of claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by redirecting an agent/receptor complex.

18. The receptor modulating agent of claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by cross-linking one or more receptors.

19. The receptor modulating agent of claim 18 wherein said receptor modulating agent is a vitamin B_{12} dimer.

20. The receptor modulating agent as in claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by anchoring a receptor in a cell membrane.

21. The receptor modulating agent as in claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by retaining an agent/receptor complex in an endosome.

22. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a lysosomotropic moiety selected from the group consisting of gentamycin, sisomicin, netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin ribostamycin butirosin, and streptomycin.

23. The receptor modulating agent as in claim 1 wherein said rerouting moiety is an intracellular polymerizing moiety selected from the group consisting of dipeptide esters and leucine zippers.

24. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a peptide sorting sequence selected from the group consisting of endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides.

25. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a conditional membrane binding peptide selected from the group consisting of charged glutamate, aspartate, and histidine.

26. A vitamin B_{12} dimer comprising a first and a second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling site h, and coupling site i.

27. The dimer of claim 26 wherein said first and second vitamin B_{12} molecules are coupled through a coupling site independently selected from the group consisting of *d-* and *e-* coupling sites on said first and said second vitamin B_{12} molecule.

28. The dimer of claim 26 wherein at least one of said first and said second vitamin B_{12} molecules is a vitamin B_{12} derivative.

29. The dimer of claim 26 wherein said first and second B_{12} molecules are coupled through at least one linker.

30. The dimer of claim 29 wherein said linker is at least 4 atoms in length.

31. The dimer of claim 30 wherein said linker is about 10 to 55 atoms in length.

32. The dimer of claim 31 wherein said linker is 35 to 45 atoms in length.

33. The dimer of claim 29 wherein said linker includes at least one amino group.

34. The dimer of claim 33 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

35. The dimer of claim 33 wherein said linker is selected from the group consisting of a diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

36. The dimer of claim 33 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x = 2-20.

37. The dimer of claim 33 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y = 3-12.

38. The dimer of claim 29 wherein said linker is a trifunctional linker.

39. A method for modulating a vitamin B_{12} receptor, comprising administering an effective amount of a receptor modulating agent to a warm-blooded animal such that a vitamin B_{12} receptor is modulated, said receptor modulating agent comprising a vitamin B_{12} molecule coupled to a rerouting moiety.

40. The method of claim 39 wherein said B_{12} molecule is coupled to said rerouting moiety by a linker.

41. The method of claim 40 wherein said linker is at least 4 atoms in length.

42. The method of claim 41 wherein said linker is 6 to 20 atoms in length.

43. The method of claim 42 wherein said linker is 12 atoms in length.

44. The method of claim 40 wherein said linker includes at least one amino group.

45. The method of claim 44 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

46. The method of claim 44 wherein said linker is selected from the group consisting of a diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

47. The method of claim 44 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x = 2-20.

48. The method of claim 44 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y = 3-12.

49. The method of claim 40 wherein said linker is coupled to said rerouting moiety through a coupling site on said vitamin B_{12} derivative selected from the group consisting of *b*-, *d*- and *e*-.

50. The method of claim 49 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites.

51. The method of claim 40 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

52. The method of claim 40 wherein said linker is a trifunctional linker.

53. The method of claim 39 wherein said rerouting moiety is selected from the group consisting of lysosomotropic moieties, intracellular polymerizing moieties, peptide sorting sequences, conditional membrane binding peptides and bi- or multi-valent receptor cross-linking moieties membrane anchors.

54. The method of claim 39 wherein said receptor modulating agent affects a receptor trafficking pathway by redirecting an agent/receptor complex.

55. The method of claim 39 wherein said receptor modulating agent affects a receptor trafficking pathway by cross-linking one or more receptors.

56. The method of claim 55 wherein said receptor modulating agent is a vitamin B_{12} dimer.

57. The method of claim 39 wherein said receptor modulating agent affects a receptor trafficking pathway by anchoring a receptor in a cell membrane.

58. The method of claim 39 wherein said receptor modulating agent affects a receptor trafficking pathway by retaining an agent/receptor complex in an endosome.

59. The method of claim 39 wherein said rerouting moiety is a lysosomotropic moiety selected from the group consisting of gentamycin, sisomicin, netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin ribostamycin butirosin, and streptomycin.

60. The method of claim 39 wherein said rerouting moiety is an intracellular polymerizing moiety selected from the group consisting of dipeptide esters and leucine zippers.

61. The method of claim 39 wherein said rerouting moiety is a peptide sorting sequence selected from the group consisting of endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides.

62. The method of claim 52 wherein said rerouting moiety is a conditional membrane binding peptide selected from the group consisting of charged glutamate, aspartate, and histidine.

63. The method of claim 56 wherein said vitamin B_{12} dimer is comprised of a first and a second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling site h, and coupling site i.

64. The method of claim 63 wherein said first and second vitamin B_{12} molecules are coupled through a coupling site independently selected from the group consisting of *d*- and *e*- coupling sites on said first and said second vitamin B_{12} molecule.

65. The method of claim 63 wherein at least one of said first and said second vitamin B_{12} molecules is a vitamin B_{12} derivative.

66. The method of claim 65 wherein said first and second B_{12} molecules are coupled through at least one linker.

67. The method of claim 66 wherein said linker is at least 4 atoms in length.

68. The method of claim 67 wherein said linker is about 10 to 55 atoms in length.

69. The method of claim 68 wherein said linker is 35 to 45 atoms in length.

70. The dimer of claim 66 wherein said linker includes at least one amino group.

71. The dimer of claim 70 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

72. The dimer of claim 70 wherein said linker is selected from the group consisting of a diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

73. The dimer of claim 70 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x = 2-20.

74. The dimer of claim 70 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y = 3-12.

75. The dimer of claim 66 wherein said linker is a trifunctional linker.

76. The method of claim 75 wherein a reactive site on said trifunctional linker is coupled to a biotin molecule.

77. The method of claim 39 wherein said vitamin B_{12} receptor modulation is sufficient to treat a neoplastic disorder.

78. The method of claim 77 wherein said neoplastic disorder is selected from the group consisting of leukemia, sarcoma, myeloma, carcinoma, neuroma, melanoma, cancers of the lung, liver, breast, brain, colon, cervix, prostrate, Hodgkin's disease, and non-Hodgkin's lymphoma.

79. A method for regulating a biological response associated with a cell surface receptor, comprising administering an effective amount of a receptor modulating agent to a warm-blooded animal such that a biological response is regulated.

80. A vitamin B_{12} derivative comprising a vitamin B_{12} molecule coupled to a biotin molecule.

81. The vitamin B_{12} derivative of claim 80 wherein said vitamin B_{12} molecule is cyanocobalamin.

82. The vitamin B_{12} derivative of claim 80 wherein said vitamin B_{12} molecule is coupled to said biotin molecule by a linker.

83. The vitamin B_{12} derivative of claim 82 wherein said linker is at least 4 atoms in length.

84. The vitamin B_{12} derivative of claim 83 wherein said linker is 6 to 20 atoms in length.

85. The vitamin B_{12} derivative of claim 84 wherein said linker is 12 atoms in length.

86. The vitamin B_{12} derivative of claim 82 wherein said linker includes at least one amino group.

87. The vitamin B_{12} derivative of claim 86 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

88. The vitamin B_{12} derivative of claim 86 wherein said linker is selected from the group consisting of a diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

89. The vitamin B_{12} derivative of claim 86 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x = 2-20.

90. The vitamin B_{12} derivative of claim 87 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y = 3-12.

91. The vitamin B_{12} derivative of claim 82 wherein said linker is coupled to said rerouting moiety through a coupling site on said vitamin B_{12} derivative selected from the group consisting of *b*-, *d*- and *e*-.

92. The vitamin B_{12} derivative of claim 91 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites on said vitamin B_{12} molecule.

93. The vitamin B_{12} derivative of claim 82 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

94. The receptor modulating agent of claim 82 wherein said linker is a trifunctional linker.

95. The vitamin B_{12} derivative of claim 80 wherein said biotin is additionally coupled to a rerouting moiety.

96. The vitamin B_{12} derivative of claim 95 wherein said biotin is coupled to said rerouting moiety by a biotin binding protein.

97. The vitamin B_{12} derivative of claim 96 wherein said biotin binding protein is selected from the group consisting of avidin and streptavidin.

98. A complex comprising a vitamin B_{12} derivative according any one of claims 80 to 97 bound to a transcobalamin II.

99. A kit for determining the presence or amount of transcobalamin in a sample using a vitamin B_{12} derivative according to any one of claims 80 to 97.

100. A pharmaceutical composition, comprising a vitamin B_{12} derivative according to any one of claims 80 to 97 and a suitable pharmaceutical carrier or diluent.

101. A receptor modulating agent, comprising a targeting moiety coupled to a rerouting moiety.

102. The receptor modulating agent as in claim 101 wherein said rerouting moiety is selected from the group consisting of lysosomotropic moieties, intracellular polymerizing moieties, peptide sorting sequences, conditional membrane binding peptides and bi- or multi-valent receptor cross-linking moieties.

103. The receptor modulating agent as in claim 101 wherein said targeting moiety is selected from the group consisting of proteins, peptides, and nonproteinacious molecules.

104. The receptor modulating agent as in claim 101 wherein the receptor modulating agent affects a receptor trafficking pathway by redirecting an agent/receptor complex.

105. The receptor modulating agent as in claim 101 wherein said receptor modulating agent affects a receptor trafficking pathway by cross-linking one or more cell surface receptors.

106. The receptor modulating agent as in claim 101 wherein said receptor modulating agent affects a receptor trafficking pathway by anchoring a cell surface receptor in a cell membrane.

107. The receptor modulating agent as in claim 101 wherein said receptor modulating agent affects a receptor trafficking pathway by retaining a receptor in an endosome.

108. The receptor modulating agent as in claim 102 wherein said lysosomotropic moiety is selected from the group consisting of gentamycin, sisomicin, netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin ribostamycin butirosin, and streptomycin.

109. The receptor modulating agent as in claim 102 wherein said intracellular polymerizing moiety is selected from the group consisting of dipeptide esters and leucine zippers.

110. The receptor modulating agent as in claim 102 wherein said peptide sorting sequence is selected from the group consisting of endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides.

111. The receptor modulating agent as in claim 102 wherein said conditional membrane binding peptide is selected from the group consisting of charged glutamate, aspartate, and histidine.

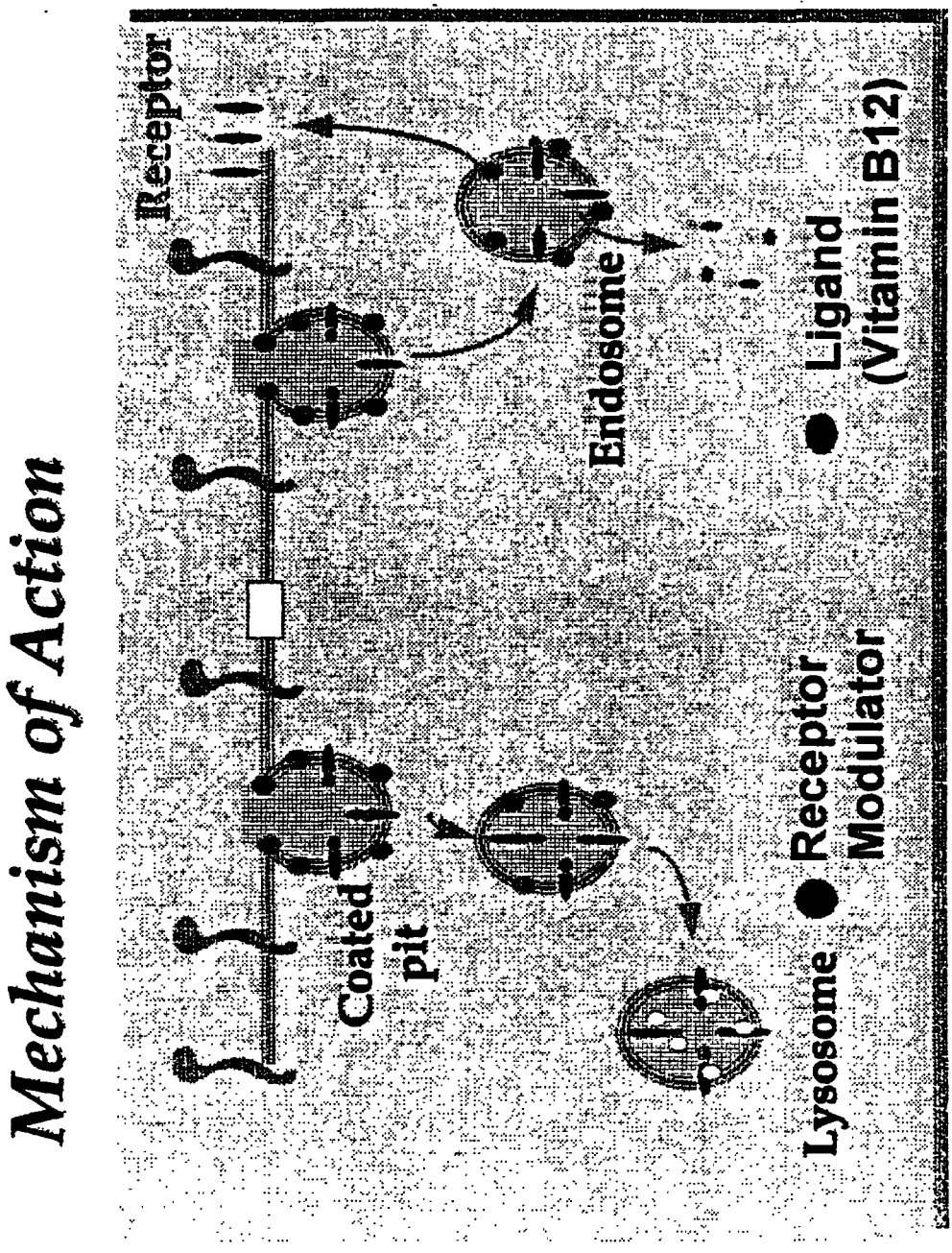
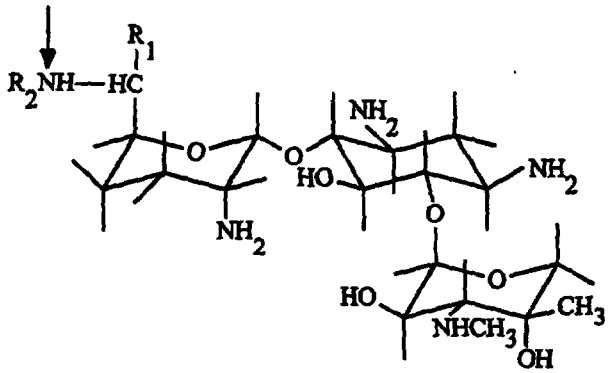
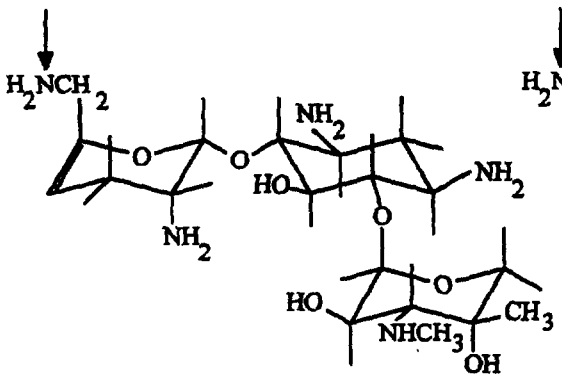


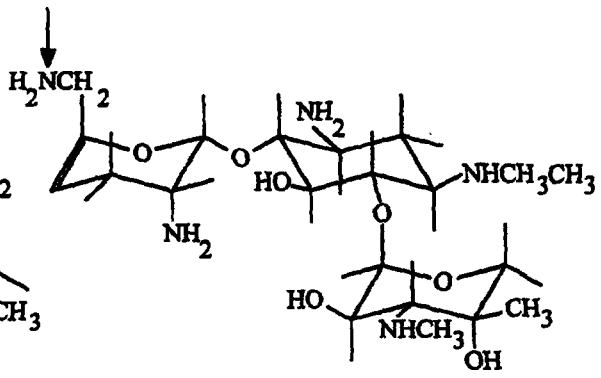
FIGURE 1



Gentamicin C_1 : $R_1 = R_2 = CH_3$
Gentamicin C_2 : $R_1 = CH_3$; $R_2 = H$
Gentamicin C_{1a}: $R_1 = R_2 = H$

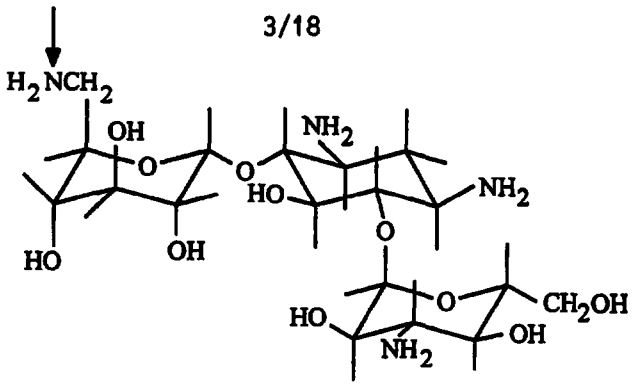


Sisomicin

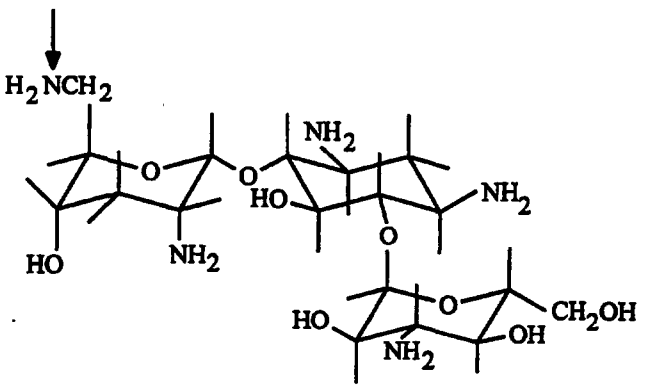


Netilmicin

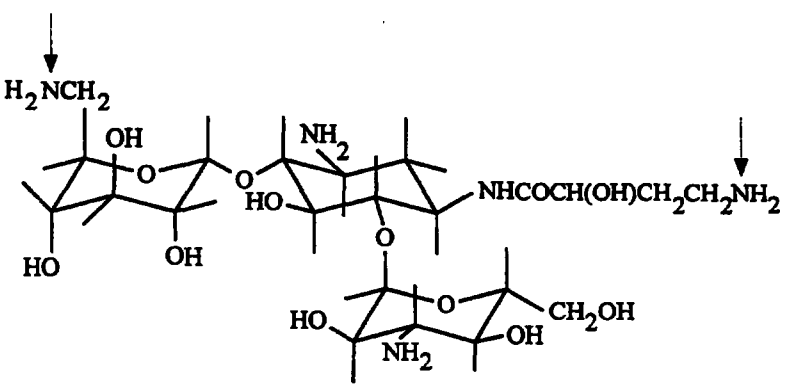
Fig. 2



Kanamycin A

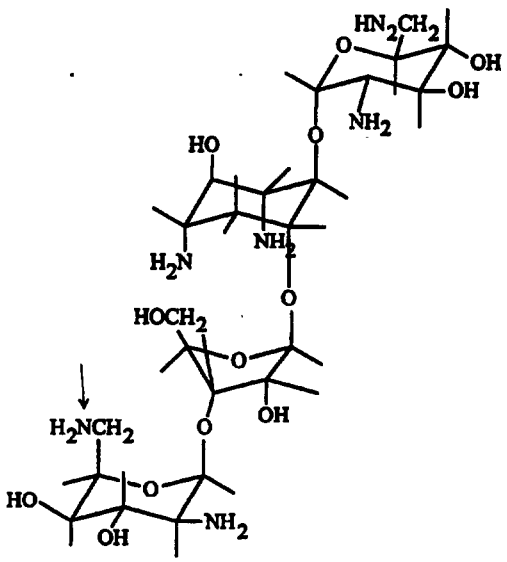


Tobramycin

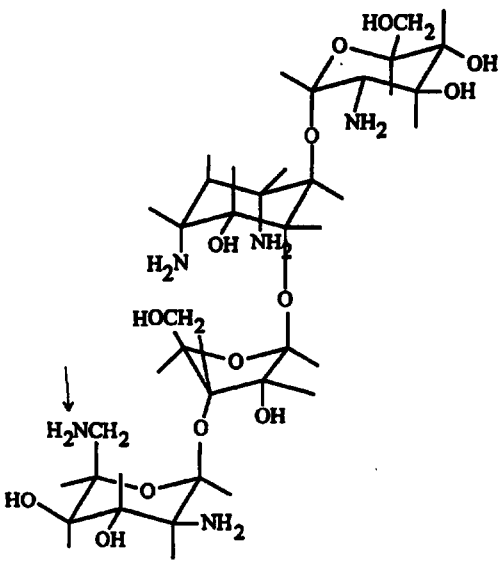


Amikacin

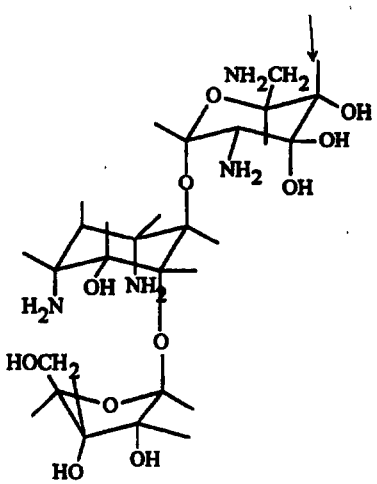
Fig. 3



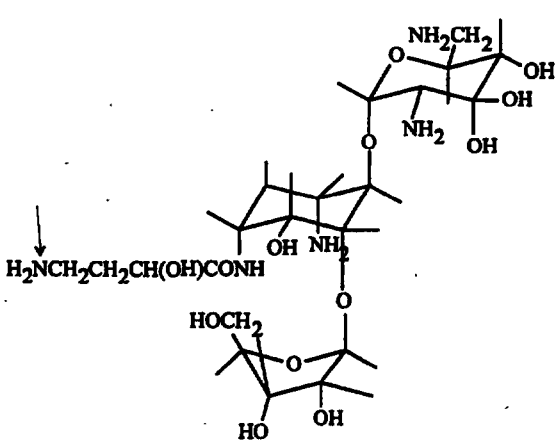
Neomycin B



Paromomycin



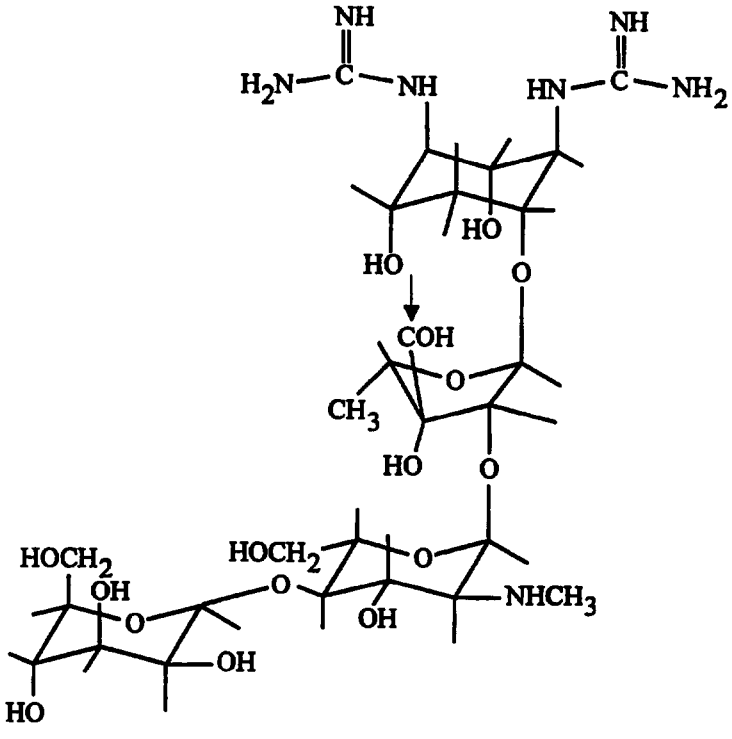
Ribostamycin



Butirosin B

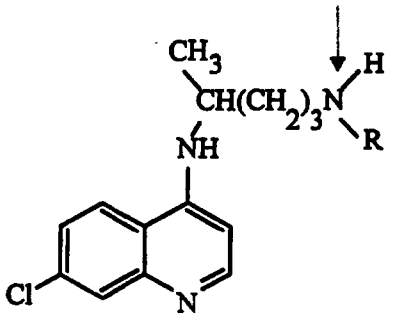
Fig. 4

Streptomycin A

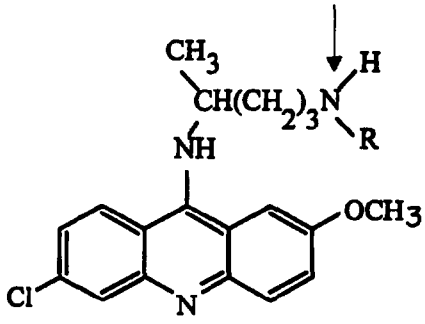


Streptomycin B

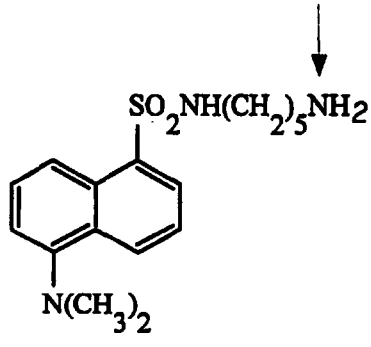
Fig. 5



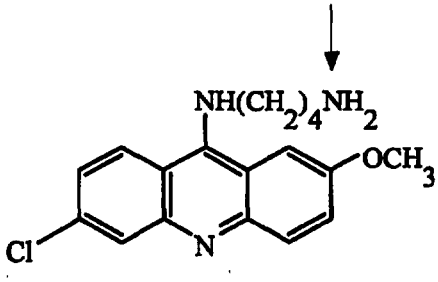
Chloroquine Derivatives



Quinacrine Derivatives



Dansyl Cadaverine



Amino Acridine

Fig. 6

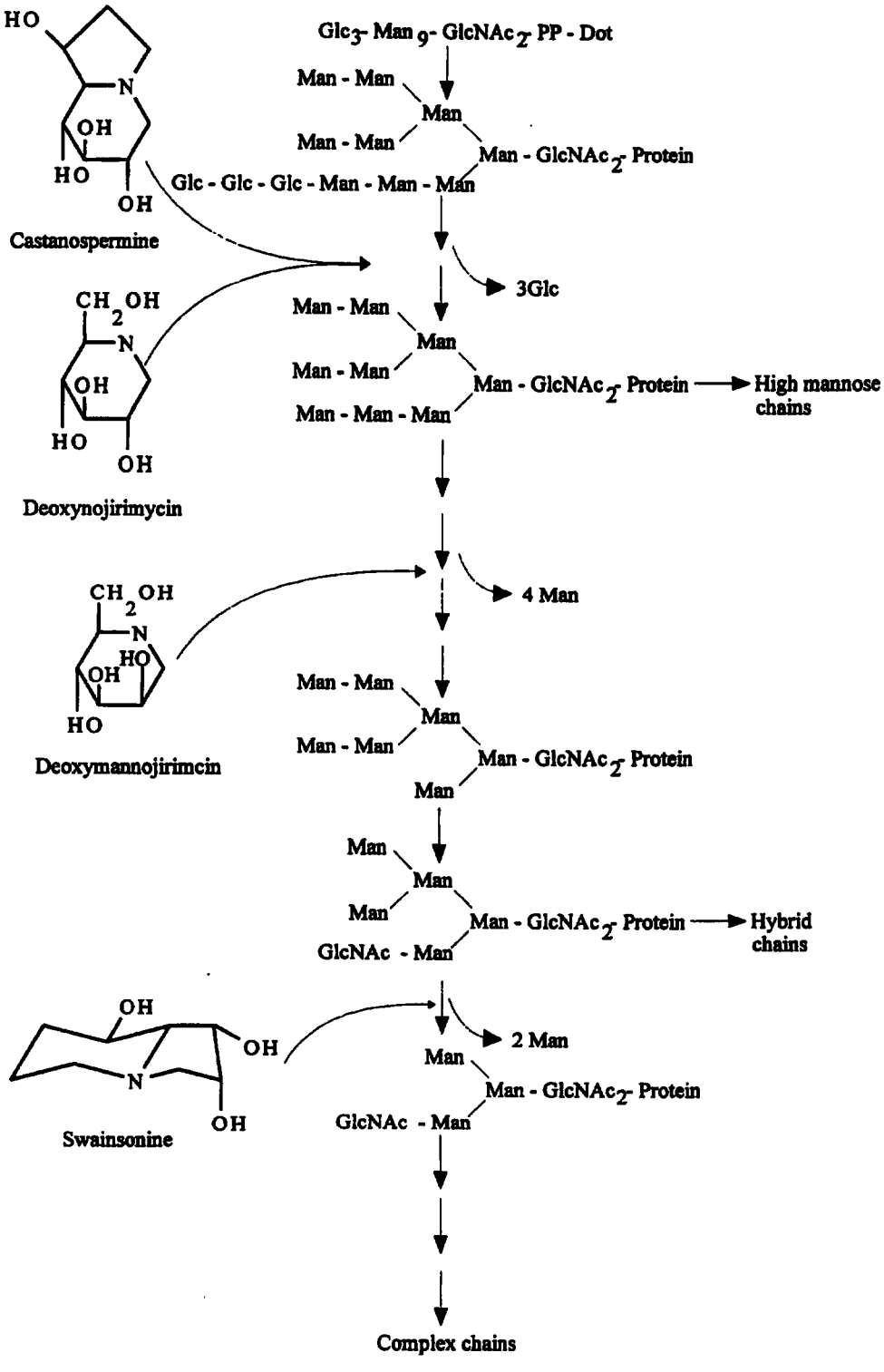
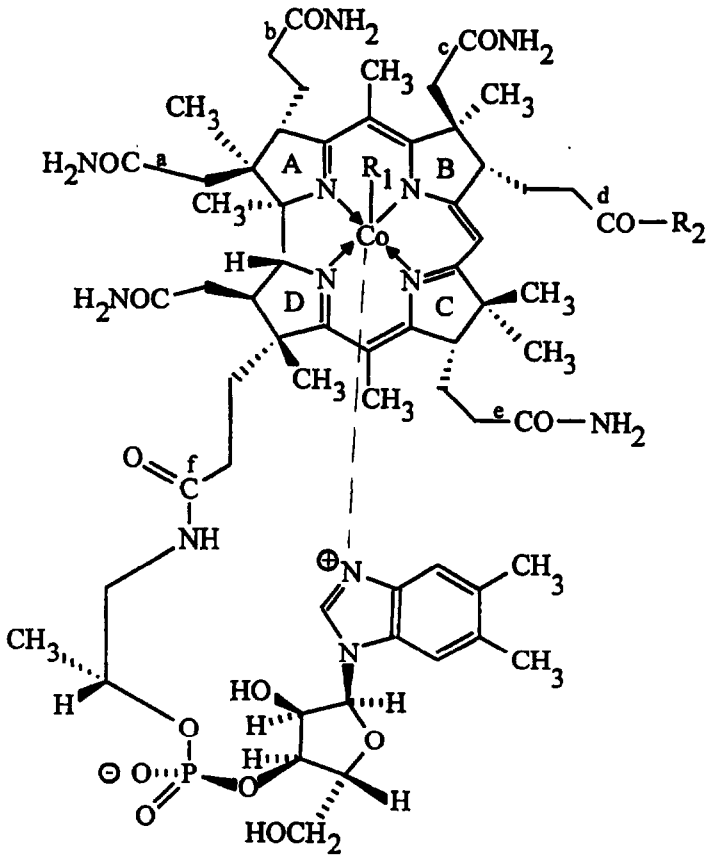


Fig. 7



$R_1 = CN$; $R_2 = NH_2$ (Cyanocobalamin)
$R_1 = CN$; $R_2 = OH$ (Cyanocobalamin -(3)-free acid)
$R_1 = CN$; $R_2 = HN\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO_2H$ (GABA adduct)
$R_1 = CN$; $R_2 =$ GABA - Peptide (where GABA = linker)
$R_1 = CN$; $R_2 =$ Peptide
$R_1 = CN$; $R_2 =$ HN-(linker)-tyramine-^{125}I
$R_1 = CN$; $R_2 =$ HN-(linker)-lysosomotropic agent
$R_1 = CN$; $R_2 =$ HN-(linker)-X-linking agent
$R_1 = CN$; $R_2 =$ HN-(linker)-biotin
$R_1 = CN$; $R_2 = NH\text{-}(CH_2)_{12}NH_2$

Fig. 8

Fig. 9

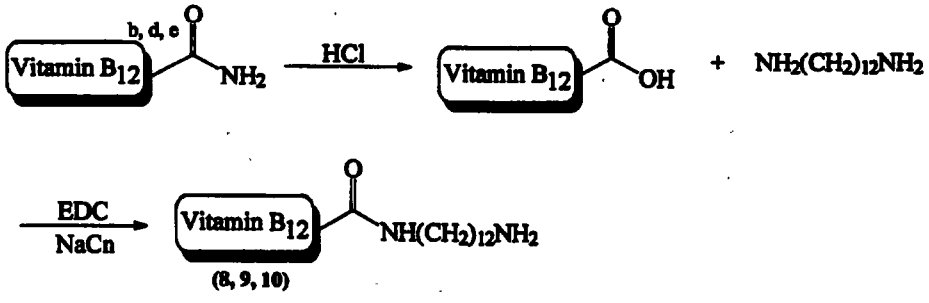


Fig. 10a

Fig. 10b

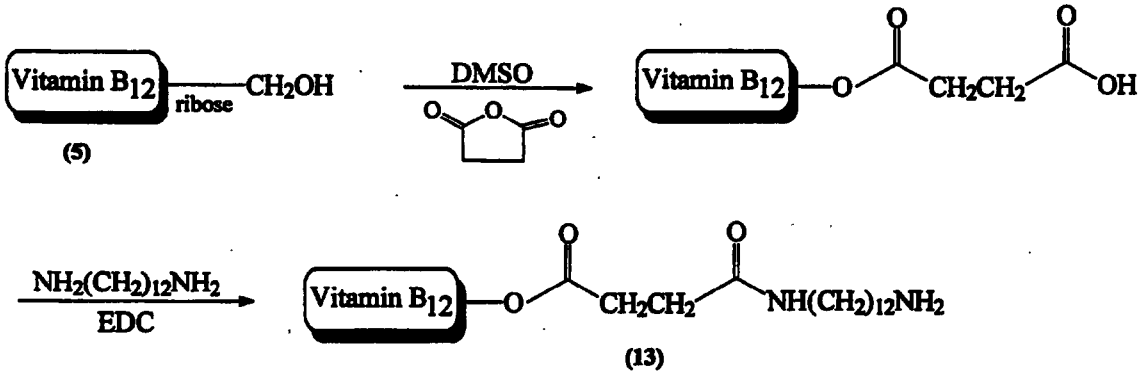


Fig. 11

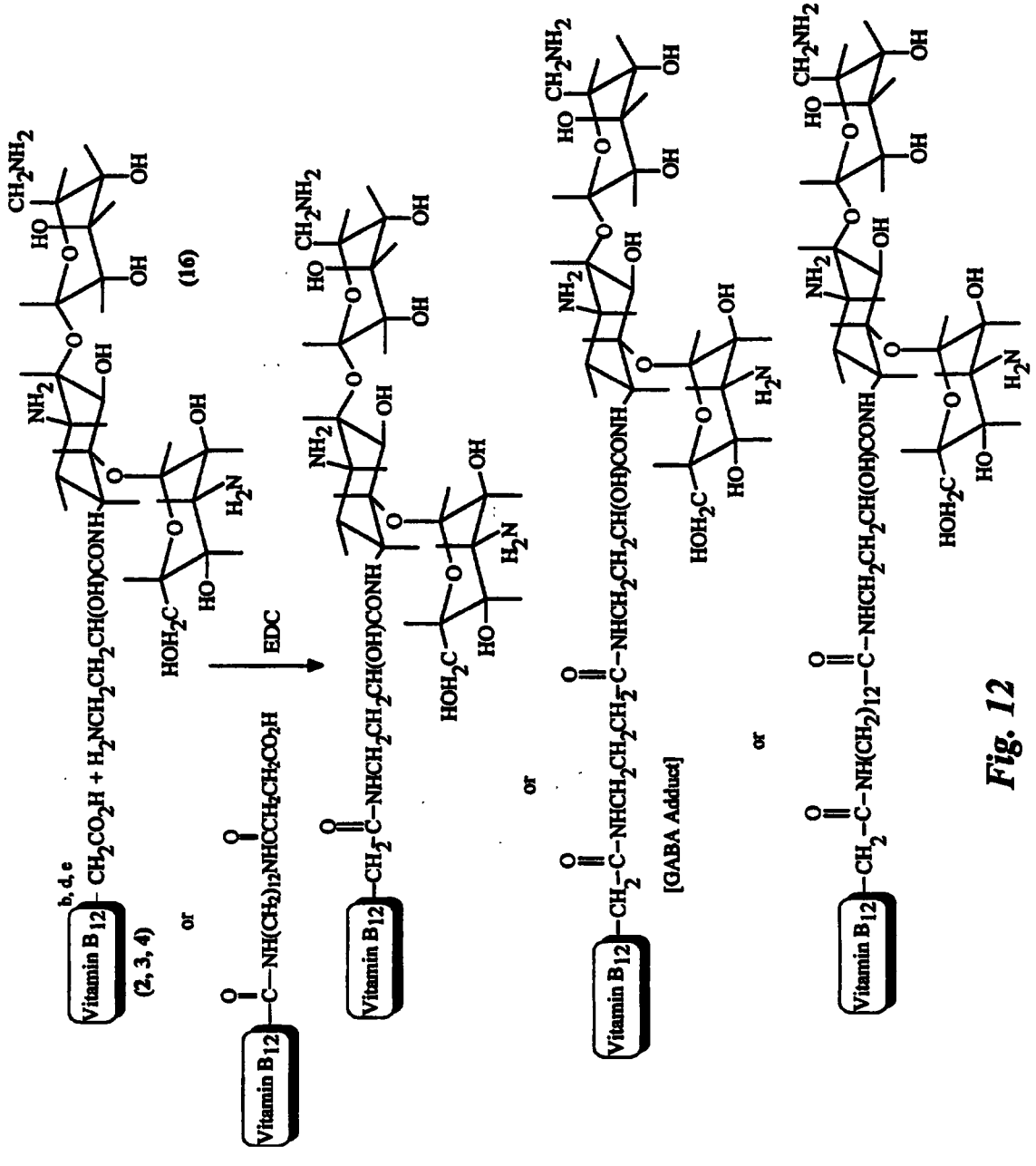


Fig. 12

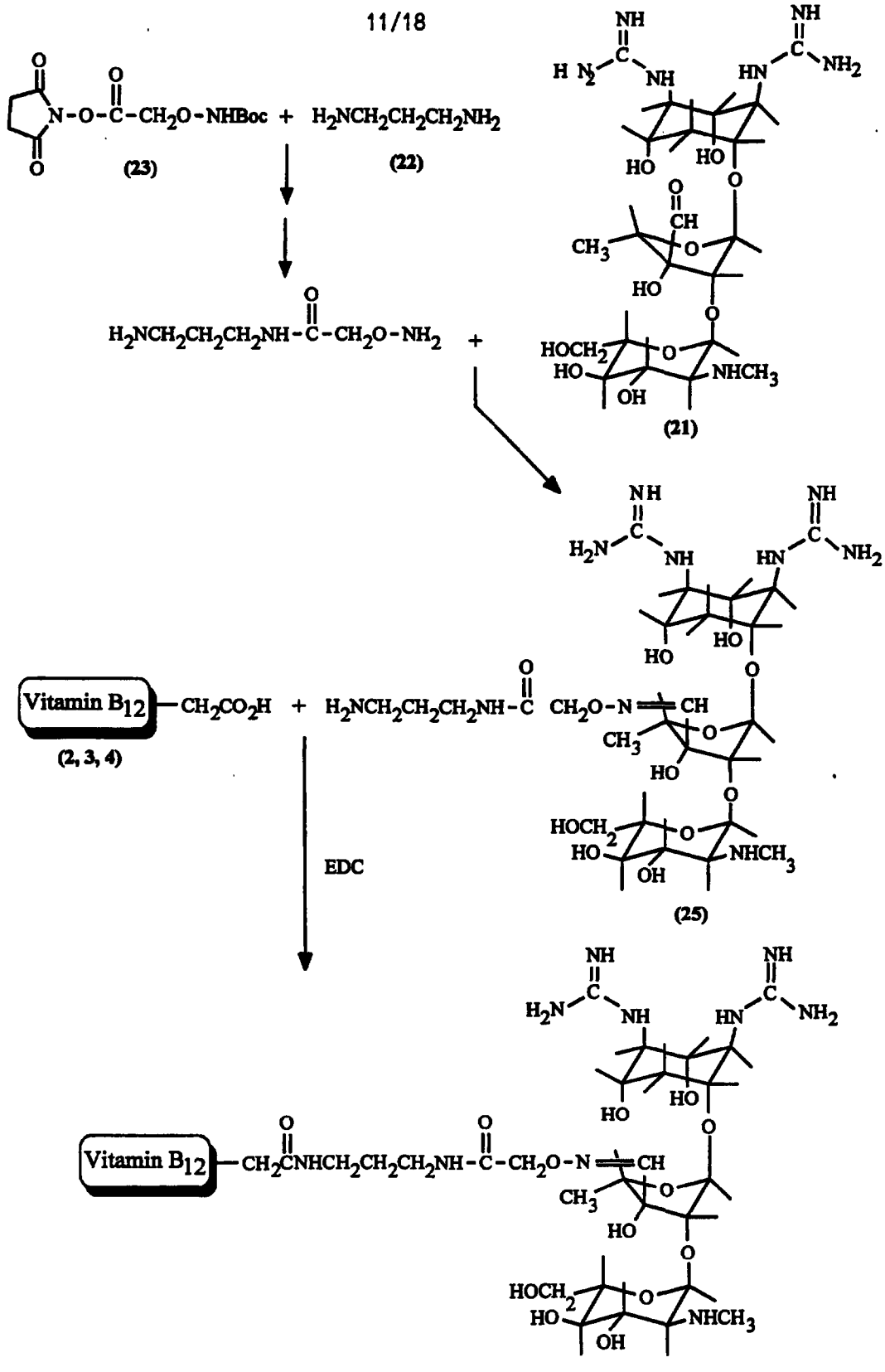


Fig. 13

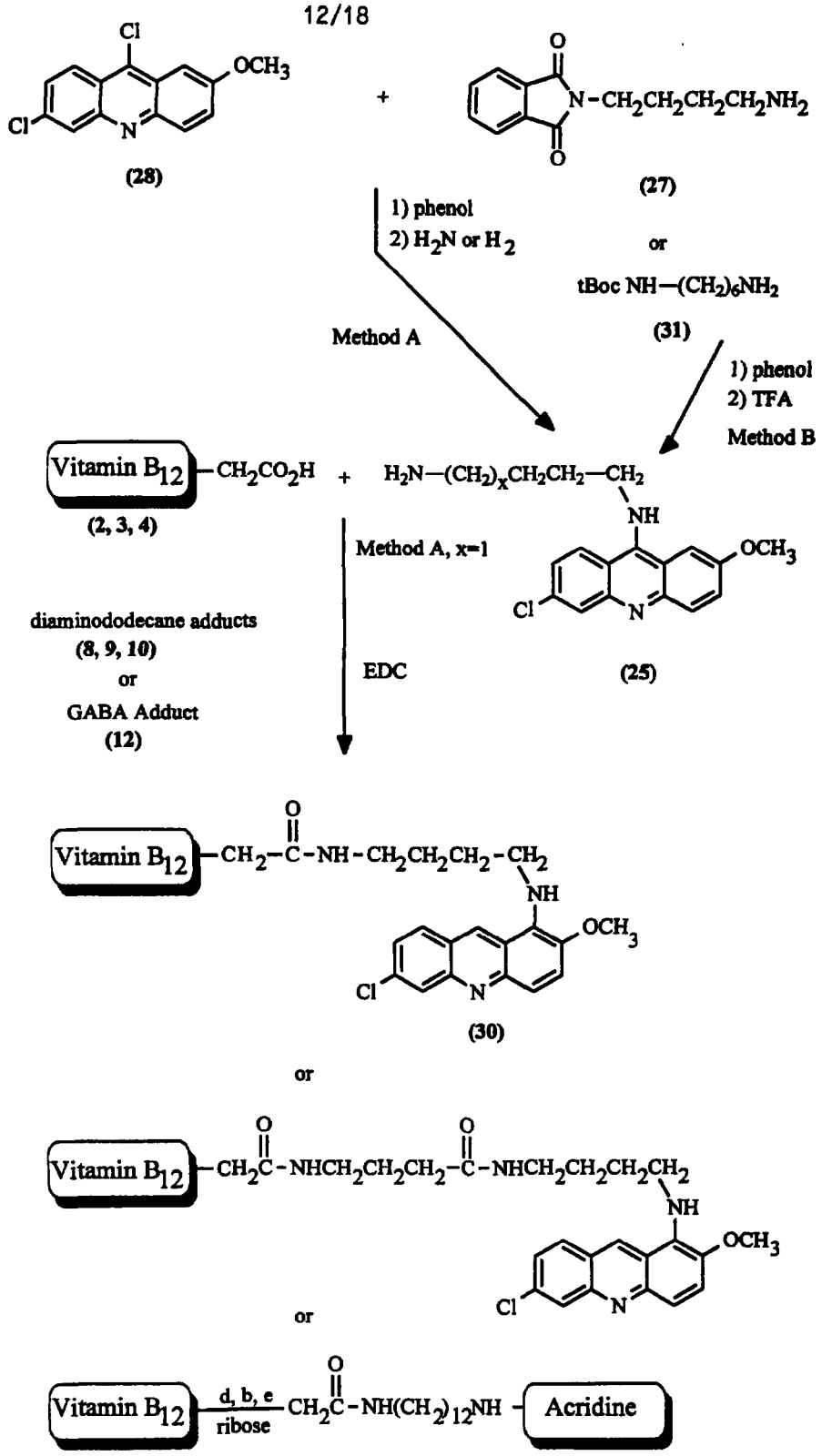


Fig. 14

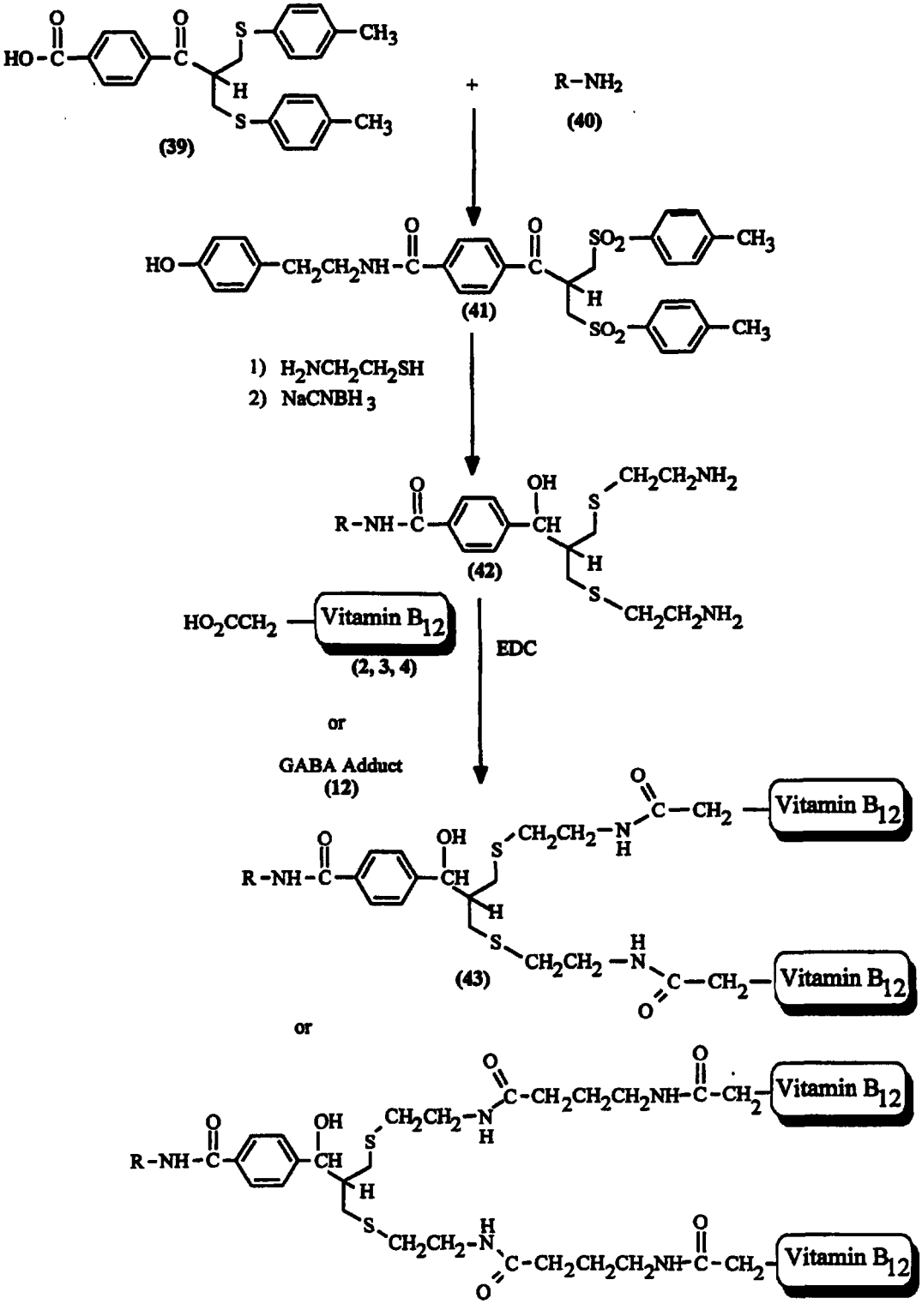
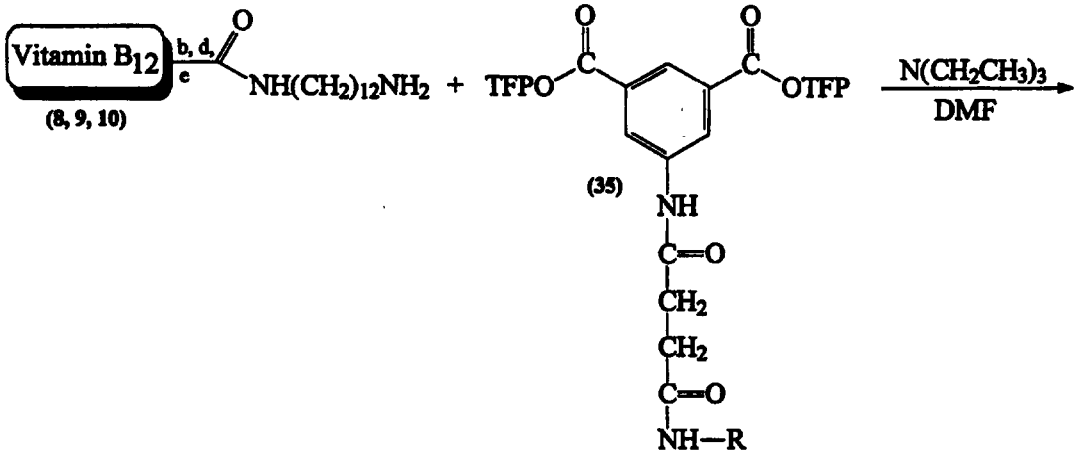


Fig. 15



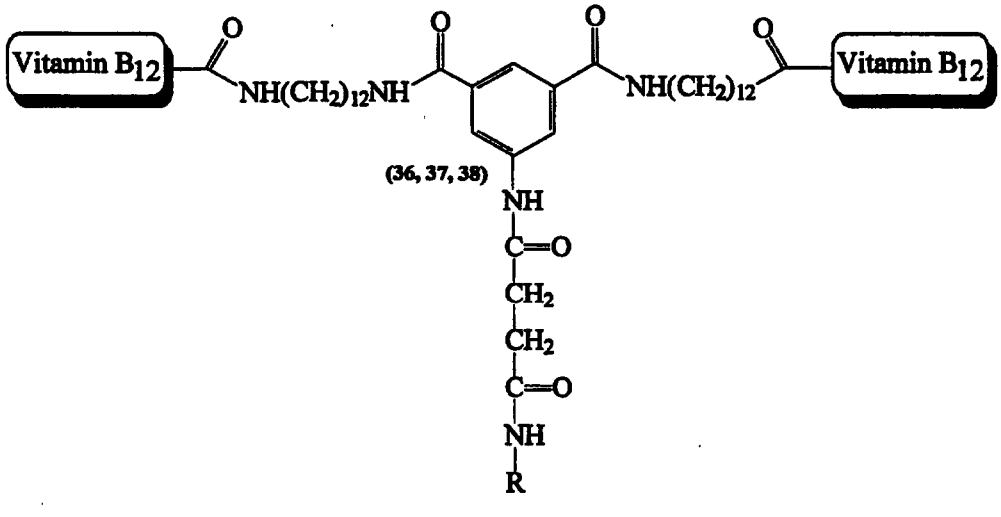


Fig. 16

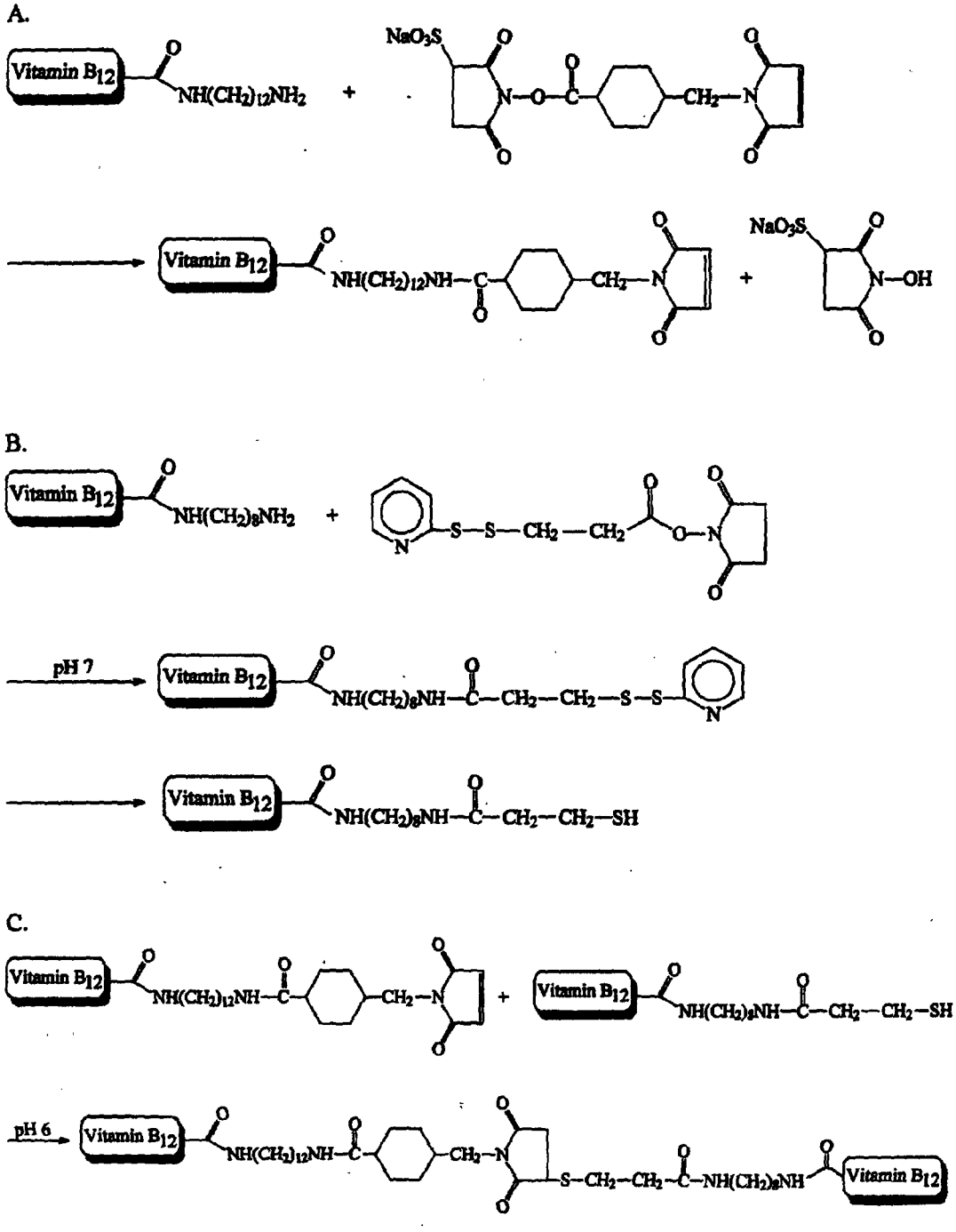


Fig. 17

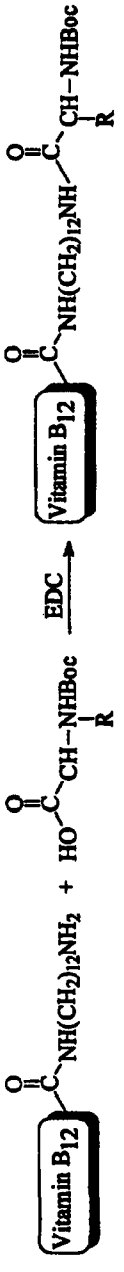


Fig. 18

Fig. 19

Fig. 20

Fig. 21

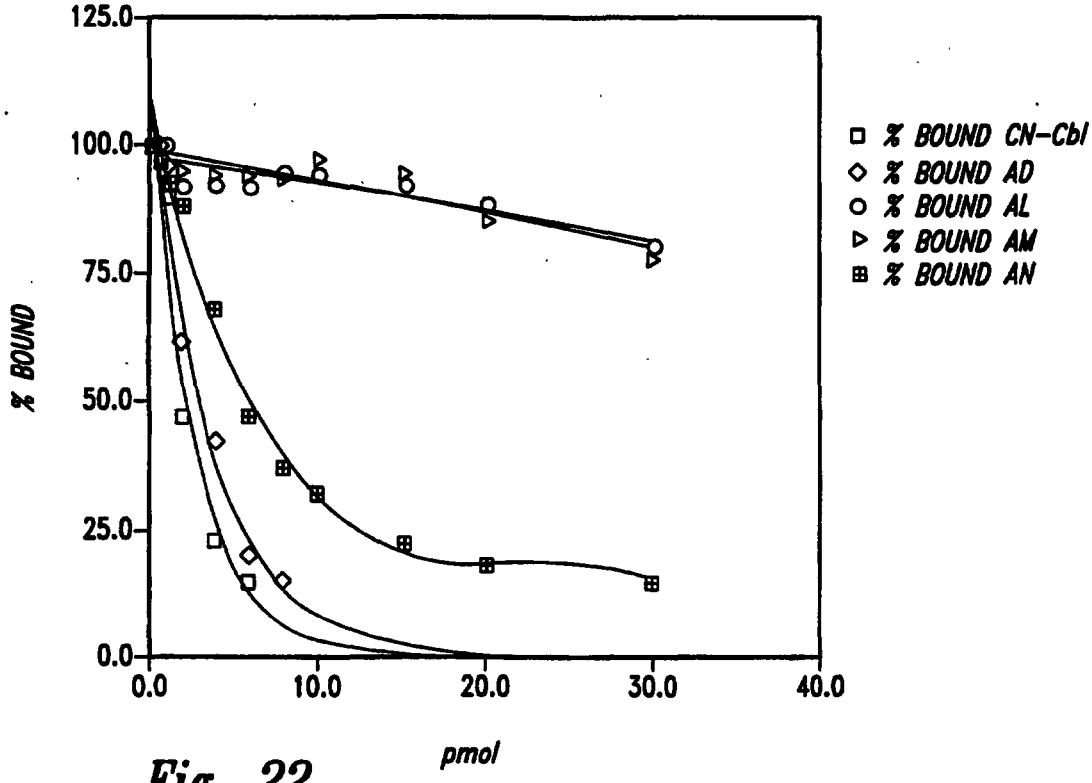


Fig. 22

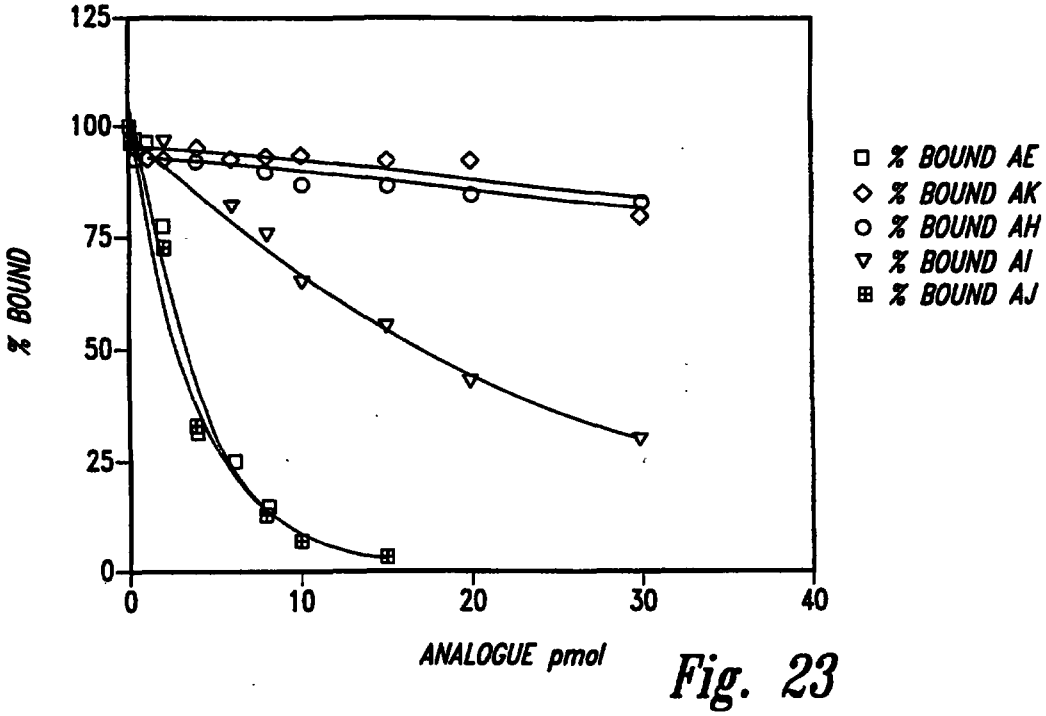


Fig. 23

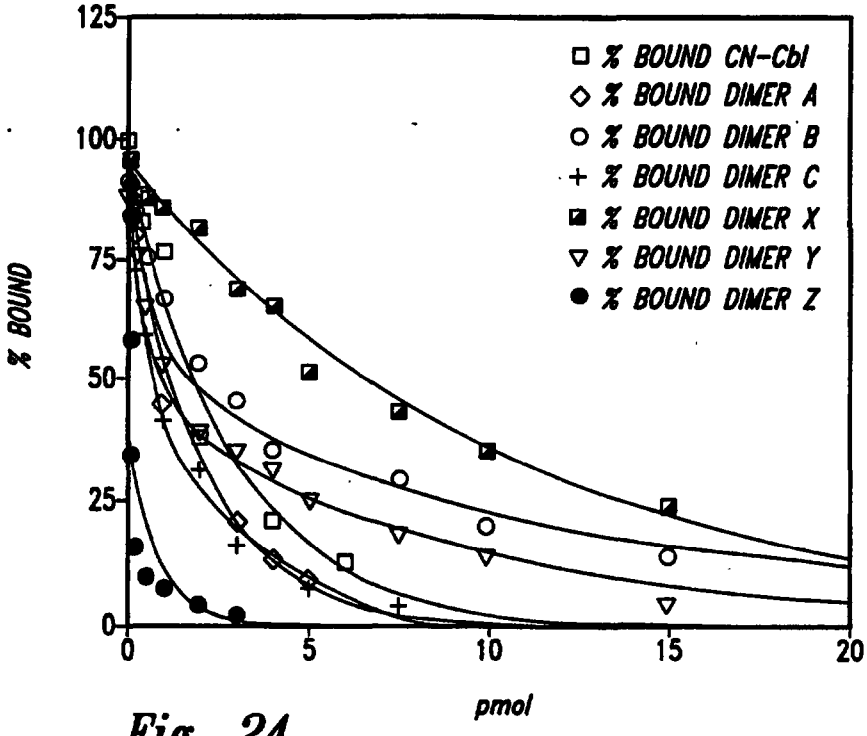


Fig. 24

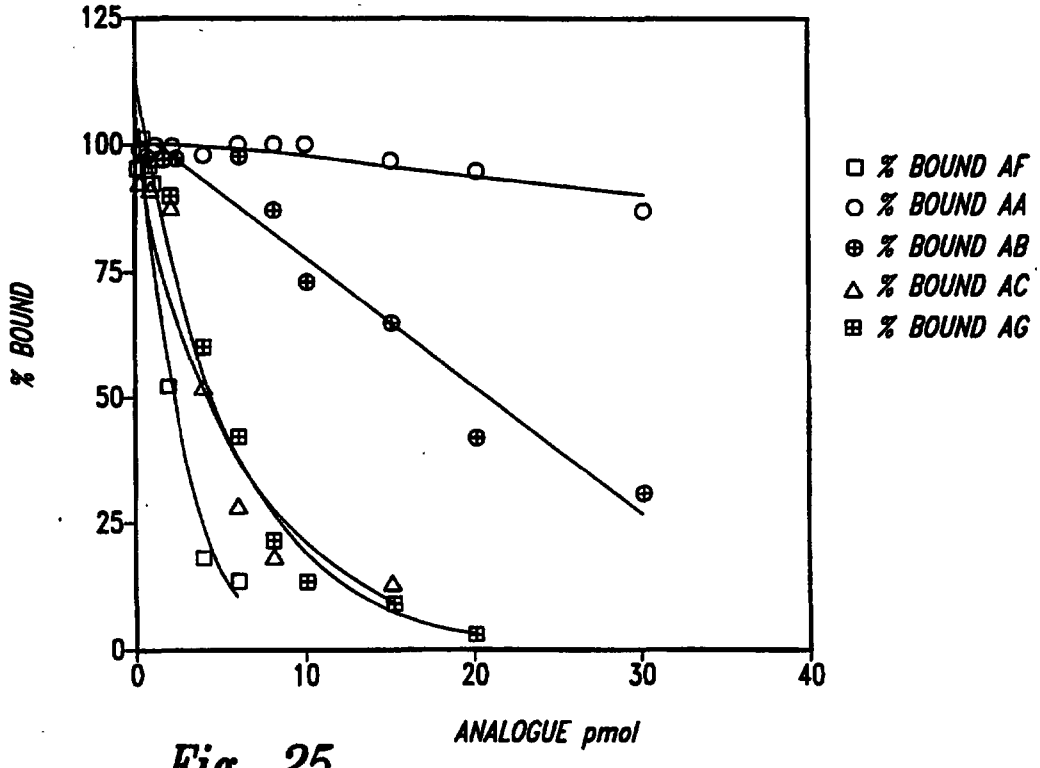


Fig. 25

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 95/04404

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H23/00 G01N33/82 A61K31/68

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 C07H G01N 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	EP,A,0 425 680 (TEIJIN LTD) 8 May 1991 see page 3 - page 5 ---	1,26,39, 79,80, 101
A	EP,A,0 069 450 (TECHNICON INSTR) 12 January 1983 see example ---	1,26,39, 79,80, 101
A	US,A,4 167 556 (SELHUB JACOB ET AL) 11 September 1979 see the whole document -----	1,26,39, 79,80, 101

☐ 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

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1995	18.08.95

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	Moreno, C

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

national application No.

PCT/US 95/ 04404

INTERNATIONAL SEARCH REPORT

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.: 39-69,77-79
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 39-69,77-79 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 searches 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

Intern al Application No
PCT/US 95/04404

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0425680	08-05-91	JP-A-	2289597	29-11-90
		WO-A-	9010014	07-09-90
		US-A-	5405839	11-04-95
EP-A-0069450	12-01-83	CA-A-	1180273	01-01-85
		JP-C-	1848006	07-06-94
		JP-A-	58000997	06-01-83
		US-A-	4465775	14-08-84
US-A-4167556	11-09-79	US-A-	4273757	16-06-81

Effect of combined ascorbic acid and B-12 on survival of mice with implanted Ehrlich carcinoma and L1210 leukemia[1,2]

M Eymard Poydock

ABSTRACT A combination of dehydroascorbic acid and hydroxycobalamin (vitamin B-12) inhibited mitoses of tumors in mice. The present study was performed to test the effect of these vitamins on the survival of mice bearing carcinomas and leukemias. In each assay 40 mice received 0.1 mL ip tumor cells ($\times 10^5$). After 24 h, 20 mice were injected with 0.2 mL (0.4 g/kg body wt) of the vitamins daily for 10 d. All controls died by day 19, but > 50% of the treated mice were alive after 60 d. In vitro findings revealed inhibition of mitoses in L1210 leukemia cells, but not in normal L929 cells. In recent research with cobalt-ascorbate plus vitamin C, we demonstrated that when B-12 is combined with vitamin C, the cobalt nucleus of B-12 attaches to a carbon on vitamin C, forming cobalt ascorbate. Tests proved that cobalt ascorbate plus vitamin C also inhibited tumor cells. *Am J Clin Nutr* 1991;54:1261S–5S.

KEY WORDS Ascorbic acid, vitamin C, hydroxycobalamin, vitamin B-12, cancer, combination therapy

Introduction

Chemotherapeutic agents such as antimetabolites and alkylating agents can be cytotoxic for normal proliferating cells as well as malignant cells. Consequently, progress in cancer therapy requires an exploration of other approaches, including biological response modifiers and agents that might be selectively toxic to malignant, but not to normal cells. The latter line of research has been conducted in our laboratory, and a series of experiments are summarized below.

Early experiments demonstrated an inhibiting effect in vitro of combined dehydroascorbic acid and vitamin B-12 on the mitotic activity of several types of tumor cells, but not on normal fibroblasts. This was followed by experiments in mice that confirmed the selective effect in vivo. These preliminary results were followed by investigations of the survival of mice bearing ascites tumor after treatment with the vitamin mixture. Finally, further explorations were conducted to elucidate the mechanism of tumor inhibition by these agents.

Methods and results

Inhibition of mitosis in vitro and in vivo by dehydroascorbic acid and B-12

In vitro experiments. These results have been reported in detail elsewhere (1). For the neoplastic material, sarcoma 37, Ehrlich carcinoma, Krebs 2, and L1210 leukemia were obtained from various sources; fibroblasts L929 were obtained for the nonneoplastic cells. Cells were propagated in Eagle, Earle's base medium with 10% calf serum. The vitamin mixture was prepared by adding 0.1 g B-12 (cyanocobalamin was equally effective) to 10 mL distilled water, and then adding 0.2 g dehydroascorbic acid (*see* Appendix). Immediately before use the mixture was diluted with three parts 5% calcium ascorbate to one part vitamin mixture. The tumor was obtained from the ascites exudate taken from a tumor stock mouse, centrifuged at 500 rpm for 10 min, and the pellet was resuspended to bring the cell suspension to 10^6 cells/mL. Then, 0.025 mL of the vitamin mixture was added to the test flasks. After incubation at 37.5 °C, mitotic counts were made at various times throughout the day (**Table 1**) by staining a drop of the cell suspension with acetocarmine. About 500 cells were counted and the mitotic index (M/C) was calculated as follows:

$$M/C = (\text{number of mitotic cells/total cells counted}) \times 1000$$

Table 1 shows the results of the in vitro experiments. The mixture of dehydroascorbic acid and vitamin B-12 inhibited mitoses in the neoplastic cells but did not affect the nonneoplastic L929 fibroblasts.

In vivo experiments. The animals were female HA/ICR Swiss mice obtained from the Roswell Park Memorial Institute (Buffalo, NY). All procedures conformed to the guidelines of the National Research Council for the care and use of laboratory animals. Transplantable sarcoma 37, Krebs 2, and Ehrlich carcinomas in the ascites form were used. Mice were injected with 0.1 mL ip washed ascites cells ($\times 10^6$), harvested on the seventh day after tumor implantation. Five experiments using a total of 50 mice were used for each tumor type. Twenty-four hours after tumor implantation the mice were divided into equal test and control groups. Test animals were treated daily with 0.2 mL ip of the vitamin mixture, 360 mg/kg body wt, for 7 d. Control animals received an equal volume of Hank's saline. After the third treatment, and for the remaining 5 d, one control and one test animal were killed at the same time each day. The ascites exudate and body cavity washings were centrifuged at 500 rpm for 10 min and were resuspended to prepare an acetocarmine smear as described above. Similar studies examined the effect of dehydroascorbic acid alone and vitamin B-12 alone.

[1] From the Cancer Research Institute, Mercyhurst College, Erie, PA.
[2] Address reprint requests to ME Poydock, Director of Cancer Research Unit, Mercyhurst College, 501 East 38th Street, Erie, PA 16546.

TABLE 1
Effect of a mixture of dehydroascorbate and vitamin B-12 (hydroxycobalamin) on mitoses of malignant cells (Sarcoma 37, Ehrlich, L1210) and nonmalignant cells (L929 fibroblasts) in vitro*

Tissue tested	Incubation time	Mitotic index	
		Test	Control
	h		
Sarcoma 37	0	48	48
	5	0	30
	6	0	75
	8.5	0	86
Ehrlich	0	78	78
	3.5	0	41
	4.5	0	55
	6.5	0	63
L1210	0	46	46
	2.5	0	40
	4.5	0	42
	6.5	0	48
L929	0	52	52
	2.5	50	48
	4.5	62	60
	6.5	64	62

* Reference 1.

Microscopic examination of the stained ascitic fluid from the mice after four treatments with the vitamin mixture revealed many lymphocytes, neutrophils, monocytes, and disintegrating tumor cells. By the sixth and seventh treatments, no tumor cells were visible in the ascitic fluid of treated mice, but numerous monocytes and macrophages were observed.

Table 2 presents the results of the effects on mitoses; the data represent an average of five experiments for each of the tumor types. Packed-cell volume of the ascites exudate from mice treated with the vitamin mixture was insignificant, and there were few tumor cells and no mitotic figures. Lymphocytes, neutrophils, and monocytes were abundant. In contrast, treatment with reduced ascorbate, calcium ascorbate, and vitamin B-12 administered separately, in the same doses as provided by the mixture, had no effect on the mitotic counts of Ehrlich ascites tumor (Table 3). Injecting a higher dose of reduced ascorbate slightly inhibited mitoses.

These results demonstrated that after three injections of the mixture of dehydroascorbic acid and vitamin B-12, mitotic activity of the ascites tumors (sarcoma 37, Krebs 2, and Ehrlich carcinoma) was completely inhibited, whereas the mitotic activity of the normal L929 fibroblasts was not. Ascites fluid taken from treated mice contained an increased concentration of lymphocytes, neutrophils, monocytes, and disintegrating tumor cells, suggesting an enhancement of immune function. Injections of ascorbic acid or of vitamin B-12 alone had no effect on mitotic activity or white blood cell population.

In further experiments, we investigated the effect of dehydroascorbic acid on mitotic activity of two different murine ascites tumors (2) and attempted to determine whether the effect was due to the acidity of dehydroascorbic acid. The methods and number of animals were the same as described above, with the following exceptions. P388 leukemia and Ehrlich carcinoma were used. Test animals were treated daily with 10 mg/0.2 mL dehydroascorbic acid ip for 7 d, and control animals received an equal volume of saline. Vitamin B-12 was not included in the mixture. Animals were killed after seven treatments. Moreover, to demonstrate that it was not the acidity of dehydroascorbic acid that caused inhibition of mitoses, a subsequent experiment was performed. By using the same procedure as above, test animals injected with tumor were treated with citric acid at the same pH (2.4) as the dehydroascorbic acid. Results proved that citric acid had no effect on mitoses.

Treatment with dehydroascorbic acid alone dramatically affected the mitotic activity (Table 4). The ascites exudate from treated mice was insignificant, and only a thin film of cells could be observed in the bottom of the tube after centrifugation, whereas packed-cell volume of control animals was 3–5 mL. Few tumor cells and no mitotic activity was observed, whereas monocytes and macrophages were numerous.

TABLE 2
Mitotic counts (M/C) and packed cell volume (PCV, in mL) taken for 5 successive days after treatment with a mixture of dehydroascorbic acid and hydroxycobalamin (vitamin B-12)*

No. of treatments	Ehrlich				Sarcoma 37				Krebs			
	M/C		PCV		M/C		PCV		M/C		PCV	
	T	C	T	C	T	C	T	C	T	C	T	C
3	0	40.5	0	0.5	0	25.2	0	0.96	0	29.6	0	1.34
4	0	39.6	0	0.96	0	32.0	0	0.92	0	22.0	0	0.66
5	0	45.0	0	1.24	0	25.6	0	0.7	0	21.0	0	0.72
6	0	31.6	0	1.54	0	25.6	0	0.94	0	20.0	0	0.72
7	0	28.0	0	2.1	0	25.2	0	0.9	0	24.0	0	1.4
Mean	0	36.9	0	1.27	0	26.7	0	0.89	0	23.3	0	0.97

* Represents the average of five experiments performed with each type of tumor. T, test; C, control. Reference 1.

TABLE 3
Mitotic counts taken for 5 successive days in Ehrlich ascites tumor after treatment with ascorbic acid and hydroxycobalamin administered separately*

No. of treatments	Ascorbic acid and calcium ascorbate (340 mg/kg)		Ascorbic acid (800 mg/kg)		Vitamin B-12 (20 mg/kg)	
	T	C	T	C	T	C
3	29.2	53.2	11.4	38.8	39.2	34.0
4	48.8	45.0	12.4	42.0	36.4	32.4
5	45.2	50.0	17.6	47.6	36.0	49.6
6	36.8	41.0	32.8	38.0	35.6	34.0
7	39.6	53.2	19.0	37.6	47.6	35.6
Mean	39.9	48.9	18.6	40.8	38.9	37.1

* Represents average data of five experiments performed with each type of treatment. The vitamin B-12 was administered in the same dosage as in the vitamin mixture whose results were presented in Table 1; the ascorbic acid/calcium ascorbate was also in the same dose as that used in the vitamin mixture, but ascorbic acid rather than dehydroascorbic acid was tested. T, test; C, control. Reference 1.

TABLE 4
Mean mitotic index taken in Ehrlich carcinoma and P388 leukemia after treatment with dehydroascorbic acid (DHA) and citric acid*

	DHA, 400 mg/kg (pH 2.4) P388 leukemia		DHA, 400 mg/kg (pH 2.4) Ehrlich carcinoma		Citric acid, 85.6 mg/kg (pH 2.4) Ehrlich carcinoma	
	T	C	T	C	T	C
	0	32	0	34	35	34
	0	34	0	32	41	36
	0	43	0	50	30	35
	0	40	0	34	30	35
	0	39	0	36	32	29
Mean	0	38	0	37	34	34

* Each group represents a sampling of 50 mice (25 test, 25 control). T, test; C, control. Reference 2.

Effect of dehydroascorbic acid and B-12 on survival of mice bearing ascites tumor

Earlier experiments in vitro and in vivo (1, 2) demonstrated an inhibitory effect on cell division of malignant cells without any comparable inhibition of normal fibroblasts or white blood cells. It remained to be answered whether these effects would be manifested in increased survival of animals with tumors. These methods and results have been reported elsewhere (3).

Transplantable P388 leukemia and Ehrlich carcinoma in the ascites form were used. P388 was maintained in DBA/2 stock mice, and Ehrlich carcinoma was maintained in HA/ICR Swiss mice. In this experiment the vitamin mixture was prepared by adding the crystals as follows: 0.01 g B-12, 0.01 g dehydroascorbic acid, and 0.01 g sodium ascorbate, into a small tube. Immediately before injection, 1 mL sterile distilled water was added to the tube.

Two experiments were conducted, one with P388 leukemia in BDF_1 mice and one with Ehrlich carcinoma in HA/ICR Swiss mice; 100 mice (50 test, 50 control) were used in each experiment. Animals were injected ip with 0.1 mL washed ascites cells ($\times 10^6$). After 24 h, mice were divided into test and control groups. Test mice received 6 mg/0.2 mL ip vitamin C and B-12 daily for 8 consecutive days. Control animals received an equal volume of saline. On the second weekend, mice received no treatment, and then injections were continued on alternate days for 3 wk for a total of 17 treatments. Treatment with a mixture of dehydroascorbic acid and B-12 dramatically increased the survival of tumor-bearing mice. **Figure 1** shows the results for mice treated with P388 leukemia, but results for Ehrlich carcinoma were virtually identical. All 50 control mice bearing P388 leukemia were dead by day 17, whereas the earliest deaths in the vitamin-treated group occurred on day 32. Median survival for untreated mice, both P388 and Ehrlich carcinoma, was 15 d; the experiments were terminated after ~70 d, at which time > 50% of the animals receiving the vitamin treatment were still alive.

Whatever the mechanism, these results demonstrate a very significant improvement in survival of tumor-bearing animals treated intraperitoneally with a mixture of dehydroascorbic acid and vitamin B-12 (hydroxycobalamin).

Effect of dehydroascorbic acid or ascorbic acid and B-12 on survival of mice bearing L1210 leukemia

Because of previous work indicating the efficacy of dehydroascorbic acid in suppressing tumor mitotic activity and the results showing increased survival in animals treated with dehydroascorbic acid plus B-12, we tested the effect of dehydroascorbic acid and B-12 on the highly resistant L1210 mouse leukemia (4).

As before, the vitamin mixture was prepared in crystalline form, 0.01 g hydroxycobalamin, 0.01 g dehydroascorbic acid, buffered to pH 5.2 with 0.01 g sodium ascorbate, dissolved with 1 mL sterile water. L1210 leukemia was supplied in DBA/2 mice from Roswell Park Memorial Institute.

A total of 140 mice were used in the three experiments conducted. Two experiments involved dehydroascorbic acid, with 40 female DBA/2 mice used in each; the third experiment involved ascorbic acid, with 60 mice used. Mice were injected intraperitoneally with 10^5 L1210 cells in 0.1 mL Tyrodes solution; this produced an ascitic leukemia, which later progressed to generalized disease. After 24 h, test mice were injected with 10 mg/0.2 mL ip (0.4 g/kg body wt) dehydroascorbate/B-12 or ascorbic acid/B-12 for 8 consecutive days. Two days later they were given another two treatments on alternate days, for a total of 10 treatments. Control mice were injected under the same conditions with saline of the same volume, pH, and osmolarity.

The two experiments involving dehydroascorbic acid, each with 40 mice, produced identical results, shown in **Figure 2.** Treatment with dehydroascorbic acid plus vitamin B-12 produced a substantial increase in survival. The median survival among control animals was 12 d, whereas for treated mice > 50% were still alive when the experiment was terminated after 60 d. Postmortem and histological examination of control mice revealed disseminated disease to liver, spleen, and lungs; treated mice killed after day 60 revealed no evidence of disease.

In the experiment involving recently purchased ascorbic acid plus B-12, the increase in survival of treated over untreated animals was minimal, and all animals were dead by day 17. (Note that our earlier published papers referred to ascorbic acid, but

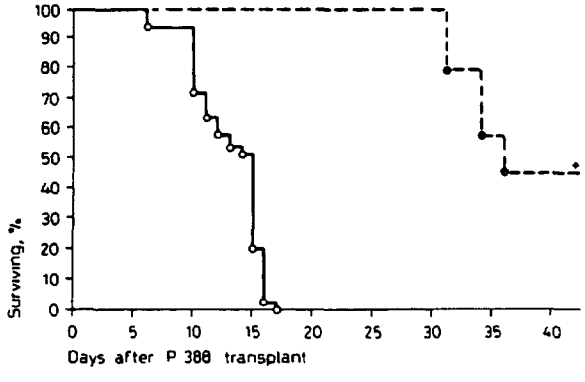


FIG 1. Survival of mice bearing P388 leukemia after treatment with dehydroascorbic acid and hydroxycobalamin (vitamin B-12). One hundred mice were sampled, 50 treated (● - - - ●) and 50 control (O —— O). Test mice were inoculated once daily with 0.2 mL of the vitamin mixture for 8 successive days; control animals received an equal volume of saline. + = study terminated.

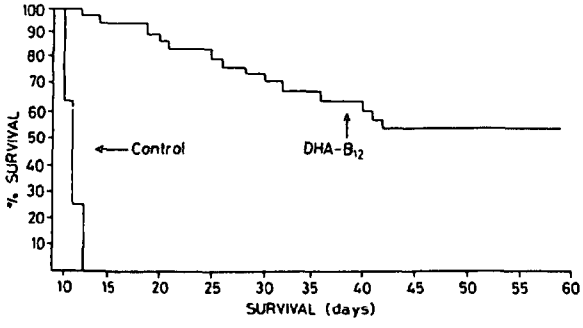


FIG 2. Survival of mice bearing leukemia L1210 after treatment with a mixture of dehydroascorbic acid and hydroxycobalamin (vitamin B-12). Eighty mice were sampled, 40 treated and 40 control. Test mice were injected with 0.2 mL ip of the vitamin mixture on days 1–8, 11, and 13. Control mice received an equal volume of a saline solution of the same pH and osmolarity.

we subsequently determined that our stock of ascorbic acid had oxidized, *see* Appendix A.)

Effect of dehydroascorbic acid and B-12 on growth of two solid tumors

Because ascites tumors are rarely found in clinical situations, we investigated the efficacy of the vitamin mixture on the growth of solid tumors (5). Two transplantable solid tumors, Krebs 2 and Ehrlich carcinoma, were used. Female HA/ICR Swiss mice were injected subcutaneously in the left flank with 0.1 mL (2×10^6) washed ascites cells in Hanks solution. (When an ascites tumor is injected subcutaneously it develops into a solid tumor.) Fifty mice received Krebs 2 carcinoma and 50 mice received Ehrlich carcinoma. The groups were then randomized to test and control. Starting 24 h after tumor implantation, test mice received, in the area of the tumor implant, 6 mg/0.2 mL sc (240 mg/kg body wt) of the dehydroascorbic acid-B-12 mixture buffered to pH 6.6 with sodium ascorbate. Control animals received an equal volume of Hank's solution. Treatment continued for 8 successive days.

Ten days after tumor transplantation, tumors were measured in three dimensions with a metric dial caliper with measurements in 0.05-mm increments. Results are shown in **Table 5.** All untreated mice developed tumors, whereas only two of the treated animals developed tumors, and those tumors were much smaller than in control animals. These results demonstrate that the combination of dehydroascorbic acid and vitamin B-12, when injected at the site of tumor implantation, almost completely inhibits the growth of these solid tumors.

Chemical investigations of the vitamin mixture

Hydroxycobalamin (vitamin B-12) contains at its center a cobalt atom bonded in a corrin ring structure. Experiments were conducted to determine whether it is the presence of the cobalt or the corrin ring that is critical to the efficacy of the vitamin mixture and whether ascorbate is critical to the efficacy (unpublished data).

The methods were similar to those described above. Ehrlich carcinoma and P388 leukemia tumors were used, and the effect on mitotic index was examined after treatment with various agents. Cobalt acetate and cobalt sulfate, when combined with sodium ascorbate, were as effective in inhibiting mitotic activity as had the hydroxycobalamin-ascorbate combination reported above. Thus, it would appear that it is the cobalt and not the corrin ring that is critical in the hydroxycobalamin. However, when cobalt acetate and cobalt sulfate were used alone, without the sodium ascorbate, there were no significant differences between the control and treated tubes. These experiments suggested that it is a compound of cobalt and ascorbate that is effective.

Further experiments in our laboratory confirmed that after mixture with dehydroascorbate and sodium ascorbate the B-12 was changed from the oxidized to the reduced form. The large stoichiometric excess of ascorbate present was sufficient to prevent the reoxidation of the Co(II) to Co(III). Fractionation experiments conducted at Battelle Labs, Columbus, OH, showed that the C-B-12 mixture contained unreacted ascorbate, reduced B-12, and a new compound showing evidence of cobalt-carbon covalent bond formation as well as preservation of the basic B-12 structure. It seems reasonable to postulate that some sort of cobalt (II) ascorbate complex is formed, and that it is this complex that is the active agent. It is also notable that cobalt (II) is much less expensive than B-12.

Discussion

This series of experiments demonstrated that a mixture of dehydroascorbate and hydroxycobalamin greatly inhibits mitotic activity of several tumor types without inhibiting the activity of normal fibroblasts; that hydroxycobalamin alone is ineffective, as are the cobalt salts, cobalt (II) acetate and cobalt (II) sulfate; that the vitamin mixture with the use of ascorbic acid instead of dehydroascorbic acid is much less effective. Most important, the experiments demonstrated that the dehydroascorbate-hydroxycobalamin vitamin mixture dramatically improves survival of tumor-bearing mice.

This series of experiments has several characteristics that may provide clues and directions for further research. *1)* It was the oxidized form, dehydroascorbic acid, which was effective in mitotic index and survival studies, not ascorbic acid itself. *2)* It appeared to be the combination of agents, dehydroascorbic acid and hydroxycobalamin, given simultaneously, that was effective. *3)* In the tumor models we used, the effect of an agent on mitotic index was a good predictor of subsequent effects on the survival of mice implanted with those tumors.

The first two of these characteristics may be useful in understanding the results of other studies of the role of ascorbate in

TABLE 5
Size (cm^2) of two types of solid tumor measured 1 wk after treatment with a mixture of hydroxycobalamin and dehydroascorbic acid buffered to pH 6.6 with sodium ascorbate*

Tumor	Test	Control†
Krebs 2	0.13‡	1.63 ± 0.47
Ehrlich	0.00§	2.19 ± 0.58

* n = 25. Reference 5.
† $\bar{x}$ ± SD. Controls all developed tumors.
‡ Mean of only two tumors that developed.
§ No tumors developed.

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relation to cancer. Some investigators have used just the reduced form, ascorbic acid; in our experiments that form was moderately effective in inhibiting mitoses only when given at very high doses and was ineffective in prolonging survival in the tumor types and animal models we used. Although other investigators have found ascorbic acid to be effective in various animal tumor models, future research should be careful to distinguish between these two forms and to examine their effectiveness separately. Furthermore, relatively few investigations have been carried out with combinations of ascorbate and other agents. Our results suggest that future research should examine the effectiveness of ascorbate when used in combination with other agents. Finally, it is notable that our research involved injection of the agents either intraperitoneally or directly in the area of the tumor. It would seem that future research might examine the effectiveness of various forms of ascorbate when administered in that way, in addition to simple administration in food or water.

Others have suggested that dehydroascorbate is an important inhibitor of cell growth and division (6, 7), and Omura et al (8) reported a 68% inhibition of growth of solid sarcoma 180 by dehydroascorbic acid. Moreover, Hogenkamp (9) reported that, in the presence of ascorbate, B-12 undergoes reactions leading to the release of the cobalt nucleus. Hollis et al (10) showed that the C2 carbon atom of vitamin C served as a binding site for transitional metals (which include both platinum and cobalt), leading to the formation of platinum-ascorbate that displayed antitumor activity. Such mechanisms and results would appear to warrant further investigation.

References

1. Poydock ME, Fardon JC, Gallina D, Ferro V, Heher C. Inhibiting effect of vitamins C and B12 on the mitotic activity of ascites tumors. Exp Cell Biol 1979;47:210–7.
2. Poydock ME, Reikert D, Rice J, Aleandri L. Inhibiting effect of dehydroascorbic acid on cell division in ascites tumors in mice. Exp Cell Biol 1982;50:34–8.
3. Poydock ME, Reikert D, Rice J. Influence of vitamins C and B12 on the survival rate of mice bearing ascites tumor. Exp Cell Biol 1982;50:88–91.
4. Poydock ME, Harguindey S, Hart T, Takita H, Kelly D. Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin. Am J Clin Oncol 1985;8:266–9.
5. Poydock ME, Phillips L, Schmitt P. Growth-inhibiting effect of hydroxycobalamin and L-ascorbic acid on two solid tumors in mice. IRCS J Med Sci 1984;12:813.
6. Edgar JA. Is dehydroascorbic acid an inhibitor in the regulation of cell division in plants and animals? Experientia 1969;25:1214–5.
7. Mills CA. Bone marrow nutrition in relation to the phagocytic activity of blood granulocytes. Effect of ascorbic acid on phagocytic activity established quantitatively. Blood 1949;4:153–9.
8. Omura H, Tomita Y, Yasuhiko N, Murakami H. Antitumor potentiality of some ascorbate derivatives. J Fac Agric Kyusu Univ 1974;18:181–9.
9. Hogenkamp HPC. The interaction between vitamin B-12 and vitamin C. Am J Clin Nutr 1980;33:1–3.
10. Hollis LS, Amundsen AR, Stern EW. Synthesis, structure and antitumor properties of platinum complexes of vitamin C. J Am Chem Soc 1985;107:274–6.

APPENDIX A

The in vitro and animal experiments reported in references 1, 3, and 5 were reported in the published papers to have used L-ascorbic acid. However, subsequent experiments in our laboratory with the use of freshly purchased ascorbic acid revealed that the active agent in the earlier work had probably been the oxidized form, dehydroascorbic acid, rather than ascorbic acid itself. The terminology used in the descriptions of that work in the present paper has therefore been corrected to refer to the active agent as dehydroascorbic acid rather than ascorbic acid.

Expl Cell Biol. 50: 88–91 (1982)

Influence of Vitamins C and B_{12} on the Survival Rate of Mice Bearing Ascites Tumor

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Mercyhurst College, Erie, Pa., USA

Key Words. Ascites tumors · Vitamin C · Vitamin B_{12}

Abstract. The survival rate of mice bearing P388 leukemia and Ehrlich carcinoma was increased after treatment with a mixture of vitamins C and B_{12}. All the mice receiving the vitamins outlived the control group. At the termination of the experiment, 30 days later, 50% of the treated mice appeared normal and healthy, whereas the remainder showed signs of tumor distention.

Introduction

Current animal experimentation and clinical findings suggest a new dimension in cancer therapy. The number of publications regarding the relationship between vitamins and cancer is steadily increasing. Nutritional deficiencies that often coexist with malignancy in humans also appear to be associated with lowered resistance [9, 10]. *Basu* et al. [1] and *Basu* [2] reported that nutrition and absorption of certain vitamins seem to improve the immunity of the body. A number of workers [8,11] have explored the efficacy of vitamin A treatment alone, and in combination with anticancer agents. *Kaplan and Busford* [12] suggested that vitamin B_{12} deficiency impairs the metabolic activity associated with phagocytosis suggesting that this vitamin is influential in augmenting the host's natural resistance. Evidence is accumulating that vitamin C may be a determinant factor in host resistance to cancer [4–7, 13, 15], and that large doses directly interfere with the metabolism of neoplastic cells. *Benade* et al. [3] found that ascorbic acid exhibited cytotoxic activity in Ehrlich carcinoma cells in tissue culture, while *Park* et al. [15] reported that ascorbic acid suppressed the growth of leukemic cells in vitro.

Previous studies in this laboratory by *Poydock* et al. [16] demonstrated complete inhibition of mitoses in sarcoma 37, P388 leukemia and Ehrlich carcinoma after treatment

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with a mixture of vitamins C and B_{12}, with no apparent toxic side-effects. More recently the author [17] reported that cell division was inhibited in two ascites tumors after injections of dehydroascorbic acid in mice. It seemed important, therefore, to investigate the effect of the vitamins on the longevity of mice bearing ascites tumors.

Methods and Materials

The transplantable P388 leukemia and Ehrlich carcinoma in the ascites form were used in this assay. To carry the ascites tumor in stock mice they were injected (i.p.) with 0.1 ml (10^6) washed ascites cells harvested on the 7th day after tumor implantation. P388 was maintained in DBA/2 stock mice, whereas Ehrlich carcinoma was propagated in HA/ICR Swiss mice.

Two experiments were designed for this study. Experiment 1 utilized P388 leukemia in BDE_1 mice and experiment 2 tested Ehrlich carcinoma in HA/ICR Swiss mice. A total of 100 mice (50 test, 50 control) were used for each experiment. All the animals were within a 3-gram weight range.

They were injected (i.p.) with 0.1 ml (10^6) washed ascites cells taken from a stock mouse. 24 h after tumor transplantation the mice in each experiment were divided equally into two groups and designated as test and control. The test mice were inoculated once daily (i.p.) with 6 mg/0.2 ml vitamin C and B_{12} mixture for 8 successive days. The control animals received an equal volume of saline. During the weekend the mice received no treatment, then the vitamin injections were continued on alternate days for 3 weeks. The total number of treatments was 17.

The vitamins, in crystalline form, were prepared by introducing 0.01 g B_{12}, 0.01 g sodium ascorbate (NaASC), and 0.01 g *L*-ascorbic acid into a small tube. A sufficient number of tubes with the dry crystals were prepared in advance. Immediately before injection, 1 ml sterile distilled H_2O was added to the tube. The solution was withdrawn into a 1-ml tuberculin syringe, a Millex-GS filter unit attached, and the needle added. Only the amount needed (1 ml) to inject 5 mice was diluted at one time.

Vitamin B_{12} (hydroxocobalamin) was received from Merck Chemical Company. The *L*-ascorbic acid and its salt NaASC, were purchased from Sigma Chemical Co. The Millex-GS filter unit was obtained from Millipore Corp.

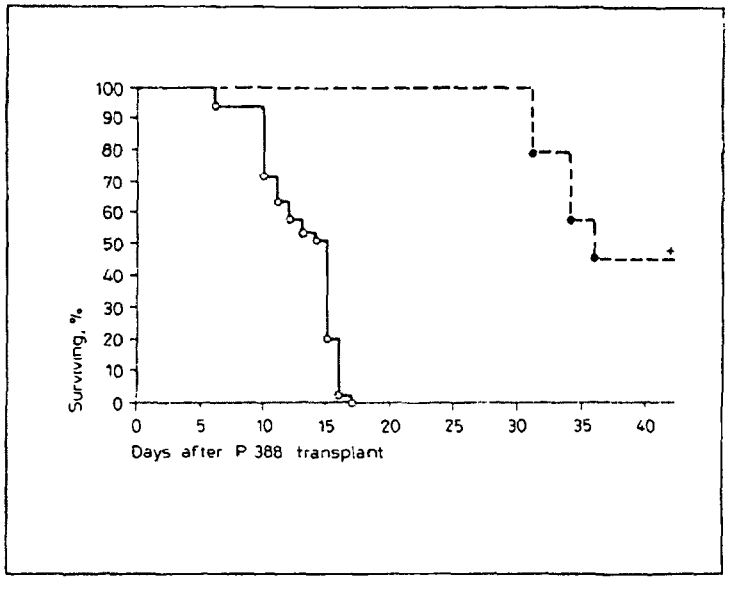

Fig. 1. Survival of mice bearing P388 leukemia after treatment with vitamins C and B_{12}. 100 mice were sampled; 50 treated (•) and 50 control (o). + = Terminated.

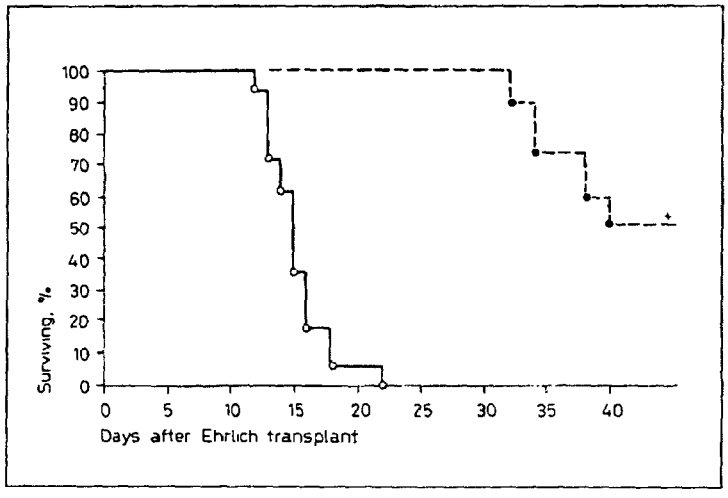

Fig. 2. Survival of mice bearing Ehrlich carcinoma after treatment with vitamins C and B_{12}. 100 mice were sampled; 50 treated (•) and 50 control (o). + = Terminated.

Results

Figures 1 and 2 show the percent survival of mice bearing P388 leukemia and Ehrlich carcinoma, respectively, after treatment with a mixture of vitamins C and B_{12}. The survival of mice receiving the vitamin mixture was significantly prolonged when compared with the control mice. The earliest deaths in the vitamin-treated group occurred on the 32nd day after tumor transplantation, whereas none of the control animals survived longer than 22 days.

The median survival time for the untreated mice, both P388 and Ehrlich, was 15 days. All the mice receiving the vitamins survived at least 30 days after tumor transplantation. Then a few, showing signs of cancer growth (distended with tumor tissue), died. After all the control mice and the test mice distended with tumors had died, the experiment was continued for another 30 days, then terminated. At this time about 50% of the animals receiving the vitamin treatment were still alive.

Discussion and Conclusion

Treatment with a mixture of vitamins C and B_{12} increased the survival rate of tumor-bearing mice. This substantiates reports published by *Cameron and Campell* [4] and *Cameron and Pauling* [5], where supplemental treatments of ascorbic acid significantly prolonged the survival time of patients with terminal cancer.

The mechanism of action which brings about the destruction of neoplastic cells is still unclear. The vitamin mixture may alter the cell membrane of malignant cells enabling the T lymphocytes to recognize them as foreign. Then, as *Old* [14] suggests, T cells kill the tumor cells by releasing a toxic factor that disrupts the cell membrane. This suggestion offered as a possible mechanism of action is supported in part by the fact that microscopic examination of the stained ascitic fluid taken from the mice after four treatments with the mixture of vitamins C and B_{12} revealed many lymphocytes, neutrophils, monocytes, and disintegrating tumor cells. When the

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1 Basu, T.D.; D
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stained ascites fluid was examined after the sixth and seventh treatments, no tumor cells, but an abundance of monocytes and macrophages were found [16]. This possible explanation, however, is hypothetical and certainly needs to be verified.

Acknowledgements

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References

1 Basu, T.D.; Dickerson, J.W.T.; Williams, D.C.: Interrelationships of nutrition and cancer. Ecol. Food Nutr. *2:* 193 (1973).

2 Basu, T.D.: Significance of vitamins in cancer. Oncology *33:* 183–187 (1976).

3 Benade, L.; Howard, T.; Burk, D.: Synergistic killing of Ehrlich ascites carcinoma cells by ascorbate and 3-amino-1,2,3,-triazole. Oncology *23:* 33–43 (1967).

4 Cameron, E.; Campell, A.: The orthomolecular treatment of cancer. II. Clinical trial of high dose ascorbic acid supplement in advanced human cancer. Biol. Interact. *9:* 285–315 (1974).

5 Cameron, E.; Pauling, L.: Supplemental ascorbic acid in the supportive treatment of cancer: prolongation of survival times in terminal human cancer. Proc. natn. Acad. Sci. USA *73:* 3685–3689 (1976).

6 Cameron, E.; Pauling, L.: The orthomolecular treatment of cancer. I. The role of ascorbic acid in host resistance. Biol. Interact. *9:* 273–283 (1974).

7 Cameron, E.; Pauling, L.; Leibovitz, B.: Ascorbic acid and cancer: a review. Cancer Res. *39:* 663–681 (1979).

8 Cohen, M.H.; Carbone, P.P.: Enhancement of the anti-tumor effects of 1,3-bis-(2-chloroethyl)-1-nitrosourea and cyclophosphamide by vitamin A. J. natn. Cancer Inst. *48:* 921 (1972).

9 Copeland, E.M.; Daly, J.M.;, Dudrick, S.J.: Nutrition as an adjunct of cancer treatment in the adult. Cancer Res. *37:* 2451–2456 (1977).

10 Danilevicius, Z.: Cancer and nutrition. J. Am. med. Ass. *238:* 1182–1183 (1977).

11 Dresser, D.W.: Adjuvanticity of vitamin A. Nature, Lond. *217:* 527 (1968).

12 Kaplan, S.S.; Busford, R.E.: Effect of vitamin B_{12} and folic acid deficiencies on neutrophil functions. Blood *47:* 801–805 (1976).

13 Lewin, S.: Evaluation of potential effects of high intake of ascorbic acid. Compar. Biochem. Physiol. *47:* 681–695 (1973).

14 Old, L.J.: Cancer immunology. Scient. Am. *236/5:* 62–69 (1977).

15 Park, C.H.; Amare, M.; Savin, M.A.; Hoogstraten, B.: Growth suppression of human leukemia cells in vitro by *L*-ascorbic acid. Cancer Res. 40: 1062: 1065 (1980).

16 Poydock, M.E.; Fardon, J.C.; Gallina, D.; Ferro, V.; Heher, C.: Inhibiting effect of vitamins C and B_{12} on the mitotic activity of ascites tumor. Expl Cell Biol. *47:* 210–217 (1979).

17 Poydock, M.E.; Reikert, D.; Rice, J.; Aleandri, L.: Inhibiting effect of dehydroascorbic acid on cell division in ascites tumors in mice. Expl Cell Biol. *50:* 34–38 (1982).

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Mini-review

Dehydroascorbic acid as an anti-cancer agent

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Abstract

Three discoveries together point the way to a potential treatment for cancer. In 1982, Poydock and colleagues found that dehydroascorbic acid has the remarkable ability to eliminate the aggressive mouse tumours, L1210, P388, Krebs sarcoma, and Ehrlich carcinoma. In 1993, Jakubowski found that cancer cells (but not normal cells) contain measurable quantities of homocysteine thiolactone. Recently, the author found that dehydroascorbic acid reacts with homocysteine thiolactone converting it to the toxic compound, 3-mercaptopropionaldehyde. Taken together, these findings suggest that rapidly-dividing tumour cells make unusually large amounts of homocysteine thiolactone and that administered dehydroascorbic acid enters the cells and converts the thiolactone to mercaptopropionaldehyde which kills the cancer cells. The effectiveness of dehydroascorbic acid might be further increased by combining it with methionine and/or methotrexate to increase the homocysteine concentration in cancer cells.

Keywords. Homocysteine thiolactone; Dehydroascorbic acid; Mercaptopropionaldehyde; Cancer treatment; Methionine auxotrophy

Twenty five years ago, the cancer-curing effect of dehydroascorbic acid was first discovered. The significance of that discovery was not appreciated until recently when it was corroborated by new findings. This review summarizes the original discovery and two recent discoveries which explain the anti-cancer effect of dehydroascorbic acid as well as the phenomenon of methionine auxotrophy in cancer cells.

Discovery 1: dehydroascorbic acid kills cancer cells

Between 1979 and 1985, Poydock and colleagues, published results showing a remarkable anti-cancer effect of dehydroascorbic acid [1–6]. Initially, they intended to study the effect of a mixture of Vitamins C and B_{12}.[1] However, after their first publication describing marked anti-cancer effects [1], they found that the "ascorbic acid"

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[1] It should be noted that Poydock continued to add Vitamin B_{12} to most treatment protocols although her own data showed that it was not needed and there was no good rationale for adding it. Her initial use of B_{12} seems to have stemmed from the orthomolecular theories of Linus Pauling and a few reports describing anti-cancer effects of megadoses of B_{12} although there were other reports showing no effect of B_{12} and some showed that B_{12} enhanced tumour growth To this day there is no rationale for giving B_{12} and no known reaction between B_{12} and ascorbic acid or dehydroascorbic acid which could explain her results. On the contrary, it is known that B_{12} administered in excess of the carrying capacity of TC I and TC II is rapidly excreted in the urine.

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which they had used was old and had oxidized to dehydroascorbic acid [2]. In subsequent studies with authentic materials, they established that dehydroascorbic acid was the active factor and that ascorbic acid had no effect [2,4]. The studies were carried out carefully with several malignant murine cell lines *in vitro* and *in vivo* and with large numbers of mice. A non-malignant cell line was used for comparison. The results are summarized briefly below.

In *in vitro* experiments, cells were cultured in MEM 10% fetal calf serum with and without 2 mM dehydroascorbic acid and 25 μM cyanocobalamin. The cultures were examined for mitotic figures at frequent intervals. Malignant cells (L1210, Sarcoma 37, and Ehrlich carcinoma) with treatment showed no mitosis after 2.5–5 h. Untreated malignant cells continued to multiply and the non-malignant lymphocyte cell line (L929) continued to multiply with or without treatment [1].

In the *in vivo* counterpart, cancer cells from ascites culture, were injected intraperitoneally into mice and, after one day, the mice were injected i.p. daily with 300 mg/kg of dehydroascorbic acid and 80 mg/kg of cyanocobalamin. Controls were injected with saline. There were 50 mice in each group. Mice were killed daily and examined for mitoses. In treated mice, mitosis of malignant cells stopped after three treatments and malignant cells were not detectable after five treatments. In control mice receiving saline, the tumour cells continued to multiply [1]. This experiment was repeated with dehydroascorbic acid alone (no B_{12}) with the same results [2].

Survival studies were carried out according to the NCI protocol which was used to develop all the early anti-cancer drugs [7]. With P388 and Ehrlich carcinoma, 50 mice received 2 mg of dehydroascorbic acid (80 mg/kg) and 2 mg of cyanocobalamin intraperitoneally daily and 50 mice received saline. The survival data were remarkable. Control mice receiving saline died on average at day 11 as expected. Treated mice with P388 and Ehrlich carcinoma did not start to die until day 32 and 50% of them went on to long-term survival with no tumour cells detectable [3].

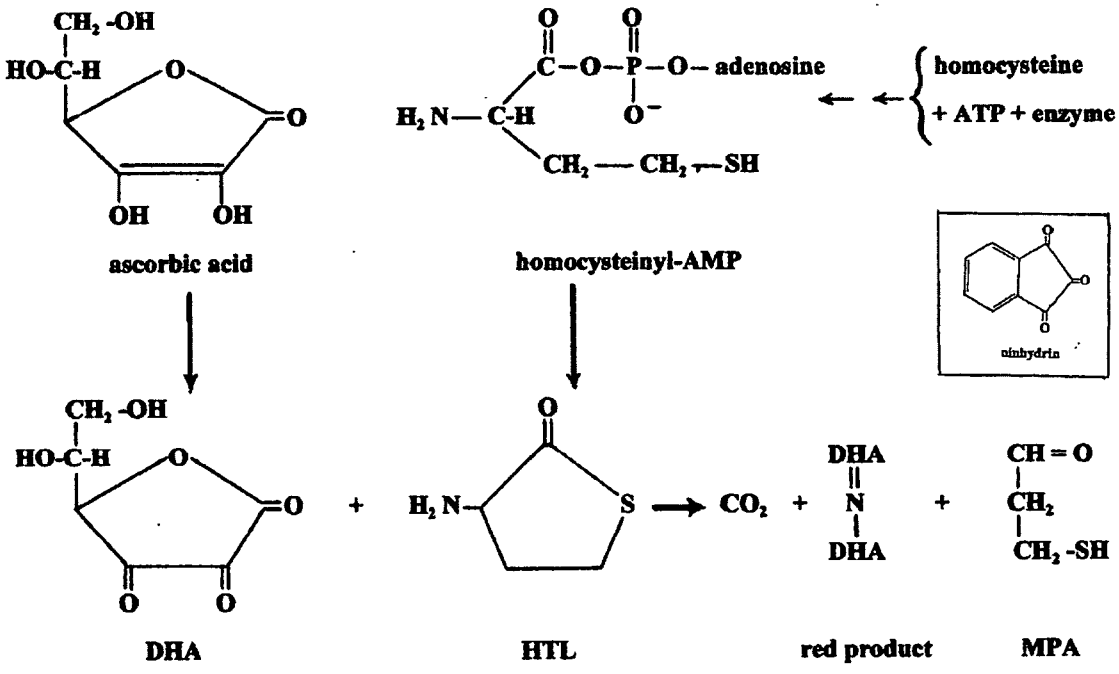
In the survival study with L1210, dehydroascorbic acid and ascorbic acid were compared. Forty mice received dehydroascorbic acid 2 mg per day (80 mg/kg) plus B_{12}, 40 mice received saline, and 60 mice received ascorbic acid 2 mg per day plus B_{12}. The control mice receiving saline and the mice receiving ascorbic acid died at the same rate with an average survival of 11–12 days. The mice receiving dehydroascorbic acid did not begin to die until day 12 and by day 42 only 50% were dead. That 50% went on to long-term survival and were free of tumour cells [4].

Solid tumours were studied using Krebs 2 sarcoma and Ehrlich carcinoma which grow as solid masses when inoculated subcutaneously [5]. Fifty mice were treated by subcutaneous injection of dehydroascorbic acid 2 mg per day (80 mg/kg) plus B_{12}. After 1 week, the control Ehrlich carcinoma tumours had an average size of $2.19 \pm 0.58\ cm^2$ while there were no tumours in the dehydroascorbic acid-treated mice. With Krebs sarcoma, the control tumours averaged $1.63 \pm 0.47\ cm^2$ while only 2 out 25 dehydroascorbic acid-treated mice had tumours and they were very small ($0.13\ cm^2$) [5,6].

These results are truly remarkable. These mouse tumours are extremely aggressive and their "cure" by a single agent was unknown at the time. In the survival tests with L1210 and P388, untreated mice normally die in a very narrow period around day 11. An increase in survival time of 20% is considered significant [7]. Long-term survival is rare in L1210 even with aggressive combination chemotherapy and then only in one or two mice out of groups of ten. P388 is more aggressive and survival even rarer. Poydock's observation of 100% cure rate and long-term survival is exceptional. These results were unexplainable at the time; they were given little credence, and have been rarely cited. However, they can be explained now on the basis of two new findings:the synthesis of homocysteine thiolactone in cancer cells and the recently-discovered reaction between dehydroascorbic (DHA) acid and homocysteine thiolactone (HTL) in which HTL is converted to toxic mercaptopropionaldehyde.

Discovery 2: HTL detected in cancer cells

HTL synthesis is mammalian cells was first demonstrated in three malignant cell lines, human cervical carcinoma (HELA), mouse renal adenocarcinoma (RAG), and Chinese hamster ovary carcinoma (CHO) [8]. In direct comparison under the same conditions, HTL could not be detected in two non-malignant cell lines, normal



human fibroblasts (GM 1374) and normal mouse fibroblasts (BALB/c 3T3). However, in normal mouse fibroblasts exposed to the folate antagonist, aminopterin, or in human fibroblast cell lines containing genetic defects in cystathionine beta synthase, HTL was formed in a linear relationship with time of culture [9]. These two conditions promote homocysteine accumulation by blocking its conversion to methionine or to cysteine, respectively. In human breast cancer cells exposed to aminopterin, there was a dramatic (ninefold) increase in HTL production [9].

The mechanism of HTL biosynthesis involves the process of amino acid activation (a preliminary step in protein synthesis which involves ATP, the amino acid, transfer RNA, and an amino acid-specific enzyme called amino acyl-tRNA synthetase). Homocysteine itself does not occur in proteins and there is no enzyme for activating it. However, homocysteine is erroneously activated by methionyl-tRNA synthetase in mammals. This is recognized as an error and the homocysteinyl-AMP is edited-out whereupon it converts to homocysteine thiolactone [8]. The chemical energy of the phosphoanhydride bond is conserved in the thiolactone bond.

The presence of HTL in cancer cells is a finding of major interest which has not yet been fully developed. It may be related to two conditions in rapidly-dividing cells:

(a) the high demand for methyl groups which leads to homocysteine formation

S-adenosylmethionine → *S*-adenosyl-homocysteine → homocysteine (1)

(b) the high rate of protein synthesis which increases the risk of homocyseine misactivation

homocysteine + ATP → homocysteinyl-AMP → homocysteine thiolactone (2)

Together these processes can explain the occurrence of HTL in rapidly-dividing cancer cells, as discussed further below.

Discovery 3: the dehydroascorbic acid–homocysteine thiolactone reaction

When DHA and HTL are combined in aqueous solution at neutral pH and room temperature, there

is a spontaneous reaction yielding CO_2, a red product, and 3-mercaptopropionaldehyde [10]. This is the Strecker degradation of amino acids with DHA replacing the traditional ninhydrin; the product from ninhydrin being blue and that from DHA being red. The remarkable feature of the DHA–HTL reaction is the facility with which it occurs. Other amino acids react with DHA (as they do with ninhydrin) but only at boiling temperature [11]. In contrast, HTL reacts at room temperature. The high reactivity of HTL can be attributed to the high energy content of the thiolactone bond since homoserine lactone also reacts with DHA at low temperature [10]. The facility of this chemical reaction suggests that it can occur *in vivo*. This is supported by the observation that, when solutions of DHA and HTL were injected separately into mice, the red product was excreted in the urine during the next 3 h [10]. This reaction may have relevance to Alzheimer's Disease [10] and connective tissue disease [12].

Poydock's results explained

One further fact is needed to set the scene for Poydock's remarkable experiments, i.e. that extracellular dehydroascorbic acid is transported very rapidly into cells by the facilitative glucose transport system [13]. Therefore, in Poydock's experiments, the injected DHA would enter the tumour cells where it would convert the endogenous HTL to 3-mercaptopropionaldehyde. The latter compound combines the toxicities of aldehydes and mercaptans and would be expected to kill the tumour cells.

Therapeutic potential

The indicated mechanism has obvious advantages for cancer therapy; the toxic agent is generated inside the cancer cells and appears to be selective for cancer cells. As noted above, the high demand for methyl groups and the rapid synthesis of proteins may account for the occurrence of HTL in rapidly-dividing cells. However, Jakubowski found measurable HTL only in malignant cells and not in non-malignant cells [8]. This coincides with Poydock's finding that DHA kills cancer cells but not normal cells. There are two possible explanations; (a) that cancer cells but not normal cells make HTL and (b) that all cells have the same ability to make HTL but normal cells have the ability to destroy it rapidly while cancer cells have lost that ability. Extracellular thiolactonase activity has been demonstrated in human serum and intracellular thiolactonase has been found in human placenta [14] but there is no information on its activity in cancer cells. The enzyme would need to be active intracellularly in order to be protective against the mechanism as proposed here.

The therapeutic potential of this mechanism might be further increased by giving methionine or a folic acid antagonist (methotrexate) along with DHA in order to increase HTL formation in cancer cells. It is well documented that methionine ingestion causes an increase in plasma homocysteine in humans [15]. The effect of folate antagonists in increasing HTL production in cancer cells is described above [9]. It may be necessary to add these agents in order to use this therapy for slow-growing cancers in which HTL production may be slow.

Methionine auxotrophy also explained

If intracellular HTL is a "marker" for malignant cells, it may explain another sulfur-related cancer "marker", methionine auxotrophy, the inability of cancer cells to survive and multiply *in vitro* in medium in which methionine is replaced by homocysteine whereas normal cells can multiply in these conditions [16]. Methionine auxotrophy may now be explainable on the basis of the recent discoveries; i.e. when homocysteine is present in excess in the culture medium and methionine availability is severely limited, the enzyme methionyl-tRNA synthetase may activate homocysteine rather than methionine. This would lead to HTL formation and its conversion to MPA which would kill the cells. In fact, in many of the studies, HTL itself was added to the medium rather than homocysteine. Again, normal cells may survive in these conditions because they have the ability to destroy HTL. Research is needed to compare the relative activity of thiolactonase in normal and malignant cells.

Relationship to Vitamin C as an anti-cancer agent

There is renewed interest in testing intravenously-administered Vitamin C as a cancer therapy agent (reviewed in [13]). The basis of this interest is that the early trials used intravenous administration with positive results while later controlled tri-

als used oral administration with negative results. Oral Vitamin C achieves a physiologically-limited blood level of 0.2 mM whereas intravenous administration can achieve concentrations of 5–6 mM. It is theorized that Vitamin C may have a toxic effect on cancer cells through reactive oxygen species, hydrogen peroxide and superoxide (ROS), which are generated during its oxidation. It is also thought that ascorbate transport may involve its prior oxidation to DHA which is then transported by the glucose transporter [13,17]. This is supported by detection of ascorbate radical in the interstitial fluid in rats under the conditions of artificially high blood ascorbate induced by i.v. administration [17]. Ascorbate radical is an intermediate in the oxidation of ascorbate to DHA. Therefore, if Vitamin C is found to have a clinical effect on cancer, the DHA–HTL mechanism outlined above will have to be considered as an alternative to the ROS mechanism.

Conclusion

The field of sulfur metabolism has yielded more differences between cancer cells and normal cells than any other area of metabolism. Aside from methionine auxotrophy and HTL formation discussed above, absence of methyhlthioadenosine phosphorylase (MTAP) and deficiency of cystathionase are major markers for cancer. MTAP absence was first found in a bank of murine tumours [18] and has been identified in a large number of human cancers [19]. Cystathionase deficiency has been found in 50–80% of neuroblastomas [20,21], 45% of hepatoblastomas [22], and 80% of acute leukemias [23]. This phenomenon is associated with cystathioninuria *in vivo* and with cysteine auxotrophy of the affected cancer cells *in vitro*. Because of the role of cystathionase in removing homocysteine, it is tempting to speculate that a deficiency of it might be related to Jakubowski's finding of HTL in cancer cells. However, congenital cystathionase deficiency is not a known cause of hyperhomocysteinemia and, therefore, the acquired defect is not likely to cause HTL formation in cancer cells. Methionine auxotrophy, MTAP absence, and cystathionase deficiency have been exploited to design and test anti-cancer therapies. HTL formation and the application of DHA have the simplest rationale and the most direct treatment design but no trials have been done yet in humans.

References

[1] M.E Poydock, J.C. Fardon, D. Gallinia, V. Ferro, C Heher, Inhibiting effect of vitamins C and B12 on the mitotic activity of ascites tumors, Exp. Cell Biol. 47 (1979) 210–217.
[2] M.E. Poydock, D. Reikert, J. Rice, L. Aleandri, Inhibiting effect of dehydroascorbic acid on cell division in ascites tumors in mice, Exp. Cell Biol. 50 (1982) 34–38.
[3] M.E. Poydock, D. Reikert, J. Rice, Influence of vitamins C and B12 on the survival rate of mice bearing ascites tumor, Exp. Cell Biol. 50 (1982) 88–91.
[4] M.E. Poydock, S. Harguindey, T. Hart, H. Takita, D. Kelly, Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin, Am. J. Clin. Oncol. 8 (1985) 266–269.
[5] M.E. Poydock, L Phillips, P. Schmitt, Growth-inhibiting effect of hydroxycobalamin and L-ascorbic acid on two solid tumors in mice, IRCS J. Med. Sci. 12 (1984) 813.
[6] M.E. Poydock, Effect of combined ascorbic acid and B12 on survival of mice with implanted Ehrlich carcinoma and L1210 leukemia, Am. J. Clin. Nutr. 54 (1991) 1261S–1265S.
[7] S. Stone, I. Miller, Statistical properties of the L1210 mouse leukaemia tumor system in primary screening, Cancer Chemother. Rep. 5 (1975) 5–13
[8] H. Jakubowski, E. Goldman, Synthesis of homocysteine thiolactone by methionyl-tRNA synthetase in cultured mammalian cells, FEBS Lett. 317 (1993) 593–598.
[9] H. Jakubowski, Metabolism of homocysteine thiolactone in human cell cultures, J. Biol. Chem. 272 (1997) 1835–1942.
[10] J.I. Toohey, Mercaptopropionaldehyde from homocysteine: implications for Alzheimer's Disease, J. Alzheimer's Dis. 12 (2007) 241–243.
[11] B. Pecherer, The preparation of dehydro-L-ascorbic acid and its methanol complex. Some reactions of dehydro-L-ascorbic acid, J. Am. Chem. Soc. 73 (1951) 3827–3830.
[12] J.I. Toohey, Homocysteine toxicity in connective tissue: theories, old and new, Connect. Tissue Res. 49 (2008) 57–61.
[13] Y. Li, H.E. Schellhorn, New developments and novel therapeutic perspectives for Vitamin C, J. Nutr. 137 (2007) 2171–2184.
[14] J. Zimny, M. Sikora, A. Gurnowski, H. Jakubowski, Protective mechanisms against homocysteine toxicity the role of bleomycin hydrolase, J. Biol. Chem. 281 (2006) 22485–22492.
[15] P.J. Garlick, Toxicity of methionine in humans, J. Nutr. 136 (2006) 1722S–1725S.
[16] E. Cellarier, X Durando, M.P. Vasson, M.C. Farges, A. Demiden, J.C. Maurizis, J.C. Madelmont, P. Chollet, Methionine dependency and cancer treatment, Cancer Treat. Rev. 29 (2003) 489–499.
[17] Q. Chen, M.G. Espey, A.Y. Sun, J. Lee, M.C. Krishna, E. Schacter, P.L. Choyke, et al., Ascorbate in pharmacological concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo, Proc. Natl. Acad. Sci. USA 104 (2007) 8749–8754.
[18] J.I. Toohey, Methylthioadenosine nucleoside phosphorylase deficiency in methylthio-dependent cancer cells, Biochem. Biophys. Res. Commun. 83 (1978) 27–35.

[19] S.A. Christopher, P. Diegelman, C.W. Porter, W.D. Kruger, Methylthioadenosine phosphorylase a gene frequently codeleted with p16cdkN2/ARF acts as a tumor suppressor in a breast cancer, Cancer Res 62 (2002) 6639–6644.
[20] C.E. Klein, B Roberts, J. Holcenberg, L.M. Glode, Cystathionase metabolism in neuroblastoma, Cancer 62 (1987) 291–298.
[21] J.R. Rajnherc, A.H. van Gennip, N. G Abeling, J.M. vander Zee, P.A. Voute, Cystathioninuria in patients with neuroblastoma, Med. Pediat. Oncol. 12 (1984) 81–84.
[22] C.F. Geiser, V.E. Shih, Cystathioninuria and its origin in children with hepatoblastoma, J. Pediat. 96 (1980) 72–75.
[23] L.M. Glode, H.L. Greene, I. Bikel, Gamma-cystathionase in normal and leukemic cells, Cancer Treat. Rep. 63 (1979) 1081–1088.

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Methylcobalamin
Vitamin B_{12}
Androgen-sensitive cell line
Estrogen-sensitive cell line

Effects of Methylcobalamin on the Proliferation of Androgen-Sensitive or Estrogen-Sensitive Malignant Cells in Culture and *in vivo*

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Summary: Methylcobalamin is one of the coenzymatically active cobalamin derivates and required for the activity of the cytoplasmic enzyme methionine synthetase catalyzing the methylation of homocysteine into methionine. The effect of methylcobalamin on the proliferation of malignant cells has been examined. Methylcobalamin inhibited the proliferation of androgen-sensitive SC-3 cells (a cloned cell line from Shionogi mouse mammary tumor, SC115) in culture at the concentration of 100–300 μg/ml. An inhibitory activity of methylcobalamin on the proliferation was also observed in other cell lines (estrogen-sensitive B-1F cells from mouse Leydig cell tumor and MCF-7 cells from human mammary tumor) at the concentration of 500 μg/ml. Moreover, large doses of methylcobalamin injected intraperitoneally (100 mg/kg body weight/day) were non-toxic and suppressed the tumor growth of SC115 and B-1F cells in mice fed a vitamin B_{12} deficient diet. These results show that methylcobalamin inhibits the proliferation of malignant cells in culture and in vivo *and propose the possibility of methylcobalamin as a candidate of potentially useful agents for the treatment for some malignant tumors.*

Introduction

Abnormalities of serum levels of vitamin B_{12} and R-binder, one of less specific vitamin B_{12} binding proteins, have been reported in patients with many types of malignant tumor [1, 2]. Serum levels of vitamin B_{12} have been elevated in patients with chronic myeloid leukemia, polycythemia vera and idiopathic thrombocythemia. The presence of abnormal R-binder has been reported in some gastric cancer extracts [3]. Effects of vitamin B_{12} on the proliferation of malignant cells have been also reported. Various reports, however, showed conflicting results. In the treatment of children with neuroblastoma vitamin B_{12} therapy was effective in some studies [4, 5], but not effective in other studies [6, 7]. An inhibitory activity of vitamin B_{12} on the proliferation was shown in experiments with mice and rats. A mixture of vitamins B_{12} and C inhibited the mitotic activity of Ehrlich sarcoma in culture as well as the growth of certain solid tumors [8–10]. In another study with combination of vitamin C, vitamin B_{12} was not effective on the growth of L9 gliosarcoma [11]. In some cases vitamin B_{12} inversely enhanced the growth of fibrosarcoma in rats [12] and exerted a procarcinogenic effect on the induction of malignant tumors in rats and hamsters [13, 14].

A recent study indicated that the metabolically active forms of cobalamins, methylcobalamin

and 5'-deoxyadenosylcobalamin, effectively elongated cell doubling time for the various malignant cells in *in vitro* study [15]. These active metabolites could significantly increase the survival time of animals implanted with the P388 leukemia cells [16]. Furthermore, we have also shown the inhibition of the proliferation of the cell line, established from an androgen-sensitive tumor, by active cobalamins [17]. In addition, cobalamins have inhibited productive human immunodeficiency virus-1 infection of hematopoietic cells *in vitro* [18]. Atypical squamous metaplasia, which is a precancerous lesion, in the lung has disappeared by the administration of the supraphysiological doses of folic acid and vitamin B_{12} to the heavy smokers [19]. In the patients with chronic myelogenous leukemia, the plasma proportion of methylcobalamin was significantly lower than in a reference group, and an association of a low plasma proportion of methylcobalamin with a poor prognosis was shown [20] In the present study, effects of methylcobalamin on the proliferation of some sex hormone-sensitive cell lines *in vitro* and *in vivo* have been examined to know whether the supraphysiological dose of methylcobalamin is a useful agent for the treatment for some malignant tumors.

Materials and Methods

Cell culture The cell lines SC-3 and B-1F were established respectively, from an androgen-sensitive mouse mammary carcinoma SC 115 [21] and from an estrogen-sensitive mouse Leydig cell tumor T124958-R [22] SC-3 cells were cultured continuously in Eagle's minimum essential medium (MEM) containing 2% dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS) and 10^{-8} M testosterone in the presence of 5% CO_2 in air at 37°C B-1F cells were maintained in MEM-Ham s F12 (1 1 vol/vol) containing 0.1% (wt/vol) bovine serum albumin (HMB medium) supplemented with 10^{-8} M 17β-estradiol (E_2) in the presence of 5% CO_2 in air at 37 C Cells were grown to confluence and passaged using trypsin (0.01%, wt/vol)-EDTA (0 02%, wt/vol) in phosphate buffered saline MCF-7 cells, an estrogen-sensitive human breast cancer cell line, were kindly supplied by Dr. R L Sutherland (Garvin Institute of Medical Research, St Vinsent's Hospital, Sidney, Australia) and maintained in Dulbecco's modified Eagle's medium (DME) containing 10% FBS. MCF-7 cells were passaged using trypsin (0 05%, wt/vol)-EDTA (0 02%, wt/vol) in phosphate buffered saline

Cell growth experiment The effects of methylcobalamin on the proliferation were examined as described previously [17, 23–25] In the present study, the basic medium used for following experiments of B-1F and MCF-7 cells was HMB medium in spite of the presence of cyanocobalamin in HMB medium Because B-1F and MCF-7 cells could not grow in HMB medium without Ham's F12 and a stimulative effect of estrogen on the proliferation of B-1F cells was not fully observed in the presence of FBS even if at the low concentration B-1F and MCF-7 cells were plated on four replicate 35-mm culture dishes at an initial cell density of 1×10^4 and 2×10^4/dish in HMB medium and DME containing 5% DCC-treated FBS, respectively On the following day (day 0), the medium was changed into HMB medium supplemented with various concentrations of methylcobalamin To remove the residual FBS in full, the dishes on which MCF-7 cells were seeded were washed twice with phosphate-buffered saline These media were changed every 2 days On day 6, the viable cells were counted as described previously.

Animals and Diets Male DS mice (seven-week-old) were obtained from Shionogi Laboratories (Osaka, Japan), housed 5/cage in a temperature-controlled (25°C) room with 12-h light/dark cycle, and given *ad libitum* access to water and pelleted diets DS mice were transplanted subcutaneously with minced SC115 tumors Starting 24 hours (day 0) after transplantation, mice were injected intraperitoneally with or without methylcobalamin (100 mg/kg body weight/day) in 0.3 ml of saline solution daily for 24 days Sterile methylcobalamin solution (10 mg/ml of saline) was used Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection Pelleted vitamin B_{12} deficient diets (Clea Japan Inc. Tokyo, Japan) (Tab I) were stored at 4°C and given with every other day replacement Palpable tumor was measured with caliper at the indicated times In case of B-1F cells, after being har-

Table I Nutrient composition of vitamin B_{12} deficient diet

Vitamin-free casein	18.0%
Sucrose	67 6%
Corn oil	8 0%
Hegsted salt mixture	4.0%
Avisel	1 5%
DL-methionine	0 3%
Choline chloride	0 1%
Vitamin mixture	0 5%
Vitamin A, D_3 (500 000 IU/100 000 IU)	2 00 mg/100 g of diet
Vitamin A (500 000 IU/g)	2 00
Vitamin E (50%)	20.00
Vitamin B_1	1 50
Vitamin B_2	0 80
Vitamin B_6	0 50
Vitamin C-Ca	24.35
Vitamin K_3	0 50
Biotin (2%)	2.00
DL-Ca-pantothenate	8.00
PABA	10 00
Nicotinic acid	5 00
Inositol	10 00
Folic acid	0 20
Lactose	413.15

vested and washed, the cells (5×10^5 cells) resuspended in MEM were implanted subcutaneously into castrated male Balb/c mice (seven-week-old). The Balb/c mice were implanted subcutaneously with a fused pellet of 5% estradiol in cholesterol and injected intraperitoneally with or without methylcobalamin on the following day (day 0) of B-1F cell implantation.

Vitamin B_{12} assay: In principle, vitamin B_{12} assay was performed as described by Gimsing [20]. On day 24 the mice described above were sacrificed under nembtal anesthesia. The whole blood samples were taken via an inferior *vena cava* by venopuncture. The serum was separated after centrifugation within 30 min after sampling. Syringes and sample containers were covered with aluminium foil. All stages of the method were carried out by red safe-light or in darkness. Tumors were cut into small pieces and washed twice with 0.15 M NaCl at 4°C. Minced tissues were homogenized in four volumes of 0.15 M NaCl by Polytron PT-10. After centrifugation (10 min, 2000 g) of homogenates the supernatants were used for vitamin B_{12} and protein assays. The concentration of protein was determined using protein assay kit. The total concentration of vitamin B_{12} was determined by radioisotope dilution method using a solid-phase intrinsic factor as the specific cobalamin-binding protein The assays were performed as directed in the manual from the supplier of the assay kit. For vitamin B_{12} the intra-assay variation amounted to a mean of 2–6% and the inter-assay variation to a mean of 5–8%. In case of established cells, cells were seeded at an initial density of 1.3 and 2.6×10^6 cells/100-mm dish in MEM containing 2% DCC-treated fFBS and HMB medium, respectively, for SC-3 and B-1F cells. On the following day (day 0), the medium was changed to 2% DCC-treated FBS-MEM supplemented with 10^{-8} M testosterone (SC-3 cells) and 10^{-8} M estradiol (B-1F cells) in the presence or absence lof methylcobalamin (final concentration of 300 µg/ml for SC-3 cells or 500 µg/ml for B-1F cells). The cells harvested on day 3 were washed four times with 20 ml of ice-cold 0.15 M NaCl and then resuspended in four volumes of 0.15 M NaCl. The viable cell number was determined with hemocytometer by trypan blue dye exclusion method. The cells lyzed by freeze-thawing were homogenized as described for minced tissues

Statistical analysis. All values were expressed as mean ± SE. Significant differences were estimated by Student's t test ($p<0.05$).

Materials: The radioinert steroids, BSA, trypsin, activated charcoal and methylcobalamin were purchased from Sigma Chemical Co (St. Louis, MO, USA). Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden) MEM, DME and Ham's F-12 were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). FBS was from Nichirei Co , Ltd (Tokyo, Japan) M-vitamin B_{12}/ folic acid kit was from CHIRON (Tokyo, Japan). Protein assay kit was from BIO-RAD (California, USA). Balb/c mice were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan). The other chemicals used in this study were of analytical grade.

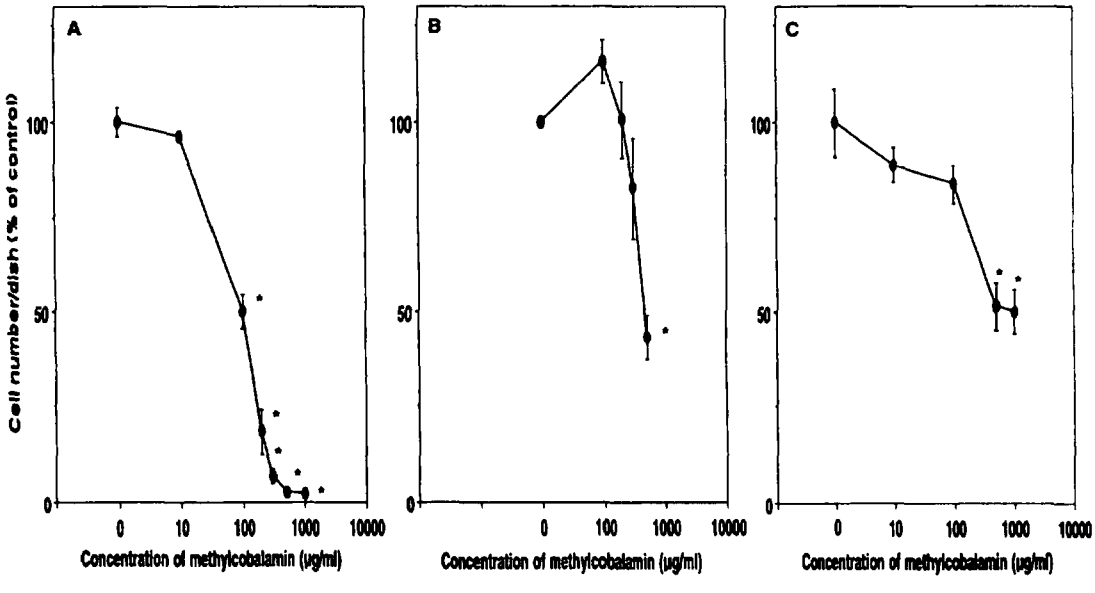

Figure 1: Effects of methylcobalamin on the proliferation of SC-3, B-1F and MCF-7 cells.
Cells were plated on four 35-mm culture dishes as described in materials and methods. On the following day (day 0), the medium was changed into the medium supplemented with various concentrations of methylcobalamin. On day 6 the viable cells were counted. The cell number was expressed as a percentage, taking the value in the absence of methylcobalamin as 100%. A, B and C correspond to SC-3, B-1F and MCF-7 cells respectively. Points, means of 4 determinations; bars, S. E.; *, $P<0.05$. Further 4 trials gave similar results.

Results

Effects of methylcobalamin on the proliferation of cultured cells: Figure 1 presents results on the cell yield of androgen-sensitive SC-3 cells and estrogen-sensitive B-1F and MCF-7 cells treated with various concentrations of methylcobalamin. In SC-3 cells (Fig. 1A) methylcobalamin inhibited the proliferation at the concentrations of 50–100 μg/ml (0.037–0.074 mM) as reported previously [17]. The proliferation of B-1F and MCF-7 cells were also inhibited (Fig. 1B and 1C) by methylcobalamin, but a higher concentration (500 μg/ml, 0.37 mM) of methylcobalamin was necessary.

Effects of methylcobalamin on the tumor growth: Effects of methylcobalamin on the tumor growth were examined at the dose of 100 mg/kg body weight/injection/day. The mice were able to tolerate the injection of methylcobalamin at this dose without any adverse effects. In the present study B-1F cells were used *in vitro* (in culture) and *in vivo*. In the experiments of androgen-sensitive tumor, tumor pieces of SC115 were subcutaneously transplanted instead of SC-3 cells, because SC-3 cells could not cause the tumor formation *in vivo*. Figure 2 shows an inhibitory effect of methylcobalamin on the tumor growth of SC115, especially in early phase, in DS mice. In B-1F cells (Fig. 3) methylcobalamin also inhibited the tumor growth. The ratio of the tumor formation was not significantly different between control and methylcobalamin injected mice (SC115 and B-1F cells). In histological findings with hematoxylin-eosin stain there was no prominent difference between tumors treated with and without methylcobalamin (data not shown), and no toxic effect on mice was observed.

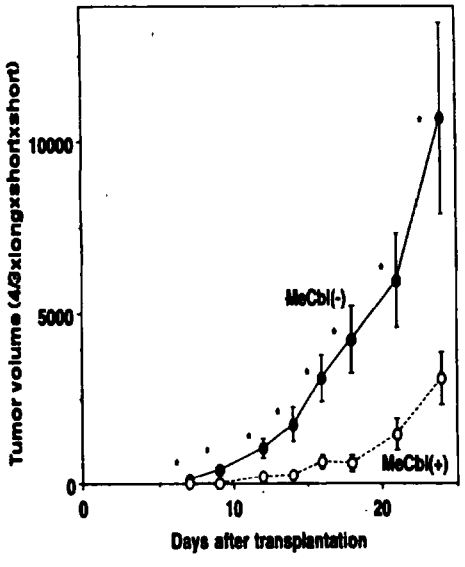

Figure 2: In vivo growth inhibition of SC115 tumor by methylcobalamin.
Male DS mice (seven-week-old) were transplanted subcutaneously with minced SC115 tumors. Starting 24 hours (day 0) after transplantation, mice were injected intraperitoneally with or without methylcobalamin (100 mg/kg body weight/day) daily for 24 days. Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection. Palpable tumor was measured at the indicated times. The tumor volume is expressed as 4/3 × long diameter (mm) × short diameter (mm) × short diameter (mm). Points, means of 10 determinations; bars, S.E.; *, $P<0.05$; MeCbl, methylcobalamin. Further 3 trials gave similar results.

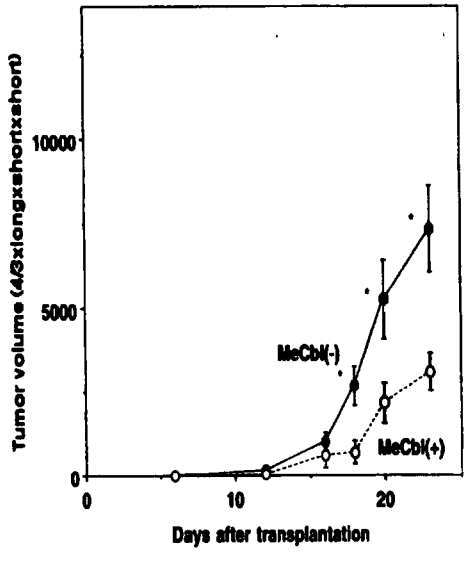

Figure 3: In vivo growth inhibition of B-1F tumor by methylcobalamin.
B-1F cells (5×10^5 cells) in MEM were implanted subcutaneously into castrated male Balb/c mice (seven-week-old). The mice were also implanted subcutaneously with a fused pellet of 5% estradiol in cholesterol and injected intraperitoneally with or without methylcobalamin on the following day (day 0) of B-1F cell implantation. Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection. Palpable tumor was measured at the indicated times. The tumor volume is expressed as 4/3 × long diameter (mm) × short diameter (mm) × short diameter (mm). Points, means of 10 determinations; bars, S.E.; *, $P<0.05$; MeCbl, methylcobalamin. Further 3 trials gave similar results.

Vitamin B_{12} assay: Sera and tissue specimens of the mice, and cultured cells treated with or without methylcobalamin were measured for total vitamin B_{12}, using M-vitamin B_{12}/folic acid kit. The concentrations of vitamin B_{12} are given in Table II. The serum, tissue and cellular concentrations of vitamin B_{12} were significantly increased in cases treated with methylcobalamin compared to the controls.

Discussion

We have found that methylcobalamin inhibits the *in vitro* proliferation of the androgen-sensitive cell line SC-3 and of the estrogen-sensitive cell lines B-1F and MCF-7 as well as the *in vivo* tumor growth of SC115 and B-1F cells. Effective dose of methylcobalamin was higher in B-1F and MCF-7 cells than in SC-3 cells. It might be partly due to the difference of the intracellular vitamin B_{12} contents among these cells. The basal level of vitamin B_{12} in SC-3 cells was extremely lower than that in B-1F cells. In the *in vitro* experiments the basic culture medium used for B-1F and MCF-7 cells was a HMB medium containing a cyanocobalamin at the concentration of 0.68 µg/ml. On the other hand the medium used for SC-3 cells contained vitamin B_{12} at the concentration of 10–30 pg/ml. Methylcobalamin seems to be also more effective for SC115 tumor than for B-1F tumor. The content of vitamin B_{12} in SC115 tumor was lower than that in B-1F tumor, while the serum levels of vitamin B_{12} were similar between Balb/c and DS mice. The metabolism of methylcobalamin might be different between SC115 and B-1F tumors, therefore between SC-3 and B-1F cells. In the *in vivo* experiments the observed maximum inhibitory activity of methylcobalamin was smaller than that in cultured systems, partly due to the solubility of methylcobalamin. It could not be solubilized in concentrations larger than 10 mg/ml in PBS. Therefore it was difficult that mice could be administered with methylcobalamin at doses larger than 100 mg/kg body weight/day in 0.3 ml of saline solution.

The precise mechanism of the inhibitory activity of methylcobalamin is not clear. In SC-3 cells, androgen induces FGF-like growth factor (AIGF) and FGF receptors [26, 27]. In MCF-7 cells, estrogen induces various growth factors

Table II: Concentration of vitamin B_{12} in tissue, serum and cells

	Methylcobalamin (–)	Methylcobalamin (+)
SC115 tumor		
Tumor	0.39±0.04 ng/mg protein (n=8)	13.28±1.34 ng/mg protein (n=6)
Serum	14276±3287 pg/ml (n=5)	204061±12506 pg/ml (n=5)
B-1F tumor		
Tumor	1.56±0.12 ng/mg protein (n=8)	22.76±3.48 ng/mg protein (n=7)
Serum	15035±970 pg/ml (n=10)	183000±16197 pg/ml (n=9)
SC-3 cell		
	0.01±0.00 ng/10^6 cells (n=5)	148.6±12.6 ng/10^6 cells (n=5)
	0.10±0.03 ng/mg protein (n=5)	1871±101 ng/mg protein (n=5)
B-1F cell		
	0.24±0.01 ng/10^6 cells (n=5)	230±13.5 ng/10^6 cells (n=5)
	1.81±0.02 ng/mg protein (n=5)	2246±120 ng/mg protein (n=5)

On day 24 the mice described in Figures 2 and 3 were sacrificed. The whole blood samples were taken via an inferior *vena cava* by venopuncture. In case of established cells, cells were seeded and treated with methylcobalamin as described in materials and methods. The total concentration of vitamin B_{12} in tumors, sera and cells was determined by radio isotope dilution method. Table shows means±S.E. "n" shows the number of assayed samples.

and their receptors [28–30]. But estrogen inhibits the activity of 5-lipoxygenase, which is a key enzyme for the production of leukotrienes that suppress the proliferation of B-1F cells [23, 24, 31, 32]. In SC-3 and MCF-7 cells, and SC115 tumor, sex-hormone seems to mostly stimulate the positive growth control system, while estrogen seems to mostly inactivate the negative growth control system in B-1F cells. The inhibitory activity of methylcobalamin on the proliferation might be mediated through the interference of these systems. In our previous report [17] the treatment of SC-3 cells with methylcobalamin decreased the activity of AIGF at the level of post androgen receptor binding. The presence of the common inhibitory mechanism by methylcobalamin among these diverse systems is unknown. Furthermore, whether the inhibitory activity is specific for methylcobalamin or not is also unknown up to this point. Nevertheless, because of the ability to achieve high blood and tissue levels *in vivo* and the lack of toxicity, methylcobalamin should be considered as a candidate of potentially useful agents for the treatment for some malignant tumors.

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References

1 Beard, B, Pitney, W R and Sanneman, E H (1954) Serum concentrations of vitamin B_{12} in patients suffering from leukemia Blood *9* 789–794
2 Nelson, R and Doctor, V M (1962) Vitamin B_{12} content of liver and serum in malignant neoplasia Gastroenterology *42* 414–418.
3 Wakatsuki, Y, Inada, M, Kudo, H, Shio, G, Masuda, T, Miyake, T and Kita, T (1989) Immunological characterization and clinical implication of cobalamin binding protein in human gastric cancer Cancer Res *49*, 3122–3128
4 Bodian M (1959) Neuroblastoma Pediatr Clin N Amer *6*, 449–472
5 Bodain, M (1963) Neuroblastoma, an evaluation of its natural history and the effects of therapy, with particular reference to treatment by massive doses of vitamin B_{12} Arch Dis Childh *38*, 609–619.
6 Sawitcky, A and Desposito, F (1965) A survey of American experience with vitamin B_{12} therapy of neuroblastoma J Pediatr. *67*, 99–103
7 Landerman, M J S (1970) Treatment of neuroblastoma with vitamin B_{12} Arch Dis Childh. *45*, 385–387
8 Poydock, M E, Fardon, J C, Gallina, D, Ferro, V and Heher, C (1979) Inhibiting effect of vitamins C and B_{12} on the mitotic activity of ascites tumors Exp. Cell Biol *47*, 210–217
9 Poydock, M E Phillips, L and Schmitt, P (1984) Growth-inhibiting effect of hydroxocobalamin and L-ascorbic acid on two solid tumors in mice I R C S. Med *12*, 813.
10 Poydock, M E, Harguindey, S, Hart, T, Takita, H and Kelly, D (1985) Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin Am. J Clin Oncol (CCT) *8*, 266–269
11 Newell Jr, S D, Kapp, J and Romfh J H (1981) Evaluation of megadose vitamin therapy in an experimental brain tumor Surg Neurol *16* 161–164
12 Rigby, C C and Bodian M (1963) Experimental study of the relationship between vitamin B_{12} and two animal tumour systems Br. J Cancer *17*, 90–99
13 Day, P L, Payne, L D and Dinning, J S (1950) Procarcinogenic effect of vitamin B_{12} on p-dimethylaminoazobenzene-fed rats Pro Soc Exp Biol N Y. *74*, 854–855
14 Georgadze, G E (1960) The influence of vitamins B_1 and B_{12} on the induction of malignant tumours in hamsters Vopr Onkol *6*, 54–58
15 Tsao, C S, Miyashita, K and Young M. (1990) Cytotoxic activity of cobalamin in cultured malignant and nonmalignant cells Pathobiology *58*, 292–296.
16 Tsao, C S and Miyashita, K (1993) Influence of cobalamin on the survival of mice bearing ascites tumor Pathobiology *61* 104–108
17 Nishizawa, Y, Yamamoto, T, Terada, N, Sato, B Matsumoto, K and Nishizawa, Y (1995) Inhibition of the proliferation of androgen-dependent SC-3 cells by methylcobalamin Oncology Reports *2* 129–132
18. Weinberg, J B, Sauls, D L, Misukonis, M A and Shugars, D C (1995) Inhibition of productive human immunodeficiency virus-1 infection by cobalamins. Blood *86*, 1281–1287
19 Heimburger, D C, Alexander, C B, Birch, R, Butterworth Jr, C E, Bailley, W C and Krumdieck, C L (1988) Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B_{12} JAMA *259*, 1525–1530.
20 Gimsing P. (1995) Cobalamin forms and analogues in plasma and myeloid cells during chronic myelogenous leukemia related to clinical condition Br J Haematology *89*, 812–819
21 Noguchi, S, Nishizawa, Y, Nakamura, N, Uchida, N, Yamaguchi, K, Sato B Kitamura, Y and Matsumoto, K (1987) Growth-stimulating effect of pharmacological doses of estrogen on androgen-dependent Shionogi carcinoma 115 *in vivo* but not in cell culture Cancer Res *47*, 263–268
22 Nishizawa, Y, Sato B, Miyashita, Y, Tsukada, S, Hirose T, Kishimoto, S, and Matsumoto, K (1988) Autocrine regulation of cell proliferation by estradiol and hydroxytamoxifen of transformed mouse Leydig cells in serum-free culture Endocrinology *122* 227–235
23 Nishizawa Y, Nishii, K, Nishizawa, Y, Koga, M, Kishimoto, S, Matsumoto, M and Sato, B (1990) Effects of estrogen on cell proliferation and leukotriene formation in transformed mouse Leydig cells cultured under serum-free conditions. Cancer Res *50*, 3866–3871
24 Nishii, K, Nishizawa, Y, Nishizawa, Y, Matsumoto, K and Sato, B (1991) Inhibition of murine transformed Leydig cell proliferation by leukotrienes in serum-free culture Cancer Res *51*, 5573–5578
25 Nishizawa, Y, Yamamoto, T, Terada, N, Fushiki, S, Amakata, Y and Nishizawa Y (1996) Effects of antialler-

gic drugs on the proliferation of estrogen-sensitive mouse Leydig cell line Anticancer Res. *16*, 1241–1246.
26. Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H and Matsumoto M. (1992) Cloing and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. Proc. Natl. Acad. Sci. USA *89*, 8928–8932.
27. Kouhara, H., Kasayama, S., Saito, H., Matsumoto, K. and Sato, S. (1991) Expression cDNA cloning of fibroblast growth factor (FGF) receptor in mouse breast cancer cells: A variant form in FGF-responsive transformed cells. Biochem. Biophys. Res. Commun. *176*, 31–37.
28. Vignon, F., Derocq, D. F., Chambon, M. and Rochefort, H. (1983) Estrogen induced proteins secreted by MCF-7 human breast cancer cells. C. R. Acad. Sci. Paris Endocrinol. *296*, 151–157.
29 Vignon, F., Capony, F., Chambon, M., Freiss, G., Marcia. M. and Rochefort, H. (1986) Autocrine growth stimulation of the MCF-7 breast cancer cell by the estrogen-regulated 52K protein. Endocrinology *118*, 1537–1545
30. Dickson, R. B., Huff, K. K., Spencer, E M. and Lippman. M. E. (1986) Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology *118*, 138–142
31. Nishizawa, Y., Nishii, K., Kihsimoto, S., Matsumoto, K and Sato, B. (1990) Regulatory role of arachidonic acid-derived matabolites for proliferation of transformed murine Leydig cell in serum-free culture condition. Anticancer Res. *10*, 317–322.
32. Nishizawa, Y., Yamamoto, T., Taniguchi, H. and Sato, B. (1992) Effects of estrogen and leukotrienes on anchorage-independent growth of transformed mouse Leydig cells under serum-free condition. Steroid Biochem. (Life Sci Adv.) *11*, 23–28.

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Influence of Cobalamin on the Survival of Mice Bearing Ascites Tumor

Key Words
Cobalamin
Ascites tumor
Mice survival

Abstract
The effect of cobalamin (vitamin B_{12}) on the survival time of mice bearing P388 leukemia has been examined. Among the three cobalamins studied, the enzymatically active derivatives, methylcobalamin and 5′-deoxyadenosylcobalamin, were able to significantly increase the survival time of mice implanted intraperitoneally with the tumor cells. The pharmaceutical form, cyanocobalamin, was not active. The antitumor activity of these cobalamins may be associated with their functions in metabolism.

Evidence has accumulated in the recent past that vitamin B_{12} is associated with tumorigenesis. Patients with many types of malignant neoplasia and leukemia had elevated serum levels of vitamin B_{12} [1, 2] and vitamin B_{12}-specific binding proteins [3–5]. Certain cancer-bearing mice synthesized vitamin B_{12} and stored more vitamin B_{12} than normal controls did [1, 6]; but several spontaneous mammary tumors were shown to destroy this vitamin [6]. Studies with mouse spleen cells have shown that the addition of methylcobalamin to culture medium enhanced the production of antibody and suppressor T cells [7].

Vitamin B_{12} had been used with apparent benefit in the treatment of young children with neuroblastoma [8, 9], but the results of two survey studies using data from several hospitals failed to confirm that vitamin B_{12} therapy was effective either when it was administered alone or in conjunction with X-ray or chemotherapeutic agents [10, 11].

Experiments with laboratory animals also showed conflicting results. The administration of vitamin B_{12} inhibited the growth of certain tumors implanted in mice [12, 13]. Mice treated with vitamin B_{12} survived longer than did untreated controls. Vitamin B_{12} also inhibited the growth of liver tumors induced by *p*-dimethylaminoazobenzene in rats [14]. A mixture of vitamin B_{12} and vitamin C was able to inhibit the growth of certain mouse ascites or solid tumors and to increase the survival rate of tumor bearing mice [15–17]. However, in another study with Fischer CDF rats, a mixture of vitamins B_{12} and C had no effects on the growth of L_9 gliosarcoma, and no differences in survival time between treatment and control group were observed [18]. Furthermore, in some cases, vitamin B_{12} enhanced the growth of fibrosarcoma in rats and of Rous sarcoma in chickens [19]. Vitamin B_{12} was also reported to be procarcinogenic in rats and hamsters [20, 21]. In addition, a deficiency of vitamin B_{12} decreased the potency of certain carcinogens in rats [22, 23].

A recent in vitro study with cultured cells indicated that cobalamins were able to inhibit the growth of several malignant cell lines [24]. In these experiments, the meta-

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bolically active forms, methylcobalamin and 5′-deoxyadenosylcobalamin, were found to be effective but the metabolically inactive cyanocobalamin had practically no effect [24]. In the present study, in vivo experiments were carried out to examine the effects of three different forms of cobalamin on the survival of mice bearing ascites tumors.

Methods and Materials

Survival Time

Drug test was performed using a murine model, under the auspices of the NCI Division of Cancer Treatment, for screening chemical agents and natural products against animal tumors, model 3PS31, In Vivo Cancer Models, US Department of Health and Human Services, National Institutes of Health Publication No. 84-2635 (1984).

Female DBA and CDF_1 mice (18–20 g) were purchased from Simonsen Laboratories, Inc. (Gilroy, Calif.). They were fed a nonpurified diet (Purina Certified Rodent Diet, No. 5001, Ralston Purina Co., St. Louis, Mo.) throughout the experiment. Feed and water were offered ad libitum. Mouse lymphoid neoplasm cells P388 were propagated in DBA mice. The ascites cells were harvested on the 7th day after tumor implantation. The CDF_1 mice were injected intraperitoneally with 10^6 washed cells, suspended in 0.1 ml phosphate-buffered saline. Mice were then randomized into various test groups of 10 animals each. Starting 24 h after implantation, test mice were injected intraperitoneally with various doses of vitamin B_{12} in 0.2 ml of saline solution daily for 10 days. The control animals received an equal volume of saline. The mice were weighed twice a week and were killed 30 days after tumor implantation and the result evaluated. When 50% or more of the animals in a test group survived to the 30-day time period after tumor implantation, the experiment was extended to a total of 60 days. The number of days that each mouse lived after the transplantation of tumor cells was recorded as the survival time.

Statistical Calculations

The Student's two tailed t test was used to determine statistical differences between the control and experimental groups. The median survival time of each group was also used as an index of comparison of the test animals to their corresponding controls. The median survival time of the untreated control groups of these experiments was 16 ± 4 days. A test-to-control ratio of survival time of 1.3 for a test agent was considered to demonstrate activity, whereas a ratio of 1.75 or greater was considered significant activity.

Calculation of Therapeutic Index

The therapeutic index, referring to the dose ratio between toxic and therapeutic effect, is expressed as the ratio LD_{50}/ED_{90}, where LD_{50} is the dosage lethal to 50% of the untreated animals and ED_{90} is the dosage that give a 90% cell kill in the experimental mice [25]. Values of ED_{90} were measured using CDF_1 mice. The mice were injected intraperitoneally with 10^6 cells, suspended in 0.1 ml phosphate-buffered saline. The mice were then randomized into various groups of 10 animals each. Starting 24 h after implantation, test mice were injected intraperitoneally with various doses of the test agents in 0.2 ml of saline solution. On the tenth day after tumor implantation, the ascites cells were harvested and washed. Then, a homogeneous representation of the cells was stained with erythrosin B. The numbers of viable cells and dead cells were counted with a hemocytometer. The value of ED_{90} was calculated by comparing viable cell numbers in treated and control animals. The survival inocculum curves from the studies of survival time described above were also used for the calculation of ED_{90}. A good agreement was obtained between the two methods of calculation.

Chemicals

Cyano-, methyl- and 5′-deoxyadenosyl cobalamin were purchased from Sigma Chemical Co., St. Louis, Mo. The cobalamin derivatives were prepared by introducing the dry crystals into small sterile tubes. A sufficient number of tubes were prepared and stored at −20 °C. Immediately before injection, sterile saline solution was added to the tubes to dissolve the compounds.

Results and Discussion

We have tested the antitumor activity of three cobalamin derivatives that were available to us, at three different dosages: 25,50 and 100 mg/kg body weight/injection. The mice were able to tolerate the injection of cobalamin at a daily dose of 100 mg/kg body weight without any apparent adverse effect. Table 1 shows the survival times of test and control mice bearing P388 leukemia after treatment with cobalamins. The data indicate a statistically significant increase in the survival of mice treated with methylcobalamin or adenosylcobalamin when compared with the controls. Adenosylcobalamin was apparently more effective than methylcobalamin. However, cyanocobalamin has practically no effect on tumor growth. These observations were in good agreement with results in previous experiments in which in vitro cell culture technique was used [24]. The ratios of median survival time of a test group to median survival time of control for these mice are shown in parentheses in table 1. These ratios are in accordance with the corresponding p values.

The therapeutic index is the ratio between toxic and therapeutic effect, or the ratio of LD_{50} and ED_{90} [25]. The values of ED_{90} for methyl- and 5′-deoxyadenosylcobalamin were 120 and 100 mg/kg body weight, respectively, whereas cyanocobalamin did not affect cell growth at a daily dose as high as 1,000 mg/kg body weight. It has been shown that the addition of vitamin B_{12} to food in amounts far in excess of need or absorbability appears to be without hazard. Cyanocobalamin has caused no toxicity in animals at levels several thousand times their nutritional requirements [29]. Toxicity tests in our laboratory indicated that these three cobalimin derivatives were

Table 1. Effect of cobalamin derivatives on the survival of mice bearing ascites tumor

Test agent	Dosage, mg/kg body weight/day				Therapeutic Index
	0	25	50	100	
Cyanocobalamin	17.8 ± 2.9	12.6 ± 7.5 (0.97)	19.3 ± 4.2 (1.03)	18.7 ± 1.5 (1.06)	-
	19.6 ± 3.5	17.1 ± 3.5 (0.94)	20.5 ± 2.7 (1.17)	20.5 ± 1.4 (1.17)	
	18.1 ± 3.6	17.9 ± 3.4 (0.97)	18.3 ± 4.5 (1.00)	18.5 ± 4.2 (1.03)	
Methylcobalamin	18.0 ± 6.0	17.5 ± 2.8 (1.00)	17.3 ± 0.5 (1.00)	38.0 ± 18.4[a] (2.12)	15
	17.0 ± 3.2	-	18.9 ± 0.3 (1.06)	22.7 ± 9.7 (1.61)	
	16.7 ± 0.9	-	17.3 ± 6.1 (0.91)	32.7 ± 10.6[c] (2.18)	
Adenosylcobalamin	9.4 ± 2.4	9.1 ± 1.4 (1.18)	8.9 ± 2.3 (1.18)	29.1 ± 1.9[c] (3.53)	20
	15.5 ± 0.9	16.7 ± 4.5 (1.11)	21.2 ± 6.2[b] (1.43)	34.3 ± 10.3[c] (2.86)	
	15.0 ± 2.1	-	28.4 ± 13.7[b] (1.33)	40.7 ± 25.2[b] (3.83)	

Values are means ± SD of survival times (days) for n = 10. Significance of the difference between control and experimental values: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$. Values in parentheses are ratios of median survival time of test group to median survival time of control group. A value of 1.75 or greater for a test agent was considered to demonstrate significant activity.

Therapeutic index is expressed as the ratio LD_{50}/ED_{90}, where LD_{50} is the dose lethal to 50% of a population and ED_{90} is the dose that gives a 90% cell kill.

remarkably nontoxic when administered intraperitoneally. The LD_{50} for methylcobalamin was 1,800 mg/kg body weight, which was 15 times the effective dose. The LD_{50} for 5'-deoxyadenosylcobalamin was higher than that for methylcobalamin. However, when a dose of 2,000 mg/kg body weight was injected into these mice, they started to lose weight. Thus, the value of 2,000 mg/kg body weight was used and the therapeutic index for 5'-deoxyadenosylcobalamin was 20. Although very large doses of cobalamins injected intraperitoneally were nontoxic, it became lethal when a much smaller dose was injected intravenously into the mouse tail vein. This may be the effect of the large volume of fluid entering the mouse bloodstream during a relatively short period of injection time.

Vitamin B_{12} and folate are involved in the process of one-carbon-unit metabolism and methylcobalamin is a source of one-carbon functional groups [26, 27]. Although the mechanism of the antitumor activity is not known, it is quite evident that the roles of vitamin B_{12} in carcinogenesis may be associated with its functions in normal metabolism, particularly in the one-carbon-unit metabolism and in the positive and negative control of DNA synthesis by normal and malignant cells [26, 27]. Vitamin B_{12} is expected to correct defective DNA-thymine synthesis in vitamin B_{12}-defective marrow [28], because thymidylate synthase requires N^5,N^{10}-methylenetetrahydrofolate as methyl donor. For the same reason, in patients with folate and vitamin B_{12} deficiency, the addition of these vitamins to marrow and lymphocyte cultures enhanced the incorporation of ^{3}H-deoxyuridine into DNA [29].

Previous in vitro findings have indicated that the metabolically active cobalamins were able to inhibit malignant cell growth, while the metabolically inactive forms had practically no effect [24]. Folic acid and vitamin B_{12} are intimately related to the synthesis of DNA and RNA; lack of either damages DNA synthesis. The primary damage is to de novo DNA synthesis, with the result that there may be a secondary increment in salvage DNA synthesis [27, 29]. In those tumors in which synthesis of DNA by the salvage pathway is relatively greater than in normal cells, as compared to the de novo pathway of DNA synthesis, it is theoretically possible that folate and vitamin B_{12}, by enhancing de novo DNA synthesis, could be relatively more helpful to normal than to tumor cells and relatively more harmful to certain tumor cells [27, 29]. It has been suggested that these vitamins and their antagonists could be involved in the control of normal gene expression and that deficiency of folate or vitamin B_{12} or any cause of failure to methylate DNA or RNA can activate malignancy by hypomethylating oncogenes, leading to such gene expression or gene amplication, and that methylating oncogenes can inhibit malignancy by making them dormant. Furthermore, these vitamins can be useful in controlling tumors that grow more rapidly as more of them are supplied, because the tumor cells can be stimulated into the DNA synthesis phase in which a number of cancer chemotherapy agents exert their deadly effects. These agents can be used in a sequence right after folate or vitamin B_{12} [27, 30]. Large doses of folic acid and vitamin B_{12} were able to potentiate cytotoxicity of fluoropyrimidine by stabilizing the ternary complex and between fluorodeoxyuridylate and thymidylate synthase [30]. The finding that adenosylcobalamin was more effective than methylcobalamin indicated that the mechanism of cobalamin in carcinogenesis involved not only methylation but also other metabolic pathways of vitamin B_{12} metabolism.

Most of the cobalamin in animals exists as the two coenzymatically active forms, methylcobalamin and adenosylcobalamin [26]. Methylcobalamin constitutes 60–80% of the total plasma cobalamin. Adenosylcobalamin is the major cobalamin in cellular tissues. The stable pharmaceutical form, cyanocobalamin, is not nutritionally active. Although animals have the biochemical machinery to convert cyanocobalamin and other cobalamins into the two metabolically active cobalamins, the difference in activity of the three cobalamins indicated that the rate of the conversion was probably very low and insufficient in view of the rather high required dosage of the active cobalamins.

In summary, these studies indicate that methyl- and 5′-deoxyadenosylcobalamin were able to significantly increase the survival time of mice implanted with the P388 tumor cells. Because there has been criticism of the use of P388 as a tumor system for drug discovery and development, other tumor systems are being studied in our laboratory for further evaluation.

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References

1 Beard B, Pitney WR, Sanneman EH: Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 1954;9:789–794.

2 Nelson R, Doctor VM: Vitamin B_{12} content of liver and serum in malignant neoplasia. Gastroenterology 1962;42:414–418.

3 Ogawa K, Kudo H, Kim YC, Nakashima Y, Ohshio G, Yamabe H: Expression of vitamin B_{12} R-binder in breast tumors: An immunohistochemical study. Arch Pathol Lab Med 1988; 112:1117–1120.

4 Gimsing P, Nexo E: Cobalamin-binding capacity of haptocorrin and transcobalamin: Age-correlated reference intervals and values from patients. Clin Chem 1989;35:1447–1451.

5 Grossowicz N, Hochman A, Aronovitch J, Izak G, Rachmilewitz M: Malignant growth in the liver and serum-vitamin B_{12} levels. Lancet 1957;i:1116–1117.

6 Woollsey DW: A further study of the apparent synthesis of vitamin B_{12} by mammary cancers of mice. Proc Natl Acad Sci Wash 1955;41: 111–118.

7 Takimoto G, Yoshimatsu K, Isomura J, Ikeda T, Morimoto T, Osawa T: The modulation of murine immune responses by methyl-B_{12}. Int J Tiss Reac 1982;IV:95–101.

8 Bodian M: Neuroblastoma. Pediatr Clin N Amer 1959;6:449–472.

9 Bodian M: Neuroblastoma, an evaluation of its natural history and the effects of therapy, with particular reference to treatment by massive doses of vitamin B_{12}. Arch Dis Childh 1963;38: 606–619.

10 Sawitcky A, Desposito F: A survey of American experience with vitamin B_{12} therapy of neuroblastoma. J Pediatr 1965;67:99–103.

11 Landerman MJS: Treatment of neuroblastoma with vitamin B_{12}. Arch Dis Childh 1970;45: 385–387.

12 Rigby CC, Bodian M: Experimental study of the relationship between vitamin B_{12} and two animal tumour systems. Br J Cancer 1963;17: 90–99.

13 Shimizu N, Hamazo R, Kanayama H, Maeta M, Koga S: Experimental study of antitumor effect of methyl-B_{12}. Oncology 1987;44:169–173.
14 Bennett MA, Ramsey J, Donnelly AJ: Retardation of induction of *p*-dimethylaminoazobenzene liver tumors by vitamin B_{12}. Int Z Vitaminforsch 1956;26:417–418.
15 Poydock ME, Reikert D, Rice J: Influence of vitamins C and B_{12} on the survival rate of mice bearing ascites tumor. Exp Cell Biol 1982;50: 88–91.
16 Poydock ME, Phillips L, Schmitt P: Growth-inhibiting effect of hydroxocobalamin and *L*-ascorbic acid on two solid tumors in mice. IRCS Med Sci 1984;12:813.
17 Poydock ME, Harguindey S, Hart T, Takita H, Kelly D: Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin. Am J Clin Oncol (CCT) 1985;8:266–269.
18 Newell SD Jr, Kapp J, Romfh JH: Evaluation of megadose vitamin therapy in an experimental brain tumor. Surg Neurol 1981;16:161–164.
19 Oleson JJ, Little PA: Effect of pteroylglutamic acid and vitamin B_{12} on growth of Rous tumor implants. Proc Soc Exp Biol NY 1949;71:226–227.
20 Day PL, Payne LD, Dinning JS: Procarcinogenic effect of vitamin B_{12} on *p*-dimethylaminoazobenzene-fed rats. Proc Soc Exp Biol NY 1950;74:854–855.
21 Georgadze GE: The influence of vitamins B_1 and B_{12} on the induction of malignant tumours in hamsters. Vopr Onkol 1960;6:10, 54–58.
22 Yamamoto RS: Effect of vitamin B_{12}-deficiency in colon carcinogenesis. Proc Soc Exp Biol Med 1980;163:350–353.
23 Miller EC, Plescia AM, Miller JA, Heidelberger C: The metabolism of methylated aminozao dyes. I. The demethylation of 3′-methyl-4-dimethyl-C^{14}-amino-azobenzene in vivo. J Biol Chem 1952;196:863–874.
24 Tsao CS, Miyashita K, Young M: Cytotoxic activity of cobalamin in cultured malignant and non-malignant cells. Pathobiology 1990; 58:292–296.
25 Berkow R (ed): The Merck Manual of Diagnosis and Therapy, ed 13. Rahway, Merck, 1978, pp 1809–1812.
26 Ellenbogen L: Vitamin B_{12}; in Machlin LJ (ed): Handbook of Vitamins. New York, Marcel Dekker, 1984, pp 497–547.
27 Herbert V: The role of vitamin B_{12} and folate in carcinogenesis. Adv Exp Med Biol 1986;206: 293–311.
28 Van Der Wayden MB, Cooper M, Firkin BG: Defective DNA synthesis in human megaloblastic bone marrow: Effects of hydroxy-B_{12} 5′-deoxyadenosyl-B_{12} and methyl-B_{12}. Blood 1973;41:299–308.
29 Das KC, Manusselis C, Herbert V: In vitro DNA synthesis by bone marrow cells and PHA-stimulated lymphocytes. Suppression by nonradioactive thymidine of the incorporation of ^{3}H-deoxyuridine into DNA: Enhancement of incorporation when inadequate vitamin B_{12} or folate is corrected. Br J Haematol 1980;44: 51–63.
30 Tisman G, Flener V, Jones ME, Buck L: Possible potentiation of fluoropyrimidine anti-tumor activity by pteroylglutamic acid (folic acid) and cyanocobalamin (B_{12}). Clin Res 1985;33:459A.

Experimental Study of the Therapeutic Effects of Folate, Vitamin A, and Vitamin B_{12} on Squamous Metaplasia of the Bronchial Epithelium

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Background. Vitamin deficiency may be related to carcinogenesis. Cytologic examinations of sputum have already found that the administration of folate and vitamin B_{12} suppressed the development of squamous metaplasia and atypia in smokers' airways. The authors investigated the effect of folic acid, vitamin B_{12}, and vitamin A on the formation of metaplasia and hyperplasia in methylcholanthrene (MCA)-treated rats.

Methods. The SD strain of rats received 10 mg of MCA intratracheally and was divided into six groups as follows: (1) vitamin A; (2) folic acid; (3) vitamin B_{12}; (4) vitamin B_{12} with folic acid; (5) a combination of vitamin A, folic acid, and vitamin B_{12}; and (6) no vitamins. The Lower respiratory tract epithelia of the rats were examined histologically 20, 32, and 36 weeks after MCA administration and at the end of the experiment.

Results. A clear difference was detected between the group receiving folic acid and that receiving vitamin A. In the former group, metaplasia was found in only one rat, atypia was not found, and hyperplasia with marked changes was present in less than 50% of other groups. In the latter group, atypia was found in all of the metaplastic foci.

Conclusions. It was suggested that the epithelial hyperplasia and metaplasia of respiratory tract induced by MCA can be suppressed by the administration of folic acid. *Cancer* 1993; 71:2477–83.

Key words: bronchial metaplasia, folate, serum folate concentration, lung cancer, vitamin A, vitamin B_{12}.

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There are numerous factors in our daily environment that are associated with carcinogenesis.[1,2] Diet and smoking, in particular, are the most important among these factors.[3] The relationship between smoking and lung cancer has already been reported,[4–6] e.g., tobacco smoke contains carcinogens that may induce lung cancer.[7,8] Airways of smokers often show epithelial hyperplasia and squamous metaplasia.[4] These changes may be induced by various stimuli, e.g., tobacco smoke, in the airways and by the persistent inflammation caused by these stimuli.

Metaplasia usually refers to the transformation of normal cellular components into completely different and thoroughly mature cells. Incomplete metaplastic foci, which consist of atypical cells mixed with well-differentiated metaplastic cells, are occasionally observed. The relationship between hyperplasia and metaplasia of the airway epithelium and pulmonary carcinogenesis has long been suggested.[9] This relationship can be particularly important when the lesion shows atypia.

On the other hand, it has been suggested that the lack of inhibitors of carcinogenesis in the human diet as well as the carcinogen itself can be an important factor in carcinogenesis.[10] Recently, some reports indicated that green and yellow vegetables contain some inhibitors of carcinogenesis.[11,12] Vitamin A and folate are considered to be the vitamins that are contained in these foods.

Vitamin A is expected to maintain the normal epithelial cell differentiation and to reinforce cellular immunity.[13] A number of investigators have reported that a deficiency of vitamin A plays a major role in pulmonary carcinogenesis and metaplasia, both epidemiologically and experimentally.[11,14–19] It is also reported that administration of vitamin A, to a certain extent, was able to suppress the development of lung cancer and metaplastic changes.[20–22]

Folate ordinarily exists in the body as a coenzyme and is an essential factor in the synthesis of nuclear

proteins. Investigations suggest that a deficiency of folate also enhances the development of preneoplastic conditions and carcinogenesis, which are suppressed when folate is supplied.[23–25] It has been reported that the serum concentrations of folate and vitamin B_{12} are decreased in smokers.[26–28] This decrease is caused by inactivation of folate and vitamin B_{12} by some element in the tobacco smoke.[24,29,30] One group reported that the serum folate concentration is lower in smokers with metaplastic lesions, which are in a potentially preneoplastic condition, than in those without them.[31] They also suggested that folate deficiency can render the bronchial epithelium more susceptible to neoplastic transformation by carcinogenic hydrocarbons of tobacco smoke.[31] Recently, they also showed, using cytologic examinations, that the administration of folate and vitamin B_{12} together significantly decreased the number of atypical metaplastic cells in the sputum of smokers.[32] Vitamin B_{12} is also considered to be a coenzyme necessary for human metabolism, and its deficiency secondarily causes folate deficiency.[33]

We examined histopathologically the effects of these vitamins, including folate, on the development of metaplasia and atypia in the airways of rats. The treatment of rats with a carcinogenic agent in the airways was followed by the systemic administration of vitamins. Histopathologic examinations were made of the airway epithelia to determine the degree of differentiation and atypia of metaplastic cells. The serum concentration of various vitamins and their changes were also studied.

Materials and Methods

One hundred seven Sprague-Dawley rats were raised on a standard feed containing 880 IU of vitamin A, 0.14 mg of folate, and 6.0 μg of vitamin B_{12} per 100 g. This standard feed was continued throughout the experiment. Ten animals were used as controls, and 97 received methylcholanthrene (MCA) at the age of 6 weeks. MCA (1200 mg) was mixed with 0.2% gelatin in physiologic saline solution (48 ml), and 0.2 ml of the suspension, containing 5 mg of MCA, was administered intratracheally to each rat under ether anesthesia twice with a 24-hour interval between doses (total dose, 10 mg). The treated animals were divided into six groups. Group 1 was given vitamin A; Group 2 received folate; Group 3 was given vitamin B_{12}; Group 4 received folic acid and vitamin B_{12}; Group 5 was given vitamin A, folate, and vitamin B_{12}; and Group 6 received no vitamins. The administration of these vitamins was initiated 9 weeks after the MCA treatment in addition to those in the standard feed. Retinol palmitate was administered as vitamin A twice a week intramuscularly, folate for injection twice a week intramuscularly, and hydroxycobalamin as vitamin B_{12} once a week intramuscularly, at weekly doses of 25,000 IU, 15 mg, and 500 μg, respectively. The blood concentration of hydroxycobalamin is known to be sustained for a long time.[33,34]

Ten animals of the MCA-treated and untreated groups were killed for evaluation 8 weeks after the administration of MCA. Measurement of the animal's body weight once a week were started 9 weeks after MCA administration. A few animals in these groups were killed 20 and 32 weeks after MCA administration and the rest of the animals, after 36 weeks. Histopathologic evaluations were done. After death, the right lung was fixed by infusing 10% formaldehyde solution, and the left lung was fixed by infusing Carnofsky solution. The fixed right lung was cut along the long axis of the bronchi in the superior, middle, inferior, and intermediate lobes, and sections in which the bronchial lumen could be observed along the long axis were studied under light microscopy with hematoxylin and eosin staining. The left lung was studied by electron microscopy. Macroscopic tumors and abscesses were also studied by preparing special sections containing them. The serum vitamin concentrations were measured in a small number of animals randomly selected at each time of death. Vitamin A was assayed as retinol using high-performance liquid chromatography and folic acid and vitamin B_{12}, by competitive protein-binding methods.

Results

Changes in Body Weight

The mean body weight of rats in each group was measured at time points during the experiment and after 36 weeks. These results are shown in Figure 1. The comparisons were expressed in terms of the percentage relative to Group 6, which did not receive extra vitamins. The body weight gains were less (95.2%) in Group 1 (vitamin A alone) and poor (85.3%) in Group 5 (vitamin A plus other vitamins, Fig. 1). The body weight gains were the similar in Group 6, the groups given folate alone (Group 2, 99.6%) and vitamin B_{12} alone (Group 3, 100.1%), and the greatest in the group given folate with vitamin B_{12} (Group 4, 108.6%).

Histopathologic Findings

Histologic examinations showed hyperplasia of various degrees and metaplasia with various degrees of differentiation and atypia. Metaplasia could be classified into the following three stages. First, there was typical metaplasia in which the process of keratinization was clear and intercellular bridges were readily observed, as in

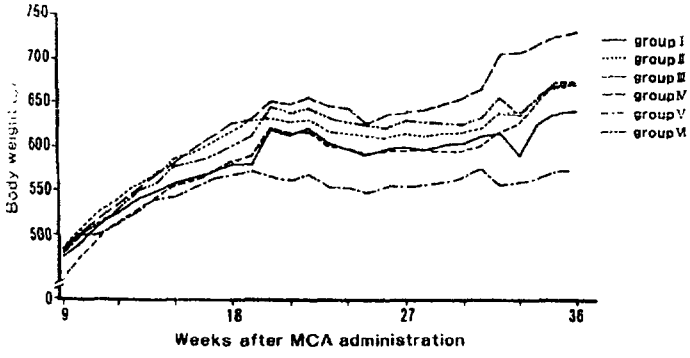

Figure 1. Effects on vitamin administration and body weight (9–36 weeks) after MCA administration. The mean weight (in grams) of each group is given at the time points indicated. The treatment groups were as follows: Group 1 (vitamin A), Group 2 (folate), Group 3 (vitamin B_{12}), Group 4 (folate plus vitamin B_{12}), Group 5 (vitamin A, folate, and vitamin B_{12}), and Group 6 (no vitamins). Relative to Group 6, the body weight in each group was 95.2% in Group 1, 99.6% in Group 2, 100.1% in Group 3, 108.6% in Group 4, and 85.3% in Group 5. The body weight gains were the greatest in Group 4, followed by Groups 2, 3, and 6. Group 1 had a slightly smaller body weight, and Group 5 showed poor body weight gains.

the stratified squamous epithelium (Fig. 2, top left). This was regarded as metaplasia without atypia. Second, there were stratified lesions in which no keratinization was detected and the degree of differentiation was obscure, but intercellular bridges were clearly observed (Fig. 2, top right). These were regarded as metaplasia with mild atypia. Third, there were stratified lesions in which the gradient of differentiation was further reduced, only a small number of intercellular bridges were present, and mitosis was occasionally observed (Fig. 2, bottom left). These were regarded as metaplasia with moderate atypia.

Because a high degree of atypia means a carcinoma in itself, the highest atypical changes in metaplasia were expressed as metaplasia with moderate atypia. Concerning hyperplasia, some lesions were hypertrophic and were not readily distinguishable from metaplastic lesions, i.e., they showed swelling of nucleoli and occasional mitosis (Fig. 2, bottom right). In contrast to the general hyperplastic changes, such as increases in goblet cells and simple stratification, these lesions were regarded as "hyperplasia with marked changes" and were examined with special attention by classifying them as moderate (++) or mild (+), according to their extent. This classification was made as follows. All slices from each rat were checked with microscopy, and the length of each marked hyperplasia seen in one slice was measured. When any one of the slices of one rat showed a length longer than 1000 μm, the sample was classified as ++. The notation + means the longest length was 500–1000 μm long, and ± means less than 500 μm long.

Frequency of Metaplasia and Hyperplasia

The frequency of metaplastic lesions observed from 20 weeks after MCA administration to the end of the study varied among groups. In Group 1, which was given vitamin A, metaplasia was observed at five sites in 4 (29%) of the 14 animals. Atypia was moderate in three (60%) and mild in two (40%) of the five sites. In the group that received folate (Group 2), metaplasia was observed in only one animal that died of bronchial pneumonia, and no atypia was noted. In Group 3, which was was given vitamin B_{12}, metaplasia was observed in 5 (38%) of the 13 animals, and mild atypia was noted in 4 (80%) of them. In the group receiving folate and vitamin B_{12} (Group 4), metaplasia was noted at six sites in 4 (31%) of the 13 animals, and mild atypia was noted at 2 sites (33%). In Group 5, which was given the three vitamins, metaplasia with mild atypia was noted in 1 (7%) of the 14 animals. In the untreated group (Group 6), metaplasia was observed at three sites in 2 (15%) of the 13 animals, and mild atypia was noted at one site (33%) (Table 1). In the 17 animals that were killed 8 weeks after MCA administration (before the beginning of vitamin administration) or died of diseases before this time, metaplasia without atypia was observed in one animal (6%), but no metaplasia was noted in the controls killed similarly after 8 weeks (not shown in Table 1). No animals died or were killed from 9 weeks (beginning of vitamin administration) to 20 weeks (12th week of vitamin administration) after MCA administration.

The frequency of the finding of hyperplasia with marked changes also varied among the groups. Moderate and mild hyperplasia was observed as follows: 5 (36%) and 3 (21%), respectively, of the 14 animals in the vitamin A group; 1 (8%) and 2 (15%), respectively, of the 13 animals in the folate group; 2 (15%) and 6 (46%), respectively, of the 13 animals in vitamin B_{12} group; 2 (15%) and 4 (31%), respectively, of the 13 animals in the folate plus vitamin B_{12} group; 6 (43%) and 2 (14%), respectively, of the 14 animals in the group that received three vitamins; and 2 (15%) and 7 (54%), respectively, of the 13 animals in the untreated group (Table 2). Moderate and mild hyperplasia with marked changes was observed in 3 (18%) and 4 (24%), respectively, of the 17 animals that were killed 8 weeks after MCA administration or died of diseases before this time, and in one (10%) and 5 (50%), respectively, of the 10 controls examined similarly after 8 weeks (not shown in Table 2).

To summarize, in animals that were killed or died of diseases after the initiation of vitamin administration (20 or more weeks after MCA administration), the following results were obtained. In the folate group, meta-

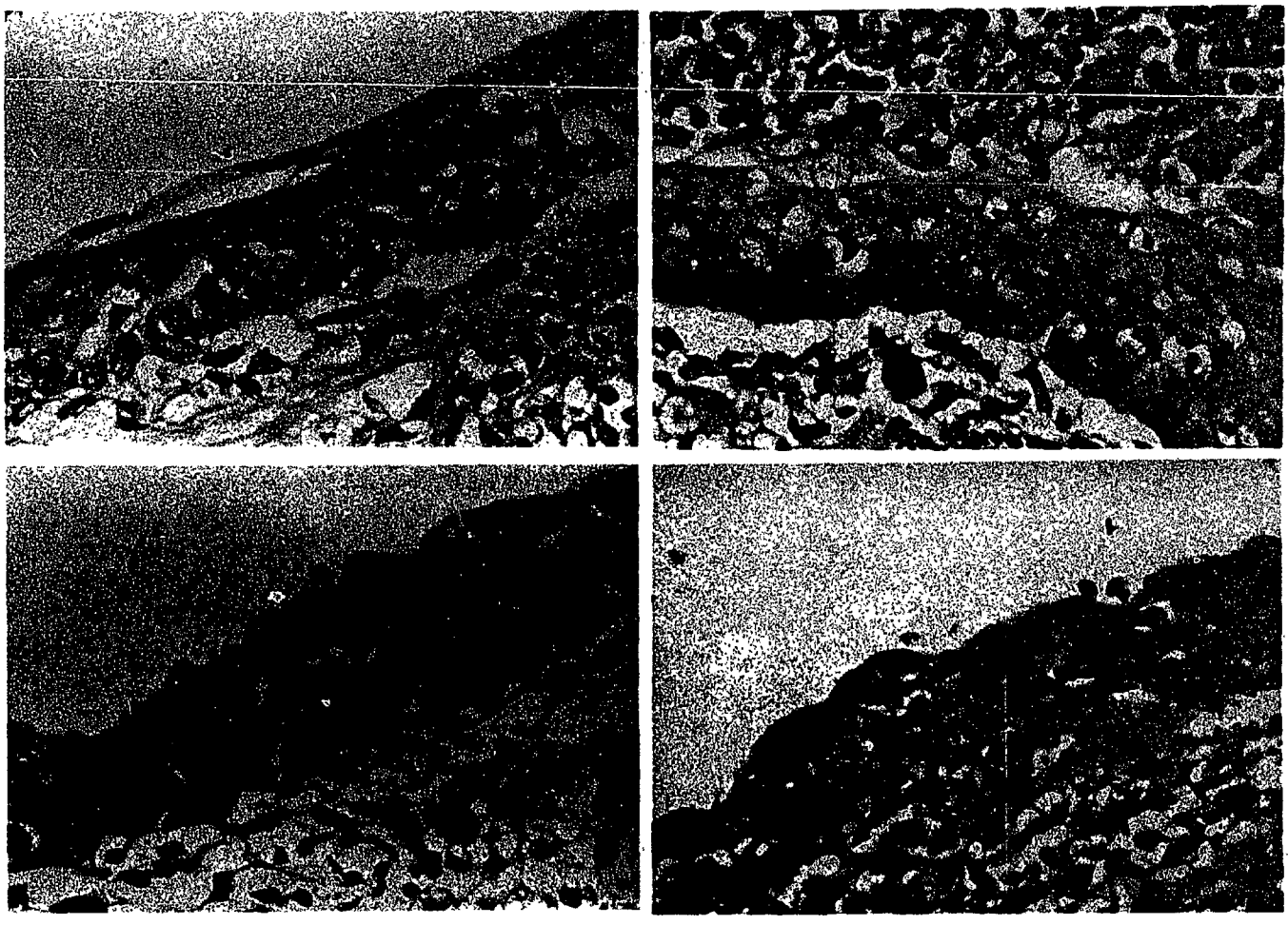

Figure 2. (Top left) Metaplasia without atypia. A typical focus of squamous metaplasia. Stages of differentiation leading to keratinization are clearly recognized, and intercellular bridges are readily confirmed. The tissue profile is that of nearly normal stratified squamous epithelium, and no atypical cells are noted. (Top right) Metaplasia with mild atypia. A multilayer lesion in which stages of differentiation are obscure. Intercellular bridges are clearly observed, but no keratinization is noted. (Bottom left) Metaplasia with moderate atypia. A multilayer lesion showing faint intercellular bridges and poor gradient of differentiation. Mitosis is observed occasionally. (Bottom right) Hyperplasia with marked changes. The lesion is not only multilayered but is accompanied by hypertrophy and is hardly distinguishable from a metaplastic lesion. Nucleoli are enlarged, and occasional mitoses are noted.

plasia was noted in only one animal that died of disease, and the cells were well differentiated with no atypia, unlike other groups, in which some atypia was noted. Mild and moderate hyperplasia with marked changes was observed in 23% of the folate-treated group and in 46% of the folic acid plus vitamin B_{12} group. It was more frequent (57–69%) in the other groups. Moderate hyperplasia was noted in 8% of the

Table 1. Frequency of Metaplasia Foci in Airway Epithelium

	Group (%)						
	1	*2*	*3*	*4*	*5*	*6*	Total
No. of rats	14	13	13	13	14	13	80
No. of rats with metaplasia	4 (29)	1 (8)	5 (38)	4 (31)	1 (7)	2 (15)	17 (21)
Total no. of metaplastic foci	5	1	5	6	1	3	21
Degree of atypism							
Metaplasia with no atypism	0 (0)	1 (100)	1 (20)	4 (67)	0 (0)	2 (67)	8
Metaplasia with mild atypism	2 (40)	0 (0)	4 (80)	2 (33)	1 (100)	1 (33)	10
Metaplasia with moderate atypism	3 (60)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3

Table 2. Frequency of Hyperplasia With Marked Changes in Airway Epithelium

	Group (%)					
	I	*II*	*III*	*IV*	*V*	*VI*
No. of rats	14	13	13	13	14	13
Hyperplasia with marked changes						
−	6 (43)	8 (62)	3 (23)	5 (38)	2 (14)	1 (8)
±	0 (0)	2 (15)	2 (15)	2 (15)	4 (29)	3 (23)
+	3 (21)	2 (15)	6 (46)	4 (31)	2 (14)	7 (54)
++	5 (36)	1 (8)	2 (15)	2 (15)	6 (43)	2 (15)

folate-treated group but in 15% or more animals in other groups. The frequency of hyperplasia with marked changes in the folate-treated group was approximately 50% or less of that in the other groups, including the untreated group.

The frequency of metaplasia without atypia was slightly higher in the group that received folate and vitamin B_{12} than in the group given vitamin B_{12} alone, in which metaplasia with mild atypia was also noted. The frequency of hyperplasia was similarly reduced in the folate plus vitamin B_{12} group. In the groups that received vitamin A with or without other vitamins, some atypia was noted in all metaplastic lesions, but both the frequency of metaplasia and the degree of atypia were less in the vitamin A, folate, plus vitamin B_{12} group than in the vitamin A-treated group. Similarly, among those with hyperplasia with marked changes, mild and moderate changes combined were noted in 57% of both groups that received vitamin A alone and with other vitamins and moderate changes, in 36% and 43% of the respective groups.

In the animals that were killed 8 weeks after MCA administration or died of diseases before this time, metaplasia without atypia was noted in one that died of disease, but no marked difference was observed compared with the control group. Moderate hyperplasia was slightly more frequent, but mild hyperplasia was less frequent.

Sites of Metaplasia

Metaplasia was classified according to its sites (Table 3). The first was metaplasia of large bronchi in the lungs observed in nine animals. It was accompanied by moderate atypia in three and mild atypia in four. The second was metaplasia of the epithelium covering the internal surface of the walls of pulmonary abscesses. It was observed in five animals and accompanied by mild atypia in two. The third was metaplasia of the peripheral airways. It morphologically resembled stratified squamous epithelium observed in metaplasia of other sites and showed nearly solid foci. It was observed in six animals and was accompanied by mild atypia in two. In two animals, metaplasia was noted in the tracheal epithelium and accompanied by mild atypia. To generalize these findings, metaplasia in the walls of abscesses or peripheral airways is considered to be less frequently associated with cell atypia than metaplasia in the bronchi.

Changes in Serum Vitamin Concentrations

Figure 3 shows the results of measurement of the serum vitamin concentrations in the animals that received MCA. The vitamin A concentration showed no marked changes with or without vitamin A administration (Fig. 3a). The folate concentration also showed no major changes in the animals given folate after compared with before administration, but it decreased markedly in the animals not receiving folate and became less than 50% of the level of the treated group (Fig. 3b). The vitamin B_{12} concentration increased markedly in the animals that received the vitamin but slightly decreased in those that did not (Fig. 3c).

Table 3. Sites of Metaplasia and Degrees of Atypism

Site of metaplasia	Metaplasia with no atypism	Metaplasia with mild atypism	Metaplasia with moderate atypism	Total
Bronchi	2	4	3	9
Wall of abscess	3	2	0	5
Peripheral airway	4	2	0	6
Trachea	0	2	0	2
Total	9	10	3	22

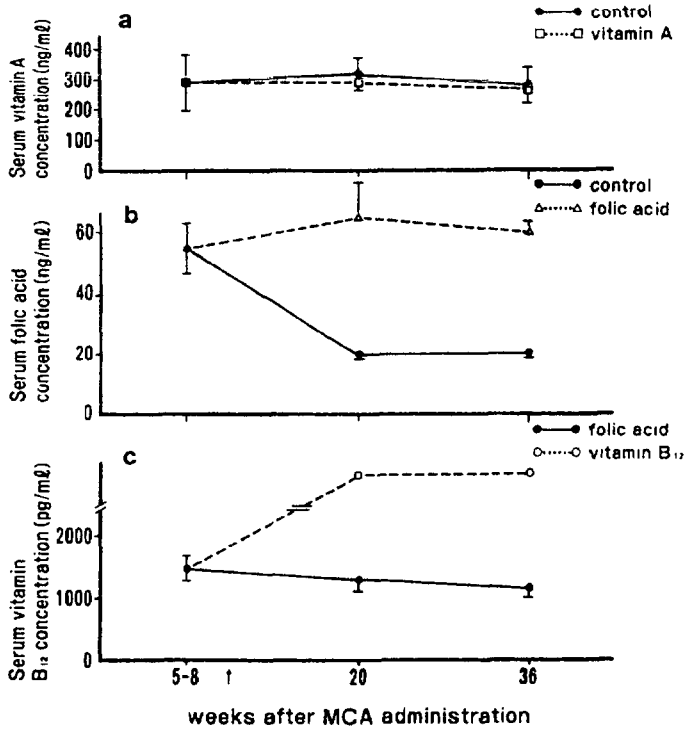

Figure 3. The serum vitamin concentrations were determined in a few animals randomly selected from each group when they were killed 5–8 weeks after MCA administration (before vitamin administration), 20 weeks after MCA administration (12 weeks after the beginning of vitamin administration), and 36 weeks after MCA administration (at the end of the study, 28 weeks after the beginning of vitamin administration). (a) Serum vitamin A concentration. The serum vitamin A concentration showed no marked changes even after vitamin A administration in the animals given it and was comparable with that of the group without Vitamin A administration. (b) Serum folate concentration. The serum folate concentration did not markedly increase in the animals that received folate but markedly decreased in the group that did not. (c) Serum vitamin B_{12}. The serum vitamin B_{12} concentration markedly increased in the animals that received vitamin B_{12} (scaled out at 2000 pg/ml) but slightly decreased in the group that did not.

Discussion

In our study, a model of pulmonary carcinogenesis was employed to show the effect of administering vitamins A, B_{12}, folate on the development of metaplasia and atypia of metaplastic cells histopathologically. It was found that metaplasia, atypia of metaplastic cells, and hyperplasia with marked changes were less frequent in the group that received folate alone than in the other groups. In the MCA-treated animals without folate administration, the serum folate concentration markedly decreased 20 or more weeks after MCA administration, whereas the folate levels were maintained around the initial level in those that were treated with folate. These findings provided an additional evidence to support the results in an earlier report on the preventative effect of folate administration on the development of atypia in airway epithelium, although their study was limited to the cytologic observations of sputum in smokers.[32]

In the experiment of vitamin B_{12} administration, some atypia, although mild, was observed in the group given vitamin B_{12} alone, and this suggested the presence of an enhancing, rather than an inhibiting, factor in the vitamin B_{12} compound to accelerate the development of atypia.

On the other hand, the preventative effect of a large amount of vitamin A against pulmonary carcinogenesis and metaplasia has been reported.[20–22] In our study, the administration of vitamin A in large doses had no preventative effect on the development of metaplasia or atypia in the airway epithelium.[22] Mild or moderate atypia was observed in all metaplastic foci in animals receiving vitamin A, and this suggested that some ingredient(s) in this vitamin A compound might work as a stronger enhancing factor than vitamin B_{12} compound.

Vitamin preparations used for oral intake have been employed in most of the earlier reports, whereas the vitamin compounds used in this study were preparations to be given by injection. The effect of ingredients other than vitamins in these preparations, e.g., solvents, relative to the development of atypia was not investigated.

Further investigations are warranted, using a greater number of animals, to detect the effect of ingredients in the vitamin preparations, to study the time course of serum folate concentrations, and to measure the timing to start folate administration.

References

1. Ames BN. Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases. *Science* 1983; 221:1256–63.
2. Miyamoto H. Lung cancer, diet and nutrition. *Kokyu To Junkan* 1990; 38:506–15.
3. Wynder EL, Gori GB. Contribution of the environment to cancer incidence: an epidemiologic exercise. *J Natl Cancer Inst* 1977; 58:825–32.
4. Auerbach O, Gere JB, Forman JB, Petrick TG, Smolin HJ, Muehsam GE, et al. Changes in the bronchial epithelium in relation to smoking and cancer of the lung. *N Engl J Med* 1957; 256:97–104.
5. Wynder EL. Etiology of lung cancer: reflections on two decades of research. *Cancer* 1972; 30:1332–9.
6. Hirayama T. Epidemiology of lung cancer with special reference to cigarette smoking. *Kokyu To Junkan* 1990; 38:3–9.
7. Wynder E, Hoffman D. Experimental tobacco carcinogenesis. *Science* 1968; 162:862–71.
8. Harris CC. Cause and prevention of lung cancer. *Semin Oncol* 1974; 1:163–6.
9. Auerbach O, Stout AP, Hammond C, Garfinkel LN. Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. *N Engl J Med* 1961; 265:253–67.
10. Carr BI. Chemical carcinogens and inhibitors of carcinogenesis in the human diet. *Cancer* 1985; 55:218–24.
11. Hirayama T. Diet and cancer. *Nutr Cancer* 1979; 1:67–81.

12. Colditz GA, Stampfer MJ, Willett WC. Diet and lung cancer: a review of the epidemiologic evidence in humans. *Arch Intern Med* 1987; 147:157–60.
13. Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. *Fed Proc* 1979; 38:2528–34.
14. Harris CC, Sporn MB, Kaufman DG, Smith JM, Jackson FE, Saffiotti U. Histogenesis of squamous metaplasia in the hamster tracheal epithelium caused by vitamin A deficiency or benzo[α]pyrene-ferric oxide. *J Natl Cancer Inst* 1972; 48:743–61.
15. Basu TK, Donaldson D, Jenner M, Williams DC, Sakula A. Plasma vitamin A in patients with bronchial carcinoma. *Br J Cancer* 1976; 33:119–21.
16. Mettlin C, Graham S, Swanson H. Vitamin A and lung cancer. *J Natl Cancer Inst* 1979; 62:1435–8.
17. Nettesheim P, Snyder C, Kim JC. Vitamin A and susceptibility of respiratory tract tissues to carcinogenic insult. *Environ Health Prospect* 1979; 29:89–93.
18. Wald N, Idle M, Boreham J, Bailey A. Low serum-vitamin A and subsequent risk of cancer. *Lancet* 1980; 2:813–5.
19. Kvale G, Bjelke E, Gart JJ. Dietary habits and lung cancer risk. *Int J Cancer* 1983; 31:397–405.
20. Saffiotti U, Montesano R, Sellakumar AR, Borg SA. Experimental cancer of the lung: inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. *Cancer* 1967; 20:857–64.
21. Cone MV, Nettesheim P. Effects of vitamin A on 3-methylcholanthrene-induced squamous metaplasias and early tumors in the respiratory tract of rats. *J Natl Cancer Inst* 1973; 50:1599–606.
22. Nettesheim P, Cone MV, Snyder C. The influence of retinyl acetate on the postinitiation phase of preneoplastic lung nodules in rats. *Cancer Res* 1976; 36:996–1002.
23. Butterworth JCE, Hatch KD, Gore H, Mueller H, Krumdieck LK. Improvement in cervical dysplasia associated with folic acid therapy in users of oral contraceptives. *Am J Clin Nutr* 1982; 35:73–82.
24. Krumdieck CL. Role of folate deficiency in carcinogenesis. In: Butterworth CE, Hutchinson ML. Nutritional factors in the induction and maintenance of malignancy. New York: Academic, 1983:225–45.
25. Eto I, Krumdieck CL. Role of vitamin B_{12} and folate deficiencies in carcinogenesis. In: Poirien LA, Poirien LA. Essential nutrients in carcinogenesis. New York: Plenum, 1986:313–30.
26. Witter FR, Blake DA, Baumgardner R, Mellits ED, Niebyl JR. Folate, carotene, and smoking. *Am J Obstet Gynecol* 1982; 144:857.
27. Nakazawa Y, Chiba K, Imatoh N, Kotorii T, Sakamoto T, Ishizaki T. Serum folic acid levels and antipyrine clearance rates in smokers and non-smokers. *Drug Alcohol Depend* 1983; 11:201–7.
28. Linnel JC, Smith ADM, Smith CL, Wilson J, Matthews DM. Effects of smoking on metabolism and excretion of vitamin B_{12}. *BMJ* 1968; 2:215–6.
29. Khald MA, Watkins CL, Krumdieck CL. Inactivation of B_{12} and folate coenzymes by butyl nitrite as observed by NMR: implications on one-carbon transfer mechanism. *Biochem Biophys Res Commun* 1986; 135:201–7.
30. Francis KT, Thompson RW, Krumdieck CL. Reaction of tetrahydrofolic acid with cyanate from urea solutions: formation of an inactive folate derivatives. *Am J Clin Nutr* 1977; 30:2028–32.
31. Heimburger DC, Krumdieck CL, Alexander CB, Birch R, Dill SR, Bailey WC. Localized folic acid deficiency and bronchial metaplasia in smokers: hypothesis and preliminary report. *Nutr Int* 1987; 3:54–60.
32. Heimburger DC, Alexander B, Birch R, Butterworth CE Jr., Bailey WC, Krumdieck CL. Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B_{12}: report of a preliminary randomized, double-blind intervention trial. *JAMA* 1988; 259:1525–30.
33. Shane B, Stokstad ELR. Vitamin B_{12}-folate interrelationships. *Annu Rev Nutr* 1985; 5:115–41.
34. Glass GBJ, Skeggs HR, Lee DH, Jones EL, Hardy WW. Hydroxocobalamin: I. Blood levels and urinary excretion of vitamin B_{12} in man after a single parental dose of aqueous hydroxocobalamin, aqueous cyanocobalamin and cyanocobalamin zinc-tannate complex. *Blood* 1961; 18:511–20.

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Experimental Study of Antitumor Effect of Methyl-B_{12}

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Key Words. Vitamin B_{12} · Methyl-B_{12} · Antitumor effect

Abstract. We examined the antitumor effect of vitamin B_{12} (methyl-B_{12}) using C3H/He, C57BL/6 and BALB/C mice for animals and MH134 hepatoma ascites cells, Lewis lung cancer cells and Ehrlich ascites tumor cells for tumor cells. At 1.0–10 μg/ml, methyl-B_{12} enhanced PHA- and Con-A-induced lymphocyte blastoformation of C3H/He mice. The growth of MH134 tumors on the backs of C3H/He mice were suppressed by the 7-day administration of 50 or 100 μg/day i.p. and their survival was longer than that of untreated mice. However, methyl-B_{12} administration did not positively affect the survival of C3H/He mice that had been irradiated with ^{60}Co 300 R on the day before tumor cell inoculation. The growth of Ehrlich ascites tumor cells inoculated into BALB/C mice was also reduced at 17 and 19 days after tumor inoculation by administration of methyl-B_{12} 50 μg/day i.p. and the mice survived longer than the untreated mice.

Introduction

Vitamin B_{12} has been used to treat pernicious anemia or malabsorption syndrome [1]. Experimental studies have shown that vitamin B_{12} promotes the regeneration of crushed peripheral nerves [14], the correction of defective DNA synthesis [12] and the maintenance and biosynthesis of RNA [5].

Takimoto et al. [11] have reported that vitamin B_{12} modulates the immune response in mice as strongly as levamisole and that the activity was strongest with methylcobalamin (CH_3–B_{12}), one of the homologues of vitamin B_{12}. We now present an experimental study on the antitumor effect of CH_3–B_{12}.

Materials and Methods

We examined the effect of CH_3–B_{12} on in vitro mitogen-stimulated lymphocyte blastoformation and on in vivo tumor growth. Male 4- to 6-week-old C3H/He, C57BL/6 and BALB/C mice (Kureha K.K., Tokyo, Japan) and MH134 ascites hepatoma cells, Lewis lung cancer cells and Ehrlich ascites tumor cells (maintained at our laboratory) were used. Methycobal® (Eisai, Tokyo, Japan) was used as CH_3–B_{12}.

Effect of CH_3–B_{12} on Mitogen-Stimulated Lymphocyte Blastoformation

Splenic lymphocytes from normal C3H/He mice were stimulated with phytohemagglutinin P (PHA, 15 μg/ml), concanavalin A (Con A, 10 μg/ml) or pokeweed mitogen (PWM, 10 μg/ml). Nunc microplates containing 1×10^5 splenic lymphocytes in RPMI 1640 medium and 20% fetal calf serum per well were incubated for 48 h at 37 °C in CO_2 incubator in the presence of mitogen. Before incubation, CH_3–B_{12} was added (0, 0.1, 1.0, 10, 50 or 100 μg/ml). After 28-hour incubation, 0.5 μCi/well of ^3H-thymidine was added to each well and the incubation was continued. The cells were harvested with a cell harvester. ^3H-thymidine uptake was measured by a liquid scintillation counter; the results are shown as the percentage, using the formula:

$$\% = \frac{\text{counts in the presence of } CH_3\text{–}B_{12} \text{ (cpm)}}{\text{counts in the absence of } CH_3\text{–}B_{12} \text{ (cpm)}} \times 100.$$

All procedures were performed under low-light conditions because light adversely affects the effect of CH_3–B_{12}.

In vivo Effect of CH_3–B_{12}

C3H/He mice were divided into five groups and in each group the tumor growth rate, the survival rate of tumor-bearing mice, and

the mitogen-stimulated splenic lymphocyte blastoformation rate were examined. The tumor growth rate was calculated as follows:

$$\text{tumor growth rate} = \frac{\sqrt{\text{longest diameter} \times \text{right-angled diameter}}}{\sqrt{\begin{matrix}\text{longest diameter} \\ \text{7 or 10 days} \\ \text{after tumor} \\ \text{inoculation}\end{matrix} \times \begin{matrix}\text{right-angled diameter} \\ \text{7 or 10 days} \\ \text{after tumor} \\ \text{inoculation}\end{matrix}}}$$

The five groups were as follows: group 1 (control): MH134 ascites tumor cells (5×10^6) were subcutaneously inoculated into the back of each mouse. Group 2 (CH_3–B_{12}, 50 μg): 1 week after MH134 ascites tumor inoculation into the back (5×10^6 cells, s.c.), CH_3–B_{12} (50 μg/mouse/day) was intraperitoneally injected for 7 consecutive days. Group 3 (CH_3–B_{12}, 100 μg): CH_3–B_{12} (100 μg/mouse/day) was injected intraperitoneally 1 week after MH134 inoculation. Group 4 (irradiated control): these mice were irradiated with ^{60}Co 300 R and the next day injected with 5×10^6 MH134 cells. Group 5 (irradiated CH_3–B_{12}, 50 μg): these mice were irradiated with ^{60}Co 300 R and the next day 5×10^6 MH134 cells were injected subcutaneously. One week later, CH_3–B_{12} (50 μg) was injected intraperitoneally for 7 consecutive days.

C57BL/6 and BALB/C mice were divided into three groups and in each group the tumor growth rate and the survival rate of tumor-bearing mice were examined, respectively.

The six groups were as follows: group 6 (control): Lewis lung cancer cells (5×10^6) were subcutaneously inoculated into the back of C57BL/6 mouse. Group 7 (CH_3–B_{12}, 50 μg): ten days after Lewis lung cancer cell inoculation into the back of C57BL/6 mice (5×10^6 cells, s.c.), CH_3–B_{12} 50 μg/mouse/day was intraperitoneally injected for 7 consecutive days. Group 8 (CH_3–B_{12}, 100 μg): CH_3–B_{12} 100 μg/mouse/day was injected intraperitoneally for 7 consecutive days 10 days after Lewis lung cancer inoculation. Group 9 (control): Ehrlich ascites tumor cells (5×10^6) were subcutaneously inoculated into the back of BALB/C mice. Group 10 (CH_3–B_{12}, 50 μg): ten days after Ehrlich ascites tumor cell inoculation into the back of BALB/C mice (5×10^6 cells, s.c.), CH_3–B_{12} 50 μg/mouse/day was injected intraperitoneally for 7 consecutive days. Group 11 (CH_3–B_{12}, 100 μg): CH_3–B_{12} 100 μg/mouse/day was injected intraperitoneally for 7 consecutive days 10 days after Ehrlich ascites tumor cell inoculation. Intergroup differences were evaluated with Student's t test.

Results

Effect of CH_3–B_{12} on Mitogen-Stimulated Splenic Lymphocyte Blastoformation

Figure 1 shows the ^3H-thymidine uptake at the different CH_3–B_{12} concentrations used. The PHA response was markedly enhanced at CH_3–B_{12} concentrations of 0.1, 1.0 and 10 μg/ml ($p < 0.05$), whereas the PWM response was not enhanced at any concentration of CH_3–B_{12}.

In vivo Effect of CH_3–B_{12}

Tumor Growth Rate. The tumor growth rates of all the groups are shown in table I. The tumor growth rates of group 3 at 12 and 15 days after tumor inoculation were slower than in group 1 ($p < 0.05$). The tumor growth rates of groups 4 and 5 were not significantly different. Between groups 6 and 7 no significant difference was observed. The tumor growth rates in

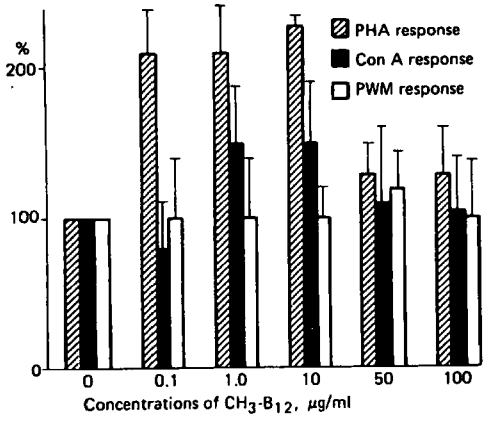

Fig. 1. Effect of CH_3–B_{12} on mitogen-stimulated splenic lymphocyte blastoformation. Each response with various concentrations of CH_3–B_{12} is shown as the percentage of the increase or decrease of ^3H-thymidine uptake when the ^3H-thymidine uptake of each response without CH_3–B_{12} is 100%. The PHA response is markedly enhanced at CH_3–B_{12} concentrations of 0.1, 1.0 and 10 μg/ml.

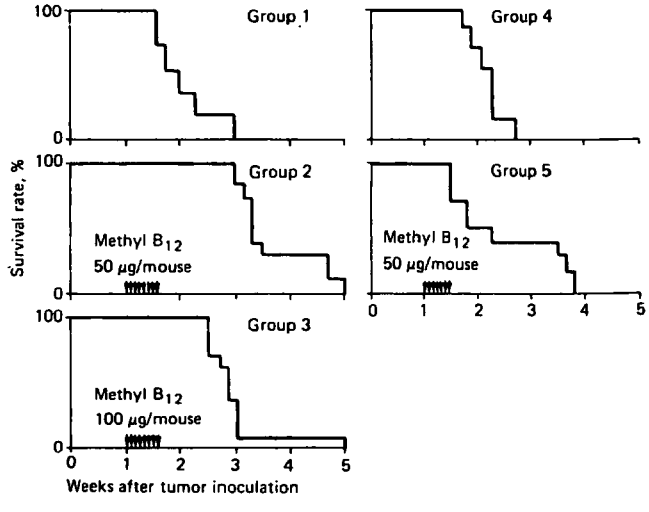

Fig. 2. Survival rate of C3H/He mice inoculated with MH134 hepatoma ascites tumor cells. The number of mice is 10 in each group. The mice in groups 2 and 3 survived longer than in group 1 ($p < 0.05$); survival in group 5 was shorter than in group 2 ($p < 0.05$). Between groups 1 and 4, and groups 4 and 5, no significant difference in survival was seen.

group 10 at 17 and 19 days after tumor inoculation were more reduced than in group 9.

Survival Rate of Tumor-Bearing Mice. The survival rate of C3H/He mice that clearly bore tumors 1 week after MH134 tumor cell inoculation is shown in figure 2. The survival period after tumor formation was 14.00 ± 3.96 days for group 1 (n = 10), 25.88 ± 6.90 days for group 2 (n = 10) and 21.78 ± 7.01 days for group 3 (n = 10) (table II). The survival of groups 2 and 3 was statistically longer than in the control group ($p < 0.05$). The survival period was 15.40 ± 2.30 days in group 4 (n = 10) and 16.44 ± 6.62 days in group 5 (n = 10) and there was no statistical difference between the two irradiated groups. The survival of mice in the irradiated group treated with CH_3–B_{12}, 50 μg (group 5) was statistically shorter than in the nonirradiated group treated with CH_3–B_{12}, 50 μg ($p < 0.05$). Between the irradiated and nonirradiated control groups, no significant difference in survival was found. Survival periods of C57BL/6 mice inoculated Lewis lung cancer and BALB/C mice inoculated Ehrlich ascites tumor were shown in table II and survival rates in figures 3 and 4. Survival periods in groups 9 and 10 were 24.7 ± 4.5 and 39.3 ± 13.2 days, respectively. Mice in group 10 survived longer than in group 9 ($p < 0.01$).

Mitogen-Stimulated Splenic Lymphocyte Blastoformation Rate. In group 1, the mitogen responses

Table I. Tumor growth rate of all groups

Days after tumor inoculation	Group 1 (control)	Group 2 (CH_3–B_{12}, 50 μg)	Group 3 (CH_3–B_{12}, 100 μg)	Group 4 (radiated control)	Group 5 (radiated, CH_3–B_{12}, 50 μg)
10th	1.49 ± 0.35 (n = 10)	1.37 ± 0.13 (n = 10)	1.29 ± 0.13 (n = 10)	1.31 ± 0.10 (n = 10)	1.52 ± 0.31 (n = 10)
12th	2.07 ± 0.75[a] (n = 10)	1.76 ± 0.20 (n = 10)	1.52 ± 0.17[a] (n = 10)	1.56 ± 0.29 (n = 10)	1.94 ± 0.63 (n = 10)
15th	2.89 ± 1.41[b] (n = 8)	2.02 ± 0.31 (n = 10)	1.73 ± 0.16[b] (n = 10)	1.99 ± 0.28 (n = 10)	2.24 ± 0.52 (n = 7)

Days after tumor inoculation	Group 6 (control)	Group 7 (CH_3–B_{12}, 50 μg)	Group 8 (CH_3–B_{12}, 100 μg)
12th	1.15 ± 0.10 (n = 10)	1.13 ± 0.16 (n = 10)	1.08 ± 0.14 (n = 10)
15th	1.33 ± 0.21 (n = 9)	1.49 ± 0.59 (n = 9)	1.24 ± 0.11 (n = 8)

Days after tumor inoculation	Group 9 (control)	Group 10 (CH_3–B_{12}, 50 μg)	Group 11 (CH_3–B_{12}, 100 μg)
12th	1.17 ± 0.15 (n = 10)	1.11 ± 0.09 (n = 10)	1.09 ± 0.10 (n = 10)
15th	1.60 ± 0.47 (n = 10)	1.24 ± 0.27 (n = 10)	1.39 ± 0.30 (n = 10)
17th	1.84 ± 0.58[c] (n = 10)	1.26 ± 0.28[c] (n = 10)	1.44 ± 0.41 (n = 10)
19th	2.22 ± 0.85[d] (n = 7)	1.44 ± 0.37[d] (n = 10)	1.83 ± 0.89 (n = 8)

[a–a], [b–b] $p < 0.05$; [c–c] $p < 0.01$; [d–d] $p < 0.02$. Group 1–5: C3H/He MH134; group 6–8: C57BL/6 Lewis lung cancer; group 9–11: BALB/C Ehrlich ascites tumor cell.

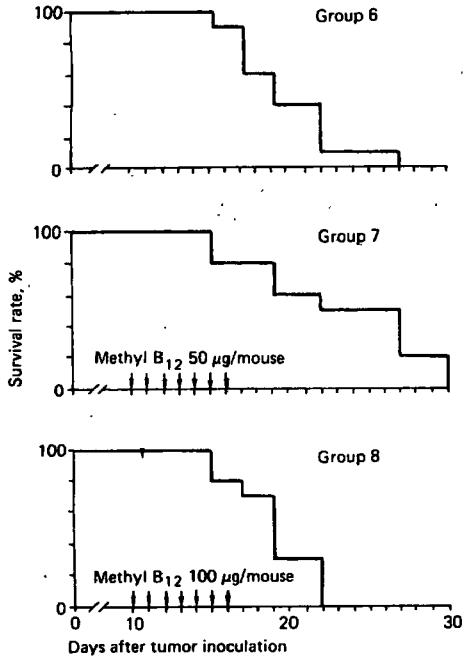

Fig. 3. Survival rate of C57BL/6 mice inoculated with Lewis lung carcinoma cells. No significant difference in the survival rates of the three groups was found.

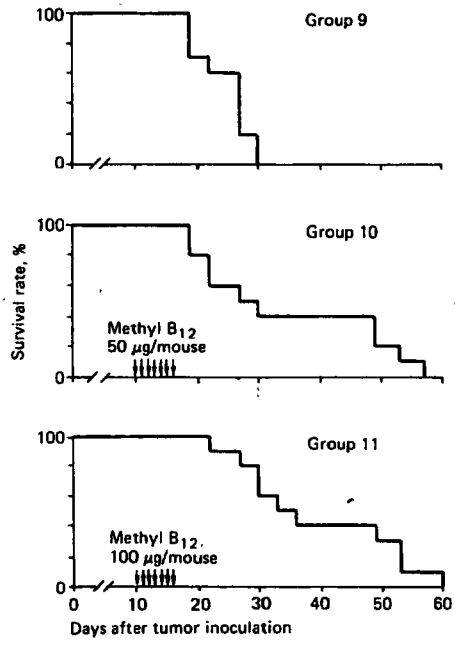

Fig. 4. Survival rate of BALB/C mice inoculated with Ehrlich ascites tumor cells. The mice in group 10 survived longer than in group 9 ($p<0.01$).

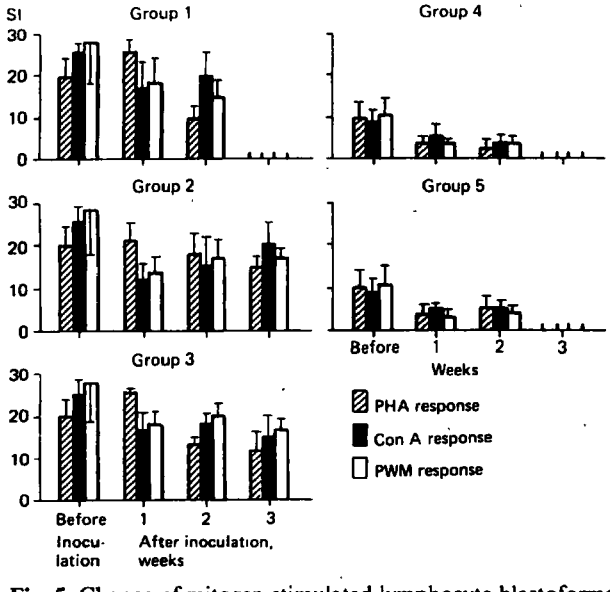

Fig. 5. Change of mitogen-stimulated lymphocyte blastoformation rate. ^{3}H-thymidine uptake of each response is shown as stimulation index (SI). In group 1, Con A and PWM responses are depressed at 1 or 2 weeks after inoculation, but PHA response is rather enhanced at 1 week and depressed at 2 weeks. In groups 2 and 3, PHA response is enhanced at 2 or 3 weeks after inoculation. In groups 4 and 5, the mitogen responses are lower before and after tumor inoculation compared with groups 1 and 2.

Table II. Survival period

Group	Days after tumor inoculation
1	$14.0 \pm 4.0^{a, b}$
2	$25.9 \pm 6.9^{a, c}$
3	21.8 ± 7.0^{b}
4	15.4 ± 2.3
5	16.4 ± 6.6^{c}
6	19.7 ± 3.6
7	23.1 ± 5.8
8	19.5 ± 3.5
9	24.7 ± 4.5^{d}
10	39.3 ± 13.2^{d}
11	24.7 ± 4.5

a–a, b–b, c–c, d–d $p<0.05$.

were depressed 1 and 2 weeks after tumor inoculation (fig. 5). Similarly, in groups 2 and 3, Con A and PWM responses were depressed at 1 or 2 weeks after inoculation, but PHA response was rather enhanced in groups 2 or 3 at 2 weeks after inoculation. In irradiated mice (groups 4 and 5), the mitogen responses were lower before and after tumor inoculation compared with the nonirradiated mice (groups 1, 2).

Discussion

Takimoto et al. [11] reported that the effect of CH_3–B_{12} on the murine immune response was as strong as that of levamisole. Our study showed that the PHA and Con A responses of murine spleen cells were enhanced after incubation in the presence of 0.1–1.0 μg/ml CH_3–B_{12} (PHA) and 1.0–10 μg/ml CH_3–B_{12} (Con A) (fig. 1).

The effectiveness of massive doses of vitamin B_{12} in the treatment of malignant human tumors (pediatric neuroblastomas) was first studied by Bodian [2], who reported remission in many patients. At present, there is no consensus on the clinical efficacy of vitamin B_{12}. Clinical and experimental studies showed both, suppression of tumor growth [4, 8–10] and ineffectiveness or carcinogenicity [3, 6, 7, 13].

We observed that MH134 tumor-bearing C3H/He mice and BALB/C mice bearing Ehrlich ascites tumor cells survived longer if they were given 50 or 100 μg/day CH_3–B_{12} for 7 consecutive days after tumor cell inoculation. Tumor growth rate of Ehrlich ascites tumor cells inoculated into BALB/C mice was slower at 17 and 19 days after tumor inoculation by administration of CH_3–B_{12} 50 μg/mouse/day for 7 consecutive days than without CH_3–B_{12}. However, the administration of CH_3–B_{12} did not prolong the life span of mice that had been irradiated prior to tumor cell inoculation. Our finding that the PHA and Con A responses were enhanced in CH_3–B_{12}-treated mice and group 2 could survive longer than group 1, but group 5 could not survive longer than group 1 or as long as group 2, suggests that the administration of CH_3–B_{12} stimulated their immune response. Immunological studies on the effect of CH_3–B_{12} in tumor-bearing mice are in progress in our laboratory.

References

1 Abe, T.; Nakajima, T.; Okamura, H.: A clinical trial of methylcobalamin in vitamin B_{12} deficiency (in Japanese with English abstract). Rinsho Ketsueki *11:* 473–477 (1970).

2 Bodian, M.: Neuroblastoma. An evaluation of its natural history and the effects of therapy, with particular reference to treatment by massive doses of vitamin B_{12}. Archs Dis. Childh. *38:* 606–619 (1963).

3 Day, P.L.; Payne, L.D.; Dinning, J.S.: Procarcinogenic effect of vitamin B_{12} on *p*-dimethylaminoazobenzene-fed rats. Proc. Soc. exp. Biol. Med. *74:* 854–855 (1950).

4 Langman, M.J.S.: Treatment of neuroblastoma with vitamin B_{12}. A report to the working party of the medical research council. Archs Dis. Childh. *45:* 385–387 (1970).

5 Nakazawa, T.; Komiya, H.; Kanno, T.: Preliminary report of RNA and DNA contents in spinal neurons of vitamin B_{12} and folic acid deficient rats (in Japanese with English abstract). Vitamins, Kyoto *42:* 275–279 (1970).

6 Newell, S.D.; John Kapp, B.S., Jr.; Romfh, J.H.: Evaluation of megadose vitamin therapy in an experimental brain tumor. Surg. Neurol. *16:* 161–164 (1981).

7 Oleson, J.J.; Little, P.A.: Effect of pteroylglutamic acid and vitamin B_{12} on growth of Rous tumor implants. Proc. Soc. exp. Biol. Med. *71:* 226–227 (1949).

8 Poydock, M.E.; Fardon, J.C.; Gallina, D.; Ferro, V.; Heher, C.: Inhibiting effect of vitamin C and B_{12} on the mitotic activity of ascites tumors. Expl Cell Biol. *47:* 210–217 (1979).

9 Rigby, C.C.; Bodian, M.: Experimental study of the relationship between vitamin B_{12} and two animal tumor systems. Br. J. Cancer *17:* 90–99 (1963).

10 Switsky, A.; Desposito, F.: A survey of American experience with vitamin B_{12} therapy of neuroblastoma. J. Pediat. *67:* 99–103 (1965).

11 Takimoto, G.; Yoshimatsu, K.; Isomura, J.; Ikeda, T.; Morimoto, T.; Osawa, T.: The modulation of murine responses by methyl-B_{12}. Int. J. Tiss. Reac. *4:* 95–101 (1982).

12 Van Der Weyden, M.B.; Cooper, M.; Firkin, B.G.: Defective DNA synthesis in human megaloblastic bone marrow: effects of hydroxy-B_{12} 5′-dioxyadenosyl-B_{12} and methyl-B_{12}. Blood *41:* 299–308 (1973).

13 Yamamoto, R.S.: Effect of vitamin B_{12}-deficiency in colon carcinogenesis. Proc. Soc. exp. Biol. Med. *163:* 350–353 (1980).

14 Yamatsu, K.; Kaneko, T.; Kitahara, A.; Ohkawa, I.: Pharmacological studies on degeneration and regeneration of peripheral nerves. I. Effects of methylcobalamin and cobamide on EMG patterns and weight-loss of denervated muscles following crush of the sciatic nerve in rats (in Japanese with English abstract). Folia pharmacol. jap. *72:* 259–268 (1976).

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22

THE ROLE OF VITAMIN B_{12} AND FOLATE IN CARCINOGENESIS*

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ABSTRACT

The roles of vitamin B_{12} and folate in carcinogenesis are largely extensions of and linked to their roles in normal metabolism, particularly 1-carbon unit metabolism. A possible key area may be hypomethylation to "switch on" genes and methylation to "switch them off." Some vitamin analogues may act as antivitamins in these reactions, as may some vitamin-binding proteins. Others may act as specific delivery proteins. Using appropriate radioactive substrates and suspensions of vitamin-dependent normal and malignant cells, it may be possible to work out their positive and negative control of DNA synthesis.

INTRODUCTION

The roles of vitamin B_{12} and folate in carcinogenesis are largely extensions of and linked to their roles in normal metabolism. One key area is the conversion of homocysteine to methionine (methyl homocysteine). This process is dependent on folate delivering its 1-carbon unit to vitamin B_{12}, which then becomes methyl-B_{12} and transfers that methyl unit to homocysteine (Fig. 1). Newberne et al.[1] recently reviewed the role of the lipotropes choline and methionine and related factors in oncogenesis, including the impaired hormonal and cell-mediated immunity in folate-deficient humans and animals, and they pointed out the synergism between high fat diets and methyl deprivation.[2] Poirier[3] reviewed the protective effect of methionine against hepatocarcinogenesis, and Farber[4] discussed the carcinogenesis promotion effect of the ethyl analogue of methionine, ethionine.

ABBREVIATIONS: azaC = 5-azacytidine; TC II = transcobalamin II; PGA = pteroglutamic acid; SAM = S-adenosylmethionine; dU = deoxyuridine; AIDS = acquired immunodeficiency syndrome; dThd = thymidine; PHA = phytohemagglutinin A.

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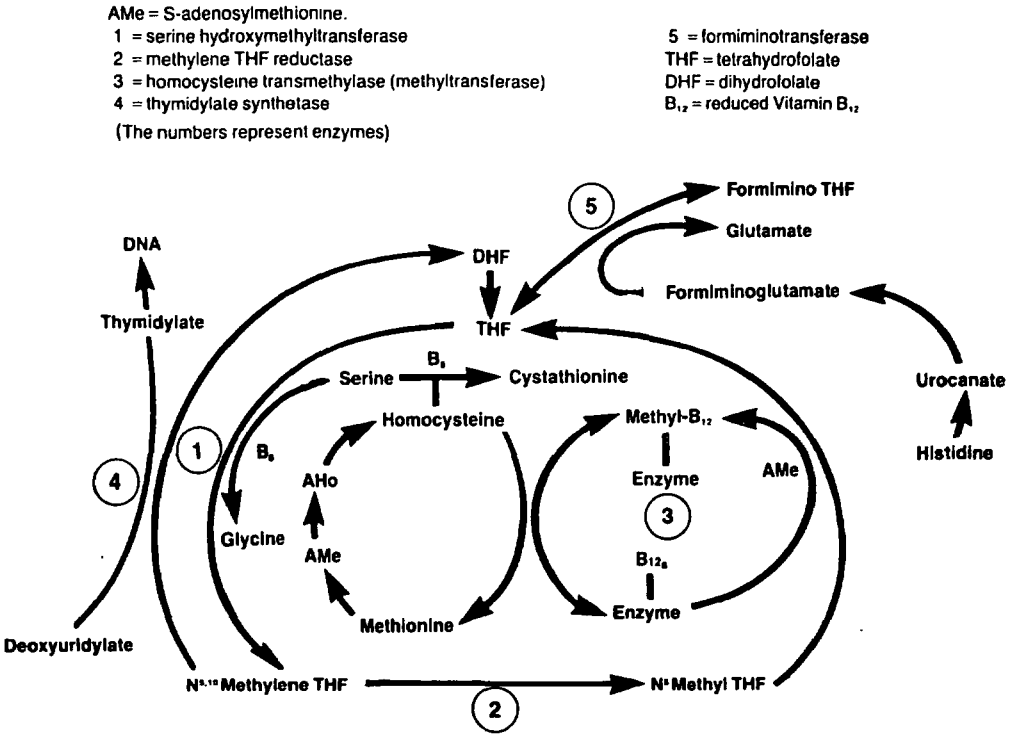


Fig. 1. Biochemical interrelationships between vitamin B_{12} and folate in human metabolism.

In a series of painstaking studies, Poirier's group[5] determined that, over a 76-week period, dietary methyl deficiency markedly promoted liver carcinogenesis and exhibited complete carcinogenic activity in this organ in the rat. They showed this in rats fed methyl-deficient, amino acid-deficient diets. When the diets were also devoid of folic acid and vitamin B_{12}, the diethylnitrosamine-initiated rats died within 23 experimental weeks, before developing hepatocellular carcinoma, but all had livers containing hepatocytes of atypical appearance and, particularly at the 2 higher dosages of diethylnitrosamine, a cirrhotic pseudonodular architecture. They also found neoplastic conversion of rat liver epithelial cells in culture by ethionine and S-adenosylethionine.[6]

Krumdieck[7] reviewed the literature pertaining to the role of folate deficiency in facilitating carcinogenesis through 1982 and Eto[8] has carried the subject through the beginning of 1985. Folate is essential in the biosynthesis of both purines and pyrimidines[9,10] and therefore is required by all dividing cells. The conversion of deoxyuridylate to thymidylate (methyldeoxyuridylate) is folate- and B_{12}-dependent, involving these 2 vitamins in a key step in DNA synthesis (Fig. 1).[9-11] These biochemical facts underlie the chromosomal abnormalities that characterize human clinical deficiency of vitamin B_{12}[9] and/or folate.[12,13] A wide range of chemical carcinogens inhibit DNA methylation in vitro.[14] It has been suggested that deficiency of folate or vitamin B_{12} or any cause of failure to methylate DNA and/or RNA can activate malignancy by hypomethylating oncogenes, leading to such gene expression and/or gene amplications, and that methylating oncogenes can inhibit malignancy by making them

dormant.[3,15] This is similar to the concept of "relaxed control" of RNA synthesis, discussed 3 decades ago by Borek and co-workers.[16] They noted that when an organism auxotrophic for methionine is deprived of methionine, it loses its ability to suppress synthesis of RNA, which is then synthesized more rapidly; they tied that observation to methylation of RNA. We speculated that vitamin B_{12} or folate deficiency could produce such "relaxed control,"[17] and we noted more recently[15] that folate, vitamin B_{12}, and their antagonists could be involved in the control of normal gene expression if in fact hypomethylation of DNA "switches on" normal genes and methylation "switches them off."[18] Although the evidence of this process is significant but inconclusive, one would expect that hypomethylation of the DNA or RNA of oncogenes would activate them and methylation would inactivate them. Perhaps some of the second cancers that develop after successful antimetabolic chemotherapy are due to the same chemotherapy that directly destroys an active cancer, demethylating an oncogene of a dormant cancer.

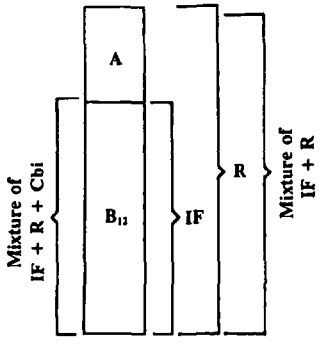
Gene amplification is a mechanism for tumor resistance to antimetabolites.[19] One can speculate that it may also be a mechanism to aid in tumor proliferation by, for example, producing gene amplification of the hepatic Phase I enzymes that activate carcinogens.[20]

Gautsch and Wilson[21] found that de novo methylation of the input provirus occurs in embryonal carcinoma cells but not in permissive, differentiated teratocarcinoma. Harrison et al.[22] demonstrated a 3-way correlation between tumorigenicity, trisomy for 3q, and specific demethylation, suggesting that decreased DNA methylation may be involved both in differentiation and in tumorigenicity and that the antileukemia drug azaC may induce chromosomal aberrations as well as altering DNA methylation. Altering DNA methylation is just one of the varied effects of azaC on cellular metabolism.[23-25] The drug reduces DNA methylation and induces theoretically therapeutically valuable differentiation of human promyelocytic leukemia cells (HL-60) in culture, although this induction is less effective than that brought about in these cells by dimethylsulfoxide and L-ethionine.[26] Anderson and colleagues[25] found that azaC selectively hypomethylates fetal globin genes, supporting work by Ley et al.[23] and Charache and associates.[27]

Patients with neoplasms excrete elevated levels of certain methylated bases in their urine, and Borek's group[28-30] has been attempting to correlate the quantity of such excretion with the degree of tumor activity. Gross's group, in collaboration with our group,[15,31,32] were unable to show any reproducible inhibitory effect of 5-methylcytidine on the development of presumably RNA virus-induced transplanted L2C leukemia-lymphoma in guinea pigs; this appears to be an animal analogue to human leukemia-lymphoma of RNA virus etiology.[33,34] After we switched to 5-iodocytidine, which seemed more promising, both groups unsuccessfully sought funding targeted to continue this work. Gross's[35] recent dramatic report of reduction in the incidence (i.e., the initial development) of radiation-induced tumors in rats after restriction of caloric food intake has been associated with renewed funding. His group previously noted that restriction of food intake will not significantly influence the growth or progress of established tumors in mice.[32] American Cancer Society statistics suggest an increased frequency of malignancy in obese persons (L. Gross, personal communication).

The roles of vitamin B_{12} and folate in carcinogenesis are not at the simple level at which serum vitamin levels correlate with extent of disease. No correlation has been found between serum folate and vitamin B_{12} levels and the extent of small cell lung cancer.[36] However, a correlation may exist between levels of certain naturally occurring folate and

B_{12} analogues in serum and/or tissues and malignancy. Some folate and B_{12} analogues may not only be vitamin-inactive for humans, but they may facilitate carcinogenesis or inhibit it by blocking normal vitamin action or in other ways. We now know, for example, that what is assayed as "B_{12}" in serum by most microbiological and radioassays is in fact a mixture of cobalamins and noncobalamin corrinoids (Fig. 2);[37] this is also true of the B_{12} in tissues[38] and multivitamin pills.[39,40] Enormous amounts of this



A=Analogues (i.e., cobalamins plus all other corrinoids)
B_{12}=Cobalamins (biologically active B_{12})
Cbi=Cobinamide (a corrinoid)
IF=Intrinsic factor
R=R-binders

Fig. 2. Serum B_{12}. The "serum B_{12}" consists of the sum of "true B_{12}" (i.e., hydroxocobalamin and other cobalamins biologically active for humans), designated B_{12}, plus the serum content of other corrinoids (i.e., molecules that have the heme-like corrin nucleus of cobalamin but differ from cobalamin in part or all of the rest of their structure), designated A (analogues). Pure gastric IF (intrinsic factor) binds only cobalamins, whereas R binders (the B_{12} binders ubiquitous in body fluids, including saliva, serum, cerebrospinal fluid, bile, and urine) bind all corrinoids (cobalamin + analogues). The brackets identify the portions of total serum corrinoids bound by each of 4 different commercially available B_{12} radioassay binders. The mixture (IF + R + $\underline{X}$ Cbi) binds a little more than B_{12} alone and, therefore, more than pure IF does, when the amount ($\underline{X}$) of preadded cobinamide exceeds the cobalamin-binding capacity of the mixture by about 100-fold; if the excess reaches 1,000-fold, there will be some binding to IF despite its specificity for cobalamins, and the mixture will therefore bind a little less than true B_{12}. Because of the specificity of its IF portion for cobalamin, (IF + R) binds a bit less than the total corrinoids that are bound by pure R.

Important note. Confusion can arise when results are reported as "serum vitamin B_{12} levels," which are actually serum total corrinoid levels, but the range of normal given with the report is for cobalamins rather than total corrinoids. For maximum reliability, each laboratory should develop its own range of normal for whatever serum B_{12} assay(s) it uses and should not use the range of normal determined in a different laboratory, because minor differences in methodology such as pH produce different values. Reprinted with permission from ref. 45.

Table 1. Cobalamin Content Versus Total Corrinoid Content of Spirulina[a]

	Corrinoid Content, μg				
			Radioassay		
B_{12} Claim on Bottle	Lactobacillus leichmanii	Euglena gracilis	Total Corrinoids	Cobalamin	Analogues
6 μg	1.24	0.615	1.63	0.09	1.54

[a]All values are for 6 tablets, which is the daily dose recommended by the manufacturer, the Earthrise Company. See ref. 38.

B_{12} have been found in the "health food" spirulina (Table 1),[41,42] in human colon, and lesser amounts in many foods.[43] Some of these analogues are antimetabolites;[40] their role in inhibiting[44] or promoting carcinogenesis, or even as direct carcinogens, remains to be determined, as do their levels in serum, tissues, bile, and colon of patients with and without various neoplasms. Furthermore, just as serum iron is attached to protein in both a "delivery" form (on transferrin) in equilibrium with parenchymal iron and a "storage" form (ferritin) in equilibrium with storage iron,[45] the same may prove true for folate[46] and B_{12}.[47] Loss of B_{12} from TC II appears to be an earlier indicator of parenchymal B_{12} deficiency than clear reduction of total serum B_{12},[48] suggesting that B_{12} on TC II is equilibrated with bioavailable tissue B_{12}, whereas the greater amount of B_{12} on TC (I + III) is not. A recent review of the macromolecules involved in the assimilation and transport of cobalamin discusses their known functions.[49]

FOLATE DEFICIENCY

Some years ago, Heller and his associates[50] observed that folate deficiency increased normal hemoglobin production in a patient with sickle cell trait. Since higher normal hemoglobin production means lower sickle cell hemoglobin, this was a desirable phenomenon. However, treatment with folic acid produced the undesirable result of lowering the normal hemoglobin.

Recognizing that the primary role of folate is in transferring 1-carbon units, one of which is the methyl unit, Heller and others focused on cytidine, the most heavily methylated of the 4 bases of the genetic code (the other sometimes methylated base is guanine). In 1982, they reported that azaC selectively activates the gene for fetal hemoglobin synthesis in patients with beta-thalassemia and sickle cell disease.[18,51] They raised the possibility that this activation results from the incorporation of azaC as cytidine into the gene for hemoglobin, which, with its 5 position blocked, cannot take on the methyl group made available in folate- (and vitamin B_{12}-) dependent reactions and thus was not methylated. It was suggested that failure to methylate the cytidine of the gene for fetal hemoglobin prevented it from becoming dormant, and it was activated to produce fetal hemoglobin. Whether or not the bases that make up the genetic code are methylated plays a major role in gene expression.[52,53] Normally, 4-5% of cytosine residues in human DNA are methylated.[54] Whether oncogene expression can similarly be prevented by increasing ordinarily

vitamin B_{12}-dependent and folate-dependent 1-carbon transfer to the genetic code of the oncogene has been the subject of subsequent studies.[21,22,24-26]

The B vitamin, folic acid, was isolated in 1943: Stokstad purified PGA and Pfiffner and associates crystallized folate from liver.[55] Within a year, Leuchtenberger and associates at Mount Sinai Hospital[56,57] reported that a form of this vitamin called folic acid concentrate [later to be known as oxidized folate triglutamate (teropterin)] inhibited the growth of transplanted sarcoma 180 in mice. This material and similar crystalline oxidized triglutamate known as fermentation Lactobacillus casei factor produced complete regression of single spontaneous breast cancers in mice.[58] One wonders if oxidized folate triglutamate could methylate sarcoma 180 and mouse breast cancer oncogenes. Leuchtenberger's first paper stated that Pollack and associates[59] had reported that fermentation L. casei factor (folic acid in triglutamate form[60]) was present in human and rat cancers at higher levels than inositol and at much higher levels than biotin or pyridoxine.

Laszlo and Leuchtenberger[61] reported that inositol (then believed to be a B vitamin for humans but more recently shown to be a B vitamin only for some bacteria, since it is synthesized adequately by humans[62]) inhibited animal tumor growth, but other B vitamins did not. Vitamin B_{12} was yet to be discovered.

Lewisohn et al.[63] reported that not only did oxidized folate monoglutamate (liver L. casei factor from Lederle Laboratory) not inhibit spontaneous breast cancers in mice, but it actually produced a more rapid growth of the primary tumors and a significant increase in lung metastases. Here was evidence that one of the monoglutamate forms of the vitamin promoted tumor growth but that one of the triglutamate forms for the same vitamin inhibited the growth of the same tumor. Recent studies were aimed at determining whether oxidized folate monoglutamate may inhibit methylation of oncogenes, thereby allowing their expression, and whether triglutamates can do the opposite. Folate triglutamates are frequently more active than monoglutamates in methylation in man.[64,65] Folate triglutamate has much greater affinity than folate monoglutamate for milk folate-binding protein,[66] which appears to be a folate delivery protein.[67] Gene amplification can lead to overproduction of certain proteins, including transport and delivery proteins as well as enzymes. Chabner's group[68] isolated a methotrexate-resistant mutant that could not polyglutamate methotrexate, causing the drug to leak out of the cell.

These and other studies suggest that one form of a vitamin can be a growth promoter, by acting as a coenzyme, while another form can attach to the same apoenzyme or other ligand, such as a vitamin-transporting protein, and interfere with the reaction, just as a key with a missing tooth can fit into a lock but will not turn. Different forms and major parts of the same basic vitamin structure, that is, analogues and congeners, exist in nature and are synthesizable; some are antagonists or antivitamins, some of which can be created from vitamins by only slightly warping their structure.

Farber et al.[60] gave pteroyltriglutamic acid (teropterin) and pteroyldiglutamic acid (diopterin), both synthesized by Y. SubbaRow and his associates at Lederle Laboratories, to 90 patients with various malignancies, noting that "in general, adult patients experienced improvement in energy, appetite, sense of well being...might be ascribed to improved morale from frequent visits, more medical attention..." They also reported inconstant temporary decreases in size of metastases in some tumors and degeneration and necrosis in others. The observation by Welch[69] of rapid deterioration of adult patients with chronic myeloge-

nous leukemia given folic acid led to the earliest attempts at therapy in 2 patients by limiting the availability of folate through the use of x-methyl folic acid, coupled with succinylsulfathathioazole and low-folate diets. Both patients went into remission, stopped following the low-folate diets, and quickly relapsed.

It is apocryphally alleged that Farber was also giving the oxidized, stable pharmaceutical form of folic acid (i.e., PGA) to children with lymphoproliferative malignancies (lymphocytic leukemia and lymphoma) until one of his residents collected sufficient data to suggest that the children receiving this new vitamin were dying faster than those children not receiving it. This observation allegedly led Farber to ask Lederle to create a warped folic acid molecule that would interfere with folate metabolism in the malignant cells; this was done by adding an NH_2 group, thereby creating aminopterin. A second alteration, methylation in the 10 position, created methotrexate, still one of our most potent anticancer agents, particularly effective against childhood lymphoproliferative disorders and trophoblastic malignancies.

Rapidly growing neoplastic tissue consumes folate so rapidly that folate deficiency megaloblastosis can occur in the host cells.[70,71] There is also evidence that tumor growth may be slowed by any form of vitamin B_{12} deficiency, including inadequate absorption or elevated levels in serum of a vitamin B_{12} binder that does not deliver the vitamin to tumor tissue[72] but does deliver it to the liver in a calcium-dependent fashion.[70,73-76] Granulocytes and liver are major sources of serum-binding proteins for both vitamin B_{12} and folic acid that tightly bind those vitamins; malignancies of granulocytes and liver theoretically can repress themselves by releasing large amounts of these binders, which could tie up supplies of the vitamins and prevent vitamin delivery to, and nourishment of, the malignancy.[47,77]

Oxidized folate (such as PGA, the stable pharmaceutical form of the vitamin) is not per se metabolically active and may even be neurotoxic; it has a structural similarity to Dilantin.[78] PGA can produce seizures in patients wtih epilepsy by blocking the protective action of Dilantin, as Butterworth's group has shown.[79] Folic acid and Dilantin compete for intestinal absorption,[80] and they probably also compete at the brain cell, where Dilantin may interfere with ATPase, as in the gut.[81] There appears to be a one-way transport system to remove noxious oxidized folates from the nervous system and a one-way transport system to deliver useful, reduced folate into the nervous system;[82] this information has been used to successfully treat with folinic acid a child with congenital folate malabsorption unresponsive to folic acid.[83,84]

Similarly, there may be a one-way transport system to remove noxious vitamin B_{12} analogues from the body[75,85] and another transport system to deliver helpful forms of the vitamin to normal tissues, as we first reported for human serum delivery of vitamin B_{12} to liver.[74] Recent evidence suggests that vitamin B_{12} analogues in human tissues may arise primarily from human colon flora.[43]

The transport systems have different affinities for different forms of vitamin B_{12} and folic acid and different delivery ability for helpful and noxious forms of the vitamins with respect to different normal cells and possibly also tumor cells.[46,47] Sutherland[86] noted that folate deficiency produces fragile chromosomes. Das[12] has noted that folate therapy will not correct folate deficiency in circulating human lymphocytes or their chromosomes for 1-2 months after the start of therapy, whereas deficiency is corrected in bone marrow cells within 6 hours after the start of folate therapy. Fragile chromosomes can persist in lymphocytes

for 1 or 2 months after the beginning of replacement therapy for folate deficiency, because circulating lymphocytes are impervious to nutrients such as the B vitamins until they are triggered to make DNA by a virus or lectin.[12,87] The role of fragile chromosomes in carcinogenesis was recently reviewed.[88]

Vitamin B_{12} is not vitamin active in its stable pharmaceutical form, cyanocobalamin. The cyanide must be removed for vitamin function to occur. Even an active form of the vitamin, hydroxocobalamin, can block vitamin B_{12} metabolism by competing with adenosylcobalamin for the binding site on the adenosylcobalamin-dependent enzyme methyl malonyl-CoA mutase.[89] Conversely, the oncogenic potential of absorbed cyanide from cassava and other foods[90] may be muted by inactivation of the cyanide by the metabolically active form of vitamin B_{12}, hydroxocobalamin. Indeed, anesthesiologists have used massive doses of hydroxocobalamin to reverse cyanide toxicity by soaking up the cyanide from the nitroprusside used in open-heart operations.[91]

Butterworth[92] noted that naturally occurring folate analogues such as pteroic acid may be lethal for rats and can displace folate from human tissues and flush it out in the urine.[93] It remains to be determined whether there is a folate analogue, such as pteroic acid, that will selectively flush folate or vitamin B_{12} out of tumor tissue or selectively deliver folate or vitamin B_{12} to tumor tissue to potentiate fluoropyrimidine or other drug antitumor activity.[94,95]

Human serum, Dilantin, and methotrexate have been reported to inhibit pteroyl monoglutamate and methyl tetrahydrofolate uptake by human bone marrow cells in vitro, but 2-deoxyglucose, an antagonist of glucose, will enhance such uptake.[96] It would be worthwhile to determine whether simultaneous administration of folate and 2-deoxyglucose are more harmful to tumor cells than to normal ones.

A folate-free diet was given for more than 4 months, with no clinical benefit, to 7 patients with disseminated cancers. The folate levels in tumor, liver, and blood all declined at the same rate.[97] On the other hand, Whitehead and colleagues[98] noted disappearance of megaloblastosis in cervical epithelial cells with folate therapy of women taking oral contraceptives, and Butterworth et al.[99] reported improvement in apparent cervical dysplasia in oral contraceptive users treated with folic acid. Longo and co-workers[100] noted human selective folate deficiency in the lymphocyte cell line after 4 months of oral contraceptive administration; lymphocytes and cervical epithelium may be similarly selectively folate deprived. Selective folate deficiency in one cell line and not another was first noted in 1962 in the first case of deliberately produced dietary folate deficiency in a volunteer, whose intestinal biopsy showed normal epithelial cells when his bone marrow had become megaloblastic.[101] Presumably, the intestine epithelial cells took up the traces of folate in the folate-deficient diet to sustain their own normality, leaving little or none to be absorbed and delivered to the bone marrow.

Selective delivery of nutrients and antimetabolites to one cell line and not another is generally a function of selective transport and delivery protein and high-affinity receptors.[102]

VITAMIN B_{12}

Shortly after Minot and Murphy reported that liver therapy cured pernicious anemia, an achievement for which they shared a Nobel prize with Whipple, Minot reported the development of a malignancy (polycythemia

vera) occurring immediately after the start of treatment for pernicious anemia. There were other sporadic reports of myeloproliferative disorders associated with therapy for pernicious anemia with liver extract and subsequently with pure vitamin B_{12}. The small number of such cases implies coincidence rather than cause and effect but does not prove it.

Warped vitamin B_{12} molecules (the anilide and ethylamide of vitamin B_{12}, synthesized by E. Lester Smith of Glaxo Laboratories) were first used a quarter of a century ago in the treatment of myelogenous leukemia refractory to all other therapy.[103] One woman given the anilide of vitamin B_{12} appeared to go into complete remission, with her bone marrow aspirate changing from florid myelogenous leukemia to a fibrotic picture. However, she continued to have a small number of leukemic cells in her peripheral blood. When she died of pneumonia 6 months later, her marrow was largely fibrotic. Whether this one case was cause and effect or coincidence is impossible to determine.

At the Great Ormond Street Hospital for Sick Children in London, Bodian[104] found that megadoses of vitamin B_{12} produced remission in neuroblastoma in children, but Sawitsky and Desposito[105] did not confirm this in 103 children. Bodian believed that megadoses of vitamin produced maturation of the tumor, but spontaneous maturation and spontaneous remission can occur in neuroblastoma with no therapy.

Day et al.[106] and Ostryanina[107] reported that vitamin B_{12} enhanced the carcinogenic effect of *p*-dimethylaminoazobenzene and 3 other carcinogens in rats consuming a methionine-deficient diet. Conversely, chemically induced tumors of the liver, colon, and esophagus are enhanced by diets deficient in folic acid, vitamin B_{12}, choline, and methionine.[108] Poirier et al.[5,6] have elegantly and laboriously delineated this phenomenon.

Folic acid and vitamin B_{12} can prove useful in those tumors that grow more rapidly as more of these vitamins are supplied, because the tumor cells can be stimulated into the DNA synthesis phase in which a number of cancer chemotherapy agents exert their deadly effects. Those agents can be used in a sequence right after folic acid and/or vitamin B_{12}.[94,95]

Analogues of vitamin B_{12} appear to be ubiquitous in human serum;[37] red blood cells, liver, and brain;[38] bile;[109] multivitamin pills;[39] a wide variety of microorganisms;[110] and human colon content and feces.[43] Two analogues from multivitamin pills have been reported to block vitamin B_{12} metabolism in normal human cells in vitro.[40] The highest known pill content of analogue of vitamin B_{12} is in the health food fad pill spirulina.[41] Such analogues must be evaluated for their ability to block vitamin B_{12} metabolism in both normal and malignant cells.[44] The enterohepatic circulation functions to get rid of vitamin B_{12} analogues,[109] a teleologic suggestion that such analogues may have undesirable effects.

The anesthetic gas nitrous oxide can produce acute vitamin B_{12} deficiency, as was first made clear by Amess et al.[111] Nitrous oxide produces a greater than 95% reduction in liver vitamin B_{12} in the fruit bat.[112] Scott and Weir in Dublin[113] and Metz's group in South Africa[114] showed that methionine can prevent the nerve damage produced by nitrous oxide-induced vitamin B_{12} deficiency in primates and the fruit bat, respectively. Chanarin et al.[115] suggested that formyl folate monoglutamate, with its formate derived from methylthioribose, is the preferred substrate for the polyglutamate forms of folate that normal cells preferentially use. Studies of the effects of nitrous oxide potentiation of cytotoxic drugs on tumors in experimental animals, with and without methionine "rescue" of normal cells, are of great interest. Kroes and associates[116,117] showed that nitrous oxide retards leukemic proliferation

in a transplantable acute nonlymphocytic leukemia in BN rats and enhanced this cytostatic effect with cycloleucine, which inactivates methionine adenosyltransferase and thereby inhibits the formation of SAM. Cycloleucine reversal of dU suppression was greater than the sum of the reversals produced by either gas or drug alone.

Differences in ability of normal versus tumor cells to take up various nutrients and antinutrients are being sought in efforts to kill tumor cells selectively. Normal cells treated with nitrous oxide to destroy vitamin B_{12} are better rescued by methionine than by SAM, even though SAM is the active form, because methionine much more readily crosses normal cell walls.[115] The relative ability of these 2 rescue agents to cross tumor versus normal cell walls requires further study.

Because folic acid and vitamin B_{12} are intimately related to the synthesis of DNA, lack of either damages DNA synthesis. The primary damage is to de novo DNA synthesis, with the result that there may be a secondary increment in salvage DNA synthesis.[118] In those tumors in which synthesis of DNA by the salvage pathway is relatively greater than in normal cells, as compared to the de novo pathway of DNA synthesis, it is theoretically possible that folic acid and/or vitamin B_{12}, by enhancing de novo DNA synthesis, could be relatively more helpful to normal than to tumor cells and relatively more harmful to certain tumor cells.[15,118]

Low serum vitamin B_{12} levels occur in multiple myeloma,[119] rheumatoid arthritis,[120] systemic lupus erythematosus,[119,121] and AIDS.[122] This may be a serum phenomenon related to transport protein abnormalities, and in AIDS it may be related to immune dysfunction. Elevation of TC II has been noted in AIDS[122] and other autoimmune disorders[121,123] and in acute leukemia and lymphoma.[119,121] The relationship of this phenomenon to increased needs for vitamin B_{12} and desaturation of TC II in vitamin B_{12} deficiency in these disorders awaits determination.[48] The diversity of disorders in which TC II is elevated suggests that it is an acute phase reactant.[123]

SUPPRESSION TESTING

Some years ago, we created the diagnostic dU suppression test to diagnose deficiencies of either vitamin B_{12} or folate; we showed that the slowing of DNA synthesis in bone marrow cells in the test tube, which Killman[124] had reported, could be corrected by adding the vitamin to the test tube.[125,126] More recently, the dThd suppression test has been added as an ancillary measure.[118]

The immediate precursors of cellular DNA synthesis are dATP, dGTP, dCTP, and dTTP. In most mammalian cells, de novo synthesis of dTMP from dUMP is a rate-limiting step and requires 5,10-methylene tetrahydrofolate as a coenzyme.[127] However, the incorporation of preformed dThd into replicating cells provides a "salvage pathway" of dTMP synthesis.[128] It was reported that an excess of dU added to culture of normal bone marrow suppressed the incorporation of [^{3}H]dThd into DNA but failed to do so in bone marrow of patients with vitamin B_{12}-[124,125] and folate-deficient[125,126] megaloblastic anemia. It was also shown that this defect (impaired dU suppression of [^{3}H]dThd into DNA) was partially corrected by adding vitamin B_{12} to the bone marrow cultures of vitamin B_{12}- but not folate-deficient patients.[125] It was almost completely corrected by large doses of folic acid and smaller doses of folinic acid (5-formyl tetrahydrofolate) in both types of deficiencies; 5-methyl tetrahydrofolate corrected only folate deficiency.[125] Further, the corrective effect of either vitamin was prevented by the folate antagonist methotrexate.[125] The main folate

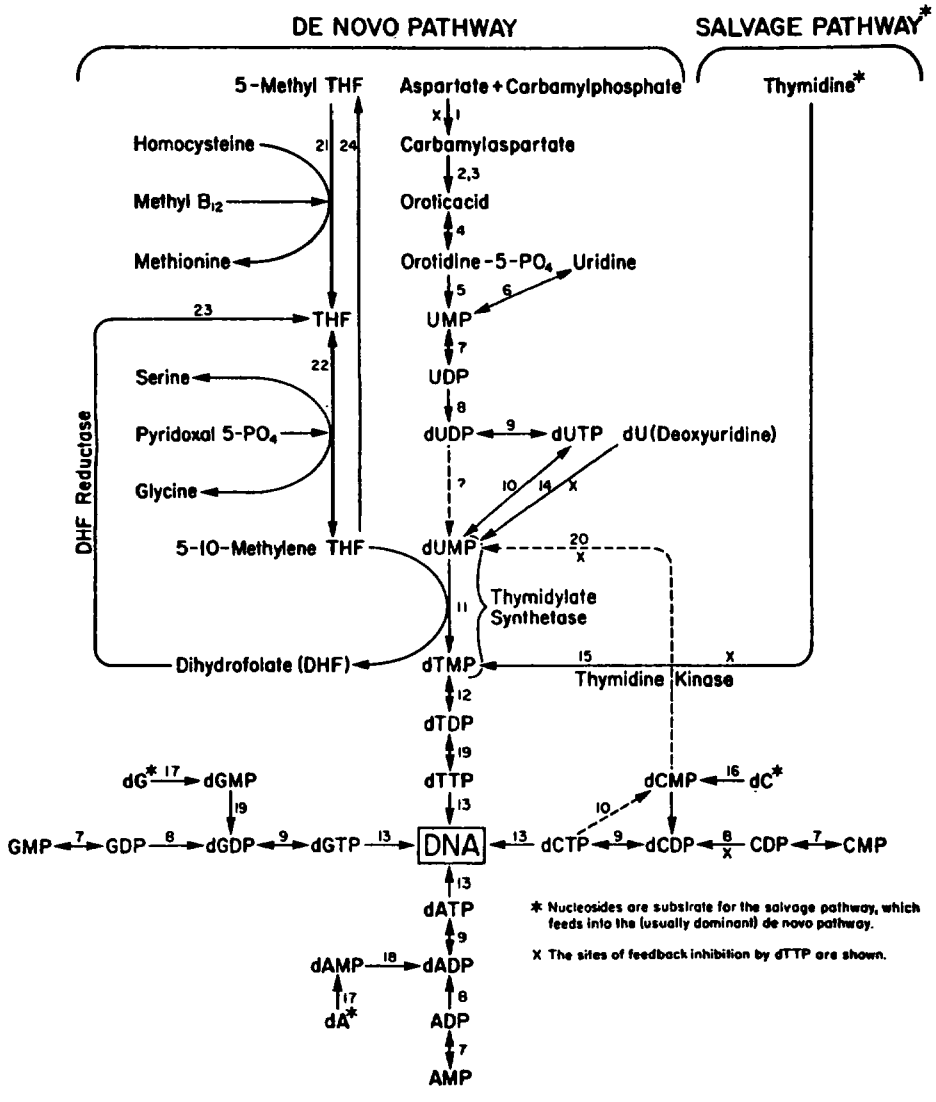


Fig. 3. Biosynthetic pathways of deoxyribonucleotides and DNA. The enzymes catalyzing the reactions are (1) aspartic transcarbamylase, (2) dihydroorotase, (3) dihydroorotate dehydrogenase, (4) OMP pyrophosphorylase, (5) OMP decarboxylase, (6) uridine kinase, (7) nucleoside monophosphokinases, (8) ribonucleotide reductase (ribonucleoside diphosphate reductase), (9) nucleoside diphosphate kinases, (10) dUTPase-dCTPase, (11) thymidylate synthetase (dTMP synthetase), (12) dTMP kinase, (13) DNA polymerase, (14) deoxyuridine kinase, (15) thymidine kinase, (16) deoxycytidine kinase, (17) deoxyguanosine-deoxyadenosine kinase, (18) dAMP kinase, (19) dGMP kinase, (20) dCMP deaminase, (21) 5-methyl THF:homocysteine methyltransferase, (22) serine transhydroxymethylase, (23) dihydrofolate reductase, (24) 5,10-methylene THF reductase. (Note: For THF read H_4PteGlu; for DHF read H_2PteGlu.)

coenzyme in plasma, 5-methyl tetrahydrofolate, which was shown by our laboratory to "pile up" in the plasma of patients with vitamin B_{12} deficiency,[129] corrected the abnormal dU suppression in folate-deficient marrows but failed to do so in vitamin B_{12}-deficient marrows unless vitamin B_{12} was also added to the in vitro system. Essentially similar abnormalities in DNA synthesis were found to occur in PHA-stimulated lymphocytes of patients with vitamin B_{12}- and folate-deficient megaloblastic anemia.[130] These findings indicated that abnormal dU suppression of [^{3}H]dThd incorporation into DNA in deficiency of vitamin B_{12} and/or folate was due to impaired de novo synthesis of thymine-DNA (impaired conversion of deoxyuridylate to thymidylate) because of reduced availability of the pertinent folate coenzyme, 5,10-methylenetetrahydrofolate.[125,128]

The 2 alternative pathways of thymine-DNA synthesis, the de novo and the salvage pathways, are interrelated by a common end product, dTPP, which exerts a regulatory influence on both pathways by a feedback inhibition and thereby presumably maintains a balanced synthesis of cellular DNA (Fig. 3). It would be expected that, just as nonradioactive dU added to cell cultures in excess inhibits the incorporation of [^{3}H]dThd into DNA (the dU suppression test),[124,125] nonradioactive dThd in excess would cause a reciprocal inhibition of [^{3}H]dU into DNA (i.e., a dThd suppression test).[118] Almost a decade ago, we reported that excess TdR added to PHA-stimulated lymphocyte cultures inhibited the incorporation into DNA of subsequently added [^{3}H]dU,[131] but a seemingly contrary claim was made (without data) by Beck.[132] We subsequently described in detail the results of such dThd suppression tests in short-term suspension cultures on bone marrow and PHA-activated lymphocytes, indicating a reciprocity of the de novo and salvage pathways in the regulation of thymine-DNA synthesis in both cell systems.[118] This supported the theoretical concept of the dU suppression test in defining biochemical megaloblastosis caused by deficiency or inhibition of folate and vitamin B_{12}. Beck[133] recently reversed his prior position and published data similar to data we had presented at 2 meetings he attended.[134,135]

Some patients have tumor shrinkage on therapy with massive doses of dThd.[136,137] It is possible that patients who will respond can be prospectively separated from those who will not respond by determining the reciprocity or lack thereof of the salvage and de novo pathways of thymine-DNA synthesis in the tumor, using in vitro dU suppression tests and dThd suppression tests.[118]

Our routine dU suppression tests now use hydroxocobalamin instead of cyanocobalamin, as recommended by van der Weyden et al.[138] and Zittoun et al.,[139,140] since it is more easily incorporated by cells, as is coenzyme B_{12}.[141] The folic acid control is no longer used, since the methyl folate control adequately identifies folate deficiency, for reasons first noted by Metz and colleagues.[118] Zittoun et al.[140] have unpublished data similar to ours indicating decreased incorporation of either labeled dThd or labeled dU after preincubation with the other, unlabeled material.

The possibility that dU and dThd suppression tests involve repression and derepression of a number of biochemical pathways, as indicated in Fig. 2 of Das et al. (Fig. 3)[142] does not reduce the value of these tests in the diagnosis of biochemical megaloblastosis. Using appropriate radioactive substrates and drug inhibitors, these tests appear to be of great value in working out the internal machinery of positive and negative controls of DNA synthesis in normal and malignant cells. The dU suppression test is being widely adopted to predict response to chemotherapy [116,117] (J. R. Bertino, personal communication); it may also be of value in assessing interferon effects.[143]

REFERENCES

1. P. M. Newberne, A. E. Rogers, and K. M. Nauss, Choline, methionine, and related factors in oncogenesis, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
2. P. M. Newberne, Lipotropic factors and oncogenesis, this volume.
3. L. A. Poirier, The role of methionine in carcinogenesis in vivo, this volume.
4. A. K. Ghoshal, D. S. R. Sarma, and E. Farber, Ethionine in the analysis of the possible separate roles of methionine and choline deficiency in carcinogenesis, this volume.
5. Y. B. Mikol, K. L. Hoover, D. Creasia, et al., Hepatocarcinogenesis in rats fed methyl-deficient, amino acid defined diets, Carcinogenesis 4:1619 (1983).
6. J. D. Brown, M. J. Wilson, and L. A. Poirier, Neoplastic conversion of rat liver epithelial cells in culture by ethionine and S-adenosylethionine, Carcinogenesis 4:173 (1983).
7. C. L. Krumdieck, Role of folate deficiency in carcinogenesis, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
8. I. Eto, Role of vitamin B_{12} and folate deficiency in carcinogenesis, this volume.
9. V. Herbert, N. Colman, and E. Jacob, Folic acid and vitamin B_{12}, in: "Modern Nutrition in Health and Disease, Sixth Edition," R. S. Goodhart and M. E. Shils, eds., Lea & Febiger, Philadelphia (1980).
10. V. Herbert and N. Colman, Folate and vitamin B_{12}, in: "Modern Nutrition in Health and Disease, Seventh Edition," M. E. Shils and V. R. Young, eds., Lea & Febiger, Philadelphia (in press, 1986).
11. D. G. Weir and J. M. Scott, Interrelationships of folates and cobalamins, Contemp. Issues Clin. Nutr. 5:121 (1983).
12. K. C. Das and V. Herbert, The lymphocyte as a marker of past nutritional status: Persistence of abnormal lymphocyte deoxyuridine (dU) suppression test and chromosomes in patients with past deficiency of folate and vitamin B_{12}, Br. J. Haematol. 38:219 (1978).
13. D. C. Arthur, T. J. Danzl, and R. F. Branda, Cytogenetic studies of a family with a hereditary defect of cellular folate uptake and high incidence of hematologic disease, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
14. V. L. Wilson and P. A. Jones, Inhibition of DNA methylation by chemical carcinogens in vitro, Cell 32:239 (1983).
15. V. Herbert, The inhibition of some cancers and the promotion of others by folic acid, vitamin B_{12}, and their antagonists, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
16. E. Borek, A. Ryan, and J. Rockenbach, Relaxed control of RNA synthesis, Fed. Proc. 13:184 (1954).
17. V. Herbert, "Megaloblastic Anemias--Mechanisms and Management," Yearbook Medical Publishers, Chicago (1965).
18. T. J. Ley, J. DeSimone, N. P. Anagnou, et al., 5-Azacytidine selectively increases γ-globin synthesis in a patient with B^+ thalassemia, N. Engl. J. Med. 307:1469 (1982).
19. J. R. Bertino, S. Srimthandaha, M. D. Carman, et al., "Mechanisms of Methotrexate Resistance in Acute Leukemia: Normal and Neoplastic Hematopoiesis," Alan R. Liss, New York (1983).
20. H. Shinozuka, S. L. Katyal, and M. I. R. Perera, Choline deficiency and chemical carcinogenesis, this volume.

21. J. W. Gautsch and M. C. Wilson, Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression, Nature 301:32 (1983).
22. J. J. Harrison, A. Anisowicz, I. K. Gadi, et al., Azacytidine-induced tumorigenesis of CHEF/18 cells: Correlated DNA methylation and chromosome changes, Proc. Natl. Acad. Sci. U.S.A. 80:6606 (1983).
23. T. L. Ley, J. DeSimone, C. T. Noguchi, et al., 5-Azacytidine increases gamma-globulin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia, Blood 62:370 (1983).
24. J. J. Mermod, S. Bourgeois, N. Defer, et al., Demethylation and expression of murine mammary tumor proviruses in mouse thymoma cell lines, Proc. Natl. Acad. Sci. U.S.A. 80:110 (1983).
25. G. Kolata, Globin gene studies create a puzzle, Science 223:470 (1984).
26. J. K. Christman, N. Mendelsohn, D. Herzog, et al., Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60), Cancer Res. 43:763 (1983).
27. S. Charache, G. Dover, K. Smith, et al., Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the gamma-delta-beta-globin gene complex, Proc. Natl. Acad. Sci. U.S.A. 80:4842 (1983).
28. E. Borek, A new family of tumor markers, Cancer Detect. Prev. 4:185 (1981).
29. D. A. Heldman, M. R. Grever, and R. W. Trewyn, Differential excretion of modified nucleosides in adult acute leukemia, Blood 61:291 (1983).
30. E. Borek, O. K. Sharma, and T. P. Waalkes, "New Applications of Nucleoside Markers: Recent Results in Cancer Research," Springer-Verlag, Berlin (1983).
31. L. Gross, "Oncogenic Viruses, 2nd ed.," Pergamon Press, New York (1970).
32. L. Gross, "Oncogenic Viruses, 3rd ed., vol. 1.," Pergamon Press, New York (1983).
33. R. C. Gallo and F. Wong-Staal, Retroviruses as etiologic agents of some animal and human leukemias and lymphomas and as tools for elucidating the molecular mechanism of leukemogenesis, Blood 60:545 (1982).
34. L. Gross, An overview of the symposium [on HTLV virus at the National Cancer Institute, December 6 and 7, 1984] and reflections about the past, present, and future, Cancer Res. 45 (Suppl.):4706, 1985.
35. L. Gross and Y. Dreyfuss, Reduction in the incidence of radiation-induced tumors in rats after restriction of food intake, Proc. Natl. Acad. Sci. U.S.A. 81:7596 (1984).
36. G. H. Clamon, R. Feld, W. K. Evans, et al., Serum folate and vitamin B_{12} levels in patients with small cell lung cancer, Cancer 53:306 (1984).
37. J. F. Kolhouse, H. Kondo, N. C. Allen, et al., Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin, N. Engl. J. Med. 298:785 (1978).
38. S. Kanazawa and V. Herbert, Noncobalamin vitamin B_{12} analogues in human red cells, liver and brain, Am. J. Clin. Nutr. 37:774 (1983).
39. V. Herbert, G. Drivas, R. Foscaldi, et al., Multivitamin/mineral food supplements containing vitamin B_{12} may also contain analogues of vitamin B_{12}, N. Engl. J. Med. 307:255 (1982).
40. H. Kondo, M. J. Binder, J. R. Kolhouse, et al., Presence and formation of cobalamin analogues in multivitamin-mineral pills, J. Clin. Invest. 70:889 (1982).
41. V. Herbert and G. Drivas, Spirulina and vitamin B_{12}, J.A.M.A. 248: 3096 (1982).

42. V. Herbert, G. Drivas, M. Chu, et al., Differential radioassays better measure cobalamin content of vitamins and "health foods" than do microbiologic assays. Some products sold to vegetarians as rich vitamin B_{12} sources are not. The official United States Pharmacopeia (USP) method (L. leichmanii) and E. gracilis assay as "vitamin B_{12}" non-cobalamin corrinoids, Blood 62:37A (1983).
43. V. Herbert, G. Drivas, C. Manusselis, et al., Are colon bacteria a major source of cobalamin analogues in human tissues? 24-hour human stool contains only about 5 µg of cobalamin but about 100 µg of apparent analogue (and 200 µg of folate), Trans. Assoc. Am. Physicians 97:161 (1984).
44. N. V. Myasishcheva, E. V. Quadros, D. M. Matthews, et al., Interference by methylcobalamin analogues with synthesis of cobalamin coenzymes in human lymphocytes in vitro, Biochim. Biophys. Acta 588:81 (1979).
45. V. Herbert, Megaloblastic anemia with two nutrient deficiencies: Two cases, Med. Grand Rounds 1:320 (1982).
46. N. Colman and V. Herbert, Folate binding proteins, in: "Annual Review of Medicine, vol. 31," W. P. Creger, C. H. Coggins, and C. W. Hancock, eds., Annual Reviews, Palo Alto, Calif. (1980).
47. E. Jacob, S. J. Baker, and V. Herbert, Vitamin B_{12}-binding proteins, Physiol. Rev. 60:918 (1980).
48. B. Herzlich, G. Drivas, and V. Herbert, A new serum test which may reliably diagnose vitamin B_{12} deficiency: Total desaturation of serum transcobalamin II (TC II), Clin. Res. 33:605A, 1985.
49. G. Marcoullis and S. P. Rothenberg, Macromolecules in the assimilation and transport of cobalamin, Contemp. Issues Clin. Nutr. 5:59 (1983).
50. P. Heller, V. N. Yakulis, R. B. Epstein, et al., Variation in amount of hemoglobin S in a patient with sickle cell trait and megaloblastic anemia, Blood 21:479 (1963).
51. E. J. Benz, Jr., Clinical management of gene expression, N. Engl. J. Med. 307:1515 (1972).
52. P. A. Jones and S. M. Taylor, Cellular differentiation, cytidine analogues, and DNA methylation, Cell 20:85 (1980).
53. S. J. Compere and R. D. Palmiter, DNA methylation controls the inducibility of the mouse metallothioneine-I gene lymphoid cells, Cell 25:233 (1981).
54. P. Tolstoshev, R. A. Berg, S. I. Rennard, et al., Procollagen production and procollagen messenger RNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth, J. Biol. Chem. 156:3135 (1981).
55. V. Herbert, Drugs effective in megaloblastic anemias, in: "The Pharmacological Basis of Therapeutics," L. S. Goodman and A. Gilman, eds., Macmillan, New York (1975).
56. C. Leuchtenberger, R. Lewisohn, D. Laszlo, et al., "Folic acid," a tumor growth inhibitor, Proc. Soc. Exp. Biol. Med. 55:204 (1944).
57. C. Leuchtenberger and R. Leuchtenberger, Growth-regulating effects of naturally occurring metabolites of folate and ascorbate on malignant cells, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
58. C. Leuchtenberger, R. Leuchtenberger, D. Laszlo, et al., Influence of "folic acid" on spontaneous breast cancer in mice, Science 101:46 (1945).
59. M. A. Pollack, A. Taylor, D. Taylor, et al., B vitamins in cancerous tissues, Cancer Res. 2:739 (1942).
60. S. Farber, E. C. Cutler, J. W. Hawkins, et al., Action of pteroylglutamic conjugates on man, Science 106:619 (1947).

61. D. Laszlo and C. Leuchtenberger, Inositol, a tumor growth inhibitor, Science 97:515 (1943).
62. Food and Nutrition Board, "Recommended Dietary Allowances," National Academy of Science, Washington, D.C. (1980).
63. R. Lewisohn, C. Leuchtenberger, R. Leuchtenberger, et al., Influence of liver L. casei factor on spontaneous breast cancer in mice, Science 104:436 (1946).
64. Food and Nutrition Board, "Folic Acid: Biochemistry and Physiology in Relation to the Human Nutrition Requirement," National Academy of Science, Washington, D.C. (1977).
65. C. L. Krumdieck, Role of folate deficiency in carcinogenesis, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
66. N. Colman and V. Herbert, Cerebrospinal fluid levels (CSF) folate levels are much higher by radioassay than by microbiologic assay, Clin. Res. 28:491A (1980).
67. N. Colman, N. Hettiarachchy, and V. Herbert, Detection of a milk factor that facilitates folate uptake by intestinal cells, Science 211:1427 (1981).
68. R. L. Schilsky, B. D. Bailey, and B. A. Chabner, Methotrexate polyglutamate synthesis by cultured human breast cancer cells, Proc. Natl. Acad. Sci. U.S.A. 77:2919 (1980).
69. A. Welch, Folic acid: Discovery and the exciting first decade, Perspect. Biol. Med. 27:64 (1983).
70. V. Herbert, "The Megaloblastic Anemias," Grune & Stratton, New York, (1959).
71. I. Chanarin, "The Megaloblastic Anemias, 2nd ed.," Blackwell, Oxford (1979).
72. J. Corcino, R. Zalusky, M. Greenberg, et al., Coexistence of pernicious anemia and chronic myeloid leukemia: An experiment of nature involving vitamin B_{12} metabolism, Br. J. Haematol. 20:511 (1971).
73. V. Herbert, Development of a possible in vitro assay for intrinsic factor, Proc. Soc. Exper. Biol. Med. 97:668 (1958).
74. V. Herbert and T. Spaet, Distribution of "intrinsic factor" activity, Am. J. Physiol. 195:194 (1958).
75. R. H. Allen, Human vitamin B_{12} transport proteins, Prog. Hematol. 9:57 (1975).
76. W. S. Beck, Biological and medical aspects of vitamin B_{12}, in: "B_{12}, vol. 2," D. Dolphin, ed., Wiley, New York (1982).
77. V. Herbert and N. Colman, Release of vitamin binding proteins from granulocytes by lithium: Vitamin B_{12} and folate binding proteins, in: "Lithium Effects on Granulopoiesis and Immune Function," A. H. Rossof and W. A. Robsin, eds., Plenum Press, New York (1980).
78. V. Herbert and R. Zalusky, Interrelations of vitamin B_{12} and folic acid metabolism: Folic acid clearance studies, J. Clin. Invest. 41:1263 (1962).
79. L. T. Ch'ien, C. L. Krumdieck, C. W. Scott, Jr., et al., Harmful effect of megadoses of vitamins: Electroencephalogram abnormalities and seizures induced by intravenous folate in drug-treated epileptics, Am. J. Clin. Nutr. 28:21 (1975).
80. C. D. Gerson, G. W. Hepner, N. Brown, et al., Inhibition by diphenylhydantoin of folic acid absorption in man, Gastroenterology 63:353 (1972).
81. G. W. Hepner, L. M. Aledort, C. D. Gerson, et al., Inhibition of intestinal ATPase by diphenylhydantoin and acetazolamide, Clin. Res. 18:382 (1970).
82. N. Colman and V. Herbert, Folate metabolism in brain, in: "Biochemistry of Brain," S. Kumar, ed., Pergamon Press, New York (1980).

83. M. Poncz, N. Colman, V. Herbert, et al., Therapy of congenital folate malabsorption, J. Pediatr. 98:76 (1981).
84. M. Poncz, N. Colman, V. Herbert, et al., Congenital folate malabsorption, J. Pediatr. 99:828 (1981).
85. S. Kanazawa, V. Herbert, B. Herzlich, et al., Removal of cobalamin analogue in bile by enterohepatic circulation of vitamin B_{12}, Lancet 1:707 (1983).
86. G. R. Sutherland, Fragile chromosomes, in: "Nutritional Factors in the Induction and Maintenance of Malignancy," C. E. Butterworth and M. L. Hutchinson, eds., Academic Press, New York (1983).
87. N. Colman and V. Herbert, Vitamin uptake by resting and transformed lymphocytes, Blood 52:132 (1978).
88. J. J. Yunis and A. L. Soreng, Constitutive fragile sites and cancer, Science 226:1199 (1984).
89. H. F. Willard and L. E. Rosenberg, Interactions of methylmalonyl CoA mutase from normal human fibroblasts with adenosylcobalamin and methylmalonyl CoA: Evidence for non-equivalent active sites, Arch. Biochem. Biophys. 200:130 (1980).
90. V. Herbert, "Nutrition Cultism: Facts and Fictions," George F. Stickley, Philadelphia (1981).
91. J. E. Cottrell, P. Casthely, J. D. Brodie, et al., Prevention of nitroprusside-induced cyanide toxicity with hydroxocobalamin, N. Engl. J. Med. 298:809 (1978).
92. C. E. Butterworth, Jr., Tropical sprue, a consideration of some possible etiologic mechanisms, in: "Malabsorption," R. H. Girdwood and N. A. Smith, eds., Williams and Wilkins, Baltimore (1969).
93. D. G. Johns and I. H. Plenderleith, Folic acid displacement in man, Biochem. Pharmacol. 12:1071 (1963).
94. G. Tisman, V. Flener, M. E. Jones, et al., Possible potentiation of fluoropyrimidine anti-tumor activity by pteroylglutamic acid (folic acid) and cyanocobalamin (B_{12}), Clin. Res. 33:495A, 1985.
95. G. Tisman, V. Flener, M. E. Jones, et al., Overcoming colon cancer resistance to hepatic artery infusional 5FUdR chemotherapy with folinic acid, Clin. Res. 33:495A, 1985).
96. G. Tisman and V. Herbert, Evidence for a serum factor enhancing incorporation of ^{3}HTdR (^{3}H thymidine) into DNA-thymine (DNA-T) of human bone marrow cells, Clin. Res. 19:433 (1971).
97. S. C. Gailani, R. W. Carey, J. R. Holland, et al., Studies of folate deficiency in patients with neoplastic diseases, Cancer Res. 30:327 (1970).
98. N. Whitehead, F. Reyner, and J. Lindenbaum, Megaloblastic changes in the cervical epithelium, association with oral contraceptive therapy and reversal with folic acid, J.A.M.A. 226:1421 (1973).
99. C. E. Butterworth, Jr., K. D. Hatch, H. Gore, et al., Improvement in cervical dysplasia associated with folic acid therapy in users of oral contraceptives, Am. J. Clin. Nutr. 35:73 (1982).
100. D. L. Longo, N. Colman, and V. Herbert, Studies on the interrelations of iron (Fe) and folate metabolism and on selective nutrient deficiency involving some but not other cell lines: Megaloblastosis in lymphocytes as proof of tissue folate deficiency in four Fe-deficient adults, and in adults taking oral contraceptives or anticonvulsant therapy, Clin. Res. 23:403A (1975).
101. V. Herbert, Experimental nutritional folate deficiency in man, Trans. Assoc. Am. Physicians 75:307 (1962).
102. B. Herzlich and V. Herbert, Role of the pancreas in cobalamin (vitamin B_{12}) absorption, Am. J. Gastroenterol. 79:489 (1984).
103. V. Herbert, Studies on the role of intrinsic factor in vitamin B_{12} absorption, transport, and storage, Am. J. Clin. Nutr. 7:433 (1959).

104. M. Bodian, Neuroblastoma, Pediatr. Clin. North Am. 6:449 (1959).
105. A. Sawitsky and F. Desposito, A survey of American experience with vitamin B_{12} therapy of neuroblastoma, J. Pediatr. 67:99 (1964).
106. P. L. Day, L. D. Payne, and J. S. Dinning, Procarcinogenic effect of vitamin B_{12} on p-dimethylaminoazobenzene-fed rats, Proc. Soc. Exp. Biol. Med. 74:854 (1950).
107. A. D. Ostryanina, Effects of some analogues of vitamin B_{12}, Vopr. Pitan. 3:25 (1974) (in Russian).
108. A. E. Rogers and P. M. Newberne, Dietary effects on chemical carcinogenesis in animal models for colon and liver tumors, Cancer Res. 35:3427 (1975).
109. S. Kanazawa, V. Herbert, B. Herzlich, et al., Removal of cobalamin analogue in bile by enterohepatic circulation of vitamin B_{12}, Lancet 1:707 (1983).
110. D. Dolphin, "B_{12}, vols.I and II," Wiley, New York (1982).
111. J. A. Amess, J. F. Burman, D. G. Nancekievill, et al., Megaloblastic haemopoiesis in patients receiving nitrous oxide, Lancet 2:339 (1978).
112. J. van der Westhuyzen, F. Fernandes-Costa, J. Metz, et al., Cobalamin (vitamin B_{12}) analogues are absent in plasma of fruit bats exposed to nitrous oxide, Proc. Soc. Exp. Biol. Med. 171:88 (1982).
113. J. M. Scott and D. G. Weir, The methyl folate trap, Lancet 2:337 (1981).
114. J. van der Westhuyzen, F. Fernandes-Costa, and J. Metz, "Abstracts, 19th Congress, International Society of Haematology," Budapest, August 1-7, 1982.
115. J. Perry, I. Chanarin, R. Deacon, et al., Chronic cobalamin inactivation impairs folate polyglutamates synthesis in the rat, J. Clin. Invest. 71:1183 (1983).
116. A. C. Kroes, J. Lindemans, A. Hagenbeek, et al., Nitrous oxide reduces growth of experimental rat leukemia, Leuk. Res. 8:441 (1984).
117. A. C. Kroes, J. Abels, and J. Lindemans, Cytostatic synergism of nitrous oxide and cycloleucine, Blood 64:115a (1984).
118. K. C. Das, C. Manusselis, and V. Herbert, In vitro DNA synthesis by bone marrow cells and PHA-stimulated lymphocytes. Suppression by nonradioactive thymidine of the incorporation of ^{3}H-deoxyuridine into DNA: Enhancement of incorporation when inadequate vitamin B_{12} or folate is corrected, Br. J. Haematol. 319:61 (1980).
119. R. Carmel and D. Hollander, Extreme elevation of transcobalamin II levels in multiple myeloma and other disorders, Blood 51:1057 (1978).
120. M. Altz-Smith, R. K. Miller, P. E. Cornwell, et al., Low serum vitamin B_{12} levels in rheumatoid arthritis, Arthritis Rheum. 25:S116 (1982).
121. B. Rachmilewitz and R. Rachmilewitz, Serum transcobalamin II concentration, N. Engl. J. Med. 296:1174 (1977).
122. R. L. Burkes, H. Cohen, R. Sinow, et al., Low serum B_{12} levels in homosexual males with AIDS or its prosyndrome, Blood 64:93a (1984).
123. M. Frater-Schroder, W. H. Hitzig, P. J. Grob, et al., Increased unsaturated transcobalamin II in active autoimmune disease, Lancet 2:238 (1978).
124. S. A. Killman, Effect of deoxyuridine on incorporation of tritiated thymidine: Difference between normoblasts and megaloblasts, Acta Med. Scand. 175:483 (1964).
125. J. Metz, A. Kelly, V. C. Swett, et al., Deranged DNA synthesis by bone marrow from vitamin B_{12}-deficient humans, Br. J. Haematol. 14:575 (1968).

126. J. Metz, The deoxyuridine suppression test, CRC Crit. Rev. Clin. Lab. Sci. 20:205 (1984).
127. M. Friedkin, Enzymatic aspects of folic acid, Annu. Rev. Biochem. 32:185 (1963).
128. L. E. Feinendegen, V. P. Bond, and R. B. Painter, Studies on the interrelationships of RNA synthesis, DNA synthesis, and precursor pool in human tissue culture cells studied with tritiated pyrimidine nucleosides, Exp. Cell Res. 22:381 (1961).
129. V. Herbert and R. Zalusky, Interrelations of vitamin B_{12} and folic acid metabolism: Folic acid clearance studies, J. Clin. Invest. 41:1134 (1962).
130. K. C. Das and A. V. Hoffbrand, Lymphocyte transformation in megaloblastic anemia: Morphology and DNA synthesis, Br. J. Haematol. 19:459 (1970).
131. K. C. Das and V. Herbert, Vitamin B_{12}-folate interrelations, Clin. Haematol. 5:697 (1976).
132. W. S. Beck, Metabolic features of cobalamin deficiency in man, in: "Cobalamin: Biochemistry and Pathophysiology," B. M. Babior, ed., Wiley, New York (1975).
133. T. T. Pelliniemi and W. S. Beck, Biochemical mechanisms in the Killman experiment: Critique of the deoxyuridine suppression test, J. Clin. Invest. 165:449 (1980).
134. K. C. Das and V. Herbert, The "dU suppression test" and "thymidine suppression test": Evidence for reciprocal relationship between the "de novo" and "salvage" pathways of DNA synthesis, in "Vitamin B_{12}," B. Zagalak and W. Freidrich, eds., Proceedings of the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Walter de Gruyter, New York (1980).
135. V. Herbert, The role of vitamin B_{12} and folate deficiencies in causing overt and covert megaloblastic anemia, in: "Proceedings of the 17th Congress, International Society of Haematology," Paris, July 23-29 (1978).
136. D. S. Zaharko, B. J. Bolton, D. Chiuten, et al., Pharmacokinetic studies during phase I trials of high-dose thymidine infusions, Cancer Res. 39:4777 (1979).
137. T. Woodcock, D. G. Martin, L. A. Damin, et al., Combination clinical trials with thymidine and fluorouracil: A phase I and clinical pharmacologic evaluation, Cancer 45:1135 (1980).
138. M. B. van der Weyden, M. Cooper, and B. G. Firkin, Defective DNA synthesis in human megaloblastic bone marrow: Effects of hydroxy-B_{12}, 5'-deoxyadenosyl-B_{12} and methyl B_{12}, Blood 41:299 (1973).
139. J. Zittoun, J. Marquet, and R. Zittoun, Effect of folate and cobalamin compounds on the deoxyuridine suppression test in vitamin B_{12} and folate deficiency, Blood 51:119 (1978).
140. J. Zittoun, J. Marquet, and R. Zittoun, in: Some biochemical aspects of the dU suppressive effect in vitamin B_{12} and folate deficiency, "Abstracts, 17th Congress, International Society Haematology," Paris, July 23-29, (1978).
141. V. Herbert and L. Sullivan, Activity of coenzyme B_{12} in man, Ann. N.Y. Acad. Sci. 112:855 (1964).
142. K. C. Das, V. Herbert, N. Colman, et al., Unmasking covert folate deficiency in iron-deficient subjects with neutrophil hypersegmentation: dU suppression tests on lymphocytes and bone marrow, Br. J. Haematol. 38:357 (1978).
143. L. M. Pfeffer and I. Tamm, Interferon inhibition of thymidine incorporation into DNA through effects of thymidine transport and uptake, J. Cell. Physiol. 121:431 (1984).

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Effects of 5-Fluorouracil Treatment of Rat Leukemia with Concomitant Inactivation of Cobalamin

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Abstract. *The cytostatic activity of 5-fluorouracil (5-FU) can be modified by the addition of reduced folates, as well as antifolates. This is indicative of the complex involvement of folate metabolism in the effects of 5-FU. In the BN rat leukemia model, 5-FU treatment was combined with the inactivation of cobalamin (vitamin B12) by nitrous oxide (N_2O). Exposure to nitrous oxide causes severe disturbance of folate metabolism through the inhibition of the cobalamin-dependent enzyme methionine synthetase, and leads to loss of folates from the cell. With regard to the effects on growth of leukemia, the addition of nitrous oxide did not antagonize 5-FU. On the contrary, therapeutic effects were enhanced by combined treatment, as was evident from a further reduction of leukemic infiltration in spleen and liver, from a decrease or even disappearance of leukemic cells in the peripheral blood, and from extended survival of rats. These findings were in accordance with metabolic studies in isolated leukemic cells of treated rats, in which combined treatment caused further impairment of thymidylate and DNA synthesis. Pretreatment with nitrous oxide, for a period of 3 days, was more effective than treatment after the administration of 5-FU. Folate levels, in plasma and intracellular, were reduced after combined treatment. It is concluded that in this leukemia, unlike observations in some models of solid tumors, the activity of 5-FU is enhanced with a depletion of folates. This effect is probably comparable to the combination of methotrexate pretreatment with 5-FU, and might be important to applications of 5-FU in combination chemotherapy of hematological neoplasms.*

The chemotherapeutic activity of 5-fluorouracil (5-FU) is closely related to folic acid metabolism. Thymidylate synthetase, the target enzyme of 5-FU with regard to inhibition of DNA synthesis, is dependent on folate coenzyme function. Recently, in several ways it has been tried to combine the inhibition of thymidylate synthetase by 5-FU wih treatment interfering with folate metabolism. 5-FU has been combined with antifolates, like methotrexate, which, through a depletion of folate coenzymes, also cause impairment of thymidylate synthesis (1). On the other hand, it has also been tried to increase the effects of 5-FU by the concomitant administration of reduced folates. This approach is based on biochemical evidence that the inhibitory complex of thymidylate synthetase and 5-FdUMP, an anabolite of 5-FU, is more stable in the presence of increased levels of folate coenzyme (2,3). Both methods are still under investigation in clinical trials, but so far varying resuls have been obtained (4-7). Clearly, the relationship between 5-FU and folate metabolism is rather complex, and more information will be useful for the optimal design of these chemotherapeutic combinations.

In this study, we present results of experiments in which 5-FU therapy is combined with inactivation of cobalamin (vitamin B12), in a rat model for acute myeloid leukemia (BNML, Brown Norway Myeloid Leukemia). Inactivation of the cobalamin coenzyme function in the enzyme methyltetrahydrofolate homocysteine methyltransferase, or methionine synthetase, is possible by exposure to nitrous oxide, or N_2O, a specific side effect of this anesthetic gas which was recognized only recently (8-10). Methionine synthetase is essential in folate metabolism, and therefore exposure to nitrous oxide represents yet another method to interfere with folate coenzyme functions. The interaction of nitrous oxide with 5-FU could, theoretically, lead to increased therapeutic effects, as well as to antagonism. We have previously described the reduction of leukemic growth by nitrous oxide in

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Abbreviations: 5-FU: 5-Fluorouracil; 5-FdUMP: 5-Fluorodeoxyuridinemonophosphate; BNML: Brown Norway Myeloid Leukemia.

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the BNML model (11), and have also reported on the therapeutic effects of 5-FU in this rat leukemia (12). The combination of both agents in the present study is directed at effects on leukemic growth and survival time of rats, and at some metabolic changes caused by this treatment, including the plasma and intracellular levels of folates, and tests of thymidylate and DNA synthesis in leukemic cells.

Materials and Methods

Animals. Male rats of the Brown Norway inbred strain were used, at the age of 12-16 weeks (body weight 200-275 g). Food and water were supplied *ad libitum* during the experiments.

Brown Norway Myeloid Leukemia (BNML). Properties and use of this transplantable rat leukemia have been described before (11,13). It is considered to be a suitable model for experimental chemotherapy (14). In this study, rats were injected with 10^7 leukemic cells i.v., which after progressive leukemic infiltration of bone marrow, liver and spleen leads to death after 20 - 24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of leukemic growth and, along with hematological determinations, can be used effectively to assess effects of chemotherapy (13).

Treatment with nitrous oxide and 5-FU. Exposure of leukemic rats to nitrous oxide was carried out in a 40 l flow chamber in which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water and contaminating volatile compounds were eliminated in a cleaning circuit (15). Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. 5-FU (Fluoro-uracil Roche, from Hoffmann-La Roche) was administered i.p., at a dose of 15 or 25 mg/kg, according to different treatment schedules. Rats not receiving 5-FU were injected wth 0.15 M NaCl i.p.

Evaluation of therapeutic effects. In most experiments, rats from all treatment groups were compared after a fixed period of leukemia of 19 days, which is just before untreated rats would die spontaneously. Rats were then killed by exsanguination, after recording their body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically and differential blood cell counts were done. Normal values for organ weights and leukocyte counts were derived from 16 comparable non-leukemic Brown Norway rats. As an alternative method, in one experiment the extension of survival time in the treatment groups was compared.

Plasma vitamin B12 and folic acid. Plasma vitamin B12 and folic acid were determined simultaneously using a radioisotope dilution assay, essentially as described by Gutcho and Mansbach (16).

Studies using leukemic spleen cells. In separate experiments, leukemic rats were examined shortly after treatment, and leukemic spleen cells were obtained to perform additional metabolic studies. After washing and resuspension in Hanks balanced salt solution, these cell suspensions were counted and used in an assay of intracellular folate levels and in deoxyuridine suppression tests.

Intracellular folate. A pellet of ca. 10^8 cells was resuspended in a total volume of 1 ml 10% (=1.3 M) mercaptoethanol, heated for 5 min at 100°C, and cooled. After incubation with hog kidney polyglutamate hydrolase (17) at room temperature for 2 hours, the samples were frozen at -20°C until assayed. After thawing and centrifugation (1500 g for 10 min at 4°C) aliquots of the supernatant were used in a folate radioisotope dilution assay (18) with ^{125}I-folic acid (Becton Dickinson, Orangeburg, NY, USA) as a tracer, and beta-lactoglobulin (Sigma, St., Louis, USA) as a folate binder. 5-Methyltetrahydrofolate was used as a standard and results are expressed as pmol of folate per 10^6 cells.

Deoxyuridine suppression test. This test is used to demonstrate impaired *de novo* synthesis of thymidylate. It measures the incorporation of ^{3}H-thymidine into DNA without and with the addition of deoxyuridine. Deoxyuridine will suppress ^{3}H-thymidine incorporation if it can be converted to thymidylate by the enzyme thymidylate synthetase. This suppression will be reduced under all circumstances with impaired activity of this enzyme (19) and the test therefore can be used to evaluate effects of chemotherapeutic agents on this metabolic pathway (20). The test was

Table I. *Effects of treatment with 5-FU and nitrous oxide (N_2O) on growth of leukemia.*

Treatment	No. of rats	Spleen weight (g ± s.e.m.)	Liver weight (g ± s.e.m.)	Peripheral leukocytes (10^9/l ± s.e.m.)	% Leukemic cells (± s.e.m.)
None (untreated controls)	9	4.10 ± 0.10	19.14 ± 0.67	23.6 ± 2.3	29 ± 4
N_2O only (12 days)	6	2.87 ± 0.14	13.57 ± 0.83	13.5 ± 1.4	23 ± 4
5-FU 3 × 15 mg/kg	10	2.91 ± 0.20	12.52 ± 0.69	15.6 ± 1.6	16 ± 3
5-FU 3 × 15 mg/kg + N_2O (12 days)	9	1.87 ± 0.74**	11.15 ± 0.81	7.0 ± 1.6*	8 ± 4*
5-FU 1 × 25 mg/kg	3	0.85 ± 0.03	8.19 ± 0.67	5.3 ± 1.0	0
5-FU 1 × 25 mg/kg + N_2O (4 d.after 5-FU)	3	0.83 ± 0.06	8.20 ± 0.25	7.2 ± 0.9	0
5-FU 1 × 25 mg/kg + N_2O (4 d.before 5-FU)	4	0.46 ± 0.04	8.49 ± 0.26	3.3 ± 0.6	0
5-FU 1 × 25 mg/kg + N_2O (8 days)	5	0.43 ± 0.01	7.28 ± 0.18	2.7 ± 0.3	0
5-FU 3 × 25 mg/kg	9	0.90 ± 0.19	9.02 ± 0.92	5.7 ± 0.8	1 ± 1
5-FU 3 × 25 mg/kg + N_2O (12 days)	9	0.49 ± 0.07**	7.48 ± 0.16*	2.7 ± 0.6**	0
Normal BN-rats	16	0.45 ± 0.02	8.25 ± 0.24	3.9 ± 0.4	0

* Significantly different from results without N_2O with $p < 0.025$
** Significantly different from results without N_2O with $p < 0.01$

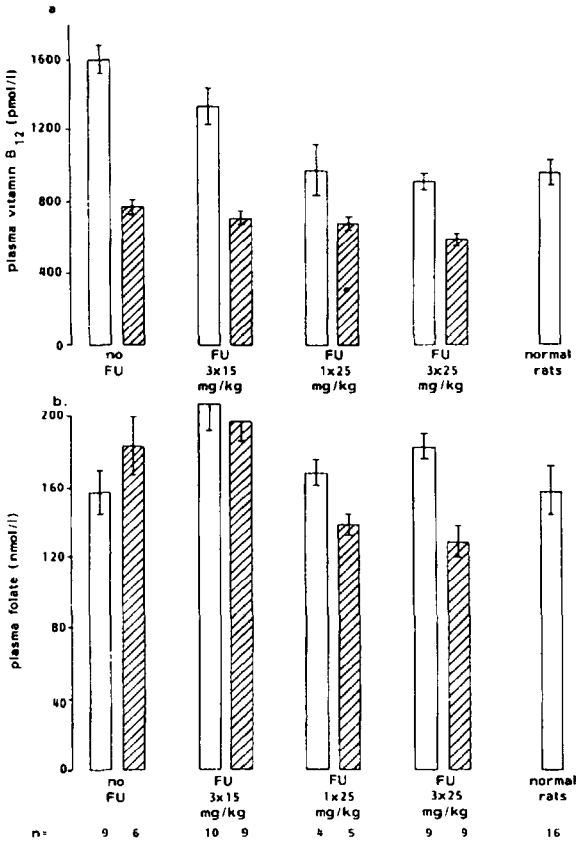

Figure 1. *Plasma levels of (a) vitamin B12 and (b) folic acid, in leukemic rats after various treatment schedules. Shaded columns indicate results with exposure to nitrous oxide during the treatment period. Values in normal (non-leukemic) BN rats are shown in the last columns. S.e.m. is indicated in each column.*

carried out essentially as described by Metz (21), with some modification as described (11). All incubations were carried out in triplicate, in each 5 × 10^6 cells were used with and without the addition of 0.1 mM deoxyuridine (Sigma, St Louis Mo, USA). Incorporation of ^{3}H-thymidine (0.3 μCi, specific activity 25 μCi/mmol, from Amersham, UK) in incubations with deoxyuridine is expressed as a percentage of the incorporation in the incubations without deoxyuridine. In one experiment, however, incorporation after treatment was too low to detect significant differences with and without deoxyuridine, and in this case only ^{3}H-thymidine incorporation in dpm/10^6 cells is given, as determined in tests without deoxyuridine.

Results

Inhibition of leukemic growth. Leukemic rats were treated with 5-FU, and the effect of concomitant exposure to nitrous oxide was investigated. After a fixed period of leukemia (19 days) several groups were compared with regard to leukemic infiltration in spleen and liver, and leukemic cells in the peripheral blood. Results of these experiments are presented together in Table I. A a dose of 3×15 mg/kg 5-FU (administered at days 7, 12, and 17 of leukemia) the addition of nitrous oxide causes a further reduction of leukemic growth,

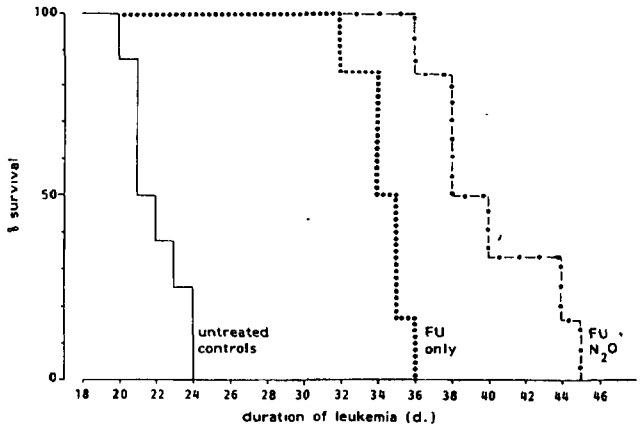

Figure 2. *Survival of leukemic rats without treatment (controls), with 5-fluorouracil (FU) only, and with the combination of 5-fluorouracil and nitrous oxide (FU + N_2O). 5-FU (25 mg/kg i.p.) was administered every 5 days. With combined therapy, rats were exposed no nitrous oxide 3 days before each administration of 5-FU.*

which is evident from all parameters that were studied. In addition, as indicated in Table I, most of these differences are statistically significant if Wilcoxon's non-parametric rank sum test is applied to values of individual rats. The same result is obtained with a combination of 3×25 mg/kg 5-FU and nitrous oxide. In this experiment the values of rats after combined treatment are not significantly increased compared to normal (non-leukemic) rats, whereas results after treatment with 5-FU alone are still significantly different from normal values ($p < 0.05$).

In one experiment with 1×25 mg/kg 5-FU, administered at day 14 of leukemia, the effects were studied of timing of exposure to nitrous oxide relative to the administration of 5-FU. It appears that results obtained with 4 days of nitrous oxide before 5-FU are better than with exposure only after 5-FU. In fact, this period of exposure does not add much to the effects of 5-FU alone, as is evident from the rats treated with nitrous oxide for both periods. In Figure 1 results are presented of determinations of plasma levels of vitamin B12 and folic acid in these experiments. It is evident that with treatment there is a dose-dependent normalization of the increased levels of vitamin B12 that are found in untreated controls. In all groups, however, the addition of nitrous oxide to 5-FU causes a further fall of vitamin B12 to subnormal levels. The determination of folic acid levels in plasma leads to more varying results, but it can be derived from Figure 1b that with the more effective doses of 5-FU the addition of nitrous oxide causes a decrease of folic acid levels to subnormal values.

Survival of leukemic rats. Leukemic rats were treated with 25 mg/kg 5-FU every 5 days, either or not preceded by a period of exposure to nitrous oxide of 3 days. This schedule was chosen as a consequence of the results in the studies presented in Table I, in which pretreatment with nitrous oxide

Table II. *Deoxyuridine suppression tests in leukemic cells after treatment of rats with 5-FU and nitrous oxide (N_2O).*

5-FU: 1 × 25 mg/kg i.p. at 3 days before examination.

Treatment	No. of rats	% Suppression*
None	4	12.6 (± 1.7)
5-FU only	4	12.8 (± 0.6)
N_2O only (3 days)	4	15.5 (± 2.1)
5-FU + N_2O (3 d., after 5-FU)	2	14.5 (13,16)
5-FU + N_2O (3 d., before 5-FU)	2	24.0 (22,26)
5-FU + N_2O (6 days)	2	35.5 (33,38)

* Deoxyuridine suppression values are expressed as percentages of the maximal incorporation of ^{3}H-thymidine, in incubations without deoxyuridine.
Mean values of each group are given, with indication of s.e.m. in groups of 4 rats, and of individual results in groups of 2 rats.
The result of each rat is the mean of triple incubations.

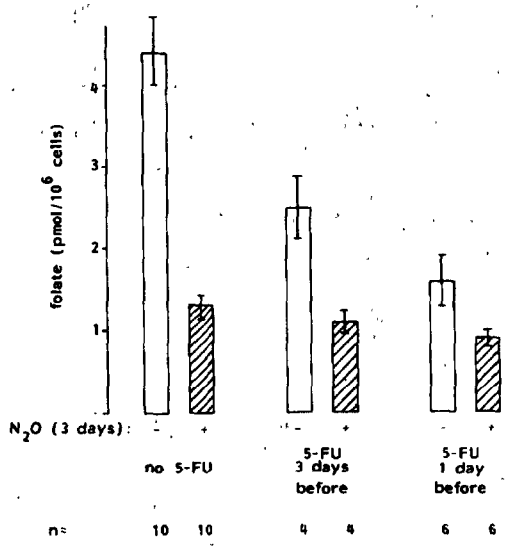

Figure 3. *Intracellular folate levels in isolated leukemic cells after* in vivo *treatment of rats. 5-FU (25 mg/kg) was administered at 1 day and 3 days before examination, respectively, either or not preceded by 3 days of exposure to nitrous oxide (shaded columns). S.e.m. is indicated in each column.*

before 5-FU was found to be most effective. Treatment was continued until death of the rats. Results of this experiment are presented in Figure 2. It appears that with addition of nitrous oxide survival of leukemic rats is extended to 40.2 ± 1.5 days (mean ± s.e.m.) compared to 22.0 ± 0.5 days for untreated rats, and 34.3 ± 0.5 days for rats treated wth 5-FU alone. The results of combined treatment are significantly different from treatment with 5-FU only, with $p < 0.005$ (Wilcoxon's rank sum test). Survival after combined treatment is increased by 18.2 days compared to untreated rats (183 %). In this leukemia model, 3 days of increased survival are eqivalent to a 10-fold reduction in tumor load (13), which in this case would mean a reduction by a factor 10^6. All rats were examined after death and without exception, leukemia was considered to be the cause of death, in view of increased spleen and liver weights. No evidence for toxicity was found.

Deoxyuridine suppression tests. Treatment of leukemic rats with a single dose of 25 mg/kg 5-FU, was conbined wth 3 days of exposure to nitrous oxide, either before or after 5-FU, or both. Rats treated with 5-FU or nitrous oxide only, and untreated rats were also included. Leukemic spleen cells of all rats were used in deoxyuridine suppression tests, 3 days after administration of 5-FU, at day 16 of leukemia. Results of these tests are presented in Table II. It appears that there is an enhanced effect of combined treatment, indicated by a higher percentage of suppression, with nitrous oxide pretreatment and with continuous treatment for 6 days, but not with nitrous oxide exposure after 5-FU. 5-FU alone, after 3 days, does not cause a disturbance of this test.

DNA synthesis in leukemic cells. In a similar experiment, rats were examined 1 day after administration of 25 mg/kg 5-FU. In this case, only pretreatment with nitrous oxide for 3 days is possible. In Table III, results are included of tumor growth in various groups, examined at day 16 of leukemia. As in other experiments, combined treatment is most effective in reducing leukemic growth. In addition, in this table results are given of ^{3}H-thymidine incorporation in isolated leukemic cells of these rats. DNA synthesis in both groups treated with 5-FU was impaired to such an extent that it was not possible to perform a deoxyuridine suppression test. A small additional effect of nitrous oxide exposure is still detectable, however.

Intracellular folate. In leukemic cell suspensions of rats treated *in vivo* with 25 mg/kg 5-FU, 1 or 3 days before examination and with or without 3 days of exposure to nitrous oxide prior to the administration of 5-FU, the intracellular levels of folate were determined. Results can be found in Figure 3. For comparison, values in untreated rats and rats treated with 3 days of nitrous oxide only, are also given. With 5-FU treatment there is some decrease of folate content, more apparent at the first day after administration, but with the addition of nitrous oxide folate loss becomes much more severe.

Discussion

Nitrous oxide distrurbs folate metabolism because of its specific interference with the coenzyme function of cobalamin, which causes inactivation of the enzyme methionine synthetase (22). This prvents the conversion of 5-methyltetrahydrofolate into tetrahydrofolate, which is essential for folate coenzyme functions. The effects of nitrous oxide develop rapidly, in a few hours after exposure is started and remain remarkably constant even with prolonged exposure of one week or more (23).

Table III. *Effects of treatment with 5-FU and nitrous oxide (N_2O) on growth of leukemia and on DNA synthesis in leukemic cells. 5-FU: 1 × 25 mg/kg i.p. at 1 day before examination. Examination at day 16 of leukemia.*

Treatment	No. of rats	Spleen weight (g ± s.e.m.)	Peripheral leukocytes (10^9/l ± s.e.m.)	^{3}H-thymidine incorporation (dpm/10^6 cells ± s.e.m.)
None	3	2.65 ± 0.18	10.8 ± 0.7	3522 ± 942
N_2O only (3 days)	3	1.83 ± 0.06	8.8 ± 1.6	2135 ± 536
5-FU only	3	1.01 ± 0.06	5.1 ± 0.1	243 ± 71
5-FU + N_2O (3 d., before 5-FU)	3	0.68 ± 0.04	4.2 ± 0.2	173 ± 17

Value of ^{3}H-thymidine incorporation in leukemic cells of each rat is the mean of triple incubations.

For normal values of spleen weight and leukocyte count in BN rats, see Table I.

In this report exposure to nitrous oxide has been used to study the effects of impaired availability of folate coenzyme forms on the activity of 5-FU towards the BN rat leukemia. It appeared that the therapeutic effects of 5-FU were increased with the addition of nitrous oxide, with regard to the reduction of leukemic infiltration in spleen and liver, as well as with regard to leukocyte counts. In addition, compared to treatment with 5-FU alone, there was a significantly increased survival of leukemic rats after combined treatment. In none of the treatment schedules there was any indication of antagonism, and no evidence of increased toxicity was found.

Plasma levels of vitamin B12 in this study are interesting for two reasons. First, in this leukemia vitamin B12 steadily increases with progression of disease (11), an effect which is also often observed in human promyelocytic leukemia (24). Treatment of leukemia will reduce vitamin B12 to about normal levels. Separately, however, the specific effect of nitrous oxide on vitamin B12 will cause an additional fall of plasma levels (11, 25). These effects explain both the dose-dependent reduction of plasma vitamin B12 with treatment, and the occurence of the lowest, subnormal, levels in rats with combined therapy.

The disturbance of folic acid metabolism by nitrous oxide is evident from the striking decrease of intracellular folates in leukemic cells after 3 days of exposure. Folate depletion with nitrous oxide exposure has often been observed (26), and is explained by a decreased synthesis of folate polyglutamates (27), as these forms are essential for cellular retention. Interestingly, 5-FU by itself also caused a less pronounced fall in intracellular folates, compared to untreated rats. This is evidence of a blockade in folate metabolism, as will indeed occur with inhibition of thymidylate synthetase, but the precise mechanism of this effect remains unclear. As would be expected, intracellular folate is lowest with combined treatment, one day after the administration of 5-FU.

The intracellular depletion of folates will ultimately also lead to reduced plasma levels of folate, as is demonstrated by the subnormal levels found after combined treatment with nitrous oxide and higher doses of 5-FU. Temporarily, however, loss of folates from the tissues can give rise to increased concentrations of folate in plasma, as has been observed before with nitrous oxide exposure (11, 28). This can explain the observation in this study of increased folate levels in rats after nitrous oxide alone and after low doses of 5-FU.

With regard to the mechanism of the interaction described in this study, an important observation is the significance of pretreatment with nitrous oxide. With nitrous oxide exposure after 5-FU, there was no enhancement of the effects of 5-FU on leukemic growth, and even some indication of a reduced effect in the deoxyuridine suppression test. A similar schedule-dependency of combination therapy is found with methotrexate and 5-FU (29, 30). In that combination, methotrexate pretreatment is essential to achieve synergistic effects. If 5-FU precedes methotrexate, antagonism will result (1). Several mechanisms have been proposed to explain these effects (31). Probably, the inhibition of purine synthesis by methotrexate is important, because this can lead to increased formation of 5-FU-nucleotides, interfering with both thymidylate (DNA) and RNA synthesis (32). This effect could also occur with nitrous oxide treatment, which can impair purine synthesis as well (33, 34). Other mechanisms may be involved however. Unlike methotrexate, nitrous oxide treatment for up to 3 days increases the activity of thymidylate synthetase (35), probably as an adaptive mechanism. Subsequent inhibition of this enzyme by 5-FU could render cells more vulnerable to the effects of nitrous oxide. This would also explain the necessity of pretreatment to achieve increased effects. Finally, it has been reported (2) that the addition of vitamin B12 and 5-methyltetrahydrofolate decreased the stability of the binding of 5-FdUMP to thymidylate synthetase. Prolonged exposure to nitrous oxide will decrease both these factors, and could therefore stabilize the inhibitory complex.

An important result of this study is the observation that some degree of folate depletion does not necessarily antago-

nize the effects of 5-FU. This conclusion obviously only applies to the leukemia model used in this study, but, interestingly, in another experimental leukemia it has been found that the addition of reduced folates to 5-FU did not increase therapeutic effects (36). Furthermore, no increased cytoxicity results from folate addition to cultured leukemic cells treated with methotrexate and 5-FU (37). In that study, it was also concluded that the interactions of 5-FU and folates were more complex and variable than was previously understood. The synergistic effect of folates and 5-FU as observed in xenografts of colon carcinomas might well be tissue specific to some degree. As discussed in another study (12), 5-FU is not used clinically in the treatment of leukemia. Recently however, sequential methotrexate / 5-FU therapy has been applied to human leukemia with favourable results (38). This report further adds to the potential importance of 5-FU, alone or in combination with other agents interfering with folate-dependent nucleotide synthesis, in the treatment of leukemia.

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References

1 Tattersall MHN: Current studies of methotrexate and 5-fluorouracil and their interaction in human tumor cells. Semin Oncol *10:* (Suppl 2) 6-14, 1983.

2 Houghton JA, Schmidt C, and Houghton PJ: The effect of derivatives of folic acid on the fluorodeoxyuridylate-thymidylate synthetase covalent complex in human colon xenografts. Eur J Cancer Clin Oncol *18:* 347-354, 1982.

3 Yin MB, Zakrzewski SF, and Hakala MT: Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol *23:* 190-197, 1983.

4 Browman GP: Clinical application of the concept of methotrexate plus 5-FU sequence dependent "synergy": How good is the evidence? Cancer Treat Rep *68:* 465-469, 1984.

5 Benz C, DeGregorio M, Saks S *et al:* Sequential infusions of methotrexate and 5-fluorouracil in advanced cancer: Pharmacology, toxicity and response. Cancer Res *45:* 3354-3358, 1985.

6 Cunningham J, Bukowski RM, Budd GT, Weick JK, and Purvis J: 5-fluorouracil and folinic acid: A phase I-II trial in gastrointestinal malignancy. Invest New Drugs *2:* 391-395, 1984.

7 Nobile MT, Sertoli MR, Bruzzone M, Tagarelli G, Rubagotti A, and Rosso R: Phase II study with high-dose N-5-10-methyltetrahydrofolate and 5-fluorouracil in advanced colorectal cancer. Eur J Cancer Clin Oncol *21:* 1175-1177, 1985.

8 Amess JAL, Burman JF, Rees GM, Nancekievill DG, and Mollin DL: Megaloblastic haemopoiesis in patients receiving nitrous oxide. Lancet *2:* 339-342, 1978.

9 Deacon R, Lumb M, Perry J *et al:* Selective inactivation of vitamin B12 in rats by nitrous oxide. Lancet *2:* 1023-1024, 1978.

10 Chanarin I: The effects of nitrous oxide on cobalamins, folates, and on related events. CRC Crit Rev Toxicol *10:* 179-213, 1982.

11 Kroes ACM, Lindemans J, Hagenbeek A, and Abels J: Nitrous oxide reduces growth of experimental rat leukemia. Leukemia Res *8:* 441-448, 1984.

12 Ermens AAM, Kroes ACM, Lindemans J, and Abels J: 5-Fluorouracil treatment of rat leukemia and a reappraisal of its application in human leukemia. Anticancer Res, 1986 (in press).

13 Hagenbeek A and Van Bekkum DW (eds.): Proceedings of a workshop on comparative evaluation of the L5222 and the BNML rat leukaemia models and their relevance for human acute leukemia. Leukemia Res *1:* 75-256, 1977.

14 Van Bekkum DW and Hagenbeek A: Relevance of the BN leukemia as a model for human acute myeloid leukemia. Blood Cells *3:* 565-579, 1977.

15 Rupreht J and Dzoljic MR: Elimination of irritating compounds during chronic exposure to gases. J Pharmacol Methods *8:* 109-114, 1982.

16 Gutcho S and Mansbach L. Simultaneous radioassay of serum vitamin B12 and folic acid. Clin Chem *23:* 1609-1614, 1977.

17 Mc Martin KE, Virayotha V, and Tephly TR: High-pressure liquid chromatography separation and determination of rat liver folates. Arch Biochem Biophys *209:* 127-136, 1981.

18 Dunn RT and Foster LB: Radioassay of serum folate. Clin Chem *19:* 1101-1105, 1973.

19 Wickramasinghe SN: The deoxyuridine suppression test: a review of its clinical and research applications. Clin Lab Haematol *3:* 1-18, 1981.

20 Bruckner HW, Schreiber C, and Waxman S: Interaction of chemotherapeutic agents with methotrexate and 5-fluorouracil and its effect on *de novo* DNA synthesis. Cancer Res *35:* 801-806, 1975.

21 Metz J, Kelly A, Sweet VC, Waxman S, and Herbert V: Deranged DNA synthesis by bone marrow from vitamin B12 deficient humans. Br J Haematol *14:* 575-592, 1968.

22 Deacon R, Lumb M, Perry J *et al:* Inactivation of methionine synthetase by nitrous oxide. Eur J Biochem *104:* 419-422, 1980.

23 Lumb M, Chanarin I, Perry J, and Deacon R: Turnover of the methyl moiety of 5-methyltetrahydropteroylglutamic acid in the cobalamin-inactivated rat. Blood *66:* 1171-1175, 1985.

24 Rachmilewitz D, Rachmilewitz EA, Polliack A, and Hershko C: Acute promyelocytic leukemia: a report of five cases with a comment on the diagnostic significance of serum vitamin B12 determination. Br J Haematol *22:* 87-92, 1972.

25 Muir M and Chanarin I: Conversion of endogenous cobalamins into microbiologically-inactive cobalamin analogues in ratys by exposure to nitrous oxide. Br J Haematol *58:* 517-523, 1984.

26 Lumb M, Perry J, Deacon R, and Chanarin I: Changes in tissues folates accompaying nitrous-oxide induced inactivation of vitamin B12 in the rat. Am J Clin Nutr *34:* 2412-2417, 1981.

27 Perry J, Chanarin I, Deacon R, and Lumb M: Folate polyglutamate synthetase activity in the cobalamin-inactivated rat. Biochem J *227:* 73-77, 1985.

28 Lumb M, Perry J, Deacon R, and Chanarin I: Changes in plasma folate levels in rats inhaling nitrous oxide. Scand J Haematol *26:* 61-64, 1981.

29 Mulder JH, Smink T, and Van Putten LM: 5-Fluorouracil and methotrexate combination chemotherapy: the effect of drug scheduling. Eur J Cancer Clin Oncol *17:* 831-837, 1981.

30 Herrmann R, Kunz W, Osswald H, Ritter M, and Port R: The effect of methotrexate on 5-fluorouracil kinetics in sarcoma 180 *in vivo*. Eur J Cancer Clin Oncol *21:* 753-758, 1985.

31 Bertino JR, Mini E, and Fernandes DJ: Sequential methotrexate and 5-fluorouracil: mechanisms of synergy. Seminars in Oncology *10:* 2-5, 1983.

32 Cadman E, Heimer R, and Davis L: Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. Science *205:* 1135-1137, 1979.

33 Deacon R, Perry J, Lumb M, and Chanarin I: Effects of cobalamin inactivation on folate-dependent transformylases involved in purine synthesis in rats. Biochem J *227:* 67-71, 1985.

34 Boss GR: Cobalamin inactivation decreases purine and methionine synthesis in cultured lymphoblasts. J Clin Invest *76:* 213-218, 1985.

35 Deacon R, Perry J, Lumb M, and Chanarin I: The effect of nitrous oxide-induced inactivation of vitamin B12 on thymidilate synthetase activity of rat bone marrow cells. Biochem Biophys Res Commun *102:* 215-218, 1981.

36 Klubes P, Cerna I, and Meldon MA: Effects of concurrent calcium leucovorin infusion on 5-fluorouracil cytotoxicity against murine L1210 leukemia. Cancer Chemother Pharmacol *6:* 121-125, 1981.

37 Danhauser LL, Heimer R, and Cadman E: Lack of enhanced cytotoxicity of cultured L1210 cells using folinic acid in combination with sequential methotrexate and fluorouracil. Cancer Chemother Pharmacol *15:* 214-219, 1985.

38 Linker CA, Ries CA, Saks SR, and Cadman EC: Treatment of refractory acute leukemia with sequential methotrexate and 5-fluorouracil. Blood *64:* (suppl 1): 169a, 1984.

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Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide

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Summary. Exposure to nitrous oxide interferes selectively with the coenzyme function of vitamin B12 and causes inactivation of methionine synthetase, with subsequent impairment of folate metabolism and reduction of cellular proliferation. In a rat leukemia model (BNML) we investigated the combined administration of nitrous oxide, inactivating vitamin B12, and methotrexate (MTX), a folate antagonist inhibiting the enzyme dihydrofolate reductase. Through different mechanisms, both agents decrease the availability of tetrahydrofolate, and subsequently of other reduced folates, with increased impairment of folate-dependent synthesis of thymidylate. Effects on leukemic growth and on hematological values in rats demonstrated enhancement of the therapeutic effect of MTX by exposure to nitrous oxide. With several treatment schedules, the results of combined treatment were seen to be better than additive when compared with the effects of single agents. In particular, pretreatment of leukemic rats with nitrous oxide for 3 days before administration of MTX appeared effective. With higher doses of MTX, concomitant exposure to nitrous oxide even resulted in toxic effects. These findings were in accordance with the results of some metabolic studies performed in leukemic rats. De novo synthesis of thymidylate in leukemic cells, when studied by means of the deoxyuridine suppression test, showed the most severe disturbance with combined treatment consisting in MTX (0.5 mg/kg) and nitrous oxide pretreatment for 3 days. Intracellular levels of folate and dTTP were lowest with 2 and 3 days' pretreatment before MTX, respectively. It is concluded that this interaction of nitrous oxide and MTX can result in enhanced metabolic and therapeutic effects of low doses of MTX. Inactivation of vitamin B12 appears to be a potentially useful addition in cancer chemotherapy.

Introduction

The similarity of hematological disturbances caused by deficiency of either folic acid or cobalamin (vitamin B12) is well established. Both vitamins are involved in pathways essential in the synthesis of nucleotides, and consequently of DNA. The important function of folic acid in cellular proliferation is also reflected in the striking cytostatic activity of folate antagonists of which methotrexate (MTX) is the best known example. This antimetabolite is widely used in cancer chemotherapy [14]. In contrast, until recently, the role of vitamin B12 in neoplastic growth has remained unclear, because no effective method was available to interfere with its coenzyme function. In 1978, however, it was recognized that selective inactivation of vitamin B12 could be achieved with exposure to the anesthetic gas nitrous oxide, or N_2O [1, 7]. Megaloblastic hematopoiesis after prolonged exposure to nitrous oxide had been observed much earlier [21], and a chemical interaction of nitrous oxide with complexes of cobalt was also known for some time [2]. It appeared that a specific oxidative action of nitrous oxide on the cobalt moiety of vitamin B12 caused a nearly complete inactivation of the methylcobalamin-requiring enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase (E. C. 2.1.1.13). Nitrous oxide effectively established a state of functional deficiency of vitamin B12, with severely disturbed folate metabolism [6] and toxic effects on hematopoiesis [38]. Methionine synthetase is essential in folate metabolism, because it provides the only pathway by which 5-methyltetrahydrofolate, the major extracellular folate, can be converted into tetrahydrofolate (THF). THF and other, subsequently formed, reduced folates can be converted into folylpolyglutamates, which are then retained in the cell and are important coenzymes in one-carbon transfer reactions. De novo synthesis of thymidylate requires such folate-dependent methylation. MTX also interferes with folate metabolism by limiting the generation of THF, but through a different mechanism. Its inhibition of dihydrofolate (DHF) reductase prevents the reconversion of DHF to THF.

It has been shown that the effect of nitrous oxide on vitamin B12 can be utilized to reduce growth of leukemia in vitro [18] and in vivo, in rats [20]. This effect is associated with a disturbance of folate-dependent de novo synthesis of thymidylate. Therefore, it appears that nitrous oxide and MTX, through inhibition of different pathways, ultimately may have similar effects on folate metabolism. These observations suggest that the inactivation of vitamin B12 can modify, and possibly enhance, the efficacy of MTX. In vitro studies on human bone marrow have demonstrated a synergistic effect of MTX and nitrous oxide

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Abbreviations used in this paper: MTX, methotrexate; THF, tetrahydrofolate; DHF, dihydrofolate; dTTP, deoxythymidine triphosphate.

with regard to impairment of nucleotide synthesis [17]. The purpose of the present study is to investigate the influence of nitrous oxide on the effects of MTX in vivo, using a rat leukemia model: the Brown Norway myeloid leukemia (BNML). This transplantable acute promyelocytic leukemia has been described in detail elsewhere [12] and is considered to be a suitable model for chemotherapeutic studies [40]. In addition to experiments intended to assess effects on leukemic growth, a number of metabolic studies were performed in leukemic rats, to investigate some effects of treatment on folate metabolism. These studies included deoxyuridine suppression tests, and determinations of intracellular folate and deoxythymidine triphosphate (dTTP) levels.

Materials and methods

Animals. Male rats of the Brown Norway inbred strain were used at the age of 12–16 weeks (body weight 200–275 g). Food and water were supplied ad libitum during the experiments.

Brown Norway myeloid leukemia (BNML). Cryopreserved leukemic cells were kindly provided by Dr. A. Hagenbeek from the Radiobiological Institute (TNO), Rijswijk, The Netherlands, where this transplantable rat leukemia model was developed. Origin, classification and proliferation kinetics were described elsewhere [12]. For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. A standard dose of 10^7 cells suspended in Hanks' balanced salt solution was injected IV, which leads to progressive leukemic infiltration of bone marrow, spleen and liver, with death after 20–24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of tumor load and, along with hematological determinations, can be used effectively to assess effects of chemotherapy [12]. To avoid a gradual change in growth properties, serial transplantations were limited to only two passages, after which spleen cells were used from rats freshly inoculated with cells from a cryopreserved stock.

Treatment with nitrous oxide and MTX. Leukemic rats were treated according to different schedules in groups of at least four. Exposure to nitrous oxide was carried out in a 40-l flow chamber into which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water, and contaminating volatile compounds were eliminated in a cleaning circuit, essentially as described by Rupreht and Dzoljic [37]. Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. Sodium methotrexate (Ledertrexate SP, from Lederle) was injected IP. Rats not receiving MTX received injections of 0.15 *M* NaCl IP instead.

Evaluation of leukemic growth. Experiments intended to assess effects on leukemic growth were all evaluated by the same procedure. To allow a simultaneous investigation of several aspects of leukemia these experiments were terminated after a fixed period of 18 days (in some instances: 19 days) of leukemia, just before death from leukemia was to be expected. Rats were killed by exsanguination, after recording of body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically, and in some experiments differential blood cell counts were done. Plasma levels of vitamin B12 were measured in a competitive radioisotope binding assay using purified intrinsic factor [22]. Normal values for organ weights, leukocyte counts and plasma vitamin B12 were derived from at least 12 comparable nonleukemic Brown Norway rats.

Metabolic studies. In separate experiments, rats with advanced leukemia were treated for short periods, after which leukemic cells were used in metabolic studies. Three similar experiments were carried out separately. In each, eight leukemic rats inoculated at day 0 were divided in four pairs. These pairs were treated with nitrous oxide for 3, 2, 1, or 0 days. Immediately afterwards one rat in each pair received MTX, 0.5 mg/kg IP, the other rat receiving saline only. At 18 h after administration of MTX, on day 15 of leukemia, leukemic cells were obtained from the spleens of all rats, washed, and resuspended in Hanks' balanced salt solution. These cell suspensions were counted electronically and used in deoxyuridine suppression tests and determinations of intracellular folate and dTTP.

Deoxyuridine suppression test. This test demonstrates impaired de novo synthesis of thymidylate. ^{3}H-Thymidine incorporation into DNA is measured with and without addition of deoxyuridine. Deoxyuridine will suppress incorporation of ^{3}H-thymidine in DNA if it can be converted to thymidylate through folate-dependent methylation. This suppression is reduced by vitamin B12 or folate deficiency [42], inactivation of vitamin B12 by nitrous oxide [28], and treatment with other agents interfering with de novo synthesis of thymidylate [5].

Leukemic spleen cells (approx. 5×10^6 per test) were used from rats of various groups, as described above. The test was carried out essentially according to Metz [30], with some modification as described elsewhere [20]. Deoxyuridine (Sigma, St. Louis, USA) was used in a concentration of 0.1 mmol/l. All incubations were performed in triplicate. Incorporation of ^{3}H-thymidine (0.3 μCi per test, specific activity 25 Ci/mmol, from Amersham, UK) is expressed as a percentage of the maximal incorporation, measured in each case in incubations without addition of deoxyuridine.

Intracellular folate. In suspensions of leukemic spleen cells, intracellular folate content was determined. After centrifugation a pellet of approx. 10^8 cells was resuspended in a total volume of 1 ml 10% (≈1.3 mol/l) mercaptoethanol, heated in a water bath of 100 °C for 5 min, and cooled. Hog kidney polyglutamate hydrolase, prepared as described by McMartin et al. [29], was added and allowed to incubate at room temperature for 2 h. The samples were frozen at −20 °C until assayed. After thawing, the extracts were centrifugated (1500 *g* for 10 min at 4 °C), and aliquots of the supernatants were used in a folate radioisotope dilution assay, essentially as described by Dunn and Foster [11], with ^{125}I-folic acid (Becton Dickinson, Orangeburg, N.Y., USA) as a tracer, and β-lactoglobulin (Sigma, St. Louis, USA) as a folate binder. 5-Methyl-THF was used as a standard, and results are expressed as picomoles of folate per 10^6 cells.

Intracellular dTTP. In leukemic cells obtained from rats as described above, dTTP was determined using the DNA polymerase assay system originally developed by Solter and Handschumacher [39], with the modifications and corrections published by Hunting and Henderson [13]. A different extraction method was used, however. After being washed once in Hanks' balanced salt solution, cell suspensions were centrifuged and the supernatant was removed. To the pellet of about 10^8 cells, 1.2 μg cyano [^{57}Co]cobalamin, or 10^5 dpm, (Amersham, UK) was added, as an internal standard for cell quantities in the assay. The pellet was then extracted with 5 ml ice-cold 60% methanol and stored at −20 °C until assayed. After centrifugation the supernatant was dried using a rotary evaporator at 25 °C, dissolved in 50 m*M* potassium cacodylate and subsequently used in the DNA polymerase catalyzed assay. DNA polymerase I from *E. coli*, dATP, dTTP, and poly(d(A,T)) were all obtained from Boehringer Mannheim (FRG) and ^{3}H-dATP and ^{3}H-dTTP were from Amersham (UK). Procedures, and calculated corrections for dilution of specific activities by the endogenous nucleotides were carried out as described elsewhere [13]. Concentrations of dTTP are expressed as picomoles per 10^6 cells, based upon counts of the internal standard and cell concentration of the initial suspension.

Results

Effects of growth of leukemia in rats

In Table 1, results of six experiments with several different treatment schedules are summarized. In all these experiments, one group of leukemic rats treated with MTX only is compared with one or more groups of rats treated with a combination of MTX and exposure to nitrous oxide. Most experiments included a group receiving nitrous oxide continuously throughout the treatment period (day 7 to day 18 or 19 of leukemia). In other groups, nitrous oxide treatment was limited to either 3 days before administration of MTX (pretreatment) or 3 days after administration of MTX (post-treatment). This is illustrated in Fig. 1, which shows the treatment schedules as applied in experiment 3 of Table 1. In addition, all experiments included rats receiving no treatment or nitrous oxide only (days 7–19), without MTX. Cumulative results in these rats are also shown in Table 1. From the data in Table 1, it follows that low doses of MTX alone do not have substantial effects on leukemic growth. In all experiments the addition of nitrous oxide enhanced the therapeutic effects of MTX. The differences between rats treated with MTX only and rats treated with both MTX and nitrous oxide (continuously) are statistically significant according to Wilcoxon's nonparametric rank sum test. For all three parameters of leukemia, results did not overlap, yielding p-values of 0.05 or less, in any of the experiments 1–4. In addition, the results of combined treatment appeared to be better than additive in these experiments, with the possible exception of experiment 1, in which the lowest dose of MTX was administered. In other cases, the reduction of leukemic growth obtained with combined treatment is often greater than the added effects of the two single agents, which is indicative of a synergistic interaction. From experiments 3 and 5 it can be derived that by far the major part of the added effect is contributed by the period of exposure to nitrous oxide before administration of MTX (pretreatment schedules). In experiment 6 the highest dose of MTX was used (4 mg/kg). In this experiment, rats treated with both MTX and nitrous oxide (continuously) died prematurely at 14–16 days after inoculation, without any evidence of leukemia. Spleen and liver weights at autopsy were subnor-

Table 1. Effects of treatment on growth of leukemia

Expt no	Treatment	No. of. rats	Spleen weight[a] (g) mean ± SEM	Liver weight[b] (g) mean ± SEM	Leukocytes[c] (10^9) mean ± SEM
	None (untreated controls)	22	3.90 ± 0.09	17.39 ± 0.45	24.8 ± 2.1
	N_2O, continuous (days 7–18/19)	14	2.89 ± 0.09	14.33 ± 0.44	11.1 ± 1.0
1	MTX, 1 × 0.5 mg/kg (day 11)	5	3.64 ± 0.10	15.60 ± 0.69	18.1 ± 1.2
	+ N_2O, continuous (days 7–19)	5	2.34 ± 0.10	12.50 ± 0.55	7.7 ± 0.7
2	MTX, 2 × 0.5 mg/kg (days 10 and 14)	4	3.29 ± 0.09	12.95 ± 0.52	16.6 ± 1.3
	+ N_2O, continuous (days 7–18)	4	1.43 ± 0.14	9.76 ± 0.47	3.8 ± 0.5
3	MTX, 2 × 0.5 mg/kg (days 10 and 16)	4	3.38 ± 0.18	16.04 ± 0.78	20.2 ± 1.4
	+ N_2O, 2 × 3 days after MTX (days 10–13/16–19)	4[d]	3.32	17.05	20.0
	+ N_2O, 2 × 3 days before MTX (days 7–10/13–16)	4	2.37 ± 0.11	13.08 ± 0.44	8.1 ± 0.6
	+ N_2O, continuous (days 7–19)	4	1.97 ± 0.17	11.68 ± 0.81	7.0 ± 1.1
4	MTX, 1 × 2 mg/kg (day 10)	4	3.44 ± 0.14	14.00 ± 0.46	20.8 ± 2.9
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	2.58 ± 0.24	12.59 ± 0.94	7.7 ± 1.0
	+ N_2O, continuous (days 7–19)	4	1.95 ± 0.08	11.47 ± 0.17	6.1 ± 0.6
5	MTX, 2 × 1 mg/kg (days 6 and 14)	4	3.19 ± 0.22	12.37 ± 0.59	15.9 ± 1.8
	+ N_2O, 2 × 3 days after MTX (days 6–9/14–17)	4	3.37 ± 0.10	14.70 ± 0.80	14.0 ± 0.7
	+ N_2O, 2 × 3 days before MTX (days 3–6/11–14)	4	2.31 ± 0.14	10.64 ± 0.51	10.8 ± 1.2
6	MTX, 1 × 4 mg/kg (day 10)	4	2.85 ± 0.32	13.30 ± 0.74	14.9 ± 1.1
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	1.47 ± 0.43	10.12 ± 0.49	4.2 ± 1.4
	+ N_2O, continuous (from day 7)	4	all rats died (without evidence of leukemia)		

[a] Normal spleen weight in comparable nonleukemic BN rats: 0.45 ± 0.07 g
[b] Normal liver weight in comparable nonleukemic BN rats: 8.25 ± 0.99 g
[c] Normal value of leukocyte count in nonleukemic BN rats: 3.9 ± 0.4 × 10^9/l
[d] 2 rats dying prematurely of leukemia are not included

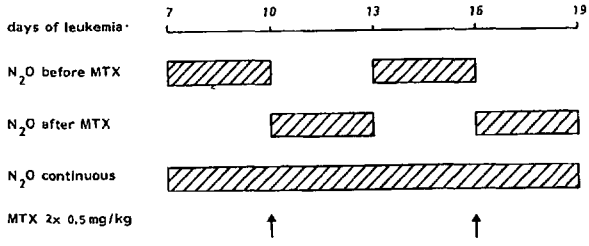

Fig. 1. Treatment of leukemic rats with nitrous oxide (N_2O) and methotrexate (MTX), as given in experiment 3 of Table 1. Periods of exposure to nitrous oxide are *shaded*, and *arrows* indicate time of administration of MTX. Rats receiving MTX or nitrous oxide only and untreated rats were also included

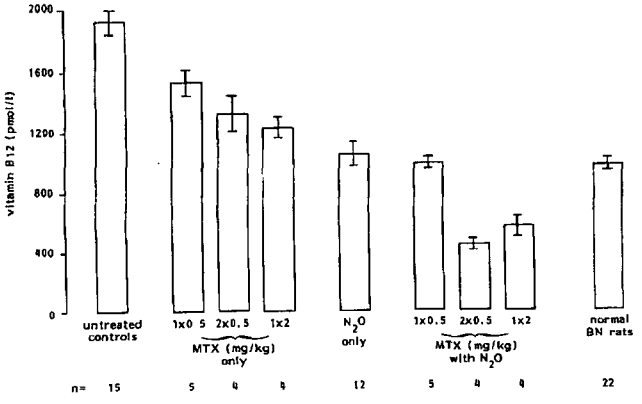

Fig. 2. Plasma levels of vitamin B12 in leukemic rats treated in several experiments with different doses of MTX. Groups with combined treatment received nitrous oxide continuously (days (7–18/19 of leukemia). Values in normal (nonleukemic) BN rats are also shown. *Bars* indicate SEM

mal, and body weight was seriously reduced (77% of weight before treatment). This was considered to be a toxic effect of treatment. In other experiments, however, no toxicity was observed and nitrous oxide treatment was well tolerated by rats without evident effects on consciousness. Even with combined treatment, loss of body weight was always less than 10%.

In some experiments differential blood cell counts were made, which demonstrated that the observed reductions in peripheral leukocyte counts were also accompanied by a striking relative decrease of leukemic cells (promyelocytes), as shown in Table 2.

Plasma levels of vitamin B12, as determined in these experiments, are presented in Fig. 2. Compared with normal BN rats, vitamin B12 levels in untreated leukemic controls are very high. With treatment these levels are reduced, as is shown for three doses of MTX. Exposure to nitrous oxide alone has marked effects on the vitamin B12 level in plasma, but much lower and even subnormal levels are found after combined treatment.

Metabolic effects

In separate experiments, pairs of leukemic rats were treated with nitrous oxide for 0, 1, 2, or 3 days. One rat of each pair subsequently received MTX (0.5 mg/kg), and 18 h afterwards leukemic cells of all rats were used for deoxyuri-

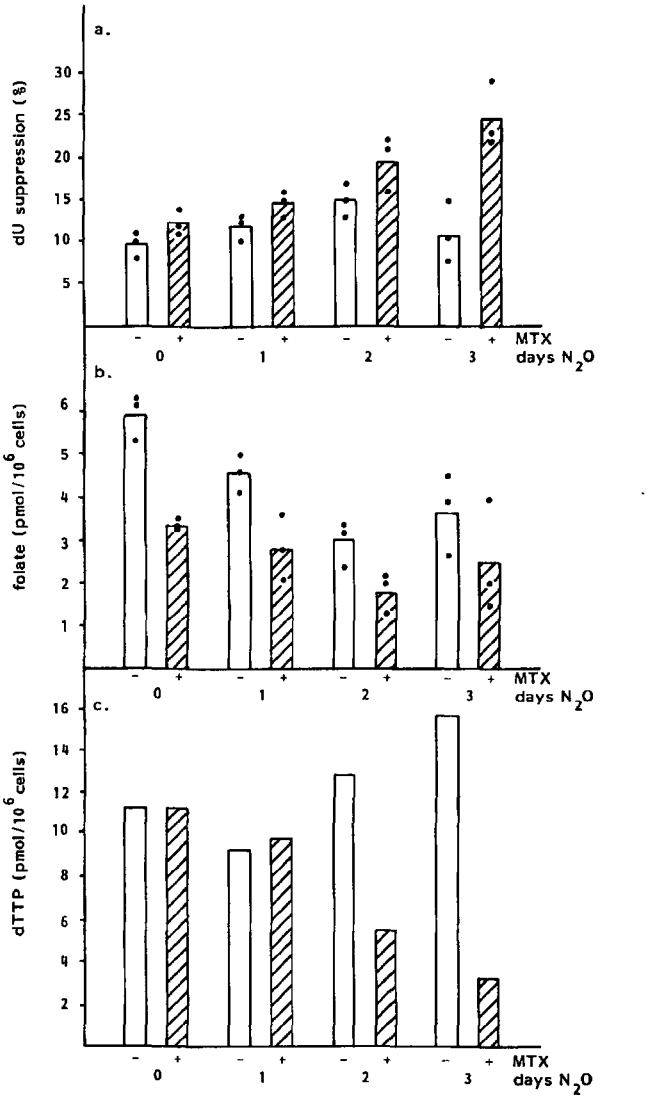

Fig. 3a–c. Results of metabolic experiments with leukemic spleen cells after in vivo treatment of rats with 0–3 days' exposure to nitrous oxide, followed or not by administration of MTX (0.5 mg/kg): **a** deoxyuridine suppression values, expressed as percentages of maximal incorporation of ^{3}H-thymidine, in incubations without deoxyuridine; **b** intracellular levels of folate (pmol/10^6 cells); **c** intracellular levels of dTTP (pmol/10^6 cells). *Shaded columns* indicate MTX treatment. The experiments were performed 18 h after administration of MTX and/or exposure to nitrous oxide

dine suppression tests and determinations of intracellular folate and dTTP. This experimental procedure was repeated twice, and results of all rats used in this study are presented together (Fig. 3).

Deoxyuridine suppression tests (Fig. 3a) show increased disturbance with longer duration of nitrous oxide exposure before the administration of MTX. Higher values in this test indicate a decreased ability of deoxyuridine to suppress the uptake of ^{3}H-thymidine, demonstrating impaired de novo synthesis of thymidylate. MTX without nitrous oxide treatment is clearly less effective: mean values are 12% without nitrous oxide and 25% with 3 days of pretreatment. Figure 3b shows the results of intracellular folate determinations. Both nitrous oxide and MTX de-

Table 2. Effect of treatment on differential blood cell counts

Treatment	No. of. rats	Promyelocytes[a] (leukemic cells)	Lymphocytes[a]	Neutrophils[a]
None (untreated controls)	8	17.4±1.9	74.4±2.0	8.3±1.6
N_2O, continuous (days 7–18/19)	6	13.5±3.4	83.2±2.7	3.3±0.8
MTX, 2×0.5 mg/kg (days 10 and 14)	4	13.5±2.2	74.5±1.8	12.0±1.0
+ N_2O, continuous (days 7–18)	4	0.8±0.2	98.3±0.6	1.0±0.4
MTX, 1×2 mg/kg (day 10)	3	30.6±1.5	65.0±1.5	4.3±1.2
+ N_2O, 1×3 days before MTX (days 7–10)	3	8.0±6.0	90.3±6.2	1.7±0.3
+ N_2O, continuous (days 7–19)	4	3.3±0.9	95.5±1.0	1.3±0.2
MTX, 1×4 mg/kg (day 10)	4	13.3±3.9	81.5±5.2	5.3±1.6
+ N_2O, 1×3 days before MTX (days 7–10)	4	2.0±0.7	97.5±0.9	0.5±0.3
Normal BN rats (nonleukemic)	5	0	91.4±1.4	6.0±1.5[b]

[a] Expressed as percentages of the total number of nucleated cells, counting 200 cells, with indication of SEM
[b] With additional 0.4% eosinophils and 2.2% monocytes

creased folate levels, and the lowest levels are found with combined treatment after 2 days of nitrous oxide exposure. With 3 days of nitrous oxide exposure it appears that folate contents recover to some extent. Figure 3c, presenting levels of intracellular dTTP, is based upon the last metabolic experiment only, involving eight rats, in contrast to the other results. From this experiment it can be concluded that dTTP levels in leukemic cells are lowest with combined treatment consisting in MTX and 2 or 3 days' pretreatment with nitrous oxide. Nitrous oxide treatment alone appears to cause increased levels of dTTP, but it should be emphasized that these and other values were obtained 18 h after exposure and some recovery may have occurred.

Discussion

Nitrous oxide interferes specifically with the coenzyme function of vitamin B12 and thereby inactivates methionine synthetase [8]. This severely affects folate metabolism, because methionine synthetase is required in the conversion of 5-methyl-THF, the predominant extracellular folate, into THF. This conversion is essential for folate coenzyme functions and also for the cellular retention of folates. In contrast to other reduced folates, 5-methyl-THF is not a substrate for synthesis of folylpolyglutamates, as is evident from metabolic studies [23, 27] and from properties of the purified enzyme [31]. The synthesis of folylpolyglutamates is decreased by nitrous oxide [27, 34, 35], which explains the serious cellular depletion of folates occurring on exposure [25]. The decreased availability of reduced folates impairs folate-dependent synthesis of thymidylate, and subsequently reduces DNA synthesis and cellular proliferation. The inhibition of leukemic growth by nitrous oxide in vitro has been demonstrated [18], and in a previous study we described in vivo antileukemic effects of nitrous oxide in rats [20]. We also showed that these effects were enhanced in combined treatment with cycloleucine, which inhibits the conversion of methionine into S-adenosylmethionine and indirectly interferes with folate metaboilism [19]. A number of studies have investigated the combination of nitrous oxide, as a vitamin B12-inactivating agent, and MTX, as a typical folate antagonist. Kano et al. [17] have demonstrated synergistic effects with regard to inhibition of thymidylate synthesis in normal human bone marrow. Black and Tephly [4] compared metabolic effects of both agents in rat liver cells. The inhibition of methionine synthetase by nitrous oxide considerably decreased the availability of THF while the inhibition of DHF reductase by MTX was much less effective. This difference probably can be explained by the low activity of thymidylate synthetase in liver cells. Dudman et al. [10] found increased sensitivity of leukemic cell lines to MTX with nitrous oxide-induced inhibition of methionine synthetase, which was further exploited by the use of 5-methyl-THF instead of 5-formyl-THF as a rescue agent.

The present study shows effects of combined therapy with nitrous oxide and MTX on in vivo growth and metabolism of rat leukemia. The exposure of rats to nitrous oxide enhanced inhibition of leukemic growth by low doses of MTX. A period of exposure before the administration of MTX (pretreatment) appeared essential for this effect. The effects on leukemic infiltration in spleen and liver correlated well with hematologic results (total and differential leukocyte counts) and with determinations of vitamin B12 in plasma. An interesting feature of this leukemia is a continuous rise of vitamin B12 levels in the course of leukemic growth [20], which is also observed in human acute promyelocytic leukemia [36]. Plasma vitamin B12 can be used as a kind of tumor marker, and treatment leads to reduced levels. The particularly striking decrease caused by nitrous oxide, however, is explained by its specific effect on cobalamin. Analogues of cobalamin are formed after oxidation and are excreted rapidly [33]. In addition to effects on leukemic growth, some metabolic aspects of this interaction were studied in leukemic cells after in vivo treatment of rats. On the basis of the results discussed before, pretreatment with nitrous oxide was administered for periods up to 3 days. Deoxyuridine suppression tests demonstrated increased disturbance of de novo synthesis of thymidylate with longer duration of nitrous oxide pretreatment before MTX. Treatment with a single agent was clearly less effective. Simultaneous determination of intracellular folates, to confirm the presumed cellular folate depletion, indeed showed a decrease in folate content, particularly with combined treatment. Some comments on these folate measurements are warranted, however. In the radioisotope competitive binding assay the

β-lactoglobulin folate binder is used, and probably not all relevant folate derivatives show the same affinity for this binder. Our own observations (not included in this study) indicated that 5-methyl-THF has slightly lower affinity than THF. As these folates are intracellularly predominant [4, 29], the observed decrease in total folate content could also be explained, at least partially, by a shift of folates from THF toward 5-methyl-THF. This, however, is functionally about equivalent, because the conversion of 5-methyl-THF is blocked on nitrous oxide exposure, as discussed before. Moreover, the results of this folate radioassay in the measurement of tissue folates were recently found to be comparable to microbiological assays [26]. Changes in cellular folate on nitrous oxide exposure in our study are similar to earlier observations [25, 35], including an indication of recovery at more than 2 days of exposure, an adaptive mechanism which is not yet understood. Finally, decreased dTTP levels show that the impairment of folate-dependent synthesis of thymidylate has noticeable effects on this direct precursor of DNA. Considered together, the findings in metabolic experiments are in accordance with the results of studies directed at inhibition of growth, showing maximum effects after about 3 days of pretreatment with nitrous oxide before MTX.

Several potential mechanisms could explain the results of this interaction. First, a reduction of intracellular folates by nitrous oxide pretreatment can obviously induce greater susceptibility to folate antagonists [16]. A second mechanism is closely related and concerns the reduced synthesis of folylpolyglutamates on nitrous oxide exposure. Polyglutamation of MTX, leading to increased activity and cellular retention [15], occurs in competition with normal folate substrates [3, 16]. In cells pretreated with nitrous oxide a larger proportion of MTX may be converted into polyglutamate forms. A third potential mechanism to explain the results relates to the observed marked increase in activity of thymidylate synthetase, after nitrous oxide exposure of up to 3 days [9]. It is well established that thymidylate synthetase has a central role in mediating the cytotoxic effects of MTX [32, 41], because this enzyme actually causes THF depletion by its conversion of reduced folates into DHF. Increased activity of this enzyme, as a possible adaptation to nitrous oxide treatment, thus may result in enhanced effects of MTX. All these mechanisms can explain the observed importance of pretreatment with nitrous oxide before MTX.

The results presented in this study demonstrate that in vivo metabolic manipulation of leukemic cells with nitrous oxide can enhance metabolic and therapeutic effects of MTX. The clinical relevance of this interaction is illustrated by the recent observation of increased toxicity of adjuvant chemotherapy involving MTX started directly postoperatively [24], which the authors attributed to inactivation of vitamin B12 by nitrous oxide during anesthesia. Our findings lend support to this suggestion. In experimental chemotherapy, the inactivation of vitamin B12 represents a new method to enhance activity of MTX. The relative contributions of increased polyglutamation of MTX, and increased activity of thymidylate synthetase, should be subjects of further research. It also remains to be demonstrated that the effects described are applicable to human leukemia, but it is known that man is more susceptible to vitamin B12 deficiency than any animal [6]. Finally, these results indicate the significance of vitamin B12 in leukemic proliferation and the value of vitamin B12-related metabolism as an additional target in cancer chemotherapy.

References

1. Amess JAL, Burman JF, Ress GM, Nancekievill DG, Mollin DL (1978 Megaloblastic haemopoiesis in patients receiving nitrous oxide. Lancet 2: 339
2. Banks RGS, Henderson RJ, Pratt JM (1968) Reactions of gases in solution: III. Some reactions of nitrous oxide with transition-metal complexes. J Chem Soc [A] 12: 2886
3. Barford PA, Blair JA, Malghani MAK (1980) The effect of methotrexate on folate metabolism in the rat. Br J Cancer 41: 816
4. Black KA, Tephly TR (1983) Effects of nitrous oxide and methotrexate administration on hepatic methionine synthetase and dihydrofolate reductase activities, hepatic folates, and formate oxidation in rats. Mol Pharmacol 23: 724
5. Bruckner HW, Schreiber C, Waxman S (1975) Interaction of chemotherapeutic agents with methotrexate and 5-fluorouracil and its effect on de novo DNA synthesis. Cancer Res 35: 801
6. Chanarin I (1982) The effects of nitrous oxide on cobalamins, folates, and on related events. CRC Crit Rev Toxicol 10: 179
7. Deacon R, Lumb M, Perry J, Chanarin I, Minty B, Halsey MJ, Nunn JF (1978) Selective inactivation of vitamin B12 in rats by nitrous oxide. Lancet 2: 1023
8. Deacon R, Lumb M, Perry J, Chanarin I, Minty B, Halsey M, Nunn J (1980) Inactivation of methionine synthetase by nitrous oxide. Eur J Biochem 104: 419
9. Deacon R, Perry J, Lumb M, Chanarin I (1981) The effect of nitrous oxide-induced inactivation of vitamin B12 on thymidylate synthetase activity of rat bone marrow cells. Biochem Biophys Res Commun 102: 215
10. Dudman NPB, Slowiaczek P, Tattersall MHN (1982) Methotrexate rescue by 5-methyltetrahydrofolate or 5-formyltetrahydrofolate in lymphoblast cell lines. Cancer Res 42: 502
11. Dunn RT, Foster LB (1973) Radioassay of serum folate. Clin Chem 19: 1101
12. Hagenbeek A, Van Bekkum DW (eds) (1977) Proceedings of a workshop on comparative evaluation of the L5222 and the BNML rat leukaemia models and their relevance for human acute leukaemia. Leukemia Res 1: 75
13. Hunting D, Henderson JF (1981) Determination of deoxyribonucleoside triphosphates using DNA polymerase: a critical evaluation. Can J Biochem 59: 723
14. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA (1983) The pharmacology and clinical use of methotrexate. N Engl J Med 309: 1094
15. Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA (1982) Synthesis, retention and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 70: 351
16. Kamen BA, Nylen PA, Camitta BM, Bertino JR (1981) Methotrexate accumulation and folate depletion in cells as a possible mechanism of chronic toxicity to the drug. Br J Haematol 49: 355
17. Kano Y, Sakamoto S, Sakuraya K, Kubota T, Hida K, Suda K, Takaku F (1981) Effect of nitrous oxide on human bone marrow cells and its synergistic effect with methionine and methotrexate on functional folate deficiency. Cancer Res 41: 4698
18. Kano Y, Sakamoto S, Sakuraya K, Kubota T, Kasahara T, Hida K, Suda K, Takaku F (1983) Effects of nitrous oxide on human cell lines. Cancer Res 43: 1493
19. Kroes ACM, Lindemans J, Abels J (1984) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia. Br J Cancer 50: 793

20. Kroes ACM, Lindemans J, Hagenbeek A, Abels J (1984) Nitrous oxide reduces growth of experimental rat leukemia. Leukemia Res 8: 441
21. Lassen HCA, Henriksen E, Neukirch F, Kristensen HS (1956) Treatment of tetanus. Severe bone marrow depression after prolonged nitrous-oxide anaesthesia. Lancet 1: 527
22. Lau KS, Gottlieb C, Wasserman LR, Herbert V (1965) Measurement of serum vitamin B12 level using radioisotope dilution and coated charcoal. Blood 26: 202
23. Lavoie A, Tripp E, Hoffbrand AV (1974) The effect of vitamin B12 deficiency on methylfolate metabolism and pteroylpolyglutamate synthesis in human cells. Clin Sci Mol Med 47: 617
24. Ludwig Breast Cancer Study Group (1983) Toxic effects of early adjuvant chemotherapy for breast cancer. Lancet 2: 542
25. Lumb M, Perry J, Deacon R, Chanarin I (1981) Changes in tissue tolates accompanying nitrous oxide-induced inactivation of vitamin B12 in the rat. Am J Clin Nutr 34: 2412
26. Mandella R, DePaola DP (1984) Measurement of tissue folates: a comparison of radioassay and microbiological methods. Nutr Res 4: 521
27. McGing P, Reed B, Weir DG, Scott JM (1978) The effect of vitamin B12 inhibition in vivo: impaired folate polyglutamate biosynthesis indicating that 5-methyltetrahydropteroylglutamate is not its usual substrate. Biochem Biophys Res Commun 82: 540
28. McKenna B, Weir DG, Scott JM (1980) The induction of functional vitamin B12-deficiency in rats by exposure to nitrous oxide. Biochim Biophys Acta 628: 314
29. McMartin KE, Virayotha V, Tephly TR (1981) High-pressure liquid chromatography separation and determination of rat liver folates. Arch Biochem Biophys 209: 127
30. Metz J, Kelly A, Sweet VC, Waxman S, Herbert V (1968) Deranged DNA synthesis by bone marrow from vitamin B12-deficient humans. Br J Haematol 14: 575
31. Moran RG, Colman PD (1984) Mammalian folyl polyglutamate synthetase: partial purification and properties of the mouse liver enyzme. Biochemistry 23: 4580
32. Moran RG, Mulkins M, Heidelberger C (1979) Role of thymidylate synthetase activity in development of methotrexate cytotoxicity. Proc Natl Acad Sci USA 76: 5924
33. Muir M, Chanarin I (1984) Conversion of endogenous cobalamins into microbiologically-inactive cobalamin analogues in rats by exposure to nitrous oxide. Br J Haematol 58: 517
34. Perry J, Chanarin I, Deacon R, Lumb M (1983) Chronic cobalamin inactivation impairs folate polyglutamate synthesis in the rat. J Clin Invest 71: 1183
35. Perry J, Chanarin I, Deacon R, Lumb M (1985) Folate polyglutamate synthetase activity in the cobalamin-inactivated rat. Biochem J 227: 73
36. Rachmilewitz D, Rachmilewitz EA, Poliack A, Hershko C (1972) Acute promyelocytic leukaemia: a report of five cases with a comment on the diagnostic significance of serum vitamin B12 determination. Br J Haematol 22: 87
37. Rupreht J, Dzoljic MR (1982) Elimination of irritating compounds during chronic exposure to gases. J Pharmacol Methods 8: 109
38. Skacel PO, Hewlett AM, Lewis JD, Lumb M, Nunn JF, Chanarin I (1983) Studies on the haemopoietic toxicity of nitrous oxide in man. Br J Haematol 53: 189
39. Solter AW, Handschumacher RE (1969) A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. Biochim Biophys Acta 174: 585
40. Van Bekkum DW, Hagenbeek A (1977) Relevance of the BN leukemia as a model for human acute myeloid leukemia. Blood Cells 3: 565
41. Washtien WL (1982) Thymidylate synthetase levels as a factor in 5-fluorodeoxyuridine and methotrexate cytotoxicity in gastrointestinal tumor cells. Mol Pharmacol 21: 723
42. Wickramasinghe SN (1981) The deoxyuridine suppression test: a review of its clinical and research applications. Clin Lab Haematol 3: 1

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Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Filer:	John A. Cleveland/Lisa Capps
Attorney Docket Number:	X14173B

Filed as Large Entity

Utility under 35 USC 111(a) 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:	5267473
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	John A. Cleveland/Lisa Capps
Filer Authorized By:	John A. Cleveland
Attorney Docket Number:	X14173B
Receipt Date:	04-MAY-2009
Filing Date:	11-JUL-2007
Time Stamp:	13:51:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	8339
Deposit Account	050840
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 1.19 (Document supply 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		X14173BResponsetoOfficeAction.pdf	107974 106bca2524c0b2c6d33ab602593f6d4a0a276cec	yes	6
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	1	
	Claims		2	3	
	Applicant Arguments/Remarks Made in an Amendment		4	6	
Warnings:					
Information:					
2	Transmittal Letter	X14173BIDS.pdf	63433 42c011576465e495e84cf4c5df64867c6d1850fc	no	2
Warnings:					
Information:					
3	Information Disclosure Statement (IDS) Filed (SB/08)	X14173BForm1449.pdf	94780 3d372e423f79d7a9748d724bad969515d7a1e065	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
4	Foreign Reference	X14173B_BA.pdf	4670342 0a1d5705afaa4d5ec80d0b2a46103efa7f978318	no	102
Warnings:					
Information:					
5	NPL Documents	X14173B_CA.pdf	489414 5067a69ab4fa754dbf578136f1ee3b9ce3197b78	no	5
Warnings:					
Information:					
6	NPL Documents	X14173B_CB.pdf	343042 e4479eafa3326478e86e3d0884af2b72d2c65df5	no	4
Warnings:					
Information:					

7	NPL Documents	X14173B_CC.pdf	263102 7b1ff750407bc7975fb0a06dfad2006fcb7dfc3d	no	4
Warnings:					
Information:					
8	NPL Documents	X14173B_CD.pdf	412088 9fc7805d20162418a566b54a310241a0c2537b58	no	6
Warnings:					
Information:					
9	NPL Documents	X14173B_CE.pdf	986409 473d33dd66f29168002f99923352a7b22963e83b	no	4
Warnings:					
Information:					
10	NPL Documents	X14173B_CF.pdf	492089 7caf2773b9a8fbefee633ca53cc54931278f0e01	no	7
Warnings:					
Information:					
11	NPL Documents	X14173B_CG.pdf	474729 f303b2797fa32ede58b67368d302f5458916e167	no	5
Warnings:					
Information:					
12	NPL Documents	X14173B_CH.pdf	805699 806647df60ac62d65838b3ca717c87b61b50223d	no	7
Warnings:					
Information:					
13	NPL Documents	X14173B_CI.pdf	355708 3284f4e625743e3617a825b45947ea8c2d195aa0	no	5
Warnings:					
Information:					
14	NPL Documents	X14173B_CJ.pdf	1173724 d888574719ea1e5c023836b7d78c99613dce8d6a	no	19
Warnings:					
Information:					
15	NPL Documents	X14173B_CK.pdf	613239 6b77230bd42df74c4255275da308fce5e6dd49ec	no	6
Warnings:					
Information:					

16	NPL Documents	X14173B_CL.pdf	741163 ae86e721fa3d454ddf74086db17c1f93445c1821	no	7
Warnings:					
Information:					
17	NPL Documents	X14173B_CM.pdf	266022 0eb5f04c1a31507c60c8135318ef8f00364e027c	no	4
Warnings:					
Information:					
18	NPL Documents	X14173B_CN.pdf	292186 61f17904afd519c8fde11fe379f6a474756c4e06	no	6
Warnings:					
Information:					
19	Fee Worksheet (PTO-875)	fee-info.pdf	30593 1a1378e5eb3d3884d62c60231ba7f3caf2d056b1	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	12675736		

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.

NOT A USPTO FORM INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X14173B	Serial No 11/776329
	First Applicant Clet Niyikiza	
	Application Date July 11, 2007 US Nat'l Entry (if applicable)	Group Art Unit 1614

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 Pages or Relevant Figures Appear
	AA	US			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number [4]- Kind Code5 (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]
	BA	WO 95/27723	10-19-1995			

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[6]
	CA	POYDOCK M. Effect of combined ascorbic acid and B-12 on survival of mice with implanted Ehrlich carcinoma and L1210 leukemia. *Am J Clin Nutr* 1991; 54: 1261S-5S,	
	CB	POYDOCK M, et al. Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin. *Am J Clin Oncol* 1985; 8: 2666-269.	
	CC	POYDOCK M, et al. Influence of Vitamins C and B12 on the Survival Rate of Mice Bearing Ascites Tumor. *Expl Cell Biol* 1982; 50:88-91.	
	CD	TOOHEY J. Dehydroascorbic acid as an anti-cancer agent. *Cancer Letters* 2008; 263:164-169.	
	CE	SALLAH S, et al. Intrathecal methotrexate-induced megaloblastic anemia in patients with acute leukemia. *Archives of Pathology & Laboratory Medicine* 1999; 123(9): 774-777.	
	CF	NISHIZAWA Y, et al. Effects of methylcobalamin on the proliferation of androgen-sensitive or estrogen-sensitive malignant cells in culture and in vivo. *International Journal for Vitamin and Nutrition Research* 1997; 67(3):164-170.	
	CG	TSAO C, et al. Influence of cobalamin on the survival of mice bearing ascites tumor. *Pathobiology* 1993; 61(2): 104-.8	
	CH	KAMEI T, et al. Experimental study of the therapeutic effects of folate, vitamin A, and vitamin B12 on squamous metaplasia of the bronchial epithelium. *Cancer* 1993; 71(8): 2477-83.	
	CI	SHIMIZU N, et al. Experimental study of antitumor effect of methyl-B12. *Oncology* 1987; 44(3): 169-73.	
	CJ	HERBERT, V. The role of vitamin B12 and folate in carcinogenesis. *Advances in Experimental Medicine and Biology* 1986; 206 (Essent. Nutr. Carcinog.), 293-311.	
	CK	KROES A, et al. Effects of 5-fluorouracil treatment of rat leukemia with concomitant inactivation of cobalamin. *Anticancer Research* 1986; 6(4): 737-42.	

NOT A USPTO FORM INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X14173B	Serial No 11/776329
	First Applicant Clet Niyikiza	
	Application Date July 11, 2007 US Nat'l Entry (if applicable)	Group Art Unit 1614

	CL	KROES A, et al. Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide. *Cancer Chemotherapy and Pharmacology* 1986; 17(2): 114-20.	
	CM	BARAK A. Vitamin B12 as a possible adjunct in prevention of methotrexate hepatotoxicity. *Biochemical Archives* 1985; 1(3): 139-42.	
	CN	HERBERT V. The inhibition and promotion of cancers by folic acid, vitamin B12, and their antagonists. ACS Symposium Series (1985); 277(Xenobiot. Metab.: Nutr. Eff.), 31-6.	
	CO		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2] See Kinds 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.

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/776,329	07/11/2007	

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	05/04/2009	(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))	* 23	Minus	** 20	= 3	X $ =		OR	X $52=	156
	Independent (37 CFR 1.16(h))	* 1	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	**156**

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		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:
/BRENDA MURPHY/

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 06/26/2009

BMURPHY SALE #00000002 Mailroom Dt: 05/04/2009 050840 11776329
01 FC : 1202 156.00 DA

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 09/08/2009

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	
ART UNIT	PAPER NUMBER
1614	

NOTIFICATION DATE	DELIVERY MODE
09/08/2009	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):

patents@lilly.com

Office Action Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

-- 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 *04 May 2009*.

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) *40-52* 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) *40-52* is/are rejected.

7)☐ Claim(s) _____ 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 *5-4-09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Claims 40-52 are presented for examination.

Applicants' amendment, response and information disclosure statement filed May 4, 2009 have been received and entered.

Accordingly, the rejection made under 35 USC 112, first paragraph (Written Description) as set forth in the previous Office action dated February 18, 2009 at pages 2-4 as applied to claim 45 is hereby withdrawn because the applicants amended claim 45 to recite the preferred folic-binding protein agent.

Accordingly, the rejection made under 35 USC 112, second paragraph as set forth in the previous Office action dated February 18, 2009 at page 4 as applied to claims 40-52 is hereby withdrawn because the applicants amended claim 40 by the insertion of –lowering agent--.

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.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

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).

Claims 40-52 are rejected under 35 U.S.C. 103(a) as being unpatentable over Taylor (5,344,932) of PTO-1449 in view of Tsao et al., "Influence of Cobalamin on the Survival of Mice Bearing Ascites Tumor", Pathobiology, Vol. 61, No. 2, pp. 104-108 (1993) of PTO-1449, further in view of Worzalla et al., Anticancer Research, Vol. 18, No. 5, pp. 3255-3239 of PTO-1449, and further in view of Cleare et al. (4,149,707).

Taylor teaches N-(pyrrolo(2,3-D)pyrimidin-3-ylacyl)-glutamic acid derivatives which includes LY 2315 (pemetrexe) and LY 231514-disodium (pemetrexed disodium) are effective an antineoplastic agents to inhibit the growth of tumors (see column 8, lines 57-63). Note particularly column 8, lines 64-68 states that other antineoplastic agents can be combined with LY 231514. Note particularly column 9, line 1 shows the various modes of administration such as parenteral routes (intramuscular) and oral.

The instant invention differs from the cited reference in that the cited reference does not teach the addition of a methylmalonic acid lowering agent. However, the

secondary reference, Tsao et al., teaches a methylmalonic acid lowering agent such as cobalamin (vitamin B_{12}) is effective as having antitumor activity (see the abstract).

The instant invention differs from the cited references in that the cited references do not teach the addition of a folic-binding-protein agent. However, the tertiary reference, Worzalla et al., teaches the supplementation of folic acid with LY 231513 to enhance LY 231514 antitumor activity.

The instant invention differs from the cited references in that the cited references do not teach the addition of cisplatin. However, the quaternary reference, Cleare et al., teaches malonato platinum anti-tumor compounds such as cisplatin to treat malignant tumors (see the abstract).

Clearly, one skilled in the art would have assumed the combination of three antineoplastic agents into a single composition would give an additive effect in the absence of evidence to the contrary.

The instant invention differ from the cited references in that the cited references do not teach the applicants' preferred dosage range for the methylmalonic acid lowering agent. However, those skilled in the art would have been readily optimized effective dosages and concurrent administration dosage forms as determined by good medical practice and the clinical condition of the individual patient. Regardless of the manner of administration, the specific dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned

formulations is routinely made by those skilled in the art and is within the ability of tasks routinely performed by them without undue experimentation.

Claims 40-52 are not allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KEVIN WEDDINGTON whose telephone number is (571)272-0587. The examiner can normally be reached on 12:30 pm - 9:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on (571)272-0718. 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.

KEVIN WEDDINGTON
Primary Examiner
Art Unit 1614

/KEVIN WEDDINGTON/
Primary Examiner, Art Unit 1614

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	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	02/11/2009	09/01/2009							
	1									
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	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	02/11/2009	09/01/2009							
	37									
	38									
	39									
	40	✓	✓							
	41	✓	✓							
	42	✓	✓							
	43	✓	✓							
	44	✓	✓							
	45	✓	✓							
	46	✓	✓							
	47	✓	✓							
	48	✓	✓							
	49	✓	✓							
	50	✓	✓							
	51	✓	✓							
	52	✓	✓							

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

SEARCHED

Class	Subclass	Date	Examiner
514	52	2/11/09	KEW
514	77	2/11/09	KEW
514	249	2/11/09	KEW
514	251	2/11/09	KEW
514	265.1	2/11/09	KEW

SEARCH NOTES

Search Notes	Date	Examiner
Consultation with parent applications, 10/297,821 and 11/288,807	2/11/09	KEW
EAST and PALM for Inventors' Names	2/11/09	KEW
CAS-ONLINE search with MEDLINE, CA and USPATALL	9/1/2009	KEW

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
5			

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* * * * * * * * * *   Welcome to STN International   * * * * * * * * * *

 NEWS  1           Web Page for STN Seminar Schedule - N. America
 NEWS  2  DEC 01   ChemPort single article sales feature unavailable
 NEWS  3  JUN 01   CAS REGISTRY Source of Registration (SR) searching
                   enhanced on STN
 NEWS  4  JUN 26   NUTRACEUT and PHARMAML no longer updated
 NEWS  5  JUN 29   IMSCOPROFILE now reloaded monthly
 NEWS  6  JUN 29   EPFULL adds Simultaneous Left and Right Truncation
                   (SLART) to AB, MCLM, and TI fields
 NEWS  7  JUL 09   PATDPAFULL adds Simultaneous Left and Right
                   Truncation (SLART) to AB, CLM, MCLM, and TI fields
 NEWS  8  JUL 14   USGENE enhances coverage of patent sequence location
                   (PSL) data
 NEWS  9  JUL 27   CA/CAplus enhanced with new citing references
 NEWS 10  JUL 16   GBFULL adds patent backfile data to 1855
 NEWS 11  JUL 21   USGENE adds bibliographic and sequence information
 NEWS 12  JUL 28   EPFULL adds first-page images and applicant-cited
                   references
 NEWS 13  JUL 28   INPADOCDB and INPAFAMDB add Russian legal status data
 NEWS 14  AUG 10   Time limit for inactive STN sessions doubles to 40
                   minutes
 NEWS 15  AUG 17   CAS REGISTRY, the Global Standard for Chemical
                   Research, Approaches 50 Millionth Registration
                   Milestone
 NEWS 16  AUG 18   COMPENDEX indexing changed for the Corporate Source
                   (CS) field
 NEWS 17  AUG 24   ENCOMPLIT/ENCOMPLIT2 reloaded and enhanced
 NEWS 18  AUG 24   CA/CAplus enhanced with legal status information for
                   U.S. patents

 NEWS EXPRESS MAY 26 09 CURRENT WINDOWS VERSION IS V8.4,
              AND CURRENT DISCOVER FILE IS DATED 06 APRIL 2009.

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FILE 'HOME' ENTERED AT 23:24:07 ON 31 AUG 2009

=> file reg
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       0.22       0.22

FILE 'REGISTRY' ENTERED AT 23:24:20 ON 31 AUG 2009
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DICTIONARY FILE UPDATES:  30 AUG 2009  HIGHEST RN 1178163-40-0
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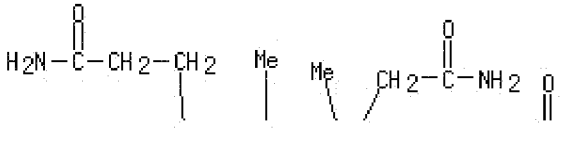
experimental property data in the original document. For information
on property searching in REGISTRY, refer to:

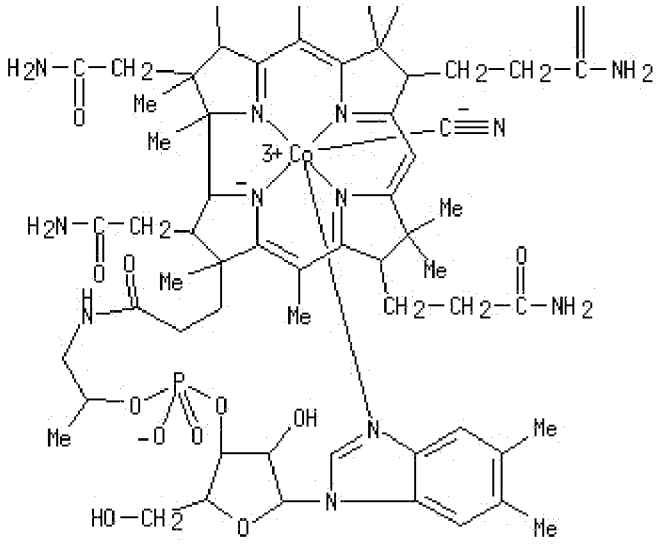
http://www.cas.org/support/stngen/stndoc/properties.html

```
=> e vitamin b12/cn
E1            1     VITAMIN B1-VITAMIN B2-VITAMIN PP COMPLEX/CN
E2            1     VITAMIN B1-VITAMIN C MIXT./CN
E3            1 --> VITAMIN B12/CN
E4            1     VITAMIN B12 (2-(METHYLTHIO)HYPOXANTHINE ANALOG)/CN
E5            1     VITAMIN B12 (BENZOTRIAZOLE ANALOG)/CN
E6            1     VITAMIN B12 5-HYDROXYBENZIMIDAZOLE ANALOG/CN
E7            1     VITAMIN B12 ABC TRANSPORT ATP-BINDING PROTEIN (SALMONELLA EN
                    TERICA TYPHI STRAIN CT18 GENE STY1768)/CN
E8            1     VITAMIN B12 ABC TRANSPORT ATP-BINDING PROTEIN (SALMONELLA EN
                    TERICA TYPHI STRAIN TY2 GENE BTUD)/CN
E9            1     VITAMIN B12 ABC TRANSPORTER, ATP-BINDING PROTEIN BTUD (PHOTO
                    BACTERIUM PROFUNDUM STRAIN SS9 GENE SF1522)/CN
E10           1     VITAMIN B12 ABC TRANSPORTER, ATP-BINDING PROTEIN BTUD (VIBRI
                    O CHOLERAE STRAIN N16961 GENE VC1245)/CN
E11           1     VITAMIN B12 ABC TRANSPORTER, ATP-BINDING PROTEIN BTUD (VIBRI
                    O PARAHAEMOLYTICUS STRAIN O3:K6 GENE VP1312)/CN
E12           1     VITAMIN B12 ABC TRANSPORTER, PERMEASE PROTEIN BTUC (PHOTOBAC
                    TERIUM PROFUNDUM STRAIN SS9 GENE SF1520)/CN

=> s e3
L1            1 "VITAMIN B12"/CN

=> d

L1   ANSWER 1 OF 1  REGISTRY  COPYRIGHT 2009 ACS on STN
RN   68-19-9  REGISTRY
ED   Entered STN:  16 Nov 1984
CN   Vitamin B12  (CA INDEX NAME)
OTHER NAMES:
CN   1H-Benzimidazole, 5,6-dimethyl-1-(3-O-phosphono-α-D-ribofuranosyl)-,
     monoester with cobinamide cyanide, inner salt
CN   5,6-Dimethylbenzimidazolyl cyanocobamide
CN   5,6-Dimethylbenzimidazolyl-Co-cyanocobamide
CN   Anacobin
CN   Antipernicin
CN   Apikobal
CN   B-Twelve
CN   B-Twelve Ora
CN   Bedodeka
CN   Bedoz
CN   Behepan
CN   Berubi
CN   Berubigen
CN   Betalin 12
CN   Betalin 12 Crystalline
CN   Betaline 12
CN   Betolvex
CN   Byladoce
CN   CN-B12
CN   Cobalamin, cyanide
CN   Cobalamin, cyano-
CN   Cobalamin, cyano-5,6-dimethylbenzimidazole-
CN   Cobalin
CN   Cobamide, α-5,6-dimethyl-1H-benzimidazolyl-, cyanide
CN   Cobamide, cyano-5,6-dimethyl-1H-benzimidazole-
CN   Cobamin
```

CN Cobinamide, cyanide, dihydrogen phosphate (ester), inner salt, 3'-ester with 5,6-dimethyl-1-α-D-ribofuranosyl-1H-benzimidazole
CN Cotel
CN Covit
CN Cromatonbic B12
CN Crystamin
CN Crystamine
CN Cyano-5,6-dimethylbenzimidazolylcobamide
CN Cyano-B12
CN Cyanocobalamin
CN Cyanocobalamine
CN Cycolamin
CN Cykobemin
CN Cykobeminet
CN Cyomin
CN Cyredin
CN Cytacon
CN Cytamen
CN Cytobion
CN Depinar
CN Dicopac Kit
CN Dobetin
CN Docemine
CN Docibin
CN Docigram
ADDITIONAL NAMES NOT AVAILABLE IN THIS FORMAT - Use FCN, FIDE, or ALL for DISPLAY
DR 8023-26-5, 8039-03-0, 11037-08-4, 24436-34-8
MF C63 H88 Co N14 O14 P
CI CCS, COM
LC STN Files: ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, AQUIRE, BEILSTEIN*, BIOSIS, BIOTECHNO, CA, CABA, CAPLUS, CASREACT, CBNB, CHEMCATS, CHEMINFORMRX, CHEMLIST, CIN, CSCHEM, CSNB, DDFU, DRUGU, EMBASE, HSDB*, IFICDB, IFIPAT, IFIUDB, IMSCOSEARCH, IPA, MEDLINE, MRCK*, MSDS-OHS, PHAR, PIRA, PROMT, PS, RTECS*, SPECINFO, SYNTHLINE, TOXCENTER, USAN, USPAT2, USPATFULL, USPATOLD, VETU
(*File contains numerically searchable property data)
Other Sources: DSL**, EINECS**, TSCA**, WHO
(**Enter CHEMLIST File for up-to-date regulatory information)



PAGE 2-A



PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

21671 REFERENCES IN FILE CA (1907 TO DATE)
401 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
21717 REFERENCES IN FILE CAPLUS (1907 TO DATE)

=> file medline

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	7.88	8.10

FILE 'MEDLINE' ENTERED AT 23:24:53 ON 31 AUG 2009

FILE LAST UPDATED: 29 Aug 2009 (20090829/UP). FILE COVERS 1949 TO DATE.

MEDLINE and LMEDLINE have been updated with the 2009 Medical Subject Headings (MeSH) vocabulary and tree numbers from the U.S. National Library of Medicine (NLM). Additional information is available at

http://www.nlm.nih.gov/pubs/techbull/nd08/nd08_medline_data_changes_2009.html.

On February 21, 2009, MEDLINE was reloaded. See HELP RLOAD for details.

This file contains CAS Registry Numbers for easy and accurate substance identification.

See HELP RANGE before carrying out any RANGE search.

```
=> s l1
L2       16339 L1

=> s (vitamin b12 or hydroxycobolamin or chlorocobolamin or aquocobolamin or cobolamin or azi
        150800 VITAMIN
         14280 B12
         11438 VITAMIN B12
                 (VITAMIN(W)B12)
             0 HYDROXYCOBOLAMIN
             0 CHLOROCOBOLAMIN
             0 AQUOCOBOLAMIN
             0 COBOLAMIN
             0 AZIDOCOBOLAMIN
L3       11438 (VITAMIN B12 OR HYDROXYCOBOLAMIN OR CHLOROCOBOLAMIN OR AQUOCOBOL
               AMIN OR COBOLAMIN OR AZIDOCOBOLAMIN)

=> s l2 or l3
L4       20105 L2 OR L3
```

```
=> s (cancer or anti-neoplast? or neoplast? or carcin? or tumor?)
        702915 CANCER
        766313 ANTI
        146280 NEOPLAST?
          1149 ANTI-NEOPLAST?
                 (ANTI(W)NEOPLAST?)
        146280 NEOPLAST?
        601058 CARCIN?
        980216 TUMOR?
L5     1707973 (CANCER OR ANTI-NEOPLAST? OR NEOPLAST? OR CARCIN? OR TUMOR?)

=> s l4 and l5
L6         773 L4 AND L5

=> s leukemia?
L7      212559 LEUKEMIA?

=> s l6 and l7
L8          66 L6 AND L7

=> d 1-66

L8   ANSWER 1 OF 66     MEDLINE on STN
Full Text
AN   2008123050     MEDLINE
DN   PubMed ID: 18280345
TI   CD4+ CD56+ hematodermic/plasmacytoid dendritic cell tumor with response
     to pralatrexate.
AU   Leitenberger Justin J; Berthelot Cindy N; Polder Kristel D; Pro Barbara;
     McLaughlin Peter; Jones Dan; Duvic Madeleine
CS   Department of Dermatology, The University of Texas MD Anderson Cancer
     Center, Houston, Texas 77030-4009, USA.
NC   CA16672 (United States NCI NIH HHS)
     K24-CA86815 (United States NCI NIH HHS)
SO   Journal of the American Academy of Dermatology, (2008 Mar) Vol. 58, No. 3,
     pp. 480-4.
     Journal code: 7907132. E-ISSN: 1097-6787.
CY   United States
DT   (CASE REPORTS)
     Journal; Article; (JOURNAL ARTICLE)
     (RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
     (RESEARCH SUPPORT, NON-U.S. GOV'T)
LA   English
FS   Priority Journals
EM   200803
ED   Entered STN: 20 Feb 2008
     Last Updated on STN: 15 Mar 2008
     Entered Medline: 14 Mar 2008

L8   ANSWER 2 OF 66     MEDLINE on STN
Full Text
AN   2007755529     MEDLINE
DN   PubMed ID: 18092842
TI   Generalized pruritus: a prospective study concerning etiology.
AU   Polat Muhterem; Oztas Pinar; Ilhan Mustafa N; Yalcin Basak; Alli Nuran
CS   1st Dermatology Department, Ankara Numune Education and Research Hospital,
     Ankara, Turkey.. drmuhterempolat@mynet.com
SO   American journal of clinical dermatology, (2008) Vol. 9, No. 1, pp. 39-44.
     Journal code: 100895290. ISSN: 1175-0561.
CY   New Zealand
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   200803
ED   Entered STN: 21 Dec 2007
     Last Updated on STN: 19 Mar 2008
     Entered Medline: 18 Mar 2008

L8   ANSWER 3 OF 66     MEDLINE on STN
Full Text
AN   2003557044     MEDLINE
DN   PubMed ID: 14636871
```

TI Significance of elevated cobalamin (**vitamin B12**) levels in blood.
AU Ermens A A M; Vlasveld L T; Lindemans J
CS Clinical Laboratory, Amphia Hospital, lokatie Langendijk, Breda, Netherlands.. aermens@amphia.nl
SO Clinical biochemistry, (2003 Nov) Vol. 36, No. 8, pp. 585-90. Ref: 42
Journal code: 0133660. ISSN: 0009-9120.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS Priority Journals
EM 200409
ED Entered STN: 26 Nov 2003
Last Updated on STN: 21 Sep 2004
Entered Medline: 17 Sep 2004

L8 ANSWER 4 OF 66 MEDLINE on STN
Full Text
AN 2003214619 MEDLINE
DN PubMed ID: 12735212
TI Erythropoietin and chronic lymphocytic **leukemia.**
AU Mauro Francesca R; Gentile Massimo; Foa Robin
CS Dipartimento di Biotecnologie Cellulari ed Ematologia, University La Sapienza, Rome, Italy.
SO Reviews in clinical and experimental hematology, (2002) Vol. Suppl 1, pp. 21-31. Ref: 58
Journal code: 9815344. ISSN: 1127-0020.
CY Italy
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS Priority Journals
EM 200307
ED Entered STN: 9 May 2003
Last Updated on STN: 13 Jul 2003
Entered Medline: 11 Jul 2003

L8 ANSWER 5 OF 66 MEDLINE on STN
Full Text
AN 2002390475 MEDLINE
DN PubMed ID: 12138901
TI A case of acute myeloid **leukemia** with t(7;11)(p15;p15) mimicking myeloid crisis of chronic myelogenous **leukemia.**
AU Kawakami Keiki; Miyanishi Setsuko; Nishii Kazuhiho; Usui Eiji; Murata Tetsuya; Shinsato Isaku; Shiku Hiroshi
CS Division of Hematology, Suzuka General Hospital, Mie, Japan.. Kawakei@cocoa.ocn.ne.jp
SO International journal of hematology, (2002 Jul) Vol. 76, No. 1, pp. 80-3.
Journal code: 9111627. ISSN: 0925-5710.
CY Ireland
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200209
ED Entered STN: 26 Jul 2002
Last Updated on STN: 14 Sep 2002
Entered Medline: 13 Sep 2002

L8 ANSWER 6 OF 66 MEDLINE on STN
Full Text
AN 2002181127 MEDLINE
DN PubMed ID: 11913109
TI [The significance of an elevated cobalamin concentration in the blood]. De betekenis van een te hoge cobalamineconcentratie in het bloed.
AU Ermens A A M; Vlasveld L Th; van Marion-Kievit J A; Lensen C J P A; Lindemans J
CS Amphia Ziekenhuis, Klinisch-Chemisch en Hematologisch Laboratorium, locatie Langendijk, Langendijk 75, 4819 EV Breda.
SO Nederlands tijdschrift voor geneeskunde, (2002 Mar 9) Vol. 146, No. 10, pp. 459-64.
Journal code: 0400770. ISSN: 0028-2162.

CY Netherlands
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Dutch
FS Priority Journals
EM 200207
ED Entered STN: 1 Apr 2002
Last Updated on STN: 12 Jul 2002
Entered Medline: 10 Jul 2002

L8 ANSWER 7 OF 66 MEDLINE on STN
Full Text
AN 2000188210 MEDLINE
DN PubMed ID: 10723243
TI Rapidly progressive, refractory eosinophilia with a 250,000/microliter eosinophil count.
AU Noguchi M; Okumura K; Kato A; Hirano T; Oshimi K
CS Department of Hematology, Juntendo University School of Medicine.
SO [Rinsho ketsueki] The Japanese journal of clinical hematology, (2000 Feb) Vol. 41, No. 2, pp. 135-9.
Journal code: 2984782R. ISSN: 0485-1439.
CY Japan
DT (CASE REPORTS)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Japanese
FS Priority Journals
EM 200005
ED Entered STN: 18 May 2000
Last Updated on STN: 18 May 2000
Entered Medline: 5 May 2000

L8 ANSWER 8 OF 66 MEDLINE on STN
Full Text
AN 1998291239 MEDLINE
DN PubMed ID: 9627769
TI Cobalamin metabolism in methionine-dependent human tumour and **leukemia** cell lines.
AU Watkins D
CS Department of Medicine, McGill University, Montreal, Que.
SO Clinical and investigative medicine. Medecine clinique et experimentale, (1998 Jun) Vol. 21, No. 3, pp. 151-8.
Journal code: 7804071. ISSN: 0147-958X.
CY Canada
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 199808
ED Entered STN: 3 Sep 1998
Last Updated on STN: 3 Sep 1998
Entered Medline: 27 Aug 1998

L8 ANSWER 9 OF 66 MEDLINE on STN
Full Text
AN 1998287116 MEDLINE
DN PubMed ID: 9625434
TI Synthesis, characterization and nitric oxide release profile of nitrosylcobalamin: a potential chemotherapeutic agent.
AU Bauer J A
CS Department of Chemistry, University of Akron, OH 44325-3601, USA.
SO Anti-cancer drugs, (1998 Mar) Vol. 9, No. 3, pp. 239-44.
Journal code: 9100823. ISSN: 0959-4973.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199807
ED Entered STN: 11 Aug 1998
Last Updated on STN: 11 Aug 1998
Entered Medline: 29 Jul 1998

L8 ANSWER 10 OF 66 MEDLINE on STN
Full Text
AN 1997450846 MEDLINE
DN PubMed ID: 9307287
TI Cobalamin analogues modulate the growth of **leukemia** cells in vitro.
AU McLean G R; Pathare P M; Wilbur D S; Morgan A C; Woodhouse C S; Schrader J W; Ziltener H J
CS The Biomedical Research Centre, University of British Columbia, Vancouver, Canada.
SO Cancer research, (1997 Sep 15) Vol. 57, No. 18, pp. 4015-22.
Journal code: 2984705R. ISSN: 0008-5472.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 199710
ED Entered STN: 5 Nov 1997
Last Updated on STN: 5 Nov 1997
Entered Medline: 20 Oct 1997

L8 ANSWER 11 OF 66 MEDLINE on STN
Full Text
AN 1997132938 MEDLINE
DN PubMed ID: 8978297
TI Antibodies to transcobalamin II block in vitro proliferation of leukemic cells.
AU McLean G R; Quadros E V; Rothenberg S P; Morgan A C; Schrader J W; Ziltener H J
CS Biomedical Research Centre, University of British Columbia, Vancouver, Canada.
NC R01-DK28561-14 (United States NIDDK NIH HHS)
SO Blood, (1997 Jan 1) Vol. 89, No. 1, pp. 235-42.
Journal code: 7603509. ISSN: 0006-4971.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 199701
ED Entered STN: 19 Feb 1997
Last Updated on STN: 19 Feb 1997
Entered Medline: 27 Jan 1997

L8 ANSWER 12 OF 66 MEDLINE on STN
Full Text
AN 1994083898 MEDLINE
DN PubMed ID: 8260900
TI Induction of differentiation of myeloid leukemic cells by busulphan: in vivo and in vitro observations.
AU Michaeli J; Fibach E; Rachmilewitz E A
CS Department of Hematology, Hadassah University Hospital, Jerusalem, Israel.
SO Leukemia & lymphoma, (1993 Oct) Vol. 11, No. 3-4, pp. 287-91.
Journal code: 9007422. ISSN: 1042-8194.
CY Switzerland
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199401
ED Entered STN: 9 Feb 1994
Last Updated on STN: 3 Feb 1997
Entered Medline: 25 Jan 1994

L8 ANSWER 13 OF 66 MEDLINE on STN
Full Text
AN 1994030584 MEDLINE
DN PubMed ID: 8216825
TI Influence of cobalamin on the survival of mice bearing ascites **tumor**.
AU Tsao C S; Myashita K
CS Linus Pauling Institute of Science and Medicine, Palo Alto, Calif. 94306.

SO Pathobiology : journal of immunopathology, molecular and cellular biology, (1993) Vol. 61, No. 2, pp. 104-8.
Journal code: 9007504. ISSN: 1015-2008.
CY Switzerland
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 199312
ED Entered STN: 17 Jan 1994
Last Updated on STN: 17 Jan 1994
Entered Medline: 17 Dec 1993

L8 ANSWER 14 OF 66 MEDLINE on STN
Full Text
AN 1993231290 MEDLINE
DN PubMed ID: 8472808
TI Misincorporation of uracil into the DNA of folate- and B12-deficient HL60 cells.
AU Wickramasinghe S N; Fida S
CS Dept. of Haematology, St. Mary's Hospital Medical School, Imperial College of Science, Technology & Medicine, London, U.K.
SO European journal of haematology, (1993 Mar) Vol. 50, No. 3, pp. 127-32.
Journal code: 8703985. ISSN: 0902-4441.
CY Denmark
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199305
ED Entered STN: 4 Jun 1993
Last Updated on STN: 3 Feb 1997
Entered Medline: 20 May 1993

L8 ANSWER 15 OF 66 MEDLINE on STN
Full Text
AN 1993043071 MEDLINE
DN PubMed ID: 1421179
TI Effects of cobalamin, cobalamin analogues and cobalamin binding proteins on P388D1 mouse leukemic cells in culture.
AU Kondo H; Iseki T; Goto S; Ohto M; Okuda K
CS Department of Medicine, Shimizu Kousei Hospital, Shizuoka, Japan.
SO International journal of hematology, (1992 Oct) Vol. 56, No. 2, pp. 167-77.
Journal code: 9111627. ISSN: 0925-5710.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 199212
ED Entered STN: 22 Jan 1993
Last Updated on STN: 3 Feb 1997
Entered Medline: 4 Dec 1992

L8 ANSWER 16 OF 66 MEDLINE on STN
Full Text
AN 1992292362 MEDLINE
DN PubMed ID: 1602609
TI Atypical **leukemia** accompanied by **vitamin B12** deficiency.
AU Tsukamoto N; Inose K; Matsushima T; Uchiyama T; Sugita Y; Takeuchi T; Sato S; Omine M; Naruse T
CS Division of Internal Medicine, Takasaki National Hospital.
SO [Rinsho ketsueki] The Japanese journal of clinical hematology, (1992 Apr) Vol. 33, No. 4, pp. 461-6.
Journal code: 2984782R. ISSN: 0485-1439.
CY Japan
DT (CASE REPORTS)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA Japanese

FS Priority Journals
EM 199207
ED Entered STN: 24 Jul 1992
Last Updated on STN: 24 Jul 1992
Entered Medline: 14 Jul 1992

L8 ANSWER 17 OF 66 MEDLINE on STN
Full Text
AN 1992159815 MEDLINE
DN PubMed ID: 2133609
TI [Chronic lymphocytic **leukemia** complicated by pernicious anemia during long-term remission].
Hronicna limfocitna leukemija komplikovana pojavom perniciozne anemije u toku dugotrajne remisije.
AU Ruvidic R; Boskovic D
CS Institute of Hematology, University Clinical Centre, Belgrade.
SO Srpski arhiv za celokupno lekarstvo, (1990 Nov-Dec) Vol. 118, No. 11-12, pp. 495-7.
Journal code: 0027440. ISSN: 0370-8179.
CY Yugoslavia
DT (CASE REPORTS)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Serbian
FS Priority Journals
EM 199203
ED Entered STN: 10 Apr 1992
Last Updated on STN: 10 Apr 1992
Entered Medline: 25 Mar 1992

L8 ANSWER 18 OF 66 MEDLINE on STN
Full Text
AN 1992074415 MEDLINE
DN PubMed ID: 1962580
TI Effect of combined ascorbic acid and B-12 on survival of mice with implanted Ehrlich **carcinoma** and L1210 **leukemia**.
AU Poydock M E
CS Cancer Research Institute, Mercyhurst College, Erie, PA 16546.
SO The American journal of clinical nutrition, (1991 Dec) Vol. 54, No. 6 Suppl, pp. 1261S-1265S.
Journal code: 0376027. ISSN: 0002-9165.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 199201
ED Entered STN: 24 Jan 1992
Last Updated on STN: 24 Jan 1992
Entered Medline: 6 Jan 1992

L8 ANSWER 19 OF 66 MEDLINE on STN
Full Text
AN 1991203220 MEDLINE
DN PubMed ID: 2016907
TI Effect of nitrous oxide and methotrexate on folate coenzyme pools of blast cells from **leukemia** patients.
AU Ermens A A; Schoester M; Lindemans J; Abels J
CS Institute of Hematology, Erasmus University, Rotterdam, The Netherlands.
SO Leukemia research, (1991) Vol. 15, No. 2-3, pp. 165-71.
Journal code: 7706787. ISSN: 0145-2126.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199105
ED Entered STN: 7 Jun 1991
Last Updated on STN: 6 Feb 1998
Entered Medline: 17 May 1991

L8 ANSWER 20 OF 66 MEDLINE on STN
Full Text
AN 1991166723 MEDLINE

DN PubMed ID: 2076192
TI Cytotoxic activity of cobalamin in cultured malignant and nonmalignant cells.
AU Tsao C S; Miyashita K; Young M
CS Linus Pauling Institute of Science and Medicine, Palo Alto, Calif.
SO Pathobiology : journal of immunopathology, molecular and cellular biology, (1990) Vol. 58, No. 5, pp. 292-6.
Journal code: 9007504. ISSN: 1015-2008.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 199104
ED Entered STN: 12 May 1991
Last Updated on STN: 3 Feb 1997
Entered Medline: 25 Apr 1991

L8 ANSWER 21 OF 66 MEDLINE on STN
Full Text
AN 1991136708 MEDLINE
DN PubMed ID: 2285461
TI [Peripheral pancytopenia].
Pancitopenia periferica.
AU Bello-Gonzalez S A; Berges-Garcia A
CS Depto. de Investigaciones Hematologicas, Hospital Infantil de Mexico Federico Gomez, Mexico, D.F.
SO Boletin medico del Hospital Infantil de Mexico, (1990 Nov) Vol. 47, No. 11, pp. 737-45. Ref: 82
Journal code: 0414106. ISSN: 0539-6115.
CY Mexico
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA Spanish
FS Priority Journals
EM 199103
ED Entered STN: 12 Apr 1991
Last Updated on STN: 12 Apr 1991
Entered Medline: 28 Mar 1991

L8 ANSWER 22 OF 66 MEDLINE on STN
Full Text
AN 1991028218 MEDLINE
DN PubMed ID: 2171697
TI [Active transport of cobalamins in leukemic cells of L-1210 mice].
Aktivnyi transport kobalaminov v leikemicheskie kletki myshei L-1210.
AU Oreshkin A E; Miasishcheva N V
SO Biulleten' eksperimental'noi biologii i meditsiny, (1990 Jul) Vol. 110, No. 7, pp. 85-7.
Journal code: 0370627. ISSN: 0365-9615.
CY USSR
DT (COMPARATIVE STUDY)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Russian
FS Priority Journals
EM 199012
ED Entered STN: 8 Feb 1991
Last Updated on STN: 3 Feb 1997
Entered Medline: 10 Dec 1990

L8 ANSWER 23 OF 66 MEDLINE on STN
Full Text
AN 1991002892 MEDLINE
DN PubMed ID: 2169922
TI Expression of transcobalamin II receptors by human **leukemia** K562 and HL-60 cells.
AU Amagasaki T; Green R; Jacobsen D W
CS Department of Laboratory Hematology, Cleveland Clinic Foundation, OH 44195-5139.
NC DK35265 (United States NIDDK NIH HHS)

SO Blood, (1990 Oct 1) Vol. 76, No. 7, pp. 1380-6.
Journal code: 7603509. ISSN: 0006-4971.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 199011
ED Entered STN: 17 Jan 1991
Last Updated on STN: 17 Jan 1991
Entered Medline: 6 Nov 1990

L8 ANSWER 24 OF 66 MEDLINE on STN
Full Text
AN 1990266154 MEDLINE
DN PubMed ID: 2189194
TI Nitrous oxide: a cause of **cancer** or chemotherapeutic adjuvant?.
AU Koblin D D
CS Department of Anesthesia, Veterans Administration Medical Center, San Francisco, CA 94121.
NC P01 AG3104 (United States NIA NIH HHS)
SO Seminars in surgical oncology, (1990) Vol. 6, No. 3, pp. 141-7. Ref: 56
Journal code: 8503713. ISSN: 8756-0437.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
General Review; (REVIEW)
LA English
FS Priority Journals
EM 199006
ED Entered STN: 10 Aug 1990
Last Updated on STN: 10 Aug 1990
Entered Medline: 29 Jun 1990

L8 ANSWER 25 OF 66 MEDLINE on STN
Full Text
AN 1990070919 MEDLINE
DN PubMed ID: 2588735
TI [Disorders of intestinal absorption in patients treated with cytostatic chemotherapy].
Storungen der intestinalen Resorption bei Patienten unter zytostatischer Chemotherapie.
AU Hurter T; Reis H E; Borchard F
CS Medizinische Klinik I an den Medizinischen Einrichtungen der RWTH Aachen.
SO Zeitschrift fur Gastroenterologie, (1989 Oct) Vol. 27, No. 10, pp. 606-10.
Journal code: 0033370. ISSN: 0044-2771.
CY GERMANY, WEST: Germany, Federal Republic of
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA German
FS Priority Journals
EM 199001
ED Entered STN: 28 Mar 1990
Last Updated on STN: 28 Mar 1990
Entered Medline: 4 Jan 1990

L8 ANSWER 26 OF 66 MEDLINE on STN
Full Text
AN 1990032992 MEDLINE
DN PubMed ID: 2553457
TI Uptake of transcobalamin II-bound cobalamin by HL-60 cells: effects of differentiation induction.
AU Lindemans J; Kroes A C; van Geel J; van Kapel J; Schoester M; Abels J
CS Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
SO Experimental cell research, (1989 Oct) Vol. 184, No. 2, pp. 449-60.
Journal code: 0373226. ISSN: 0014-4827.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English

FS Priority Journals
EM 198912
ED Entered STN: 28 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 1 Dec 1989

L8 ANSWER 27 OF 66 MEDLINE on STN
Full Text
AN 1989336663 MEDLINE
DN PubMed ID: 2758400
TI Spontaneous chromosome fragility in band 3q21, 11p11, or 11q13 of cultured bone marrow cells from two patients with hematologic disorders.
AU Abe S; Nishida-Umehara C; Tamura T; Mikuni C; Sasaki M
CS Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo, Japan.
SO Cancer genetics and cytogenetics, (1989 Jul 1) Vol. 40, No. 1, pp. 47-53.
Journal code: 7909240. ISSN: 0165-4608.
CY United States
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198909
ED Entered STN: 9 Mar 1990
Last Updated on STN: 29 Jan 1999
Entered Medline: 20 Sep 1989

L8 ANSWER 28 OF 66 MEDLINE on STN
Full Text
AN 1989276217 MEDLINE
DN PubMed ID: 2543552
TI Detection and characteristics of DNA polymerase activity in serum from patients with malignant, viral, or B12-deficiency disease.
AU Neumuller M; Kallander C F; Gronowitz J S
CS Department of Medical Virology, Biomedical Center, Uppsala University, Sweden.
SO Enzyme, (1989) Vol. 41, No. 1, pp. 6-16.
Journal code: 1262265. ISSN: 0013-9432.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198907
ED Entered STN: 9 Mar 1990
Last Updated on STN: 6 Feb 1998
Entered Medline: 27 Jul 1989

L8 ANSWER 29 OF 66 MEDLINE on STN
Full Text
AN 1989275033 MEDLINE
DN PubMed ID: 2731156
TI Nitrous oxide selectively reduces the proliferation of the malignant cells in experimental rat **leukemia**.
AU Ermens A A; Vink N; Schoester M; van Lom K; Lindemans J; Abels J
CS Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
SO Cancer letters, (1989 May) Vol. 45, No. 2, pp. 123-8.
Journal code: 7600053. ISSN: 0304-3835.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198907
ED Entered STN: 9 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 20 Jul 1989

L8 ANSWER 30 OF 66 MEDLINE on STN
Full Text
AN 1989111624 MEDLINE
DN PubMed ID: 3216671

TI Effect of cobalamin inactivation on folate metabolism of leukemic cells.
AU Ermens A A; Kroes A C; Schoester M; van Lom K; Lindemans J; Abels J
CS Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
SO Leukemia research, (1988) Vol. 12, No. 11-12, pp. 905-10.
Journal code: 7706787. ISSN: 0145-2126.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198903
ED Entered STN: 8 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 1 Mar 1989

L8 ANSWER 31 OF 66 MEDLINE on STN
Full Text
AN 1986321824 MEDLINE
DN PubMed ID: 3752954
TI Effects of 5-fluorouracil treatment of rat **leukemia** with concomitant inactivation of cobalamin.
AU Kroes A C; Ermens A A; Lindemans J; Abels J
SO Anticancer research, (1986 Jul-Aug) Vol. 6, No. 4, pp. 737-42.
Journal code: 8102988. ISSN: 0250-7005.
CY Greece
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198610
ED Entered STN: 21 Mar 1990
Last Updated on STN: 21 Mar 1990
Entered Medline: 10 Oct 1986

L8 ANSWER 32 OF 66 MEDLINE on STN
Full Text
AN 1986247319 MEDLINE
DN PubMed ID: 3720639
TI [Kinetics of 57Co-cyanocobalamin distribution in the organs and tissues of mice with transplanted **tumors**].
Kinetika raspredeleniia 57Co-tsianokobalamina v organakh i tkaniakh myshei s perevivaemymi opukholiami.
AU Vares Iu V; Miasishcheva N V
SO Eksperimental nai a onkologii a, (1986) Vol. 8, No. 3, pp. 33-6.
Journal code: 8406659. ISSN: 0204-3564.
CY USSR
DT (COMPARATIVE STUDY)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Russian
FS Priority Journals
EM 198608
ED Entered STN: 21 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 14 Aug 1986

L8 ANSWER 33 OF 66 MEDLINE on STN
Full Text
AN 1986217806 MEDLINE
DN PubMed ID: 3458528
TI Factors influencing leukemic transformation in refractory anemias with excess of blasts, with ringed sideroblasts, and without ringed sideroblasts.
AU Oguma S; Yoshida Y; Uchino H; Maekawa T
SO Cancer research, (1986 Jul) Vol. 46, No. 7, pp. 3698-700.
Journal code: 2984705R. ISSN: 0008-5472.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198607
ED Entered STN: 21 Mar 1990

Last Updated on STN: 21 Mar 1990
Entered Medline: 23 Jul 1986

L8 ANSWER 34 OF 66 MEDLINE on STN
Full Text
AN 1986022753 MEDLINE
DN PubMed ID: 4050746
TI Mitogenic inhibition and effect on survival of mice bearing L1210 **leukemia** using a combination of dehydroascorbic acid and hydroxycobalamin.
AU Poydock M E; Harguindey S; Hart T; Takita H; Kelly D
SO American journal of clinical oncology, (1985 Jun) Vol. 8, No. 3, pp. 266-9.
Journal code: 8207754. ISSN: 0277-3732.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198511
ED Entered STN: 21 Mar 1990
Last Updated on STN: 21 Mar 1990
Entered Medline: 14 Nov 1985

L8 ANSWER 35 OF 66 MEDLINE on STN
Full Text
AN 1984280758 MEDLINE
DN PubMed ID: 6590092
TI Acute myelogenous leukaemia modulated by B12 deficiency: a case with bone marrow blast cell assay corroboration.
AU Ahmann F R; Durie B G
SO British journal of haematology, (1984 Sep) Vol. 58, No. 1, pp. 91-4.
Journal code: 0372544. ISSN: 0007-1048.
CY ENGLAND: United Kingdom
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198410
ED Entered STN: 20 Mar 1990
Last Updated on STN: 20 Mar 1990
Entered Medline: 24 Oct 1984

L8 ANSWER 36 OF 66 MEDLINE on STN
Full Text
AN 1984228545 MEDLINE
DN PubMed ID: 6731467
TI Unusual case of acute **leukemia**. Coexisting acute **leukemia** and pernicious anemia.
AU Vogelsang G B; Spivak J L
SO The American journal of medicine, (1984 Jun) Vol. 76, No. 6, pp. 1144-50.
Journal code: 0267200. ISSN: 0002-9343.
CY United States
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 198407
ED Entered STN: 20 Mar 1990
Last Updated on STN: 20 Mar 1990
Entered Medline: 17 Jul 1984

L8 ANSWER 37 OF 66 MEDLINE on STN
Full Text
AN 1984196444 MEDLINE
DN PubMed ID: 6326284
TI [Changes in the mean corpuscular volume during the cytotoxic treatment of **cancer** and risk of secondary **leukemia**. Preliminary results].
L'evolution du volume globulaire moyen pendant le traitement cytotoxique des cancers et le risque de leucemie secondaire. Resultats preliminaires.
AU de Gramont A; Rioux E; Drolet Y; Barry A; Delage J M
SO La semaine des hopitaux : organe fonde par l'Association d'enseignement

medical des hopitaux de Paris, (1984 Mar 29) Vol. 60, No. 14, pp. 961-6.
Journal code: 9410059.
CY France
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA French
FS Priority Journals
EM 198405
ED Entered STN: 19 Mar 1990
Last Updated on STN: 19 Mar 1990
Entered Medline: 30 May 1984

L8 ANSWER 38 OF 66 MEDLINE on STN
Full Text
AN 1982264737 MEDLINE
DN PubMed ID: 7107216
TI Production of transcobalamin II by various murine and human cells in culture.
AU Rabinowitz R; Rachmilewitz B; Rachmilewitz M; Schlesinger M
SO Israel journal of medical sciences, (1982 Jul) Vol. 18, No. 7, pp. 740-5.
Journal code: 0013105. ISSN: 0021-2180.
CY Israel
DT (COMPARATIVE STUDY)
(IN VITRO)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198210
ED Entered STN: 17 Mar 1990
Last Updated on STN: 17 Mar 1990
Entered Medline: 29 Oct 1982

L8 ANSWER 39 OF 66 MEDLINE on STN
Full Text
AN 1982187527 MEDLINE
DN PubMed ID: 7075860
TI Influence of vitamins C and B12 on the survival rate of mice bearing ascites **tumor**.
AU Poydock M E; Reikert D; Rice J
SO Experimental cell biology, (1982) Vol. 50, No. 2, pp. 88-91.
Journal code: 7701827. ISSN: 0304-3568.
CY Switzerland
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS Priority Journals
EM 198207
ED Entered STN: 17 Mar 1990
Last Updated on STN: 17 Mar 1990
Entered Medline: 8 Jul 1982

L8 ANSWER 40 OF 66 MEDLINE on STN
Full Text
AN 1981018502 MEDLINE
DN PubMed ID: 6932166
TI Erythremia with special reference to sideroblastic anemia.
AU Taki T; Wakabayashi T; Kishimoto H
SO Acta pathologica japonica, (1980 Jul) Vol. 30, No. 4, pp. 565-78.
Journal code: 0372637. ISSN: 0001-6632.
CY Japan
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198011
ED Entered STN: 16 Mar 1990
Last Updated on STN: 16 Mar 1990
Entered Medline: 24 Nov 1980

L8 ANSWER 41 OF 66 MEDLINE on STN

Full Text
AN 1978172794 MEDLINE
DN PubMed ID: 274499
TI The identification and measurement of a folate-binding protein in human serum by radioimmunoassay.
AU da Costa M; Rothenberg S P; Fischer C; Rosenberg Z
SO The Journal of laboratory and clinical medicine, (1978 Jun) Vol. 91, No. 6, pp. 901-7.
Journal code: 0375375. ISSN: 0022-2143.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197807
ED Entered STN: 14 Mar 1990
Last Updated on STN: 14 Mar 1990
Entered Medline: 26 Jul 1978

L8 ANSWER 42 OF 66 MEDLINE on STN
Full Text
AN 1978142124 MEDLINE
DN PubMed ID: 416709
TI **Vitamin B12**-binding proteins in serum and plasma in various disorders. Effect of anticoagulants.
AU Carmel R
SO American journal of clinical pathology, (1978 Mar) Vol. 69, No. 3, pp. 319-25.
Journal code: 0370470. ISSN: 0002-9173.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197805
ED Entered STN: 14 Mar 1990
Last Updated on STN: 14 Mar 1990
Entered Medline: 17 May 1978

L8 ANSWER 43 OF 66 MEDLINE on STN
Full Text
AN 1978117789 MEDLINE
DN PubMed ID: 607423
TI **Vitamin B12** and **vitamin B12** binding proteins in liver diseases.
AU Areekul S; Panatampon P; Doungbarn J
SO The Southeast Asian journal of tropical medicine and public health, (1977 Sep) Vol. 8, No. 3, pp. 322-8.
Journal code: 0266303. ISSN: 0125-1562.
CY Thailand
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197804
ED Entered STN: 14 Mar 1990
Last Updated on STN: 14 Mar 1990
Entered Medline: 26 Apr 1978

L8 ANSWER 44 OF 66 MEDLINE on STN
Full Text
AN 1978076371 MEDLINE
DN PubMed ID: 339530
TI [Analysis of the cobalamin coenzymes in mouse splenic **tumor** cells]. Analiz kobalaminovykh kofermentov v opukholevykh kletkakh selezenki myshei.
AU Vares Iu V; Miasishcheva N V
SO Voprosy medit sinskoi khimii, (1977 Sep-Oct) Vol. 23, No. 5, pp. 681-4.
Journal code: 0416601. ISSN: 0042-8809.
CY USSR
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Russian
FS Priority Journals
EM 197802

ED Entered STN: 14 Mar 1990
Last Updated on STN: 14 Mar 1990
Entered Medline: 23 Feb 1978

L8 ANSWER 45 OF 66 MEDLINE on STN
Full Text
AN 1977131707 MEDLINE
DN PubMed ID: 265135
TI Hemoglobin A2 levels in health and various hematologic disorders.
AU Alperin J B; Dow P A; Petteway M B
SO American journal of clinical pathology, (1977 Mar) Vol. 67, No. 3, pp. 219-26.
Journal code: 0370470. ISSN: 0002-9173.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197704
ED Entered STN: 13 Mar 1990
Last Updated on STN: 13 Mar 1990
Entered Medline: 30 Apr 1977

L8 ANSWER 46 OF 66 MEDLINE on STN
Full Text
AN 1977080713 MEDLINE
DN PubMed ID: 1006164
TI Pernicious anaemia and lymphoproliferative disease.
AU Parker A C; Bennett M
SO Scandinavian journal of haematology, (1976 Nov) Vol. 17, No. 5, pp. 395-7.
Journal code: 0404507. ISSN: 0036-553X.
CY Denmark
DT (CASE REPORTS)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197702
ED Entered STN: 13 Mar 1990
Last Updated on STN: 13 Mar 1990
Entered Medline: 24 Feb 1977

L8 ANSWER 47 OF 66 MEDLINE on STN
Full Text
AN 1977019051 MEDLINE
DN PubMed ID: 9787
TI B12 -- dependent methionine synthetase as a potential target for **cancer** chemotherapy.
AU Huennekens F M; DiGirolamo P M; Fujii K; Jacobsen D W; Vitols K S
SO Advances in enzyme regulation, (1976) Vol. 14, pp. 187-205. Ref: 51
Journal code: 0044263. ISSN: 0065-2571.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
General Review; (REVIEW)
LA English
FS Priority Journals
EM 197611
ED Entered STN: 13 Mar 1990
Last Updated on STN: 6 Feb 1998
Entered Medline: 21 Nov 1976

L8 ANSWER 48 OF 66 MEDLINE on STN
Full Text
AN 1976244023 MEDLINE
DN PubMed ID: 951181
TI [Acute or subacute myelofibrosis].
Les myelofibroses aigues ou subaigues.
AU Briere J; Castro-Malaspina H; Briere J F; Bernard J
SO Nouvelle revue francaise d'hematologie, (1976 Jun) Vol. 16, No. 1, pp. 3-22.
Journal code: 7909092.
CY France

DT (CASE REPORTS)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA French
FS Priority Journals
EM 197610
ED Entered STN: 13 Mar 1990
Last Updated on STN: 13 Mar 1990
Entered Medline: 2 Oct 1976

L8 ANSWER 49 OF 66 MEDLINE on STN
Full Text
AN 1976080662 MEDLINE
DN PubMed ID: 812175
TI Granulocyte release of **vitamin B12**-binders in vivo and in vitro in leukaemia and non-**neoplastic** leucocytosis.
AU Gullberg R; Riezenstein P
SO Scandinavian journal of haematology, (1975 Dec) Vol. 15, No. 5, pp. 377-83.
Journal code: 0404507. ISSN: 0036-553X.
CY Denmark
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197603
ED Entered STN: 13 Mar 1990
Last Updated on STN: 13 Mar 1990
Entered Medline: 1 Mar 1976

L8 ANSWER 50 OF 66 MEDLINE on STN
Full Text
AN 1976078390 MEDLINE
DN PubMed ID: 1081693
TI New approach to antifolate treatment of certain cancers as demonstrated in tissue culture.
AU Halpern R M; Halpern B C; Clark B R; Ashe H; Hardy D N; Jenkinson P Y; Chou S C; Smith R A
SO Proceedings of the National Academy of Sciences of the United States of America, (1975 Oct) Vol. 72, No. 10, pp. 4018-22.
Journal code: 7505876. ISSN: 0027-8424.
Report No.: NLM-PMC433129.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197603
ED Entered STN: 13 Mar 1990
Last Updated on STN: 13 Mar 1990
Entered Medline: 1 Mar 1976

L8 ANSWER 51 OF 66 MEDLINE on STN
Full Text
AN 1976024988 MEDLINE
DN PubMed ID: 1176445
TI Human plasma R-type **vitamin B12**-binding proteins. II. The role of transcobalamin I, transcobalamin III, and the normal granulocyte **vitamin B12**-binding protein in the plasma transport of **vitamin B12**.
AU Burger R L; Schneider R J; Mehlman C S; Allen R H
SO The Journal of biological chemistry, (1975 Oct 10) Vol. 250, No. 19, pp. 7707-13.
Journal code: 2985121R. ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Priority Journals
EM 197512
ED Entered STN: 13 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 23 Dec 1975

L8 ANSWER 52 OF 66 MEDLINE on STN

Full Text
AN 1976018381 MEDLINE
DN PubMed ID: 1164397
TI Differentiation of Friend virus-induced **leukemia** cells.
AU Sugano H; Kawaguchi T; Furusawa M; Ikawa Y
SO Bibliotheca haematologica, (1975) No. 40, pp. 221-8.
Journal code: 0372513. ISSN: 0067-7957.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197512
ED Entered STN: 13 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 4 Dec 1975

L8 ANSWER 53 OF 66 MEDLINE on STN
Full Text
AN 1975083933 MEDLINE
DN PubMed ID: 4445153
TI Delivery of 57Co B12 to lymphoblasts derived from mice with transplanted 1210 ascites **tumor** cells by transcobalamins I, II, and III.
AU Meyer L M; Gams R A; Ryel E M; Miller I E; Kumar S
SO Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), (1974 Dec) Vol. 147, No. 3, pp. 679-80.
Journal code: 7505892. ISSN: 0037-9727.
CY United States
DT (IN VITRO)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS Priority Journals
EM 197503
ED Entered STN: 10 Mar 1990
Last Updated on STN: 10 Mar 1990
Entered Medline: 26 Mar 1975

L8 ANSWER 54 OF 66 MEDLINE on STN
Full Text
AN 1975082263 MEDLINE
DN PubMed ID: 1053806
TI Extreme elevation of serum transcobalamin I in patients with metastatic **cancer**.
AU Carmel R
SO The New England journal of medicine, (1975 Feb 6) Vol. 292, No. 6, pp. 282-4.
Journal code: 0255562. ISSN: 0028-4793.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197504
ED Entered STN: 10 Mar 1990
Last Updated on STN: 10 Mar 1990
Entered Medline: 11 Apr 1975

L8 ANSWER 55 OF 66 MEDLINE on STN
Full Text
AN 1974287001 MEDLINE
DN PubMed ID: 4367719
TI Characteristics of a novel serum **vitamin-B12**-binding protein associated with hepatocellular **carcinoma**.
AU Wasman S; Gilbert H S
SO British journal of haematology, (1974 Jun) Vol. 27, No. 2, pp. 229-39.
Journal code: 0372544. ISSN: 0007-1048.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197410

ED Entered STN: 10 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 7 Oct 1974

L8 ANSWER 56 OF 66 MEDLINE on STN
Full Text
AN 1974170781 MEDLINE
DN PubMed ID: 4524624
TI The effect of replacement of methionine by homocystine on survival of malignant and normal adult mammalian cells in culture.
AU Halpern B C; Clark B R; Hardy D N; Halpern R M; Smith R A
SO Proceedings of the National Academy of Sciences of the United States of America, (1974 Apr) Vol. 71, No. 4, pp. 1133-6.
Journal code: 7505876. ISSN: 0027-8424.
Report No.: NLM-PMC388177.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197407
ED Entered STN: 10 Mar 1990
Last Updated on STN: 10 Mar 1990
Entered Medline: 31 Jul 1974

L8 ANSWER 57 OF 66 MEDLINE on STN
Full Text
AN 1974004406 MEDLINE
DN PubMed ID: 4126370
TI A **tumor**-related **vitamin B12** binding protein in adolescent hepatoma.
AU Waxman S; Gilbert H S
SO The New England journal of medicine, (1973 Nov 15) Vol. 289, No. 20, pp. 1053-6.
Journal code: 0255562. ISSN: 0028-4793.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197312
ED Entered STN: 10 Mar 1990
Last Updated on STN: 10 Mar 1990
Entered Medline: 11 Dec 1973

L8 ANSWER 58 OF 66 MEDLINE on STN
Full Text
AN 1972200957 MEDLINE
DN PubMed ID: 4555534
TI Unfavorable signs in patients with chronic myelocytic **leukemia**.
AU Theologides A
SO Annals of internal medicine, (1972 Jan) Vol. 76, No. 1, pp. 95-9. Ref: 54
Journal code: 0372351. ISSN: 0003-4819.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197208
ED Entered STN: 10 Mar 1990
Last Updated on STN: 10 Mar 1990
Entered Medline: 7 Aug 1972

L8 ANSWER 59 OF 66 MEDLINE on STN
Full Text
AN 1972041358 MEDLINE
DN PubMed ID: 5000872
TI Gastric secretory and serologic studies on patients with **neoplastic** and immunologic disorders.
AU Twomey J J; Laughter A H; Villanueva N D; Kao Y S; Lidsky M D; Jordan P H Jr
SO Archives of internal medicine, (1971 Nov) Vol. 128, No. 5, pp. 746-9.
Journal code: 0372440. ISSN: 0003-9926.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)

LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197201
ED Entered STN: 10 Mar 1990
Last Updated on STN: 6 Feb 1998
Entered Medline: 25 Jan 1972

L8 ANSWER 60 OF 66 MEDLINE on STN
Full Text
AN 1971281351 MEDLINE
DN PubMed ID: 5284678
TI Increased transcobalamin I in a leukemoid reaction.
AU Hall C A; Wanko M
SO The Journal of laboratory and clinical medicine, (1971 Aug) Vol. 78, No. 2, pp. 298-301.
Journal code: 0375375. ISSN: 0022-2143.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 197111
ED Entered STN: 1 Jan 1990
Last Updated on STN: 1 Jan 1990
Entered Medline: 3 Nov 1971

L8 ANSWER 61 OF 66 MEDLINE on STN
Full Text
AN 1970113051 MEDLINE
DN PubMed ID: 5740509
TI [The mechanism of the emergence of hematological remissions (on the problem of **tumor** regression)].
O mekhanizme vozniknoveniia gematologicheskikh remissii (K voprosu ob opukholevoi regressii).
AU Alekseev G A
SO Terapevticheskii arkhiv, (1968 Apr) Vol. 40, No. 4, pp. 16-25.
Journal code: 2984818R. ISSN: 0040-3660.
CY USSR
DT Journal; Article; (JOURNAL ARTICLE)
LA Russian
FS Priority Journals
EM 197004
ED Entered STN: 1 Jan 1990
Last Updated on STN: 1 Jan 1990
Entered Medline: 2 Apr 1970

L8 ANSWER 62 OF 66 MEDLINE on STN
Full Text
AN 1969175359 MEDLINE
DN PubMed ID: 5252793
TI Uptake of labelled vitamin B 12 and 4-iodophenylalanine in some **tumors** of mice.
AU Blomquist L; Flodh H; Ullberg S
SO Experientia, (1969 Mar 15) Vol. 25, No. 3, pp. 294-6.
Journal code: 0376547. ISSN: 0014-4754.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 196906
ED Entered STN: 1 Jan 1990
Last Updated on STN: 1 Jan 1990
Entered Medline: 19 Jun 1969

L8 ANSWER 63 OF 66 MEDLINE on STN
Full Text
AN 1969057044 MEDLINE
DN PubMed ID: 5724527
TI Accumulation of labelled **vitamin B12** in some transplanted tumours.
AU Flodh H; Ullberg S
SO International journal of cancer. Journal international du cancer, (1968 Sep 15) Vol. 3, No. 5, pp. 694-9.
Journal code: 0042124. ISSN: 0020-7136.

CY Denmark
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 196901
ED Entered STN: 1 Jan 1990
    Last Updated on STN: 1 Jan 1990
    Entered Medline: 30 Jan 1969

L8 ANSWER 64 OF 66 MEDLINE on STN
Full Text
AN 1966098269 MEDLINE
DN PubMed ID: 4159695
TI Excretion of formiminoglutamic acid in reticulosis and **carcinoma**.
AU Noeypatimanond S; Watson-Williams E J; Israels M C
SO Lancet, (1966 Feb 26) Vol. 1, No. 7435, pp. 454-6.
    Journal code: 2985213R. ISSN: 0140-6736.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 196605
ED Entered STN: 1 Jan 1990
    Last Updated on STN: 6 Feb 1998
    Entered Medline: 23 May 1966

L8 ANSWER 65 OF 66 MEDLINE on STN
Full Text
AN 1965135871 MEDLINE
DN PubMed ID: 14331187
TI ADENOSYLMETHIONINE ELEVATION IN LEUKEMIC WHITE BLOOD CELLS.
AU BALDESSARINI R J
SO Science (New York, N.Y.), (1965 Aug 6) Vol. 149, pp. 644-5.
    Journal code: 0404511. ISSN: 0036-8075.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS OLDMEDLINE; NONMEDLINE
EM 199612
ED Entered STN: 16 Jul 1999
    Last Updated on STN: 16 Jul 1999
    Entered Medline: 1 Dec 1996

L8 ANSWER 66 OF 66 MEDLINE on STN
Full Text
AN 1960104214 MEDLINE
DN PubMed ID: 13783966
TI Co58B12 absorption, plasma transport and excretion in patients with
    myeloproliferative disorders, solid **tumors** and non-**neoplastic** diseases.
AU WEINSTEIN I B; WATKIN D M
SO The Journal of clinical investigation, (1960 Nov) Vol. 39, pp. 1667-74.
    Journal code: 7802877. ISSN: 0021-9738.
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS OLDMEDLINE; NONMEDLINE
OS NLMPMC293407
EM 199811
ED Entered STN: 16 Jul 1999
    Last Updated on STN: 16 Jul 1999
    Entered Medline: 1 Nov 1998

=> d his

    (FILE 'HOME' ENTERED AT 23:24:07 ON 31 AUG 2009)

    FILE 'REGISTRY' ENTERED AT 23:24:20 ON 31 AUG 2009
              E VITAMIN B12/CN
L1   1 S E3

    FILE 'MEDLINE' ENTERED AT 23:24:53 ON 31 AUG 2009
L2   16339 S L1

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L3        11438 S (VITAMIN B12 OR HYDROXYCOBOLAMIN OR CHLOROCOBOLAMIN OR AQUOCO
L4        20105 S L2 OR L3
L5      1707973 S (CANCER OR ANTI-NEOPLAST? OR NEOPLAST? OR CARCIN? OR TUMOR?)
L6          773 S L4 AND L5
L7       212559 S LEUKEMIA?
L8           66 S L6 AND L7

=> d an ti au si ab kwic 18 47
'SI' IS NOT A VALID FORMAT FOR FILE 'MEDLINE'

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The default display format is BIB.

ABS ---- AB
ALL ---- AN, DN, TI, AU, AUGR, AUCL, CS, NC, SO, CM, CY, DT, LA,
         FS, NCT, OS, EM, ED, AB, ST, CT, NA, RN, CN, GEN
BIB ---- AN, DN, TI, AU, AUGR, AUCL, CS, NC, SO, CY, DT, LA, FS, NCT, OS, EM, ED
CBIB --- AN, DN, TI, AU, AUGR, AUCL, CS, NC, SO, CY, DT, LA, FS, NCT, OS, EM, ED
DALL --- ALL, delimited for post processing
IABS --- ABS, with a text label
IALL --- ALL, indented with text labels
IBIB --- BIB, indented with text labels
IND ---- ST, CT, NA, RN, CN, GEN
TRIAL -- TI, ST, CT, NA, RN, CN, GEN
 (SAM, TRI, FREE)
HIT ---- All fields containing hit terms
HITIND - IND
KWIC --- All hit terms plus 20 words on either side
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PY.

To display a particular field or fields,  enter the display field
codes.  For a list of display field codes, enter 'HELP DFIELDS'
at an arrow prompt (=>).  Examples of formats include: 'BIB';
'AB'; 'SO,ST'.  You may specify the format fields in any order,
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The same formats (except for HIT, HITIND, KWIC, and OCC) may be
used with the DISPLAY ACC command to display the record for a
specified Accession Number.
ENTER DISPLAY FORMAT (BIB):end

=> d an ti au so ab kwic 18 47

L8   ANSWER 18 OF 66     MEDLINE on STN
Full Text
AN   1992074415     MEDLINE
TI   Effect of combined ascorbic acid and B-12 on survival of mice with
     implanted Ehrlich carcinoma and L1210 leukemia.
AU   Poydock M E
SO   The American journal of clinical nutrition, (1991 Dec) Vol. 54, No. 6
     Suppl, pp. 1261S-1265S.
     Journal code: 0376027. ISSN: 0002-9165.
AB   A combination of dehydroascorbic acid and hydroxycobalamin (vitamin B-12)
     inhibited mitoses of tumors in mice.  The present study was performed to
     test the effect of these vitamins on the survival of mice bearing
     carcinomas and leukemias.  In each assay 40 mice received 0.1 mL ip
     tumor cells (x10(5)).  After 24 h, 20 mice were injected with 0.2 mL
     (0.4 g/kg body wt) of the vitamins daily for 10 d.  All controls died by
     day 19, but greater than 50% of the treated mice were alive after 60 d.
     In vitro findings revealed inhibition of mitoses in L1210 leukemia
     cells, but not in normal L929 cells.  In recent research with
     cobalt-ascorbate plus vitamin C, we demonstrated that when B-12 is
     combined with vitamin C, the cobalt nucleus of B-12 attaches to a carbon
     on vitamin C, forming cobalt ascorbate.  Tests proved that cobalt
     ascorbate plus vitamin C also inhibited tumor cells.
TI   Effect of combined ascorbic acid and B-12 on survival of mice with
     implanted Ehrlich carcinoma and L1210 leukemia.
```

AB A combination of dehydroascorbic acid and hydroxycobalamin (vitamin B-12) inhibited mitoses of **tumors** in mice. The present study was performed to test the effect of these vitamins on the survival of mice bearing **carcinomas** and **leukemias.** In each assay 40 mice received 0.1 mL ip **tumor** cells (x10(5)). After 24 h, 20 mice were injected with 0.2 mL (0.4 g/kg body wt) of the vitamins daily. . . than 50% of the treated mice were alive after 60 d. In vitro findings revealed inhibition of mitoses in L1210 **leukemia** cells, but not in normal L929 cells. In recent research with cobalt-ascorbate plus vitamin C, we demonstrated that when B-12. . . attaches to a carbon on vitamin C, forming cobalt ascorbate. Tests proved that cobalt ascorbate plus vitamin C also inhibited **tumor** cells.
CT Check Tags: Female
Animals
*Ascorbic Acid: PD, pharmacology
***Carcinoma, Ehrlich Tumor: MO, mortality**
Carcinoma, Ehrlich Tumor: PA, pathology
Dehydroascorbic Acid: PD, pharmacology
Drug Combinations
***Leukemia, Experimental: MO, mortality**
Mice
Mice, Inbred ICR
Neoplasm Transplantation
Survival Analysis
*Vitamin B 12: PD, pharmacology
RN 490-83-5 (Dehydroascorbic Acid); 50-81-7 (Ascorbic Acid); **68-19-9 (Vitamin B 12)**

L8 ANSWER 47 OF 66 MEDLINE on STN
Full Text
AN 1977019051 MEDLINE
TI B12 -- dependent methionine synthetase as a potential target for **cancer** chemotherapy.
AU Huennekens F M; DiGirolamo P M; Fujii K; Jacobsen D W; Vitols K S
SO Advances in enzyme regulation, (1976) Vol. 14, pp. 187-205. Ref: 51
Journal code: 0044263. ISSN: 0065-2571.
TI B12 -- dependent methionine synthetase as a potential target for **cancer** chemotherapy.
CT . . . S-Methyltransferase: IP, isolation & purification
*5-Methyltetrahydrofolate-Homocysteine S-Methyltransferase: ME, metabolism
Animals
Cells, Cultured
Cobamides: BI, biosynthesis
Enzyme Activation
Flavoproteins: ME, metabolism
Leukemia L1210: EN, enzymology
Leukemia L1210: ME, metabolism
Methionine: BI, biosynthesis
*Methyltransferases: ME, metabolism
Mice
NADP: ME, metabolism
*Neoplasms: ME, metabolism
S-Adenosylmethionine: ME, metabolism
. . .
RN 29908-03-0 (S-Adenosylmethionine); 53-59-8 (NADP); 63-68-3 (Methionine); **68-19-9 (Vitamin B 12)**

=> file ca
COST IN U.S. DOLLARS SINCE FILE ENTRY TOTAL SESSION
FULL ESTIMATED COST 18.48 26.58

FILE 'CA' ENTERED AT 23:34:40 ON 31 AUG 2009
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FILE COVERS 1907 - 27 Aug 2009 VOL 151 ISS 10
FILE LAST UPDATED: 27 Aug 2009 (20090827/ED)
REVISED CLASS FIELDS (/NCL) LAST RELOADED: Jun 2009
USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Jun 2009

CA now includes complete International Patent Classification (IPC)
reclassification data for the third quarter of 2009.

CAS Information Use Policies apply and are available at:

http://www.cas.org/legal/infopolicy.html

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substance identification.

The ALL, BIB, MAX, and STD display formats in the CA/CAplus family
of databases have been updated to include new citing references
information. This enhancement may impact record import into
database management software. For additional information, refer
to NEWS 9.

```
=> s l1
L9       21671 L1

=> s (vitamin b12 or hydroxycobolamin or chlorocobolamin or aquocobolamin or cobolamin or azi
        217802 VITAMIN
         40353 B12
         25073 VITAMIN B12
                 (VITAMIN(W)B12)
             1 HYDROXYCOBOLAMIN
             0 CHLOROCOBOLAMIN
             0 AQUOCOBOLAMIN
             3 COBOLAMIN
             0 AZIDOCOBOLAMIN
L10      25074 (VITAMIN B12 OR HYDROXYCOBOLAMIN OR CHLOROCOBOLAMIN OR AQUOCOBOL
               AMIN OR COBOLAMIN OR AZIDOCOBOLAMIN)

=> s l9 or l10
L11      26800 L9 OR L10

=> s (cancer or anti-neoplast? or neoplast? or carcin? or tumor?)
        385602 CANCER
        525123 ANTI
         69871 NEOPLAST?
          1018 ANTI-NEOPLAST?
                 (ANTI(W)NEOPLAST?)
         69871 NEOPLAST?
        307373 CARCIN?
        553203 TUMOR?
L12     881426 (CANCER OR ANTI-NEOPLAST? OR NEOPLAST? OR CARCIN? OR TUMOR?)

=> s l11 and l12
L13        959 L11 AND L12

=> s leukemia?
L14     121003 LEUKEMIA?

=> s l13 and l14
L15         88 L13 AND L14

=> d 1-88

L15  ANSWER 1 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   151:214450  CA
```

TI Substrate-selective inhibition of pappalysin activity against insulin-like growth factor-binding protein 4 using substrate-binding site ligands
IN Oxvig, Claus; Mikkelsen, Jakob Hauge; Nielsen, Claus Gyrup
PA Aarhus Universitet, Den.
SO PCT Int. Appl., 219pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2009092806	A2	20090730	WO 2009-EP50796	20090123
	W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
	RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
PRAI	US 2008-23631P	P	20080125		
	DK 2008-148	A	20080201		
	US 2008-25545P	P	20080201		

L15 ANSWER 2 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 150:555809 CA
TI Lipid compositions for the treatment and prevention of proliferative diseases and for the reduction of incidences of mutagenesis and **carcinogenesis**
IN Bar Yosef, Fabiana
PA Enzymotec Ltd., Israel
SO U.S. Pat. Appl. Publ., 16pp.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 20090131523	A1	20090521	US 2008-285806	20081014
PRAI	US 2007-960798P	P	20071015		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS MARPAT 150:555809

L15 ANSWER 3 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 150:464210 CA
TI Naphthalene-based inhibitors of anti-apoptotic proteins
IN Pellecchia, Maurizio; Reed, John C.
PA Burnham Institute for Medical Research, USA
SO PCT Int. Appl., 114pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2009052443	A1	20090423	WO 2008-US80386	20081017
	W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
	RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,				

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             TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW,
             AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
     US 20090105319       A1    20090423    US 2008-253918         20081017
PRAI US 2007-981400P      P     20071019
     US 2008-35969P       P     20080312
     US 2008-97171P       P     20080915
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS   MARPAT 150:464210
RE.CNT  4     THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 4 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   150:395435  CA
TI   Studies on similarity of hepatocarcinogenesis in liver cirrhosis to
     leukomogenesis
AU   Feng, Baozhang; Lei, Jianling; Fu, Yu; Liu, Fangjie; Zhou, Yingjie
CS   V-erb Lab, V-erb Gene Therapy Co., Ltd., Tianjin, 300020, Peop. Rep. China
SO   Zhongliu Yanjiu Yu Linchuang (2007), 19(6), 393-394
     CODEN: ZYLIFJ; ISSN: 1006-9801
PB   Zhongliu Yanjiu Yu Linchuang Zazhi Bianjibu
DT   Journal
LA   Chinese

L15  ANSWER 5 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   150:268020  CA
TI   Transfer factor compositions and methods for therapeutic use thereof
IN   Ramaekers, Joseph C.
PA   USA
SO   U.S. Pat. Appl. Publ., 21pp.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 2
     PATENT NO.           KIND   DATE        APPLICATION NO.         DATE
     ---------------      ----   --------    --------------------    --------
PI   US 20090053197        A1    20090226    US 2007-762727          20070613
     WO 2007149287         A2    20071227    WO 2007-US13903         20070614
     WO 2007149287         A3    20081002
          W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
              CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI,
              GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG,
              KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
              MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL,
              PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN,
              TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
          RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
              IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, BF,
              BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW,
              GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ,
              BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA
PRAI US 2006-814777P      P     20060614
     US 2006-834739P      P     20060731
     US 2007-762727       A     20070613

L15  ANSWER 6 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   149:386609  CA
TI   Cobalamin taxane bioconjugates useful as oral anti-cancer or
     anti-angiogenic drugs
IN   Gebhard, John R.; Vollmer, David; Patel, Dinesh; Daugherty, Claire
PA   Inflabloc Pharmaceuticals, Inc., USA
SO   PCT Int. Appl., 42pp.
     CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 1
     PATENT NO.           KIND   DATE        APPLICATION NO.         DATE
     ---------------      ----   --------    --------------------    --------
PI   WO 2008115805        A2    20080925    WO 2008-US57038         20080314
     WO 2008115805        A3    20090115
```

W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES,
FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH,
PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU,
IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK,
TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW,
AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA

US 20080233135 A1 20080925 US 2008-77060 20080314
PRAI US 2007-919121P P 20070319
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS CASREACT 149:386609

L15 ANSWER 7 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 149:119595 CA
TI Diagnosis and treatment of **cancer** related to human dormancy
IN Powell, Michael
PA USA
SO U.S. Pat. Appl. Publ., 27pp.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 20080160007	A1	20080703	US 2008-6462	20080102
PRAI US 2007-878343P	P	20070103		

L15 ANSWER 8 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 149:111963 CA
TI **Vitamin B12**-mediated transport: a potential tool for **tumor** targeting of antineoplastic drugs and imaging agents
AU Gupta, Yashwant; Kohli, Dharm Veer; Jain, Sanjay K.
CS Pharmaceutics Research Projects Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Vishwavidyalaya, Sagar, 470003, India
SO Critical Reviews in Therapeutic Drug Carrier Systems (2008), 25(4), 347-379
CODEN: CRTSEO; ISSN: 0743-4863
PB Begell House, Inc.
DT Journal; General Review
LA English
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)
RE.CNT 153 THERE ARE 153 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 9 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 148:375932 CA
TI Markers of increased angiogenesis and their correlation with biological parameters identifying high-risk patients in early B-cell chronic lymphocytic **leukemia**
AU Molica, Stefano; Cutrona, Giovanna; Vitelli, Gaetano; Mirabelli, Rosanna; Molica, Matteo; Digiesi, Giovanna; Ribatti, Domenico; Ferrarini, Manlio; Vacca, Angelo
CS Hematology/Oncology Department, Azienda Ospedaliera Pugliese-Ciaccio, Catanzaro, 88100, Italy
SO Leukemia Research (2007), 31(11), 1575-1578
CODEN: LEREDD; ISSN: 0145-2126
PB Elsevier Ltd.
DT Journal
LA English
OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)
RE.CNT 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 10 OF 88 CA COPYRIGHT 2009 ACS on STN

Full Text
AN 148:186576 CA
TI Method of detecting and/ or measuring hepcidin in a sample
IN Li, Hongyan; Breau, Alan; Sasu, Barbra
PA Amgen Inc., USA
SO PCT Int. Appl., 42pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2008011158	A2	20080124	WO 2007-US16477	20070720
	WO 2008011158	A3	20080605		
	W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
	RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA				
	AU 2007275638	A1	20080124	AU 2007-275638	20070720
	CA 2657307	A1	20080124	CA 2007-2657307	20070720
	EP 2057472	A2	20090513	EP 2007-810654	20070720
	R: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, AL, BA, HR, MK, RS				
	US 20090173876	A1	20090709	US 2007-880313	20070720
PRAI	US 2006-832625P	P	20060721		
	WO 2007-US16477	W	20070720		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

L15 ANSWER 11 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 148:106222 CA
TI Pharmaceutical compositions containing inhibitors of histone deacetylase and B vitamins, and methods of use thereof in the treatment of histone deacetylase dependent diseases
IN Shultz, Michael
PA Novartis AG, Switz.; Novartis Pharma GmbH
SO PCT Int. Appl., 58 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2008002862	A1	20080103	WO 2007-US72004	20070625
	W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
	RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	AU 2007265190	A1	20080103	AU 2007-265190	20070625
	CA 2660782	A1	20080103	CA 2007-2660782	20070625
	EP 2034978	A1	20090318	EP 2007-798994	20070625
	R: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, AL, BA, HR, MK, RS				

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     IN 2008DN10353       A     20090320    IN 2008-DN10353        20081215
     MX 2008016125        A     20090115    MX 2008-16125          20081216
     KR 2009023631        A     20090305    KR 2008-731346         20081224
     CN 101478959         A     20090708    CN 2007-80024079       20081226
PRAI US 2006-816459P      P     20060626
     WO 2007-US72004      W     20070625
RE.CNT  4     THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 12 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   148:85733  CA
TI   Transfer factor compositions and methods
IN   Ramaekers, Joseph C.
PA   Ramaekers Nutrition, LLC, USA
SO   PCT Int. Appl., 45pp.
     CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 2
     PATENT NO.           KIND   DATE        APPLICATION NO.        DATE
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PI   WO 2007149287         A2    20071227    WO 2007-US13903        20070614
     WO 2007149287         A3    20081002
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
             CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI,
             GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG,
             KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
             MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL,
             PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN,
             TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
         RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
             IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR, BF,
             BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW,
             GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ,
             BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA
     US 20090053197        A1    20090226    US 2007-762727         20070613
PRAI US 2006-814777P      P     20060614
     US 2006-834739P      P     20060731
     US 2007-762727       A     20070613

L15  ANSWER 13 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   147:491621  CA
TI   Nutraceutical composition comprising
     2,3-dimethoxy-5-methyl-1,4-benzoquinone and method of use for
     treatment/prevention of cancer
IN   Mazzio, Elizabeth; Soliman, Karam
PA   USA
SO   U.S. Pat. Appl. Publ., 31pp., Cont.-in-part of U.S. Ser. No. 233,279.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 3
     PATENT NO.           KIND   DATE        APPLICATION NO.        DATE
     ---------------      ----   --------    -------------------    --------
PI   US 20070248693        A1    20071025    US 2007-711883         20070227
     US 20060035981        A1    20060216    US 2005-233279         20050920
PRAI US 2003-491841P      P     20030802
     US 2004-540525P      P     20040129
     US 2004-909590       B2    20040802
     US 2005-233279       A2    20050920

L15  ANSWER 14 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   147:181566  CA
TI   Dietary and pharmaceutical compositions using
     N-acetyl-glucosamine-N-acetylmuramyl peptides for management and treatment
     of oxidative stress and conditions with elevated γ-glutamyl
     transferase activity and alterations of NF-κB expression
IN   Ellithorpe, Rita R.; Slesarev, Vladimir I.; Dimitrov, Todor V.
PA   USA
```

SO U.S. Pat. Appl. Publ., 11pp., Cont.-in-part of U.S. Ser. No. 794,285.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 2

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 20070167355	A1	20070719	US 2006-581623	20061017
	US 20040258779	A1	20041223	US 2003-455123	20030606
	US 20050059579	A1	20050317	US 2004-794285	20040308
PRAI	US 2003-455123	A2	20030606		
	US 2004-794285	A2	20040308		

L15 ANSWER 15 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 147:125831 CA
TI Transdermal delivery of pharmaceutical agent comprising genetic molecule
IN Russell-Jones, Gregory J.; Luke, Michael R.; Himes, Stewart R.
PA Apollo Life Sciences Limited, Australia
SO PCT Int. Appl., 121pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2007070983	A1	20070628	WO 2006-AU1999	20061222
	W: 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, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
	RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	AU 2006326870	A1	20070628	AU 2006-326870	20061222
	US 20070243132	A1	20071018	US 2006-645122	20061222
	EP 1978997	A1	20081015	EP 2006-840407	20061222
	R: 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				
PRAI	US 2005-753454P	P	20051222		
	AU 2006-905107	A	20060915		
	WO 2006-AU1999	W	20061222		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (7 CITINGS)
RE.CNT 2 THERE ARE 2 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 16 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 146:476726 CA
TI Protein and cDNA sequences of vWFA (von Willebrand factor type A), collagen, and Kunitz - domains containing proteins INSP150, and therapeutic and diagnostic use thereof
IN Davies, Mark Douglas; Fagan, Richard Joseph; Yorke, Melanie; Power, Christine
PA Ares Trading S. A., Switz.
SO PCT Int. Appl., 146 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2007049065	A2	20070503	WO 2006-GB4041	20061027
	WO 2007049065	A3	20070809		
	W: 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, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN,
KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK,
MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO,
RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT,
TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ,
CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY,
KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA
PRAI GB 2005-21958 A 20051027

L15 ANSWER 17 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 145:432186 CA
TI Use of PT523 for treating cancers
IN Weiser, Michael; Serbin, Jeff; Rosenwald, Lindsay A.
PA Hana Biosciences, Inc., USA
SO PCT Int. Appl., 57 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 2006113536	A2	20061026	WO 2006-US14250	20060413
WO 2006113536	A3	20061207		

W: 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,
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KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX,
MZ, NA, NG, 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
RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ,
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GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY,
KG, KZ, MD, RU, TJ, TM

PRAI US 2005-671891P P 20050414
US 2005-735336P P 20051110
RE.CNT 2 THERE ARE 2 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 18 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 145:348597 CA
TI Use of phenylmethimazoles, methimazole derivatives, and tautomeric cyclic thiones for the treatment of autoimmune/inflammatory diseases associated with toll-like receptor overexpression
IN Kohn, Leonard D.; Harii, Norikazu; Benavides-Peralta, Uruguaysito; Gonzalez-Murguiondo, Mariana; Lewis, Christopher J.; Napolitano, Giorgio; Giuliani, Cesidio; Malgor, Ramiro; Goetz, Douglas J.
PA The Interthyr Corporation, USA
SO U.S. Pat. Appl. Publ., 102 pp., Cont.-in-part of U.S. Ser. No. 912,948.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 3

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 20060211752	A1	20060921	US 2005-130922	20050517
US 20050209295	A1	20050922	US 2004-801986	20040316
AU 2004317993	A1	20051013	AU 2004-317993	20040316
CA 2559712	A1	20051013	CA 2004-2559712	20040316
EP 1725230	A1	20061129	EP 2004-821836	20040316

R: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
IT, LI, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR

JP 2007529510	T	20071025	JP 2007-503869	20040316
US 20060058365	A1	20060316	US 2004-912948	20040806
AU 2006247504	A1	20061123	AU 2006-247504	20060511
CA 2606769	A1	20061123	CA 2006-2606769	20060511

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     WO 2006124676         A1    20061123    WO 2006-US18554         20060511
         W:  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, KM, KN, KP, KR,
             KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX,
             MZ, NA, NG, 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
         RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,
             IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ,
             CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH,
             GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY,
             KG, KZ, MD, RU, TJ, TM
     EP 1896015            A1    20080312    EP 2006-770302          20060511
         R:  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
     JP 2008545651         T     20081218    JP 2008-512377          20060511
PRAI US 2004-801986        A2    20040316
     US 2004-912948        A2    20040806
     WO 2004-US7888        A     20040316
     US 2005-130922        A     20050517
     WO 2006-US18554       W     20060511
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS   MARPAT 145:348597
OSC.G   3     THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)

L15  ANSWER 19 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   144:286212  CA
TI   Diagnosis and treatment of human dormancy-related sequellae
IN   Powell, Michael
PA   USA
SO   U.S. Pat. Appl. Publ., 35 pp., Cont.-in-part of U. S. Ser. No. 444,845.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 3
     PATENT NO.          KIND   DATE        APPLICATION NO.         DATE
     ---------------     ----   --------    --------------------    --------
PI   US 20060052278       A1    20060309    US 2005-206564          20050818
     US 7485298           B2    20090203
     US 20030228628       A1    20031211    US 2003-444845          20030523
     US 7288257           B2    20071030
     US 20090163448       A1    20090625    US 2009-322488          20090202
PRAI US 2002-382913P      P     20020523
     US 2002-383271P      P     20020524
     US 2003-444845       A2    20030523
     US 2005-206564       A1    20050818
RE.CNT  11    THERE ARE 11 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 20 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   144:219302  CA
TI   Composition comprising mixture of ubiquinones, lactic acid dehydrogenase
     inhibitor, compound capable of augmenting oxidative phosphorylation and
     compound that antagonize gluconeogenesis from non-glucose carbon based
     substrates for treatment of cancer
IN   Mazzio, Elizabeth Anne; Soliman, Karam F.
PA   USA
SO   U.S. Pat. Appl. Publ., 20 pp., Cont.-in-part of U.S. Ser. No. 909,590,
     abandoned.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 3
     PATENT NO.          KIND   DATE        APPLICATION NO.         DATE
     ---------------     ----   --------    --------------------    --------
PI   US 20060035981       A1    20060216    US 2005-233279          20050920
     US 20070248693       A1    20071025    US 2007-711883          20070227
PRAI US 2003-491841P      P     20030802
     US 2004-540525P      P     20040129
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     US 2004-909590       B2    20040802
     US 2005-233279       A2    20050920

L15  ANSWER 21 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   143:139157  CA
TI   Preparation of rigid liposomal cochleate
IN   Krause-Elsmore, Sara L.; Mannino, Raphael J.
PA   Biodelivery Sciences International, Inc., USA
SO   PCT Int. Appl., 50 pp.
     CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 1
     PATENT NO.          KIND   DATE        APPLICATION NO.         DATE
     ---------------     ----   --------    --------------------    --------
PI   WO 2005063213        A1    20050714    WO 2004-US42927         20041220
         W:  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, SY,
             TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW
         RW: BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM,
             AZ, BY, KG, KZ, MD, RU, TJ, TM, 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML,
             MR, NE, SN, TD, TG
PRAI US 2003-531546P      P     20031219
     US 2004-565120P      P     20040423
OSC.G   4     THERE ARE 4 CAPLUS RECORDS THAT CITE THIS RECORD (4 CITINGS)
RE.CNT  12    THERE ARE 12 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 22 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   142:291352  CA
TI   Cobalamin conjugates with antitumor drugs, their preparation, and their
     use in antitumor therapy
IN   Weinshenker, Ned M.; West, Frederick G.; Araneo, Barbara A.; Li, Weiping
PA   Inflabloc Pharmaceuticals, Inc., USA
SO   U.S. Pat. Appl. Publ., 41 pp.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 1
     PATENT NO.          KIND   DATE        APPLICATION NO.         DATE
     ---------------     ----   --------    --------------------    --------
PI   US 20050054607       A1    20050310    US 2003-659501          20030910
     US 7232805           B2    20070619
     AU 2004272105        A1    20050324    AU 2004-272105          20040910
     CA 2538748           A1    20050324    CA 2004-2538748         20040910
     WO 2005025512        A2    20050324    WO 2004-US29879         20040910
     WO 2005025512        A3    20050728
         W:  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, SY,
             TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW
         RW: BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM,
             AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK,
             EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE,
             SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
             SN, TD, TG
     EP 1672978           A2    20060628    EP 2004-783919          20040910
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, SI, FI, RO, CY, TR, BG, CZ, EE, HU, PL, SK
     JP 2007505144        T     20070308    JP 2006-526379          20040910
     KR 2007019942        A     20070216    KR 2006-704844          20060309
PRAI US 2003-659501       A     20030910
     WO 2004-US29879      W     20040910
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RE.CNT 49 THERE ARE 49 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 23 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 141:384286 CA
TI Novel encochleation methods, cochleates and methods of use
IN Mannino, Raphael J.; Gould-Fogerite, Susan; Krause-Elsmore, Sara L.; Delmarre, David; Lu, Ruying
PA Biodelivery Sciences International, Inc., USA; University of Medicine and Dentistry of New Jersey
SO PCT Int. Appl., 195 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2004091578	A2	20041028	WO 2004-US11026	20040409
	WO 2004091578	A3	20050331		
	W: 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, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW				
	RW: BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG				
	US 20050013854	A1	20050120	US 2004-822230	20040409
	EP 1624858	A2	20060215	EP 2004-759375	20040409
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, FI, RO, CY, TR, BG, CZ, EE, HU, PL, SK				
	US 20070237814	A1	20071011	US 2007-653434	20070111
	US 20080009457	A1	20080110	US 2007-653093	20070111
PRAI	US 2003-461483P	P	20030409		
	US 2003-463076P	P	20030415		
	US 2003-499247P	P	20030828		
	US 2003-502557P	P	20030911		
	US 2003-532755P	P	20031224		
	US 2004-537252P	P	20040115		
	US 2004-556192P	P	20040324		
	US 2004-822230	A1	20040409		
	US 2004-822235	B1	20040409		
	WO 2004-US11026	W	20040409		

OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)

L15 ANSWER 24 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 141:342745 CA
TI Vitamin-mediated targeting as a potential mechanism to increase drug uptake by **tumors**
AU Russell-Jones, Gregory; McTavish, Kirsten; McEwan, John; Rice, John; Nowotnik, David
CS Targeted Delivery, Access Pharmaceuticals Australia Pty Ltd., Sydney, 2067, Australia
SO Journal of Inorganic Biochemistry (2004), 98(10), 1625-1633
CODEN: JIBIDJ; ISSN: 0162-0134
PB Elsevier B.V.
DT Journal; General Review
LA English
OSC.G 22 THERE ARE 22 CAPLUS RECORDS THAT CITE THIS RECORD (22 CITINGS)
RE.CNT 44 THERE ARE 44 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 25 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 141:21306 CA
TI Clinical and molecular features of FIP1L1-PDFGRA (+) chronic eosinophilic **leukemias**

AU Vandenberghe, P.; Wlodarska, I.; Michaux, L.; Zachee, P.; Boogaerts, M.; Vanstraelen, D.; Herregods, M-C.; Van Hoof, A.; Selleslag, D.; Roufosse, F.; Maerevoet, M.; Verhoef, G.; Cools, J.; Gilliland, D. G.; Hagemeijer, A.; Marynen, P.
CS The Center for Human Genetics, University Hospital Leuven, Louvain, B-3000, Belg.
SO Leukemia (2004), 18(4), 734-742
CODEN: LEUKED; ISSN: 0887-6924
PB Nature Publishing Group
DT Journal
LA English
OSC.G 57 THERE ARE 57 CAPLUS RECORDS THAT CITE THIS RECORD (58 CITINGS)
RE.CNT 21 THERE ARE 21 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 26 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 140:241008 CA
TI Drug delivery and targeting with **vitamin B12** conjugates
IN Wilson, Stephen; Reinhard, Kathryn S.; Gao, Xiang
PA USA
SO U.S. Pat. Appl. Publ., 22 pp.
CODEN: USXXCO
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 20040047917	A1	20040311	US 2002-235857	20020906
US 20070066561	A1	20070322	US 2006-601809	20061120
PRAI US 2002-235857	A3	20020906		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

L15 ANSWER 27 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 140:178997 CA
TI Significance of elevated cobalamin (**vitamin B12**) levels in blood
AU Ermens, A. A. M.; Vlasveld, L. T.; Lindemans, J.
CS Clinical Laboratory, Lokatie Langendijk, Amphia Hospital, Breda, Neth.
SO Clinical Biochemistry (2003), 36(8), 585-590
CODEN: CLBIAS; ISSN: 0009-9120
PB Elsevier Science Inc.
DT Journal; General Review
LA English
OSC.G 12 THERE ARE 12 CAPLUS RECORDS THAT CITE THIS RECORD (12 CITINGS)
RE.CNT 42 THERE ARE 42 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 28 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 138:314549 CA
TI Combination therapies using methyl donors or methyl donor enhancers and therapeutic agents for treatment of viral, proliferative and inflammatory diseases
IN Cruz, Tony; Pastrak, Aleksandra
PA Transition Therapeutics Inc., Can.
SO PCT Int. Appl., 70 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 6

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 2003030929	A1	20030417	WO 2002-CA1503	20021004

W: 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, US, UZ, VC, VN, YU, ZA, ZM, ZW
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES,

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             CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
     WO 2002100428        A1    20021219    WO 2002-CA895          20020611
         W:  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, US, UZ, VN, YU, ZA, ZM, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, CH,
             CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR,
             BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
     WO 2002100429        A1    20021219    WO 2002-CA896          20020611
         W:  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, US, UZ, VN, YU, ZA, ZM, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, CH,
             CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR,
             BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
     US 20030086901       A1    20030508    US 2002-167765         20020611
     US 6908611           B2    20050621
     US 20030152552       A1    20030814    US 2002-167752         20020611
     US 6894033           B2    20050517
     AU 2002331483        A1    20030422    AU 2002-331483         20021004
PRAI US 2001-327700P      P     20011005
     US 2001-334535P      P     20011203
     US 2002-366539P      P     20020325
     US 2002-167752       A2    20020611
     US 2002-167765       A2    20020611
     WO 2002-CA895        A2    20020611
     WO 2002-CA896        A2    20020611
     US 2001-297514P      P     20010611
     US 2001-908298       A     20010717
     US 2001-971068       A     20011003
     WO 2002-CA1503       W     20021004
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OSC.G   2     THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)
RE.CNT  7     THERE ARE 7 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 29 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   138:95595  CA
TI   Compositions containing a transfer factor for treating animal diseases and
     syndromes
IN   Ramaekers, Joseph C.
PA   USA
SO   U.S., 13 pp.
     CODEN: USXXAM
DT   Patent
LA   English
FAN.CNT 2
     PATENT NO.           KIND   DATE        APPLICATION NO.        DATE
     ---------------      ----   --------    --------------------   --------
PI   US 6506413           B1     20030114    US 2001-847036         20010430
     CA 2448580           A1     20021107    CA 2002-2448580        20020430
     WO 2002087599        A1     20021107    WO 2002-US13650        20020430
         W:  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, TR, TT, TZ, UA,
             UG, UZ, VN, YU, ZA, ZM, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY,
             KG, KZ, MD, RU, TJ, TM, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB,
             GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA,
             GN, GQ, GW, ML, MR, NE, SN, TD, TG
     AU 2002311871        A1     20021111    AU 2002-311871         20020430
     AU 2002311871        B2     20080131
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     US 20030077254       A1    20030424    US 2002-136854         20020430
     US 6962718           B2    20051108
     EP 1390049           A1    20040225    EP 2002-739205         20020430
     EP 1390049           B1    20060705
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, SI, LT, LV, FI, RO, MK, CY, AL, TR
     AT 332142            T     20060715    AT 2002-739205         20020430
     ES 2268048           T3    20070316    ES 2002-739205         20020430
     US 20060029585       A1    20060209    US 2005-237316         20050927
     AU 2008200364        A1    20080221    AU 2008-200364         20080124
PRAI US 2001-847036       A     20010430
     AU 2002-311871       A3    20020430
     US 2002-136854       A3    20020430
     WO 2002-US13650      W     20020430
OSC.G   5     THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)
RE.CNT  4     THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 30 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   138:35768  CA
TI   Preparation of fluorescent cobalamins and uses for tumor tissue staining
IN   Grissom, Charles B.; West, Frederick G.; Mcgreevy, James; Bentz, Joel S.;
     Cannon, Michelle J.
PA   University of Utah Research Foundation, USA
SO   U.S. Pat. Appl. Publ., 31 pp., Cont.-in-part of Appl. No. PCT/US00/29370.
     CODEN: USXXCO
DT   Patent
LA   English
FAN.CNT 3
     PATENT NO.           KIND   DATE        APPLICATION NO.        DATE
     ---------------      ----   --------    --------------------   --------
PI   US 20020192683        A1    20021219    US 2002-97646          20020315
     US 6797521            B2    20040928
     WO 2001030967         A2    20010503    WO 2000-US29370        20001026
     WO 2001030967         A3    20020221
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CR, CU, CZ, DE, DK, DM, DZ, 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, PL, PT, RO, RU,
             SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN,
             YU, ZA, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY,
             DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ,
             CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
     AU 2002258546         A1    20021003    AU 2002-258546         20020315
     AU 2002258546         B2    20060907
     JP 2004535371         T     20041125    JP 2002-572885         20020315
     US 20040224921        A1    20041111    US 2004-866988         20040615
     US 6905884            B2    20050614
     AU 2008200058         A1    20080131    AU 2008-200058         20080104
PRAI US 1999-161368P       P     19991026
     WO 2000-US29370       A2    20001026
     US 2001-276036P       P     20010316
     US 2001-336316P       P     20011030
     AU 2002-255730        A3    20020315
     US 2002-97646         A1    20020315
     WO 2002-US8285        W     20020315
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS   MARPAT 138:35768
OSC.G   1     THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)
RE.CNT  21    THERE ARE 21 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 31 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   137:89412  CA
TI   Detection of variations in the DNA methylation profile of genes in the
     determining the risk of disease
IN   Berlin, Kurt; Piepenbrock, Christian; Olek, Alexander
PA   Epigenomics A.-G., Germany
SO   PCT Int. Appl., 636 pp.
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CODEN: PIXXD2
DT Patent
LA German
FAN.CNT 69

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2001077373	A2	20011018	WO 2001-XA1486	20010406
	W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DK, DM, DZ, 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, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW				
	RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, CF, CG, CI, CM, GA, GW, ML, MR, NE, SN, TD, TG				
	DE 10019058	A1	20011220	DE 2000-10019058	20000406
	WO 2001077373	A2	20011018	WO 2001-DE1486	20010406
	W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DK, DM, DZ, 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, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW				
	RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
	AU 2001077487	A	20011023	AU 2001-77487	20010406
	EP 1360319	A2	20031112	EP 2001-955278	20010406
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO, MK, CY, AL, TR				
	EP 2014776	A2	20090114	EP 2008-12765	20010406
	R: AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE, TR				
	AT 339520	T	20061015	AT 2002-90203	20020605
	ES 2272636	T3	20070501	ES 2002-90203	20020605
	US 20040067491	A1	20040408	US 2003-240454	20030311
	AU 2003204553	A1	20040108	AU 2003-204553	20030605
	AU 2003204553	B2	20071129		
	JP 2004008217	A	20040115	JP 2003-160375	20030605
	US 20040023279	A1	20040205	US 2003-455212	20030605
	AU 2006203475	A1	20060831	AU 2006-203475	20060811
	AU 2006213968	A1	20061019	AU 2006-213968	20060915
	AU 2006225250	A1	20061026	AU 2006-225250	20061005
PRAI	DE 2000-10019058	A	20000406		
	WO 2001-DE1486	W	20010406		
	DE 2000-10019173	A	20000407		
	DE 2000-10032529	A	20000630		
	DE 2000-10043826	A	20000901		
	AU 2001-275663	A	20010406		
	AU 2001-276331	A3	20010406		
	AU 2001-75663	A	20010406		
	EP 2001-969303	A3	20010406		
	WO 2001-EP4016	W	20010406		
	EP 2002-90203	A	20020605		
	AU 2006-230475	A	20060811		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

L15 ANSWER 32 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 135:71265 CA
TI Combinations of a receptor tyrosine kinase inhibitor with an organic compound capable of binding to α1-acidic glycoprotein
IN Gambacorti-Passerini, Carlo; Lecoutre, Philipp
PA Novartis A.-G., Switz.; Novartis-Erfindungen
SO PCT Int. Appl., 79 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2001047507	A2	20010705	WO 2000-EP13161	20001222

```
     WO 2001047507         A3    20020404
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CR, CU, CZ, DE, DK, DM, DZ, 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, PL, PT, RO, RU,
             SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN,
             YU, ZA, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY,
             DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF,
             BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
     IT 99MI2711           A1    20010627    IT 1999-MI2711          19991227
     TW 246917             B     20060111    TW 2000-89126229        20001208
     CA 2394944            A1    20010705    CA 2000-2394944         20001222
     BR 2000016817         A     20021001    BR 2000-16817           20001222
     EP 1250140            A2    20021023    EP 2000-985244          20001222
     EP 1250140            B1    20090527
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, SI, LT, LV, FI, RO, MK, CY, AL, TR
     JP 2003523325         T     20030805    JP 2001-548102          20001222
     CN 1304005            C     20070314    CN 2000-817897          20001222
     AT 432069             T     20090615    AT 2000-985244          20001222
     US 20030125343        A1    20030703    US 2002-169035          20021007
PRAI IT 1999-MI2711        A     19991227
     WO 2000-EP13161       W     20001222
     WO 2000-EP31361       W     20001222
OS   MARPAT 135:71265
OSC.G   10    THERE ARE 10 CAPLUS RECORDS THAT CITE THIS RECORD (10 CITINGS)
RE.CNT  1     THERE ARE 1 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15  ANSWER 33 OF 88  CA  COPYRIGHT 2009 ACS on STN
Full Text
AN   134:323120  CA
TI   Fluorescent cobalamins and uses thereof
IN   Grissom, Charles B.; West, Frederick G.; Mcgreevy, James; Bentz, Joel S.
PA   University of Utah Research Foundation, USA
SO   PCT Int. Appl., 32 pp.
     CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 3
     PATENT NO.            KIND   DATE        APPLICATION NO.         DATE
     ----------------      ----   --------    --------------------    --------
PI   WO 2001030967         A2     20010503    WO 2000-US29370         20001026
     WO 2001030967         A3     20020221
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CR, CU, CZ, DE, DK, DM, DZ, 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, PL, PT, RO, RU,
             SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN,
             YU, ZA, ZW
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY,
             DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ,
             CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
     CA 2387503            A1     20010503    CA 2000-2387503         20001026
     AU 2001012300         A      20010508    AU 2001-12300           20001026
     AU 784424             B2     20060330
     EP 1226153            A2     20020731    EP 2000-973834          20001026
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, SI, LT, LV, FI, RO, MK, CY, AL
     JP 2003528041         T      20030924    JP 2001-533951          20001026
     NZ 519129             A      20060630    NZ 2000-519129          20001026
     US 20020192683        A1     20021219    US 2002-97646           20020315
     US 6797521            B2     20040928
     US 20040224921        A1     20041111    US 2004-866988          20040615
     US 6905884            B2     20050614
PRAI US 1999-161368P       P      19991026
     WO 2000-US29370       W      20001026
     US 2001-276036P       P      20010316
     US 2002-97646         A1     20020315
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS   MARPAT 134:323120
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OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)
RE.CNT 1 THERE ARE 1 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 34 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 134:37051 CA
TI Method for immune-system strengthening and development of a lipid transporter for anti-HIV and antibacterial gene therapy
IN Worm, Richard; Correa, Michel; Mavoungou, Donatien
PA Can.
SO Fr. Demande, 16 pp.
CODEN: FRXXBL
DT Patent
LA French
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	FR 2792201	A1	20001020	FR 1999-4706	19990415
	FR 2792201	B1	20011102		
PRAI	FR 1999-4706		19990415		

OSC.G 4 THERE ARE 4 CAPLUS RECORDS THAT CITE THIS RECORD (4 CITINGS)
RE.CNT 4 THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 35 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 132:58824 CA
TI Compounds of **vitamin B12** and its derivatives combined with ascorbic acid as potential antitumor agents
AU Vol'pin, M. E.; Krainova, N. Yu.; Levitin, I. Ya.; Mityaeva, Z. Ya.; Novodarova, G. N.; Oganezov, V. K.; Pankratov, A. A.; Chissov, V. I.; Yakubovskaya, R. I.
CS Inst. Elementoorg. Soedin. im. A. N. Nesmeyanova, RAN, Moscow, 117813, Russia
SO Rossiiskii Khimicheskii Zhurnal (1998), 42(5), 116-127
CODEN: RKZHEZ; ISSN: 1024-6215
PB Rossiiskoe Khimicheskoe Obshchestvo im. D. I. Mendeleeva
DT Journal
LA Russian
OSC.G 8 THERE ARE 8 CAPLUS RECORDS THAT CITE THIS RECORD (9 CITINGS)

L15 ANSWER 36 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 131:208725 CA
TI Intrathecal methotrexate-induced megaloblastic anemia in patients with acute **leukemia**
AU Sallah, Sabah; Hanrahan, L. Robert, Jr.; Phillips, Debra L.
CS Department of Medicine, Division of Hematology/Oncology, East Carolina University, School of Medicine, Greenville, NC, USA
SO Archives of Pathology & Laboratory Medicine (1999), 123(9), 774-777
CODEN: APLMAS; ISSN: 0003-9985
PB College of American Pathologists
DT Journal
LA English
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)
RE.CNT 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 37 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 131:120695 CA
TI Targeting **leukemia** cells with cobalamin bioconjugates
AU Mitchell, Alice M.; Bayomi, Ashraf; Natarajan, Ettaya; Barrows, Louis R.; West, Frederick G.; Grissom, Charles B.
CS Department of Chemistry, University of Utah, Salt Lake City, UT, 84112-0850, USA
SO Biomedical and Health Research (1999), 27(Enzymatic Mechanisms), 150-154
CODEN: BIHREN; ISSN: 0929-6743
PB IOS Press
DT Journal
LA English

OSC.G 9 THERE ARE 9 CAPLUS RECORDS THAT CITE THIS RECORD (9 CITINGS)
RE.CNT 18 THERE ARE 18 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 38 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 129:12414 CA
OREF 129:2551a,2554a
TI Synthesis, characterization and nitric oxide release profile of nitrosylcobalamin: a potential chemotherapeutic agent
AU Bauer, Joseph A.
CS Dep. Chem., Univ. Akron, Akron, OH, 44325-3601, USA
SO Anti-Cancer Drugs (1998), 9(3), 239-244
CODEN: ANTDEV; ISSN: 0959-4973
PB Rapid Science Ltd.
DT Journal
LA English
OSC.G 14 THERE ARE 14 CAPLUS RECORDS THAT CITE THIS RECORD (14 CITINGS)
RE.CNT 31 THERE ARE 31 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 39 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 128:226232 CA
OREF 128:44693a,44696a
TI Cobalt complex bioconjugates, preparation thereof, and delivery of bioactive agents
IN Grissom, Charles B.; West, Frederick G.; Howard, W. Allen, Jr.
PA University of Utah Research Foundation, USA; Grissom, Charles B.; West, Frederick G.; Howard, W. Allen, Jr.
SO PCT Int. Appl., 91 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9808859	A1	19980305	WO 1997-US14140	19970822
	W: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, 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, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU				
	RW: GH, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	CA 2264592	A1	19980305	CA 1997-2264592	19970822
	AU 9741482	A	19980319	AU 1997-41482	19970822
	AU 738431	B2	20010920		
	EP 1007533	A1	20000614	EP 1997-939382	19970822
	EP 1007533	B1	20050622		
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
	NZ 334870	A	20001222	NZ 1997-334870	19970822
	JP 2001501596	T	20010206	JP 1998-511674	19970822
	AT 298344	T	20050715	AT 1997-939382	19970822
	ES 2244006	T3	20051201	ES 1997-939382	19970822
	US 6315978	B1	20011113	US 1999-202328	19991022
	US 20020049154	A1	20020425	US 2001-982968	20011022
	US 6777237	B2	20040817		
	US 20020111294	A1	20020815	US 2001-982940	20011022
	US 6790827	B2	20040914		
	US 20020115595	A1	20020822	US 2001-982892	20011022
	US 6776976	B2	20040817		
PRAI	US 1996-24430P	P	19960827		
	US 1996-25036P	P	19960827		
	WO 1997-US14140	W	19970822		
	US 1999-202328	A3	19991022		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS MARPAT 128:226232
OSC.G 15 THERE ARE 15 CAPLUS RECORDS THAT CITE THIS RECORD (20 CITINGS)
RE.CNT 4 THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 40 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 128:70422 CA
OREF 128:13599a,13602a
TI Experimental study evaluating the effect of combined methotrexate and fluorouracil therapy on anemia in mice with L1210 lymphoid **leukemia**
AU Graczyk, Julia
CS Dep. Pharmacology, Medical Univ. Lodz, Lodz, 90151, Pol.
SO Pteridines (1997), 8(3), 216-227
CODEN: PTRDEO; ISSN: 0933-4807
PB International Society of Pteridinology
DT Journal
LA English

L15 ANSWER 41 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 127:328691 CA
OREF 127:64461a,64464a
TI Immortalized human colon epithelial cell lines
IN Blum, Stephanie; Pfeifer, Andrea; Troumvoukis, Yvonne
PA Societe Des Produits Nestle S.A., Switz.
SO Eur. Pat. Appl., 19 pp.
CODEN: EPXXDW
DT Patent
LA French
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI EP 802257	A1	19971022	EP 1996-201064	19960419
EP 802257	B1	20020821		
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, PT, IE, SI, LT, LV				
AT 222598	T	20020915	AT 1996-201064	19960419
ES 2180689	T3	20030216	ES 1996-201064	19960419
CA 2202923	A1	19971019	CA 1997-2202923	19970416
CA 2202923	C	20080610		
RU 2220201	C2	20031227	RU 1997-106170	19970416
FI 9701628	A	19971020	FI 1997-1628	19970417
NO 9701757	A	19971020	NO 1997-1757	19970417
NO 319494	B1	20050822		
AU 9718933	A	19971023	AU 1997-18933	19970417
US 6194203	B1	20010227	US 1997-839271	19970417
JP 10028580	A	19980203	JP 1997-102172	19970418
JP 3931212	B2	20070613		
US 6395542	B1	20020528	US 2000-593134	20000614
US 6399381	B1	20020604	US 2000-593135	20000614
PRAI EP 1996-201064	A	19960419		
US 1997-839271	A3	19970417		
US 1998-6886	B3	19980114		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OSC.G 7 THERE ARE 7 CAPLUS RECORDS THAT CITE THIS RECORD (7 CITINGS)

L15 ANSWER 42 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 125:164537 CA
OREF 125:30763a,30766a
TI Apoptosis in blood diseases. Review - new data
AU Binet, J. L.; Mentz, F.; Merle-Beral, H.
CS Department Hematology, Hopital Pitie-Salpetriere, Paris, F-75651/13, Fr.
SO Hematology and Cell Therapy (1996), 38(3), 253-264
CODEN: HCTHFA; ISSN: 1430-2772
PB Springer
DT Journal; General Review
LA English
OSC.G 11 THERE ARE 11 CAPLUS RECORDS THAT CITE THIS RECORD (11 CITINGS)

L15 ANSWER 43 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 125:8488 CA
OREF 125:1955a,1958a
TI Anti-receptor and growth blocking agents to the **vitamin**

B12/transcobalamin II receptor and binding sites
IN Morgan, A. Charles, Jr.; Quadros, Edward V.; Rothenberg, Sheldon P.
PA Receptagen Corporation, USA; State University of New York
SO PCT Int. Appl., 65 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9608515	A1	19960321	WO 1995-US12207	19950913
	W: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, UZ, VN				
	RW: KE, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	US 5688504	A	19971118	US 1994-306504	19940913
	AU 9536833	A	19960329	AU 1995-36833	19950913
	EP 783526	A1	19970716	EP 1995-934520	19950913
	EP 783526	B1	20060301		
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE				
	JP 10508831	T	19980902	JP 1995-510437	19950913
PRAI	US 1994-306504	A	19940913		
	US 1995-381522	A	19950131		
	US 1995-476440	A	19950607		
	US 1992-880540	B2	19920508		
	WO 1995-US12207	W	19950913		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
RE.CNT 1 THERE ARE 1 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 44 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 124:176815 CA
OREF 124:32818h,32819a
TI Preparation of **vitamin B12** derivatives as receptor modulating agents for treating cancers
IN Morgan, A. Charles; Wilbur, D. Scott; Pathare, Pradip M.
PA USA
SO PCT Int. Appl., 101 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 6

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9527723	A1	19951019	WO 1995-US4404	19950407
	W: AU, CA, JP, KR, NO, NZ				
	RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE				
	US 5739287	A	19980414	US 1995-406192	19950316
	US 5840880	A	19981124	US 1995-406191	19950316
	US 5869465	A	19990209	US 1995-406194	19950316
	AU 9522835	A	19951030	AU 1995-22835	19950407
	EP 754189	A1	19970122	EP 1995-916284	19950407
	EP 754189	B1	20021009		
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE				
	JP 10502334	T	19980303	JP 1995-526497	19950407
	AT 225799	T	20021015	AT 1995-916284	19950407
	US 6083926	A	20000704	US 1998-200422	19981123
PRAI	US 1994-224831	A	19940408		
	US 1995-406191	A	19950316		
	US 1995-406192	A	19950316		
	US 1995-406194	A	19950316		
	WO 1995-US4404	W	19950407		
	US 1995-545151	A3	19951019		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OS MARPAT 124:176815
OSC.G 9 THERE ARE 9 CAPLUS RECORDS THAT CITE THIS RECORD (10 CITINGS)
RE.CNT 3 THERE ARE 3 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 45 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 120:227009 CA
OREF 120:40121a,40124a
TI Prevention of birth defects and childhood **cancer** with fluoride
IN Grogan, Jack R., Jr.
PA USA
SO Can. Pat. Appl., 17 pp.
CODEN: CPXXEB
DT Patent
LA English
FAN.CNT 2

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	CA 2071378	A1	19931217	CA 1992-2071378	19920616
	GB 2267824	A	19931222	GB 1992-12672	19920615
PRAI	CA 1992-2071378		19920616		

L15 ANSWER 46 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 119:131055 CA
OREF 119:23285a,23288a
TI Influence of cobalamin on the survival of mice bearing ascites **tumor**
AU Tsao, Constance S.; Myashita, Koichi
CS Linus Pauling Inst. Sci. Med., Palo Alto, CA, 94306, USA
SO Pathobiology (1993), 61(2), 104-8
CODEN: PATHEF; ISSN: 1015-2008
DT Journal
LA English
OSC.G 5 THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)

L15 ANSWER 47 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 119:39993 CA
OREF 119:7079a,7082a
TI Vitamins as chemotherapeutic and chemopreventive agents
AU Ryan, Donna H.; Starr, Barry
CS Pennington Biomed. Res. Cent., Baton Rouge, LA, 70808, USA
SO Pennington Center Nutrition Series (1993), 3(Vitamins and Cancer Prevention), 147-60
CODEN: PCNSEW; ISSN: 1063-8822
DT Journal; General Review
LA English

L15 ANSWER 48 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 116:75807 CA
OREF 116:12671a,12674a
TI Effect of combined ascorbic acid and B-12 on survival of mice with implanted Ehrlich **carcinoma** and L1210 **leukemia**
AU Poydock, M. Eymard
CS Cancer Res. Inst., Mercyhurst Coll., Erie, PA, 16546, USA
SO American Journal of Clinical Nutrition (1991), 54(6, Suppl.), 1261S-1265S
CODEN: AJCNAC; ISSN: 0002-9165
DT Journal
LA English
OSC.G 4 THERE ARE 4 CAPLUS RECORDS THAT CITE THIS RECORD (4 CITINGS)

L15 ANSWER 49 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 115:126995 CA
OREF 115:21549a,21552a
TI New **vitamin B12** derivatives, production thereof, and applications thereof
IN Toraya, Tetsuo; Ishida, Atsuhiko; Uejima, Yasuhide; Fujii, Katsuhiko
PA Teijin Ltd., Japan
SO PCT Int. Appl., 49 pp.
CODEN: PIXXD2
DT Patent
LA Japanese
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE

PI WO 9010014 A1 19900907 WO 1990-JP253 19900228
W: US
RW: CH, DE, FR, GB, IT
JP 02289597 A 19901129 JP 1990-45905 19900228
JP 2962755 B2 19991012
EP 425680 A1 19910508 EP 1990-903929 19900228
R: CH, DE, FR, GB, IT, LI
US 5405839 A 19950411 US 1993-104606 19930811
PRAI JP 1989-45172 A 19890228
WO 1990-JP253 W 19900228
US 1990-601778 B1 19901026
OS MARPAT 115:126995
OSC.G 9 THERE ARE 9 CAPLUS RECORDS THAT CITE THIS RECORD (11 CITINGS)
RE.CNT 2 THERE ARE 2 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L15 ANSWER 50 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 106:98888 CA
OREF 106:16133a,16136a
TI Rapid determination of serum transcobalamins
AU Hu, Jiuru; Wang, Fumin; Dou, Huanfu; Wang, Liangxu
CS Nav. Gen. Hosp., Peop. Rep. China
SO Zhonghua Xueyexue Zazhi (1986), 7(7), 431-3
CODEN: CHTCD7; ISSN: 0253-2727
DT Journal
LA Chinese

L15 ANSWER 51 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 105:126980 CA
OREF 105:20333a,20336a
TI Effects of 5-fluorouracil treatment of rat **leukemia** with concomitant inactivation of cobalamin
AU Kroes, A. C. M.; Ermens, A. A. M.; Lindemans, J.; Abels, J.
CS Inst. Hematol., Erasmus Univ., Rotterdam, Neth.
SO Anticancer Research (1986), 6(4), 737-42
CODEN: ANTRD4; ISSN: 0250-7005
DT Journal
LA English
OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)

L15 ANSWER 52 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 105:108097 CA
OREF 105:17335a,17338a
TI Enhanced therapeutic effect of methotrexate in experimental rat **leukemia** after inactivation of cobalamin (**vitamin B12**) by nitrous oxide
AU Kroes, A. C. M.; Lindemans, J.; Schoester, M.; Abels, J.
CS Inst. Hematol., Erasmus Univ., Rotterdam, 3000 DR, Neth.
SO Cancer Chemotherapy and Pharmacology (1986), 17(2), 114-20
CODEN: CCPHDZ; ISSN: 0344-5704
DT Journal
LA English
OSC.G 5 THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)

L15 ANSWER 53 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 105:76826 CA
OREF 105:12445a,12448a
TI Kinetics of 57Co-cyanocobalamin distribution in organs and tissues of mice with transplanted **tumors**
AU Vares, Yu. V.; Myasishcheva, N. V.
CS Res. Inst. Carcinogen., Moscow, 115478, USSR
SO Eksperimental'naya Onkologiya (1986), 8(3), 33-6
CODEN: EKSODD; ISSN: 0204-3564
DT Journal
LA Russian

L15 ANSWER 54 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 104:84931 CA

OREF 104:13417a,13420a
TI Simultaneous multiple assays and compounds and compositions useful in them
IN Olson, Douglas Richard
PA Micromedic Systems, Inc., USA
SO Eur. Pat. Appl., 26 pp.
CODEN: EPXXDW
DT Patent
LA English
FAN.CNT 2

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	EP 165716	A1	19851227	EP 1985-303564	19850521
	EP 165716	B1	19900131		
	R: AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE				
	US 4672028	A	19870609	US 1984-612979	19840523
	AT 50066	T	19900215	AT 1985-303564	19850521
	AU 8542798	A	19851128	AU 1985-42798	19850523
	AU 582970	B2	19890413		
	JP 61000092	A	19860106	JP 1985-111312	19850523
PRAI	US 1984-612979	A	19840523		
	EP 1985-303564	A	19850521		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OSC.G 8 THERE ARE 8 CAPLUS RECORDS THAT CITE THIS RECORD (9 CITINGS)

L15 ANSWER 55 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 103:213903 CA
OREF 103:34477a,34480a
TI Mitogenic inhibition and effect on survival of mice bearing L1210 **leukemia** using a combination of dehydroascorbic acid and hydroxycobalamin
AU Poydock, M. E.; Harguindey, S.; Hart, T.; Takita, H.; Kelly, D.
CS Cancer Res. Unit, Mercyhurst Coll., Erie, PA, USA
SO American Journal of Clinical Oncology (1985), 8(3), 266-9
CODEN: AJCODI; ISSN: 0277-3732
DT Journal
LA English
OSC.G 7 THERE ARE 7 CAPLUS RECORDS THAT CITE THIS RECORD (7 CITINGS)

L15 ANSWER 56 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 99:35419 CA
OREF 99:5533a,5536a
TI Studies of the radioimmunoassay of serum haptocorrin and its clinical application
AU Saito, Kainosuke
CS Dep. Intern. Med., Sapporo Med. Coll., Sapporo, Japan
SO Sapporo Igaku Zasshi (1983), 52(2), 237-52
CODEN: SIZSAR; ISSN: 0036-472X
DT Journal
LA Japanese

L15 ANSWER 57 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 97:107723 CA
OREF 97:17883a,17886a
TI Production of transcobalamin II by various murine and human cells in culture
AU Rabinowitz, R.; Rachmilewitz, B.; Rachmilewitz, M.; Schlesinger, M.
CS Hadassah Med. Sch., Hebrew Univ., Jerusalem, 91010, Israel
SO Israel Journal of Medical Sciences (1982), 18(7), 740-5
CODEN: IJMDAI; ISSN: 0021-2180
DT Journal
LA English
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)

L15 ANSWER 58 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 97:5040 CA
OREF 97:987a,990a
TI Influence of vitamins C and B12 on the survival rate of mice bearing ascites **tumor**
AU Poydock, M. Eymard; Reikert, D.; Rice, J.

CS Mercyhurst Coll., Erie, PA, 16546, USA
SO Experimental Cell Biology (1982), 50(2), 88-91
CODEN: ECEBDI; ISSN: 0304-3568
DT Journal
LA English
OSC.G 4 THERE ARE 4 CAPLUS RECORDS THAT CITE THIS RECORD (4 CITINGS)

L15 ANSWER 59 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 95:93426 CA
OREF 95:15687a,15690a
TI Determination of transcobalamins
IN Selhub, Jacob; Rachmilewitz, Bracha; Grossowicz, Nathan
PA Yissum Research Development Co., Israel
SO U.S., 8 pp. Cont.-in-part of U.S. 4,167,556.
CODEN: USXXAM
DT Patent
LA English
FAN.CNT 4

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 4273757	A	19810616	US 1978-961771	19781117
	CA 1092956	A1	19810106	CA 1977-278950	19770520
	US 4167556	A	19790911	US 1977-802379	19770602
PRAI	US 1977-802379	A2	19770602		
	IL 1976-49662	A	19760526		
	US 1978-961771	A	19781117		

OSC.G 13 THERE ARE 13 CAPLUS RECORDS THAT CITE THIS RECORD (16 CITINGS)

L15 ANSWER 60 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 90:99501 CA
OREF 90:15677a,15680a
TI The identification and measurement of a folate-binding protein in human serum by radioimmunoassay
AU Da Costa, Maria; Rothenberg, Sheldon P.; Fischer, Craig; Rosenberg, Zoltan
CS Dep. Med., New York Med. Coll., New York, NY, USA
SO Journal of Laboratory and Clinical Medicine (1978), 91(6), 901-10
CODEN: JLCMAK; ISSN: 0022-2143
DT Journal
LA English
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

L15 ANSWER 61 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 89:40483 CA
OREF 89:6263a,6266a
TI **Vitamin B12**-binding proteins in serum and plasma in various disorders. Effect of anticoagulants
AU Carmel, Ralph
CS Dep. Med., Univ. Southern California Sch. Med., Los Angeles, CA, USA
SO American Journal of Clinical Pathology (1978), 69(3), 319-25
CODEN: AJCPAI; ISSN: 0002-9173
DT Journal
LA English
OSC.G 5 THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)

L15 ANSWER 62 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 88:150028 CA
OREF 88:23630h,23631a
TI **Vitamin B12** and **vitamin B12** binding proteins in liver diseases
AU Areekul, Suvit; Panatampon, Piangporn; Doungbarn, Jiraporn
CS Fac. Trop. Med., Mahidol Univ., Bangkok, Thailand
SO Southeast Asian Journal of Tropical Medicine and Public Health (1977), 8(3), 322-8
CODEN: SJTMAK; ISSN: 0125-1562
DT Journal
LA English
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)

L15 ANSWER 63 OF 88 CA COPYRIGHT 2009 ACS on STN

Full Text
AN 88:20262 CA
OREF 88:3251a,3254a
TI Analysis of cobalamin coenzymes in **tumor** cells of mice spleen
AU Vares, Yu. V.; Myasishcheva, N. V.
CS Oncol. Res. Cent., Moscow, USSR
SO Voprosy Meditsinskoi Khimii (1977), 23(5), 681-4
CODEN: VMDKAM; ISSN: 0042-8809
DT Journal
LA Russian

L15 ANSWER 64 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:153564 CA
OREF 86:24107a,24110a
TI Hemoglobin A2 levels in health and various hematologic disorders
AU Alperin, Jack B.; Dow, Patricia A.; Petteway, Mozellar B.
CS Dep. Intern. Med., Univ. Texas, Galveston, TX, USA
SO American Journal of Clinical Pathology (1977), 67(3), 219-26
CODEN: AJCPAI; ISSN: 0002-9173
DT Journal
LA English
OSC.G 6 THERE ARE 6 CAPLUS RECORDS THAT CITE THIS RECORD (6 CITINGS)

L15 ANSWER 65 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:137655 CA
OREF 86:21624h,21625a
TI Determination of the unsaturated **vitamin B12** binding capacity in normal and physiopathological conditions
AU Areekul, Suvit; Vongtapvanish, Srisuda
CS Fac. Trop. Med., Mahidol Univ., Bangkok, Thailand
SO Southeast Asian Journal of Tropical Medicine and Public Health (1976), 7(3), 496-8
CODEN: SJTMAK; ISSN: 0125-1562
DT Journal
LA English

L15 ANSWER 66 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 86:3 CA
OREF 86:1a
TI B12-dependent methionine synthetase as a potential target for **cancer** chemotherapy
AU Huennekens, F. M.; DiGirolamo, P. M.; Fujii, K.; Jacobsen, D. W.; Vitols, K. S.
CS Dep. Biochem., Scripps Clin. Res. Found., La Jolla, CA, USA
SO Advances in Enzyme Regulation (1976), 14, 187-205
CODEN: AEZRA2; ISSN: 0065-2571
DT Journal; General Review
LA English
OSC.G 5 THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)

L15 ANSWER 67 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 82:29483 CA
OREF 82:4708h,4709a
TI Granulocyte colony stimulating activity and **vitamin B12** binding proteins in human urine
AU Gibson, Emma L.; Herbert, Victor; Robinson, William A.
CS Med. Cent., Univ. Colorado, Denver, CO, USA
SO British Journal of Haematology (1974), 28(2), 191-7
CODEN: BJHEAL; ISSN: 0007-1048
DT Journal
LA English

L15 ANSWER 68 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 81:89342 CA
OREF 81:14171a,14174a
TI Characteristics of a novel serum **vitamin B12**-binding protein associated with hepatocellular **carcinoma**

AU Waxman, Samuel; Gilbert, Harriet S.
CS Mt. Sinai Sch. Med., City Univ. New York, New York, NY, USA
SO British Journal of Haematology (1974), 27(2), 229-39
CODEN: BJHEAL; ISSN: 0007-1048
DT Journal
LA English

L15 ANSWER 69 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:131413 CA
OREF 80:21193a,21196a
TI N5-Methyltetrahydrofolate:homocysteine methyltransferase activity in extracts from normal, malignant, and embryonic tissue culture cells
AU Ashe, Hilary; Clark, Brian R.; Chu, Fred; Hardy, Dorothy N.; Halpern, Barbara C.; Halpern, Richard M.; Smith, Roberts A.
CS Mol. Biol. Inst., Univ. California, Los Angeles, CA, USA
SO Biochemical and Biophysical Research Communications (1974), 57(2), 417-25
CODEN: BBRCA9; ISSN: 0006-291X
DT Journal
LA English
OSC.G 6 THERE ARE 6 CAPLUS RECORDS THAT CITE THIS RECORD (6 CITINGS)

L15 ANSWER 70 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 80:25638 CA
OREF 80:4234h,4235a
TI Glutathione peroxidase in human red cells in health and disease
AU Hopkins, J.; Tudhope, G. R.
CS Dep. Pharmacol. Ther., Univ. Dundee, Dundee, UK
SO British Journal of Haematology (1973), 25(5), 563-75
CODEN: BJHEAL; ISSN: 0007-1048
DT Journal
LA English
OSC.G 49 THERE ARE 49 CAPLUS RECORDS THAT CITE THIS RECORD (49 CITINGS)

L15 ANSWER 71 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 77:138108 CA
OREF 77:22717a,22720a
TI Leukemogenesis by Rauscher virus in mice
AU Irino, Shozo; Miyoshi, Isao; Sezaki, Tatsuo; Nagao, Tadami; Taguchi, Hirokuni; Hara, Koichi; Hiraki, Kiyoshi
CS Med. Sch., Okayama Univ., Okayama, Japan
SO Exp. Leukemogenesis, Pap. Jap. Cancer Ass. Symp. Exp. Leuk. Res. Jap. (1972), Meeting Date 1970, 47-63. Editor(s): Yamamoto, Tadashi. Publisher: Univ. Park Press, Baltimore, Md.
CODEN: 25POAE
DT Conference
LA English

L15 ANSWER 72 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 76:70733 CA
OREF 76:11401a,11404a
TI Formiminoglutamic acid excretion after histidine loading in folic acid-**vitamin B12** metabolic disturbances
AU Wilmanns, W.
CS Med. Universitaetsklin., Tuebingen, Fed. Rep. Ger.
SO Wissenschaftliche Veroeffentlichungen der Deutschen Gesellschaft fuer Ernaehrung (1971), 19, 30-46
CODEN: WVGEAP; ISSN: 0043-6828
DT Journal
LA German

L15 ANSWER 73 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 75:96679 CA
OREF 75:15287a,15290a
TI Increased transcobalamin I in a leukemoid reaction
AU Hall, Charles A.; Wanko, Maxine
CS Hematol. Res. Lab., Albany Veterans Adm. Hosp., Albany, NY, USA
SO Journal of Laboratory and Clinical Medicine (1971), 78(2), 298-301

CODEN: JLCMAK; ISSN: 0022-2143
DT Journal
LA English
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)

L15 ANSWER 74 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 74:40522 CA
OREF 74:6517a,6520a
TI Acquired aplastic anemia
AU Keiser, G.
CS Med. Abt., Buergerspital, Zug, Switz.
SO Deutsche Medizinische Wochenschrift (1970), 95(40), 2032-4
CODEN: DMWOAX; ISSN: 0012-0472
DT Journal
LA German

L15 ANSWER 75 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 71:28714 CA
OREF 71:5289a,5292a
TI Determination of blood folate activity in humans in healthy and in various pathological states
AU Karlin, Rosalie
CS Inst. Pasteur, Lyons, Fr.
SO Internationale Zeitschrift fuer Vitaminforschung (1969), 39(1), 44-64
CODEN: IZVIAK; ISSN: 0020-9406
DT Journal
LA French

L15 ANSWER 76 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 71:11249 CA
OREF 71:2051a,2054a
TI **Vitamin B12** and some indexes of nucleic acid metabolism in **leukemia**
AU Sheremet, Z. I.; Myasishcheva, N. V.
CS Inst. Eksp. Klin. Onkol., Moscow, USSR
SO Probl. Leikozov (1967), 164-70. Editor(s): Rostovtsev, N. F. Publisher: Izd. "Kolos", Moscow, USSR.
CODEN: 20XPAO
DT Conference
LA Russian

L15 ANSWER 77 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 70:94909 CA
OREF 70:17731a,17734a
TI Uptake of labeled **vitamin B12** and 4-iodophenylalanine in some **tumors** of mice
AU Blomquist, Lars; Flodh, H.; Ullberg, Sven
CS Dep. Pharmacol., Roy. Vet. Coll., Stockholm, Swed.
SO Experientia (1969), 25(3), 294-6
CODEN: EXPEAM; ISSN: 0014-4754
DT Journal
LA English
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)

L15 ANSWER 78 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 69:84990 CA
OREF 69:15874h,15875a
TI Determination of formiminoglutamic acid excretion as a functional test for disturbances in folic acid and **vitamin B12** metabolism
AU Wilmanns, W.; Burgmann, T.
CS Med. Universitaetsklin. Tuebingen, Tuebingen, Fed. Rep. Ger.
SO Deutsche Medizinische Wochenschrift (1968), 93(38), 1801-6
CODEN: DMWOAX; ISSN: 0012-0472
DT Journal
LA German

L15 ANSWER 79 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text

AN 63:91925 CA
OREF 63:16915d-f
TI Adenosylmethionine elevation in leukemic white blood cells
AU Baldessarini, Ross J.; Carbone, Paul P.
CS Natl. Cancer Inst., Bethesda, MD
SO Science (Washington, DC, United States) (1965), 149(3684), 644-5
CODEN: SCIEAS; ISSN: 0036-8075
DT Journal
LA English

L15 ANSWER 80 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 61:71260 CA
OREF 61:12425g-h
TI Some investigations of folic acid deficiency
AU Kershaw, P. W.; Girdwood, R. H.
CS Roy. Infirmary, Edinburgh
SO Scot. Med. J. (1964), 9(5), 201-12
DT Journal
LA Unavailable

L15 ANSWER 81 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 60:41018 CA
OREF 60:7258h,7259a
TI Serum protein changes and organ dye concentrations in trypan blue **carcinogenesis**
AU Brown, D. V.; Norlind, L. M.; Adamovics, A.; Bowen, A.
CS Univ. of Washington, Seattle
SO Proceedings of the Society for Experimental Biology and Medicine (1963), 114, 290-3
CODEN: PSEBAA; ISSN: 0037-9727
DT Journal
LA Unavailable

L15 ANSWER 82 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 60:5296 CA
OREF 60:961a-d
TI Red cell enzymes in anemia
AU Vuopio, Pekka
CS Finnish Red Cross Blood Transfusion Serv., Helsinki
SO Scandinavian Journal of Clinical and Laboratory Investigation (1963), Suppl. 15(72), 90 pp.
CODEN: SJCLAY; ISSN: 0036-5513
DT Journal
LA Unavailable

L15 ANSWER 83 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 55:18970 CA
OREF 55:3798e-h
TI Co58-[**Vitamin**]**B12** absorption, plasma transport, and excretion in patients with myeloproliferative disorders, solid **tumors**, and non-**neoplastic** disease
AU Weinstein, I. Bernard; Watkin, Donald M.
CS Natl. Cancer Inst. Bethesda, MD
SO Journal of Clinical Investigation (1960), 39, 1667-74
CODEN: JCINAO; ISSN: 0021-9738
DT Journal
LA Unavailable

L15 ANSWER 84 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 54:131385 CA
OREF 54:25240i,25241a
TI Clearance of intravenously injected radioactive cobalt-labeled **vitamin B12** in chronic myeloid **leukemia** and other conditions
AU Ritz, Norton D.; Meyer, Leo M.
CS Maimonides Hosp., Brooklyn, NY
SO Cancer (1960), 13, 1000-7
DT Journal

LA Unavailable

L15 ANSWER 85 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 52:115884 CA
OREF 52:20584a-b
TI The diagnostic value of the determination of **vitamin B12** in body fluids in diseases of the blood and liver
AU Rachmilewitz, M.; Stein, Y.
CS Rothschild Hadassah Univ. Hosp., Jerusalem, Israel
SO Harefuah (1958), 54, 167-70
CODEN: HAREA6; ISSN: 0017-7768
DT Journal
LA Unavailable

L15 ANSWER 86 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 52:78440 CA
OREF 52:13964a-c
TI Serum **vitamin B12** concentrations determined by Lactobacillus leichmannii assay in patients with **neoplastic** disease
AU Mendelsohn, Robert S.; Watkin, Donald M.
CS Natl. Insts. Health, Bethesda, MD
SO Journal of Laboratory and Clinical Medicine (1958), 51, 860-6
CODEN: JLCMAK; ISSN: 0022-2143
DT Journal
LA Unavailable
OSC.G 2 THERE ARE 2 CAPLUS RECORDS THAT CITE THIS RECORD (2 CITINGS)
L15 ANSWER 87 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 52:46370 CA
OREF 52:8346c-f
TI Chromatography of serum proteins in normal and pathologic serums: the distribution of protein-bound carbohydrate and cholesterol, siderophilin, thyroxine-binding protein, **vitamin B12**-binding protein, alkaline and acid phosphatases, radioiodinated albumin, and myeloma proteins
AU Fahey, John L.; McCoy, Patricia F.; Goulian, Mehran
CS Natl. Insts. of Health, Bethesda, MD
SO Journal of Clinical Investigation (1958), 37, 272-84
CODEN: JCINAO; ISSN: 0021-9738
DT Journal
LA Unavailable

L15 ANSWER 88 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 50:90938 CA
OREF 50:17113g-i,17114a
TI Pathology and physiology of zinc metabolism
AU Wolff, H. P.
CS Univ. Marburg a.d. Lahn, Germany
SO Klinische Wochenschrift (1956), 34, 409-18
CODEN: KLWOAZ; ISSN: 0023-2173
DT Journal
LA Unavailable
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

=> d an ti in au so pi ab kwic 44 47

L15 ANSWER 44 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 124:176815 CA
OREF 124:32818h,32819a
TI Preparation of **vitamin B12** derivatives as receptor modulating agents for treating cancers
IN Morgan, A. Charles; Wilbur, D. Scott; Pathare, Pradip M.
IN Morgan, A. Charles; Wilbur, D. Scott; Pathare, Pradip M.
SO PCT Int. Appl., 101 pp.
CODEN: PIXXD2
PATENT NO. KIND DATE APPLICATION NO. DATE
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PI WO 9527723 A1 19951019 WO 1995-US4404 19950407
W: AU, CA, JP, KR, NO, NZ
RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE
US 5739287 A 19980414 US 1995-406192 19950316
US 5840880 A 19981124 US 1995-406191 19950316
US 5869465 A 19990209 US 1995-406194 19950316
AU 9522835 A 19951030 AU 1995-22835 19950407
EP 754189 A1 19970122 EP 1995-916284 19950407
EP 754189 B1 20021009
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE
JP 10502334 T 19980303 JP 1995-526497 19950407
AT 225799 T 20021015 AT 1995-916284 19950407
US 6083926 A 20000704 US 1998-200422 19981123

AB Receptor modulating agents comprising a **vitamin B12** targeting mol. coupled to a rerouting moiety (I; R1 - R7 = a linker, through which a rerouting moiety is coupled), which are capable of modulating cell surface receptors by affecting the cell surface receptor trafficking pathway via retaining an agent/receptor complex in an endosome, are prepd. Said rerouting moiety is preferably (1) a lysosomotropic moiety selected from aminoglycoside antibiotics such as gentamycin, sisomicin, netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin, ribostamycin, butirosin, and streptomycin, (2) a peptide sorting sequence selected from endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides, and clathrin-binding peptides., and (3) a conditional membrane binding peptide selected from charged glutamate, aspartate, and histidine. These receptor modulating agents are useful for treating **neoplastic** disorders such as **leukemia**, sarcoma, myeloma, **carcinoma**, neuroma, melanoma, cancers of the lung, liver, breast, colon, cervix, and prostate, Hodgkin's disease, and non-Hodgkin's lymphoma. Thus, a mixt. of 500 mg cyanocobalamin monocarboxylic acids I (R1 = R7 = OH, R2 - R6 = NH2; R1 = R3 - R6 = NH2, R2 = R7 = OH; R1 - R3 = R5 = R6 = NH2, R4 = R7 = OH) (prepn. given) and 3.6 g 1,12-diaminododecane in 100 mL H2O was adjusted to pH 6 with 1 N HCl, treated with 726 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and stirred at room temp. for 22 h to give cyanocobalamin monocarboxylic acid N-(12-aminododecyl)amides I [R1 = NH(CH2)12NH2, R2 - R6 = NH2, R7 = OH] and I [R1 = R3 - R6 = NH2, R2 = NH(CH2)12NH2, R7 = OH] (II). II at 10 μM in vitro killed 85% K562 cells.

TI Preparation of **vitamin B12** derivatives as receptor modulating agents for treating cancers

AB Receptor modulating agents comprising a **vitamin B12** targeting mol. coupled to a rerouting moiety (I; R1 - R7 = a linker, through which a rerouting moiety is. . . a conditional membrane binding peptide selected from charged glutamate, aspartate, and histidine. These receptor modulating agents are useful for treating **neoplastic** disorders such as **leukemia**, sarcoma, myeloma, **carcinoma**, neuroma, melanoma, cancers of the lung, liver, breast, colon, cervix, and prostate, Hodgkin's disease, and non-Hodgkin's lymphoma. Thus, a mixt.. . .

ST **vitamin B12** deriv prepn receptor modulating; anticancer **vitamin B12** deriv; aminoglycoside antibiotic conjugate **vitamin B12**; peptide conjugate **vitamin B12**; conditional membrane binding peptide

IT Peptides, preparation
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(peptide sorting sequence (e.g. endoplasmic retention peptides) or conditional membrane binding peptide; prepn. of **vitamin B12**-peptide conjugates as receptor modulating agents for treating cancers)

IT Receptors
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(prepn. of **vitamin B12** derivs. as receptor modulating agents affecting cell surface receptor trafficking pathway for treating cancers)

IT Neoplasm inhibitors
(prepn. of **vitamin B12** derivs. as receptor modulating agents for treating cancers)

IT Antibiotics
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)

(aminoglycoside, prepn. of **vitamin B12**
-aminoglycoside antibiotic conjugates as receptor modulating agents for treating cancers)

IT 57-92-1DP, Streptomycin, **vitamin B12** conjugate
59-01-8DP, Kanamycin, **vitamin B12** conjugate
1403-66-3DP, Gentamycin, **vitamin B12** conjugate
1404-04-2DP, Neomycin, **vitamin B12** conjugate
7542-37-2DP, Paromomycin, **vitamin B12** conjugate
12772-35-9DP, Butirosin, **vitamin B12** conjugate
25546-65-0DP, Ribostamycin, **vitamin B12** conjugate
32385-11-8DP, Sisomicin, **vitamin B12** conjugate
32986-56-4DP, Tobramycin, **vitamin B12** conjugate
37517-28-5DP, Amikacin, **vitamin B12** conjugate
56391-56-1DP, Netilmicin, **vitamin B12** conjugate
160927-56-0P 173341-36-1P 173341-37-2P 173341-38-3P 173341-39-4P
173341-40-7P 173341-41-8P 173341-42-9P 173341-43-0P 173341-44-1P
173341-45-2P 173341-46-3P 173341-47-4P 173341-48-5P 173341-52-1P
173341-53-2P 173341-54-3P
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(prepn. of **vitamin B12** derivs. as receptor modulating agents for treating cancers)

IT **68-19-9**, Cyanocobalamin 99-31-0, 5-Aminoisophthalic acid
99-63-8, 1,3-Benzenedicarbonyl dichloride 108-30-5, reactions
769-39-1, 2,3,5,6-Tetrafluorophenol 813-19-4, Bis(tributyltin)
1711-02-0, 4-Iodobenzoyl chloride 2783-17-7, 1,12-Diaminododecane
35013-72-0 110079-43-1
RL: RCT (Reactant); RACT (Reactant or reagent)
(prepn. of **vitamin B12** derivs. as receptor modulating agents for treating cancers)

IT 72040-64-3P 173341-22-5P 173341-23-6P 173341-24-7P 173341-25-8P
173341-26-9P 173341-27-0P 173341-28-1P 173341-29-2P 173341-30-5P
173341-31-6P 173341-32-7P 173341-33-8P 173341-34-9P 173341-35-0P
173341-49-6P 173341-50-9P 173341-51-0P 173341-59-8P
RL: RCT (Reactant); SPN (Synthetic preparation); PREP (Preparation); RACT (Reactant or reagent)
(prepn. of **vitamin B12** derivs. as receptor modulating agents for treating cancers)

IT 173341-56-5P 173341-57-6P 173341-58-7P
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); SPN (Synthetic preparation); THU (Therapeutic use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(prepn. of **vitamin B12**-aminoglycoside antibiotic conjugates as receptor modulating agents for treating cancers)

IT 86-38-4, 6,9-Dichloro-2-methoxyacridine 51857-17-1 99008-43-2
RL: RCT (Reactant); RACT (Reactant or reagent)
(prepn. of **vitamin B12**-aminoglycoside antibiotic conjugates as receptor modulating agents for treating cancers)

IT 7657-92-3P 121714-48-5P
RL: RCT (Reactant); SPN (Synthetic preparation); PREP (Preparation); RACT (Reactant or reagent)
(prepn. of **vitamin B12**-aminoglycoside antibiotic conjugates as receptor modulating agents for treating cancers)

L15 ANSWER 47 OF 88 CA COPYRIGHT 2009 ACS on STN
Full Text
AN 119:39993 CA
OREF 119:7079a,7082a
TI Vitamins as chemotherapeutic and chemopreventive agents
AU Ryan, Donna H.; Starr, Barry
SO Pennington Center Nutrition Series (1993), 3(Vitamins and Cancer Prevention), 147-60
CODEN: PCNSEW; ISSN: 1063-8822
AB A review with 45 refs. Therapy with retinoids has produced objective responses in patients with some types of skin **cancer**, and tretinoin is effective in producing terminal differentiation and complete remission in acute promyelocytic **leukemia**. **Cancer** chemoprevention trails are under way evaluating the activity of multiple vitamin prepns., beta-carotene, retinoids, vitamin C, vitamin E, **vitamin B12**, vitamin B6, and folate. Since **carcinogenesis** is a multistage process that can occur over decades in humans, efficient evaluation of chemopreventive agents requires

research strategies utilizing intermediate biol. end points. Preneoplasia, classically defined histol. cellular change, is being redefined by advances in mol. and cell biol. Vitamins have been exploited as unproven remedies to vulnerable **cancer** patients, but now vitamins and their derivs. have an emerging role in **cancer** chemotherapy and chemoprevention.

AB A review with 45 refs. Therapy with retinoids has produced objective responses in patients with some types of skin **cancer**, and tretinoin is effective in producing terminal differentiation and complete remission in acute promyelocytic **leukemia**. **Cancer** chemoprevention trails are under way evaluating the activity of multiple vitamin prepns., beta-carotene, retinoids, vitamin C, vitamin E, **vitamin B12**, vitamin B6, and folate. Since **carcinogenesis** is a multistage process that can occur over decades in humans, efficient evaluation of chemopreventive agents requires research strategies utilizing. . . change, is being redefined by advances in mol. and cell biol. Vitamins have been exploited as unproven remedies to vulnerable **cancer** patients, but now vitamins and their derivs. have an emerging role in **cancer** chemotherapy and chemoprevention.

IT Vitamins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(**cancer** chemotherapeutic and chemopreventive activity of)

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=> file uspatall
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                     149.18     175.76

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)          SINCE FILE      TOTAL
                                                         ENTRY    SESSION
CA SUBSCRIBER PRICE                                      -1.56      -1.56

FILE 'USPATFULL' ENTERED AT 23:42:50 ON 31 AUG 2009
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FILE 'USPATOLD' ENTERED AT 23:42:50 ON 31 AUG 2009
CA INDEXING COPYRIGHT (C) 2009 AMERICAN CHEMICAL SOCIETY (ACS)

FILE 'USPAT2' ENTERED AT 23:42:50 ON 31 AUG 2009
CA INDEXING COPYRIGHT (C) 2009 AMERICAN CHEMICAL SOCIETY (ACS)

=> s l1
L16       2261 L1

=> s (vitamin b12 or hydroxycobolamin or chlorocobolamin or aquocobolamin or cobolamin or azi
L17       6738 (VITAMIN B12 OR HYDROXYCOBOLAMIN OR CHLOROCOBOLAMIN OR AQUOCOBOL
               AMIN OR COBOLAMIN OR AZIDOCOBOLAMIN)

=> s (vitamin b12 or hydroxycobolamin or chlorocobolamin or aquocobolamin or cobolamin or azi
L18        888 (VITAMIN B12 OR HYDROXYCOBOLAMIN OR CHLOROCOBOLAMIN OR AQUOCOBOL
               AMIN OR COBOLAMIN OR AZIDOCOBOLAMIN)/CLM

=> s l16 or l17
L19       7872 L16 OR L17

=> s l16 or l18
L20       2538 L16 OR L18

=> s (cancer or anti-neoplast? or neoplast? or carcin? or tumor?)
L21     271712 (CANCER OR ANTI-NEOPLAST? OR NEOPLAST? OR CARCIN? OR TUMOR?)

=> s (cancer or anti-neoplast? or neoplast? or carcin? or tumor?)/clm
L22      59768 (CANCER OR ANTI-NEOPLAST? OR NEOPLAST? OR CARCIN? OR TUMOR?)/CLM

=> s l19 and l21
L23       4265 L19 AND L21

=> s l20 and l22
L24        254 L20 AND L22

=> s leukemia?
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```
L25      72327 LEUKEMIA?

=> s leukemia?/clm
L26       8743 LEUKEMIA?/CLM

=> s l23 and l25
L27       1851 L23 AND L25

=> s l24 and l26
L28         24 L24 AND L26

=> d 1-24

L28  ANSWER 1 OF 24  USPATFULL on STN
Full Text
AN     2009:145928  USPATFULL
TI     Lipid compositions for the treatment and prevention of proliferative
       diseases and for the reduction of incidences of mutagenesis and
       carinogenesis
IN     Yosef, Fabiana Bar, Haifa, ISRAEL
PA     Enzymotec Ltd., Migdal Haemek, ISRAEL (non-U.S. corporation)
PI     US 20090131523      A1  20090521
AI     US 2008-285806      A1  20081014 (12)
PRAI   US 2007-960798P         20071015 (60)
DT     Utility
FS     APPLICATION
LN.CNT 1226
INCL   INCLM: 514/558.000
       INCLS: 426 2
NCL    NCLM:  514/558.000
       NCLS:  426/002.000
IC     IPCI   A61K0031-20 [I,A]; A61K0031-185 [I,C*]; A23D0007-005 [I,A];
              A23D0007-04 [I,A]; A23D0007-02 [I,C*]; A23L0001-29 [I,A]
       IPCR   A61K0031-185 [I,C]; A61K0031-20 [I,A]; A23D0007-005 [I,C];
              A23D0007-005 [I,A]; A23D0007-02 [I,C]; A23D0007-04 [I,A];
              A23L0001-29 [I,C]; A23L0001-29 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 2 OF 24  USPATFULL on STN
Full Text
AN     2009:58740  USPATFULL
TI     Transfer Factor Compositions and Methods
IN     Ramaekers, Joseph C., Aptos, CA, UNITED STATES
PI     US 20090053197      A1  20090226
AI     US 2007-762727      A1  20070613 (11)
PRAI   US 2006-814777P         20060614 (60)
       US 2006-834739P         20060731 (60)
DT     Utility
FS     APPLICATION
LN.CNT 1798
INCL   INCLM: 424/130.100
NCL    NCLM:  424/130.100
IC     IPCI   A61K0039-395 [I,A]; A61P0003-00 [I,A]
       IPCR   A61K0039-395 [I,C]; A61K0039-395 [I,A]; A61P0003-00 [I,C];
              A61P0003-00 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 3 OF 24  USPATFULL on STN
Full Text
AN     2008:253184  USPATFULL
TI     Advanced drug development and manufacturing
IN     Birnbaum, Eva R., Los Alamos, NM, UNITED STATES
       Koppisch, Andrew T., Flagstaff, AZ, UNITED STATES
       Baldwin, Sharon M., Santa Fe, NM, UNITED STATES
       Warner, Benjamin P., Los Alamos, NM, UNITED STATES
       McCleskey, T. Mark, Los Alamos, NM, UNITED STATES
       Stewart, Jeffrey Joseph, Los Alamos, NM, UNITED STATES
       Berger, Jennifer A., Los Alamos, NM, UNITED STATES
       Harris, Michael N., Los Alamos, NM, UNITED STATES
       Burrell, Anthony K., Los Alamos, NM, UNITED STATES
PI     US 20080220441      A1  20080911
AI     US 2007-974156      A1  20071010 (11)
```

RLI Continuation-in-part of Ser. No. US 2001-859701, filed on 16 May 2001, PENDING Continuation-in-part of Ser. No. US 2002-206524, filed on 25 Jul 2002, ABANDONED Continuation-in-part of Ser. No. US 2003-621825, filed on 16 Jul 2003, Pat. No. US 6858148
PRAI US 2006-850594P 20061010 (60)
DT Utility
FS APPLICATION
LN.CNT 10199
INCL INCLM: 435/071.000
INCLS: 436/501.000; 436/172.000; 436/086.000; 378/045.000
NCL NCLM: 435/007.100
NCLS: 378/045.000; 436/086.000; 436/172.000; 436/501.000
IC IPCI G01N0033-53 [I,A]; G01N0021-76 [I,A]; G01N0033-68 [I,A]; G01N0023-223 [I,A]; G01N0023-22 [I,C*]
IPCR G01N0033-53 [I,C]; G01N0033-53 [I,A]; G01N0021-76 [I,C]; G01N0021-76 [I,A]; G01N0023-22 [I,C]; G01N0023-223 [I,A]; G01N0033-68 [I,C]; G01N0033-68 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 4 OF 24 USPATFULL on STN
Full Text
AN 2007:328349 USPATFULL
TI Modulation of Hyaluronan Synthesis and Degradation in the Treatment of Disease
IN Brown, Tracey Jean, Flemington, AUSTRALIA
Brownlee, Gary Russell, East Burwood, AUSTRALIA
PA ALCHEMIA ONCOLOGY LIMITED, Eight Mile Plains, AUSTRALIA, 4113 (non-U.S. corporation)
PI US 20070286856 A1 20071213
AI US 2004-574903 A1 20041011 (10)
WO 2004-AU1383 20041011
20070228 PCT 371 date
PRAI AU 2003-905551 20031010
AU 2003-3906658 20031201
DT Utility
FS APPLICATION
LN.CNT 8892
INCL INCLM: 424/133.100
INCLS: 424/130.100; 424/142.100; 514/044.000; 530/387.100; 530/387.300; 530/388.100; 530/389.100; 536/022.100; 536/023.200; 536/024.500
NCL NCLM: 424/133.100
NCLS: 424/130.100; 424/142.100; 514/044.000A; 530/387.100; 530/387.300; 530/388.100; 530/389.100; 536/022.100; 536/023.200; 536/024.500
IC IPCI A61K0048-00 [I,A]; A61K0039-395 [I,A]; A61P0043-00 [I,A]; C07H0021-04 [I,A]; C07H0021-00 [I,C*]; C07K0016-18 [I,A]
IPCR A61K0048-00 [I,C]; A61K0048-00 [I,A]; A61K0031-395 [I,C*]; A61K0031-395 [I,A]; A61K0031-7105 [I,C*]; A61K0031-7105 [I,A]; A61K0031-711 [I,C*]; A61K0031-711 [I,A]; A61K0031-7115 [I,C*]; A61K0031-7115 [I,A]; A61K0031-712 [I,C*]; A61K0031-712 [I,A]; A61K0031-7125 [I,C*]; A61K0031-7125 [I,A]; A61K0039-395 [I,C]; A61K0039-395 [I,A]; A61P0035-00 [I,C*]; A61P0035-00 [I,A]; A61P0043-00 [I,C]; A61P0043-00 [I,A]; C07H0021-00 [I,C]; C07H0021-02 [I,A]; C07H0021-04 [I,A]; C07K0016-18 [I,C]; C07K0016-18 [I,A]; C07K0016-40 [I,C*]; C07K0016-40 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 5 OF 24 USPATFULL on STN
Full Text
AN 2007:284140 USPATFULL
TI Nutraceutical composition and method of use for treatment / prevention of cancer
IN Mazzio, Elizabeth, Tallahassee, FL, UNITED STATES
Soliman, Karam, Tallahassee, FL, UNITED STATES
PI US 20070248693 A1 20071025
AI US 2007-711883 A1 20070227 (11)
RLI Continuation-in-part of Ser. No. US 2005-233279, filed on 20 Sep 2005, ABANDONED Continuation-in-part of Ser. No. US 2004-909590, filed on 2 Aug 2004, ABANDONED
PRAI US 2003-491841P 20030802 (60)
US 2004-540525P 20040129 (60)
DT Utility
FS APPLICATION

LN.CNT 2576
INCL INCLM: 424/725.000
NCL NCLM: 424/725.000
IC IPCI A61K0036-00 [I,A]; A61P0035-00 [I,A]
IPCR A61K0036-00 [I,C]; A61K0036-00 [I,A]; A61P0035-00 [I,C]; A61P0035-00 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 6 OF 24 USPATFULL on STN
Full Text
AN 2007:257306 USPATFULL
TI COBALAMIN COMPOSITIONS FOR THE TREATMENT OF CANCER
IN Brown, Chad, Newport Beach, CA, UNITED STATES
PA BEBAAS, INC. (U.S. corporation)
PI US 20070225250 A1 20070927
AI US 2007-627816 A1 20070126 (11)
PRAI US 2006-762131P 20060126 (60)
DT Utility
FS APPLICATION
LN.CNT 699
INCL INCLM: 514/052.000
NCL NCLM: 514/052.000
IC IPCI A61K0031-714 [I,A]; A61K0031-7135 [I,C*]
IPCR A61K0031-7135 [I,C]; A61K0031-714 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 7 OF 24 USPATFULL on STN
Full Text
AN 2007:161483 USPATFULL
TI Composition and procedure for tissue creation, regeneration and repair by a cell-bearing biological implant enriched with platelet concentrate and supplements
IN Gorrochategui Barrueta, Alberto, Bilbao, SPAIN
Simon Elizundia, Josu, Bilbao, SPAIN
PI US 20070141036 A1 20070621
AI US 2007-704784 A1 20070209 (11)
RLI Continuation-in-part of Ser. No. US 2003-475866, filed on 24 Oct 2003, PENDING A 371 of International Ser. No. WO 2002-EP7, filed on 9 Jan 2002
DT Utility
FS APPLICATION
LN.CNT 1406
INCL INCLM: 424/093.700
NCL NCLM: 424/093.700
IC IPCI A61K0035-14 [I,A]
IPCR A61K0035-14 [I,C]; A61K0035-14 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 8 OF 24 USPATFULL on STN
Full Text
AN 2007:155116 USPATFULL
TI Therapeutic molecules
IN Collier, Greg, Victoria, AUSTRALIA
Walder, Ken, Victoria, AUSTRALIA
Kerr-Bayles, Lyndal, Victoria, AUSTRALIA
PA Autogen Research Pty Ltd., North Brighton, Victoria, AUSTRALIA (non-U.S. corporation)
Deakin University, Waurn Ponds, Victoria, AUSTRALIA (non-U.S. corporation)
PI US 20070135335 A1 20070614
AI US 2004-545099 A1 20040210 (10)
WO 2004-AU147 20040210
20060504 PCT 371 date
PRAI US 2003-446191P 20030210 (60)
DT Utility
FS APPLICATION
LN.CNT 6649
INCL INCLM: 514/012.000
INCLS: 514/044.000; 530/350.000
NCL NCLM: 514/012.000
NCLS: 514/044.000R; 530/350.000
IC IPCI A61K0038-17 [I,A]; A61K0048-00 [I,A]; C07K0014-705 [I,A]; C07K0014-435 [I,C*]

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       IPCR   A61K0038-17 [I,C]; A61K0038-17 [I,A]; A61K0048-00 [I,C];
              A61K0048-00 [I,A]; C07K0014-435 [I,C]; C07K0014-705 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 9 OF 24  USPATFULL on STN
Full Text
AN     2007:30123  USPATFULL
TI     Detection of variations in the dna methylation profile
IN     Berlin, Kurt, Stahnsdorf, GERMANY, FEDERAL REPUBLIC OF
       Piepenbrock, Christian, Berlin, GERMANY, FEDERAL REPUBLIC OF
       Olek, Alexander, Berlin, GERMANY, FEDERAL REPUBLIC OF
PI     US 20070026393      A1  20070201
AI     US 2001-240970      A1  20010406 (10)
       WO 2001-DE1486          20010406
                               20030711  PCT 371 date
PRAI   DE 2000-100190588       20000406
DT     Utility
FS     APPLICATION
LN.CNT 16100
INCL   INCLM: 435/006.000
       INCLS: 536/024.300
NCL    NCLM:  435/006.000
       NCLS:  536/024.300
IC     IPCI   C12Q0001-68 [I,A]; C07H0021-04 [I,A]; C07H0021-00 [I,C*]
       IPCR   C12Q0001-68 [I,C]; C12Q0001-68 [I,A]; C07H0021-00 [I,C];
              C07H0021-04 [I,A]; C07K0014-435 [I,C*]; C07K0014-47 [I,A];
              C07K0014-82 [I,C*]; C07K0014-82 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 10 OF 24  USPATFULL on STN
Full Text
AN     2006:248357  USPATFULL
TI     Use of phenylmethimazoles, methimazole derivatives, and tautomeric
       cyclic thiones for the treatment of autoimmune/inflammatory diseases
       associated with toll-like receptor overexpression
IN     Kohn, Leonard D., Athens, OH, UNITED STATES
       Harii, Norikazu, Yaminashi, JAPAN
       Benavides-Peralta, Uruguaysito, Montevideo, URUGUAY
       Gonzalez-Murguiondo, Mariana, Montevideo, URUGUAY
       Lewis, Christopher J., Athens, OH, UNITED STATES
       Napolitano, Giorgio, Pescara, ITALY
       Giuliani, Cesidio, Roccamonce, ITALY
       Malgor, Ramiro, Athens, OH, UNITED STATES
       Goetz, Douglas J., Athens, OH, UNITED STATES
PI     US 20060211752      A1  20060921
AI     US 2005-130922      A1  20050517 (11)
RLI    Continuation-in-part of Ser. No. US 2004-912948, filed on 6 Aug 2004,
       PENDING Continuation-in-part of Ser. No. US 2004-801986, filed on 16 Mar
       2004, PENDING
DT     Utility
FS     APPLICATION
LN.CNT 8384
INCL   INCLM: 514/389.000
NCL    NCLM:  514/389.000
IC     IPCI   A61K0031-4166 [I,A]; A61K0031-4164 [I,C*]
       IPCR   A61K0031-4164 [I,C]; A61K0031-4166 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 11 OF 24  USPATFULL on STN
Full Text
AN     2006:41329  USPATFULL
TI     Inhibition of anaerobic glucose metabolism and corresponding composition
       as a natural non-toxic approach to cancer treatment
IN     Mazzio, Elizabeth Anne, Tallahassee, FL, UNITED STATES
       Soliman, Karam F., Tallahassee, FL, UNITED STATES
PI     US 20060035981      A1  20060216
AI     US 2005-233279      A1  20050920 (11)
RLI    Continuation-in-part of Ser. No. US 2004-909590, filed on 2 Aug 2004,
       ABANDONED
PRAI   US 2003-491841P         20030802 (60)
       US 2004-540525P         20040129 (60)
DT     Utility
```

FS APPLICATION
LN.CNT 1613
INCL INCLM: 514/690.000
INCLS: 514/045.000; 514/051.000; 514/027.000; 514/251.000; 424/725.000; 424/748.000; 424/756.000; 424/745.000; 424/746.000; 424/729.000
NCL NCLM: 514/690.000
NCLS: 424/725.000; 424/729.000; 424/745.000; 424/746.000; 424/748.000; 424/756.000; 514/027.000; 514/045.000; 514/051.000; 514/251.000
IC IPCI A61K0031-12 [I,A]; A61K0031-7072 [I,A]; A61K0031-7076 [I,A]; A61K0031-7042 [I,C*]; A61K0031-525 [I,A]; A61K0031-519 [I,C*]; A61K0036-328 [I,A]; A61K0036-23 [I,A]; A61K0036-185 [I,C*]; A61K0036-906 [I,A]; A61K0036-88 [I,C*]
IPCR A61K0031-12 [I,A]; A61K0031-12 [I,C]; A61K0031-519 [I,C]; A61K0031-525 [I,A]; A61K0031-7042 [I,C]; A61K0031-7072 [I,A]; A61K0031-7076 [I,A]; A61K0036-185 [I,C]; A61K0036-23 [I,A]; A61K0036-328 [I,A]; A61K0036-537 [I,A]; A61K0036-82 [I,A]; A61K0036-88 [I,C]; A61K0036-906 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 12 OF 24 USPATFULL on STN
Full Text
AN 2005:69438 USPATFULL
TI Dietary and pharmaceutical compositions for management and treatment of oxidative stress
IN Ellithorpe, Rita R., Santa Ana, CA, UNITED STATES
Slesarev, Vladimir I., Coeur d'Alene, CA, UNITED STATES
Dimitrov, Todor, Chestnut Hill, MA, UNITED STATES
PI US 20050059579 A1 20050317
AI US 2004-794285 A1 20040308 (10)
PRAI SN 2003-10455123 20030506
DT Utility
FS APPLICATION
LN.CNT 835
INCL INCLM: 514/008.000
NCL NCLM: 514/008.000
IC [7]
ICM A61K038-16
IPCI A61K0038-16 [ICM,7]
IPCR A23L0001-305 [I,C*]; A23L0001-305 [I,A]; A61K0031-01 [I,C*]; A61K0031-015 [I,A]; A61K0031-352 [I,C*]; A61K0031-352 [I,A]; A61K0036-185 [I,C*]; A61K0036-185 [I,A]; A61K0038-16 [I,C*]; A61K0038-16 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 13 OF 24 USPATFULL on STN
Full Text
AN 2004:18482 USPATFULL
TI Additive method of standardized drinks and potable water production
IN Costa, Fortunato, Linda-a-Velha, PORTUGAL
PI US 20040013784 A1 20040122
AI US 2003-239621 A1 20030127 (10)
WO 2001-PT3 20010315
PRAI PT 2000-102430 20000316
DT Utility
FS APPLICATION
LN.CNT 1215
INCL INCLM: 426/590.000
NCL NCLM: 426/590.000
IC [7]
ICM C12C001-00
IPCI C12C0001-00 [ICM,7]
IPCR A23L0001-29 [I,C*]; A23L0001-29 [I,A]; A23L0002-52 [I,C*]; A23L0002-52 [I,A]; C02F0001-68 [I,C*]; C02F0001-68 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 14 OF 24 USPATFULL on STN
Full Text
AN 2003:282627 USPATFULL
TI Genostics
IN Roberts, Gareth Wyn, Cambs, UNITED KINGDOM
PA GENOSTIC PHARMA LIMITED (non-U.S. corporation)
PI US 20030198970 A1 20031023

```
AI     US 2002-206568      A1  20020729 (10)
RLI    Continuation of Ser. No. US 1999-325123, filed on 3 Jun 1999, ABANDONED
PRAI   GB 1998-12098           19980606
       GB 1998-28289           19981223
DT     Utility
FS     APPLICATION
LN.CNT 4299
INCL   INCLM: 435/006.000
       INCLS: 536/024.300
NCL    NCLM:  435/006.000
       NCLS:  536/024.300
IC     [7]
       ICM    C12Q001-68
       ICS    C07H021-04
       IPCI   C12Q0001-68 [ICM,7]; C07H0021-04 [ICS,7]; C07H0021-00 [ICS,7,C*]
       IPCR   C07K0016-18 [I,C*]; C07K0016-18 [I,A]; C12Q0001-68 [I,C*];
              C12Q0001-68 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 15 OF 24  USPATFULL on STN
Full Text
AN     2003:112524  USPATFULL
TI     Compositions for treating animal diseases and syndromes
IN     Ramaekers, Joseph C., Aptos, CA, UNITED STATES
PI     US 20030077254      A1  20030424
       US 6962718          B2  20051108
AI     US 2002-136854      A1  20020430 (10)
RLI    Continuation-in-part of Ser. No. US 2001-847036, filed on 30 Apr 2001,
       PENDING
DT     Utility
FS     APPLICATION
LN.CNT 2396
INCL   INCLM: 424/093.300
       INCLS: 424/617.000; 424/602.000; 424/094.500; 424/703.000; 514/168.000;
              514/558.000; 514/251.000; 514/393.000; 514/356.000; 514/276.000
NCL    NCLM:  424/535.000; 424/093.300
       NCLS:  424/093.400; 424/093.510; 424/400.000; 424/520.000; 424/725.000;
              424/094.500; 424/602.000; 424/617.000; 424/703.000; 514/168.000;
              514/251.000; 514/276.000; 514/356.000; 514/393.000; 514/558.000
IC     [7]
       ICM    A61K045-00
       ICS    A61K038-52; A61K031-525
       IPCI   A61K0045-00 [ICM,7]; A61K0038-52 [ICS,7]; A61K0038-43 [ICS,7,C*];
              A61K0031-525 [ICS,7]; A61K0031-519 [ICS,7,C*]
       IPCI-2 A61K0035-20 [ICM,7]; A61K0035-72 [ICS,7]; A61K0035-74 [ICS,7];
              A61K0035-66 [ICS,7,C*]; A61K0035-78 [ICS,7]
       IPCR   A61K0038-19 [I,C*]; A61K0038-19 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28  ANSWER 16 OF 24  USPATFULL on STN
Full Text
AN     2002:337325  USPATFULL
TI     Fluorescent cobalamins and uses thereof
IN     Grissom, Charles B., Salt Lake City, UT, UNITED STATES
       West, Frederick G., Salt Lake City, UT, UNITED STATES
       McGreevy, James, Salt Lake City, UT, UNITED STATES
       Bentz, Joel S., Salt Lake City, UT, UNITED STATES
       Cannon, Michelle J., Price, UT, UNITED STATES
PI     US 20020192683      A1  20021219
       US 6797521          B2  20040928
AI     US 2002-97646       A1  20020315 (10)
RLI    Continuation-in-part of Ser. No. WO 2000-US29370, filed on 26 Oct 2000,
       UNKNOWN
PRAI   US 1999-161368P         19991026 (60)
       US 2001-276036P         20010316 (60)
DT     Utility
FS     APPLICATION
LN.CNT 1337
INCL   INCLM: 435/006.000
       INCLS: 536/026.440
NCL    NCLM:  436/505.000; 435/006.000
       NCLS:  435/004.000; 435/007.100; 435/007.210; 435/007.230; 436/063.000;
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436/064.000; 436/164.000; 436/172.000; 514/052.000; 536/026.440
IC [7]
ICM C12Q001-68
ICS C07H023-00
IPCI C12Q0001-68 [ICM,7]; C07H0023-00 [ICS,7]
IPCI-2 G01N0033-567 [ICM,7]; A61K0031-70 [ICS,7]; C07H0023-00 [ICS,7]
IPCR A61B0001-04 [I,C*]; A61B0001-04 [I,A]; A61B0001-313 [N,C*];
A61B0001-313 [N,A]; A61B0005-00 [N,C*]; A61B0005-00 [N,A];
A61B0019-00 [N,C*]; A61B0019-00 [N,A]; A61K0047-48 [I,C*];
A61K0047-48 [I,A]; A61K0049-00 [I,C*]; A61K0049-00 [I,A];
C07F0015-00 [I,C*]; C07F0015-06 [I,A]; C09K0011-06 [I,C*];
C09K0011-06 [I,A]; G01N0021-64 [N,C*]; G01N0021-64 [N,A];
G01N0033-52 [I,C*]; G01N0033-52 [I,A]; G01N0033-574 [I,C*];
G01N0033-574 [I,A]; G01N0033-58 [I,C*]; G01N0033-58 [I,A];
G02B0021-00 [I,C*]; G02B0021-00 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 17 OF 24 USPATFULL on STN
Full Text
AN 2002:206597 USPATFULL
TI Bioconjugates and delivery of bioactive agents
IN Grissom, Charles B., Salt Lake City, UT, UNITED STATES
West, Frederick G., Salt Lake City, UT, UNITED STATES
Howard, Allen W., JR., Dexter, MI, UNITED STATES
PI US 20020111294 A1 20020815
US 6790827 B2 20040914
AI US 2001-982940 A1 20011022 (9)
RLI Division of Ser. No. US 1999-202328, filed on 22 Oct 1999, PATENTED A
371 of International Ser. No. WO 1997-US14140, filed on 22 Aug 1997,
UNKNOWN
PRAI US 1996-24430P 19960827 (60)
US 1996-25036P 19960827 (60)
DT Utility
FS APPLICATION
LN.CNT 2337
INCL INCLM: 514/006.000
INCLS: 514/044.000; 424/043.000
NCL NCLM: 514/006.000
NCLS: 424/001.110; 424/001.530; 424/001.690; 435/091.100; 435/091.310;
435/181.000; 435/455.000; 514/001.000; 514/002.000; 514/004.000;
536/023.100; 536/024.500; 424/043.000; 514/044.000A
IC [7]
ICM A61K048-00
ICS A61K051-00; A61K038-17; A61K009-00
IPCI A61K0048-00 [ICM,7]; A61K0051-00 [ICS,7]; A61K0038-17 [ICS,7];
A61K0009-00 [ICS,7]
IPCI-2 A61K0038-16 [ICM,7]; A61K0051-00 [ICS,7]; C12N0011-06 [ICS,7];
C12N0011-00 [ICS,7,C*]; C12P0019-34 [ICS,7]; C12P0019-00
[ICS,7,C*]; C07H0021-04 [ICS,7]; C07H0021-00 [ICS,7,C*]
IPCR A61K0041-00 [I,C*]; A61K0041-00 [I,A]; A61K0047-48 [I,C*];
A61K0047-48 [I,A]; C07H0021-00 [I,C*]; C07H0021-00 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 18 OF 24 USPATFULL on STN
Full Text
AN 2002:92630 USPATFULL
TI Bioconjugates and delivery of bioactive agents
IN Grissom, Charles B., Salt Lake City, UT, UNITED STATES
West, Frederick G., Salt Lake City, UT, UNITED STATES
Howard, W. Allen, JR., Dexter, MN, UNITED STATES
PA University of Utah Research Foundation, Salt Lake City, UT, UNITED
STATES, 84108 (U.S. corporation)
PI US 20020049154 A1 20020425
US 6777237 B2 20040817
AI US 2001-982968 A1 20011022 (9)
RLI Division of Ser. No. US 1999-202328, filed on 22 Oct 1999, GRANTED, Pat.
No. US 6315978 A 371 of International Ser. No. WO 1997-US14140, filed on
22 Aug 1997, UNKNOWN
PRAI US 1996-24430P 19960827 (60)
US 1996-25036P 19960827 (60)
DT Utility
FS APPLICATION

LN.CNT 2360
INCL INCLM: 514/006.000
INCLS: 514/044.000; 604/020.000
NCL NCLM: 435/455.000; 514/006.000
NCLS: 424/001.110; 424/001.530; 424/001.690; 424/001.730; 435/091.100; 435/091.310; 435/181.000; 514/001.000; 514/002.000; 514/004.000; 514/006.000; 536/023.100; 536/024.500; 514/044.000A; 604/020.000
IC [7]
ICM A61K038-16
ICS A61K048-00; A61N001-30
IPCI A61K0038-16 [ICM,7]; A61K0048-00 [ICS,7]; A61N0001-30 [ICS,7]
IPCI-2 A61K0051-00 [ICM,7]; A61K0038-16 [ICS,7]; C12N0011-06 [ICS,7]; C12N0011-00 [ICS,7,C*]; C12P0019-34 [ICS,7]; C12P0019-00 [ICS,7,C*]; C07H0021-04 [ICS,7]; C07H0021-00 [ICS,7,C*]
IPCR A61K0041-00 [I,C*]; A61K0041-00 [I,A]; A61K0047-48 [I,C*]; A61K0047-48 [I,A]; C07H0021-00 [I,C*]; C07H0021-00 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 19 OF 24 USPATFULL on STN
Full Text
AN 87:41588 USPATFULL
TI Compositions and method for simultaneous multiple array of analytes using radioisotope chelate labels
IN Olson, Douglas R., Doylestown, PA, United States
PA ICN Micromedic Systems, Inc., Costa Mesa, CA, United States (U.S. corporation)
PI US 4672028 19870609
AI US 1984-612979 19840523 (6)
DT Utility
FS Granted
LN.CNT 784
INCL INCLM: 435/005.000
INCLS: 435/007.000; 435/017.000; 435/026.000; 435/810.000; 436/500.000; 436/505.000; 436/510.000; 436/536.000; 436/542.000; 436/545.000; 436/804.000; 436/808.000; 436/811.000; 436/813.000; 436/814.000; 436/816.000; 436/817.000; 436/818.000; 436/820.000; 436/826.000
NCL NCLM: 435/005.000
NCLS: 435/007.230; 435/007.400; 435/017.000; 435/026.000; 435/810.000; 435/973.000; 435/975.000; 436/500.000; 436/505.000; 436/510.000; 436/536.000; 436/542.000; 436/545.000; 436/804.000; 436/808.000; 436/811.000; 436/813.000; 436/814.000; 436/816.000; 436/817.000; 436/818.000; 436/820.000; 436/826.000
IC [4]
ICM G01N033-53
ICS G01N033-567; G01N033-536
IPCI G01N0033-53 [ICM,4]; G01N0033-567 [ICS,4]; G01N0033-536 [ICS,4]
IPCR A61K0035-66 [I,C*]; A61K0035-74 [I,A]; A61K0038-00 [I,C*]; A61K0038-00 [I,A]; A61K0038-22 [I,C*]; A61K0038-22 [I,A]; A61K0038-24 [I,C*]; A61K0038-24 [I,A]; C07F0015-00 [I,C*]; C07F0015-00 [I,A]; C07H0015-00 [I,C*]; C07H0015-00 [I,A]; C07H0023-00 [I,C*]; C07H0023-00 [I,A]; G01N0033-534 [I,C*]; G01N0033-534 [I,A]; G01N0033-60 [I,C*]; G01N0033-60 [I,A]; G01N0033-74 [I,C*]; G01N0033-74 [I,A]
EXF 436/536; 436/542; 436/545; 436/500; 436/505; 436/510; 436/804; 436/808; 436/811; 436/813; 436/814; 436/817; 436/818; 436/816; 436/820; 436/826; 435/5; 435/7; 435/4; 435/17; 435/26; 435/810
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 20 OF 24 USPAT2 on STN
Full Text
AN 2005:49435 USPAT2
TI Methods of increasing delivery of active agents to brain comprising administering receptor associated protein (RAP) fragments conjugated to active agents
IN Zankel, Todd, San Francisco, CA, UNITED STATES
Starr, Christopher M., Sonoma, CA, UNITED STATES
PA Raptor Pharmaceutical Inc., Novato, CA, UNITED STATES (U.S. corporation)
PI US 7569544 B2 20090804
AI US 2004-812849 20040330 (10)
RLI Continuation-in-part of Ser. No. US 2003-600862, filed on 20 Jun 2003, ABANDONED
DT Utility

FS GRANTED
LN.CNT 5335
INCL INCLM: 514/012.000
NCL NCLM: 514/012.000
IC IPCI A61K0048-00 [ICM,7]; A61K0039-395 [ICS,7]
IPCI-2 A61K0038-18 [I,A]; C07K0019-00 [I,A]; C07K0014-435 [I,A]; C07K0014-48 [I,A]; C07K0014-485 [I,A]; C07K0014-50 [I,A]
IPCR A61K0038-17 [I,C*]; A61K0038-17 [I,A]; A61K0039-395 [I,C*]; A61K0039-395 [I,A]; A61K0048-00 [I,C*]; A61K0048-00 [I,A]; C07K0014-435 [I,C*]; C07K0014-705 [I,A]
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 21 OF 24 USPAT2 on STN
Full Text
AN 2003:93594 USPAT2
TI Use of multiple antioxidant micronutrients as systemic biological radioprotective agents against potential ionizing radiation risks
IN Prasad, Kedar N., Denver, CO, UNITED STATES
Haase, Gerald M., Greenwood Village, CO, UNITED STATES
Cole, William C., Centennial, CO, UNITED STATES
PA Premier Micronutrient Corporation, Nashville, TN, UNITED STATES (U.S. corporation)
PI US 7449451 B2 20081111
AI US 2002-229274 20020828 (10)
DT Utility
FS GRANTED
LN.CNT 1344
INCL INCLM: 514/052.000
INCLS: 514/251.000; 514/184.000; 514/393.000; 514/350.000; 514/167.000; 514/474.000; 514/458.000; 514/440.000; 514/552.000; 514/276.000; 514/562.000; 514/494.000; 514/574.000; 514/763.000
NCL NCLM: 514/052.000
NCLS: 514/167.000; 514/184.000; 514/251.000; 514/276.000; 514/350.000; 514/393.000; 514/440.000; 514/458.000; 514/474.000; 514/494.000; 514/552.000; 514/562.000; 514/574.000; 514/763.000
IC IPCI A61K0031-714 [ICM,7]; A61K0031-7135 [ICM,7,C*]; A61K0031-59 [ICS,7]; A61K0031-555 [ICS,7]; A61K0031-525 [ICS,7]; A61K0031-519 [ICS,7,C*]; A61K0031-51 [ICS,7]; A61K0031-506 [ICS,7,C*]; A61K0031-4184 [ICS,7]; A61K0031-4164 [ICS,7,C*]; A61K0031-015 [ICS,7]; A61K0031-01 [ICS,7,C*]
IPCI-2 A61K0031-714 [I,A]; A61K0031-7135 [I,C*]; A61K0031-59 [I,A]; A61K0031-555 [I,A]; A61K0031-525 [I,A]; A61K0031-519 [I,C*]; A61K0031-51 [I,A]; A61K0031-506 [I,C*]; A61K0031-4184 [I,A]; A61K0031-4164 [I,C*]; A61K0031-015 [I,A]; A61K0031-01 [I,C*]
IPCR A61K0031-7135 [I,C]; A61K0031-714 [I,A]; A61K0031-01 [I,C]; A61K0031-015 [I,A]; A61K0031-4164 [I,C]; A61K0031-4184 [I,A]; A61K0031-506 [I,C]; A61K0031-51 [I,A]; A61K0031-519 [I,C]; A61K0031-525 [I,A]; A61K0031-555 [I,C]; A61K0031-555 [I,A]; A61K0031-59 [I,C]; A61K0031-59 [I,A]
EXF 514/52; 514/167; 514/184; 514/251; 514/276; 514/350; 514/393; 514/440; 514/458; 514/474; 514/494; 514/552; 514/562; 514/574; 514/763; 514/188; 514/725
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 22 OF 24 USPAT2 on STN
Full Text
AN 2002:337325 USPAT2
TI Fluorescent cobalamins and uses thereof
IN Grissom, Charles B., Salt Lake City, UT, United States
West, Frederick G., Salt Lake City, UT, United States
McGreevy, James, Salt Lake City, UT, United States
Bentz, Joel S., Salt Lake City, UT, United States
Cannon, Michelle J., Price, UT, United States
PA University of Utah Research Foundation, Salt Lake City, UT, United States (U.S. corporation)
PI US 6797521 B2 20040928
AI US 2002-97646 20020315 (10)
RLI Continuation-in-part of Ser. No. WO 2000-US29370, filed on 26 Oct 2000
PRAI US 1999-161368P 19991026 (60)
US 2001-276036P 20010316 (60)
DT Utility
FS GRANTED

LN.CNT 1187
INCL INCLM: 436/505.000
INCLS: 514/052.000; 536/026.440; 435/004.000; 435/007.100; 435/007.210; 435/007.230; 436/063.000; 436/064.000; 436/164.000; 436/172.000
NCL NCLM: 436/505.000; 435/006.000
NCLS: 435/004.000; 435/007.100; 435/007.210; 435/007.230; 436/063.000; 436/064.000; 436/164.000; 436/172.000; 514/052.000; 536/026.440
IC [7]
ICM G01N033-567
ICS A61K031-70; C07H023-00
IPCI C12Q0001-68 [ICM,7]; C07H0023-00 [ICS,7]
IPCI-2 G01N0033-567 [ICM,7]; A61K0031-70 [ICS,7]; C07H0023-00 [ICS,7]
IPCR A61B0001-04 [I,C*]; A61B0001-04 [I,A]; A61B0001-313 [N,C*]; A61B0001-313 [N,A]; A61B0005-00 [N,C*]; A61B0005-00 [N,A]; A61B0019-00 [N,C*]; A61B0019-00 [N,A]; A61K0047-48 [I,C*]; A61K0047-48 [I,A]; A61K0049-00 [I,C*]; A61K0049-00 [I,A]; C07F0015-00 [I,C*]; C07F0015-06 [I,A]; C09K0011-06 [I,C*]; C09K0011-06 [I,A]; G01N0021-64 [N,C*]; G01N0021-64 [N,A]; G01N0033-52 [I,C*]; G01N0033-52 [I,A]; G01N0033-574 [I,C*]; G01N0033-574 [I,A]; G01N0033-58 [I,C*]; G01N0033-58 [I,A]; G02B0021-00 [I,C*]; G02B0021-00 [I,A]
EXF 536/26.44; 514/52; 436/505
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 23 OF 24 USPAT2 on STN
Full Text
AN 2002:206597 USPAT2
TI Bioconjugates and delivery of bioactive agents
IN Grissom, Charles B., Salt Lake City, UT, United States
West, Frederick G., Salt Lake City, UT, United States
Howard, Jr., W. Allen, Dexter, MI, United States
PA University of Utah Research Foundation, Salt Lake City, UT, United States (U.S. corporation)
PI US 6790827 B2 20040914
AI US 2001-982940 20011022 (9)
RLI Division of Ser. No. US 202328, now patented, Pat. No. US 6315978
PRAI US 1996-24430P 19960827 (60)
US 1996-25036P 19960827 (60)
DT Utility
FS GRANTED
LN.CNT 2388
INCL INCLM: 514/006.000
INCLS: 424/001.110; 424/001.530; 424/001.690; 435/091.310; 435/091.100; 435/181.000; 435/455.000; 514/001.000; 514/002.000; 514/004.000; 536/023.100; 536/024.500
NCL NCLM: 514/006.000
NCLS: 424/001.110; 424/001.530; 424/001.690; 435/091.100; 435/091.310; 435/181.000; 435/455.000; 514/001.000; 514/002.000; 514/004.000; 536/023.100; 536/024.500; 424/043.000; 514/044.000A
IC [7]
ICM A61K038-16
ICS A61K051-00; C12N011-06; C12P019-34; C07H021-04
IPCI A61K0048-00 [ICM,7]; A61K0051-00 [ICS,7]; A61K0038-17 [ICS,7]; A61K0009-00 [ICS,7]
IPCI-2 A61K0038-16 [ICM,7]; A61K0051-00 [ICS,7]; C12N0011-06 [ICS,7]; C12N0011-00 [ICS,7,C*]; C12P0019-34 [ICS,7]; C12P0019-00 [ICS,7,C*]; C07H0021-04 [ICS,7]; C07H0021-00 [ICS,7,C*]
IPCR A61K0041-00 [I,C*]; A61K0041-00 [I,A]; A61K0047-48 [I,C*]; A61K0047-48 [I,A]; C07H0021-00 [I,C*]; C07H0021-00 [I,A]
EXF 424/1.11; 424/1.69; 424/1.53; 424/9.361; 424/193.1; 435/6; 435/91.1; 435/91.31; 435/455; 435/181; 514/1; 514/2; 514/4; 514/6; 514/44; 536/23.1; 536/24.5
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 24 OF 24 USPAT2 on STN
Full Text
AN 2002:92630 USPAT2
TI Bioconjugates and delivery of bioactive agents
IN Grissom, Charles B., Salt Lake City, UT, United States
West, Frederick G., Salt Lake City, UT, United States
Howard, Jr., Allen W., Dexter, MI, United States
PA University of Utah Research Foundation, Salt Lake City, UT, United

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       States (U.S. corporation)
PI     US 6777237          B2  20040817
AI     US 2001-982968          20011022 (9)
RLI    Division of Ser. No. US 202328, now patented, Pat. No. US 6315978
PRAI   US 1996-24430P          19960827 (60)
       US 1996-25036P          19960827 (60)
DT     Utility
FS     GRANTED
LN.CNT 2410
INCL   INCLM: 435/455.000
       INCLS: 424/001.690; 424/001.110; 424/001.730; 424/001.530; 435/091.100;
              435/091.310; 435/181.000; 514/001.000; 514/002.000; 514/004.000;
              514/006.000; 536/023.100; 536/024.500
NCL    NCLM:  435/455.000; 514/006.000
       NCLS:  424/001.110; 424/001.530; 424/001.690; 424/001.730; 435/091.100;
              435/091.310; 435/181.000; 514/001.000; 514/002.000; 514/004.000;
              514/006.000; 536/023.100; 536/024.500; 514/044.000A; 604/020.000
IC     [7]
       ICM    A61K051-00
       ICS    A61K038-16; C12N011-06; C12P019-34; C07H021-04
       IPCI   A61K0038-16 [ICM,7]; A61K0048-00 [ICS,7]; A61N0001-30 [ICS,7]
       IPCI-2 A61K0051-00 [ICM,7]; A61K0038-16 [ICS,7]; C12N0011-06 [ICS,7];
              C12N0011-00 [ICS,7,C*]; C12P0019-34 [ICS,7]; C12P0019-00
              [ICS,7,C*]; C07H0021-04 [ICS,7]; C07H0021-00 [ICS,7,C*]
       IPCR   A61K0041-00 [I,C*]; A61K0041-00 [I,A]; A61K0047-48 [I,C*];
              A61K0047-48 [I,A]; C07H0021-00 [I,C*]; C07H0021-00 [I,A]
EXF    435/6; 435/91.1; 435/91.31; 435/181; 435/455; 514/1; 514/2; 514/4;
       514/6; 514/44; 424/1.11; 424/1.53; 424/9.361; 424/193.1; 536/23.1;
       536/24.5
CAS INDEXING IS AVAILABLE FOR THIS PATENT.


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COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                      ENTRY    SESSION
FULL ESTIMATED COST                                    32.58     208.34

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)          SINCE FILE      TOTAL
                                                      ENTRY    SESSION
CA SUBSCRIBER PRICE                                     0.00      -1.56

STN INTERNATIONAL LOGOFF AT 23:45:29 ON 31 AUG 2009
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NOT A USPTO FORM INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X14173B	Serial No 11/776329
	First Applicant Clet Niyikiza	
	Application Date July 11, 2007 US Nat'l Entry (if applicable)	Group Art Unit 1614

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 Pages or Relevant Figures Appear
	AA	US			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No. [1]	Foreign Patent Document Country Code [3]-Number [4]-Kind Code5 (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]
/K.W./	BA	WO 95/27723	10-19-1995			

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 [6]
/K.W./	CA	POYDOCK M. Effect of combined ascorbic acid and B-12 on survival of mice with implanted Ehrlich carcinoma and L1210 leukemia. *Am J Clin Nutr* 1991; 54: 1261S-5S,	
	CB	POYDOCK M, et al. Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin. *Am J Clin Oncol* 1985; 8: 2666-269.	
	CC	POYDOCK M, et al. Influence of Vitamins C and B12 on the Survival Rate of Mice Bearing Ascites Tumor. *Expl Cell Biol* 1982; 50:88-91.	
	CD	TOOHEY J. Dehydroascorbic acid as an anti-cancer agent. *Cancer Letters* 2008; 263:164-169.	
	CE	SALLAH S, et al. Intrathecal methotrexate-induced megaloblastic anemia in patients with acute leukemia. *Archives of Pathology & Laboratory Medicine* 1999; 123(9): 774-777.	
	CF	NISHIZAWA Y, et al. Effects of methylcobalamin on the proliferation of androgen-sensitive or estrogen-sensitive malignant cells in culture and in vivo. *International Journal for Vitamin and Nutrition Research* 1997; 67(3):164-170.	
	CG	TSAO C, et al. Influence of cobalamin on the survival of mice bearing ascites tumor. *Pathobiology* 1993; 61(2): 104-.8	
	CH	KAMEI T, et al. Experimental study of the therapeutic effects of folate, vitamin A, and vitamin B12 on squamous metaplasia of the bronchial epithelium. *Cancer* 1993; 71(8): 2477-83.	
	CI	SHIMIZU N, et al. Experimental study of antitumor effect of methyl-B12. *Oncology* 1987; 44(3): 169-73.	
	CJ	HERBERT, V. The role of vitamin B12 and folate in carcinogenesis. *Advances in Experimental Medicine and Biology* 1986; 206 (Essent. Nutr. Carcinog.), 293-311.	
/K.W./	CK	KROES A, et al. Effects of 5-fluorouracil treatment of rat leukemia with concomitant inactivation of cobalamin. *Anticancer Research* 1986; 6(4): 737-42.	

/Kevin Weddington/ 08/30/2009

NOT A USPTO FORM INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X14173B	Serial No 11/776329
	First Applicant Clet Niyikiza	
	Application Date July 11, 2007 US Nat'l Entry (if applicable)	Group Art Unit 1614

/K.W./	CL	KROES A, et al. Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide. *Cancer Chemotherapy and Pharmacology* 1986; 17(2): 114-20.	
/K.W./	CM	BARAK A. Vitamin B12 as a possible adjunct in prevention of methotrexate hepatotoxicity. *Biochemical Archives* 1985; 1(3): 139-42.	
/K.W./	CN	HERBERT V. The inhibition and promotion of cancers by folic acid, vitamin B12, and their antagonists. ACS Symposium Series (1985); 277(Xenobiot. Metab.: Nutr. Eff.), 31-6.	
	CO		

Examiner Signature	/Kevin Weddington/	Date Considered	08/30/2009

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2] See Kinds 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.

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PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	Clet Niyikiza	Group Art Unit: 1614	
Serial No.:	11/776,329	Examiner:	Kevin E. Weddington
Application Date:	July 11, 2007	Confirmation No.:	6568
For:	NOVEL ANTIFOLATE COMBINATION THERAPIES		
Docket No.:	X14173B		

REPLY UNDER 37 C.F.R. 1.111 & AMENDMENT UNDER 37 C.F.R. 1.121

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Office action of September 8, 2009, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims, which begin on page 2 of this paper.

Remarks begin on page 5 of this paper.

Remarks

Thank you for taking the time to discuss this case with me earlier today. I look forward to a timely allowance of this case. Please call me at the number provided below if during final review of the files an issue presents itself.

Claims 1-39, 45, and 46 have been cancelled. Claim 40 has been amended to a) introduce a new limitation, pretreatment with folic acid, b) remove the requirement for cyclic administration, c) to include cobalamin and cyanocobalamin in the Markush group, and d) correct spelling errors. Applicants submit that no new material has been introduced through this amendment. This amendment finds support at least at page 7, lines 5-8, page 9, lines 1-11, and page 15, line 20. Claim 41 has been amended to include a space between "vitamin" and "B12" and to add the term "acid" to the phrase "methylmalonic lowering agent." Claims 47, 49, and 52 have been amended to correct claim dependency. Applicants submit that no new material has been introduced through these amendments. Claims 53 - 62 are new and find support at least at page 13, lines 21 to 25, page 6, lines 3-5; page 7, lines 20-27; and page 14, line 3. No Claims are allowed and all claims stand rejected under 35 U.S.C. 103(a). In view of the reasons set forth below, Applicants submit that the rejection is improper and should be withdrawn. Entry of the amendments and reconsideration and allowance of the present application are respectfully requested.

Rejections Under 35 USC §103(a)

All claims stand rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Taylor (5,344,932) in view of Tsao et al., "Influence of Cobalamin on the Survival of Mice Bearing Ascites Tumor," Pathobiology, Vol. 61, No. 2, pp. 104-108 (1993), further in view of Worzalla et al., Anticancer Research, Vol. 18, No. 5, pp. 3235-3239, and further in view of Cleare et al. (4,149,707). Applicants submit that the Examiner meant to cite to Cleare et al. at 4,140,707 ("Malonato Platinum Anti-Tumor Compounds") and not 4,149,707 ("Spring Device"). Applicants address the Examiner's concerns below based upon the belief that Cleare et al. refers to US Patent #4,140,707. If this is incorrect, Applicants reserve the right to address the new art in a future communication.

The presently claimed invention is directed to improving the therapeutic utility of pemetrexed disodium by administering to a patient a methylmalonic acid lowering agent and folic acid followed by administering an effective amount of pemetrexed disodium. Applicants have discovered that the claimed method reduces mortality and nonhematologic events, such as skin rashes and fatigue events without compromising pemetrexed disodium's efficacy, see page 3,

lines 5-15 of the Specification. Prior to Applicant's invention a skilled artisan would not have been motivated to combine pemetrexed disodium with a methylmalonic acid lowering agent, such as vitamin B12, and folic acid and there would have been no reasonable expectation in the art that the claimed treatment method would provide a viable chemotherapy regimen, let alone reduce toxic events related to administration of pemetrexed disodium.

The Examiner alleges that in view of Taylor, Tsao, Worzalla, and Cleare a skilled artisan would have "assumed the combination of three antineoplastic agents into a single composition would give an additive effect in the absence of evidence to the contrary." *Office Action* (OA) dated 9/8/2009, page 4, paragraph 3. Applicants respectfully assert that the Examiner's obviousness rejection is inappropriate and should be withdrawn.

The *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966), factors control an obviousness inquiry. *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007). Those factors are: 1) "the scope and content of the prior art"; 2) the "differences between the prior art and the claims"; 3) "the level of ordinary skill in the pertinent art"; and 4) objective evidence of nonobviousness. *KSR*, 127 S. Ct. at 1734 (quoting *Graham*, 383 U.S. at 17-18).

The Court in *KSR* acknowledged the importance of identifying "a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does" in an obviousness determination. *KSR*, 127 S. Ct. at 1731. *KSR* also did not disturb the longstanding requirement that an obviousness determination requires that a skilled artisan would have perceived a reasonable expectation of success in making the invention in light of the prior art. *In re Kubin*, 561 F.3d 1351, 1352+ (Fed. Cir. 2009) (quoting *In re O'Farrell,* 853 F.2d 894, 903-904 (Fed. Cir. 1988)).

Rejection based upon Taylor in view of Tsao

The Examiner alleges that pemetrexed disodium and vitamin B12 were known to be "antineoplastic agents" and therefore could be combined "into a single composition [that] would give an additive effect." OA, page 4, paragraph 3. However, the Examiner appears to have misinterpreted the understanding in the art concerning vitamin B12 antineoplastic activity and the teachings of Taylor. Particularly, the rejection overstates what Tsao as a whole fairly discloses. Tsao teaches that conflicting results have been found for the use of vitamin B12 as an antitumor agent in animals and in man (see page 104, column 1 at about line 13 through column 2 at about line 18). Tsao states:

> "the results of two survey studies using data from several hospitals failed to confirm that B12 therapy was effective either when it was

> administered alone of in conjunction with X-ray or chemotherapeutic agents…Experiments with laboratory animals also showed conflicting results."

(see p. 104, col. 1, lines 15-21). In fact, Tsao reports that cyanocobalamin "did not affect cell growth at a daily dose as high as 1,000 mg/kg body weight." Tsao, page 105, last paragraph. It is therefore submitted that when viewed as a whole, a person of ordinary skill in the art reading Tsao would not have perceived a reasonable expectation of success in making Applicant's invention in view of the scientific uncertainty concerning vitamin B12 and its use as an antitumor agent. In fact, Applicants submit that the activity of B12 as a potential antitumor therapeutic is still inconclusive even as of today (see Volkov 2008, attached, introductory paragraph, page 324, "Researchers have attempted to correlate vitamin B12 with malignancy ever since the multifunctional role of cobalamin has begun to be understood…There are many hypotheses about the role of vitamin B12 in growth of malignancy, but we still have many more questions than we have answers.").

Additionally, page 3 of the OA asserts that Taylor discloses certain glutamic acid derivatives, including pemetrexed disodium, as effective antineoplastic agents and that pemetrexed disodium can be combined with other antineoplastic agents. The OA admits that Taylor "does not teach the addition of a methylmalonic acid lowering agent." However, the OA goes on to suggest that "the secondary reference, Tsao et al., teaches a methylmalonic acid lowering agent such as cobalamin (vitamin B12) is effective as having antitumor activity (see the abstract)." *OA*, pp 3-4. The rejection particularly notes column 8, lines 64-68, of Taylor, which merely states the compounds of the invention "can be administered … with other therapeutic agents, including antineoplastic agents [which is another genus of compounds], steroids, etc. to a mammal suffering from neoplasm …" As discussed *supra*, at the time of Applicant's invention there was scientific uncertainty concerning vitamin B12 and its use as an antitumor agent. In fact, as will be further discussed below, the skilled artisan would have expected a decrease in the antineoplastic activity of pemetrexed disodium when administered in combination with vitamin B12, see Specification page 3, lines 7-8, not an additive or even a synergistic effect for antineoplastic activity, see Specification page 16, lines 6-9.

Applicants respectfully assert the Examiner has not made a *prima facie* showing of obviousness, at least because the rejection lacks support for why a skilled artisan would have combined pemetrexed disodium with a methylmalonic acid lowering agent and folic acid as claimed and that there would have been any reasonable expectation the claimed method would provide a viable chemotherapy regimen and reduce toxicity associated with pemetrexed disodium

administration. In view of the comments made *supra*, Applicants respectfully request reconsideration and allowance of the present application.

Although the Examiner has not set forth a *prima facie* showing of obviousness, to expedite allowance of the application, Applicants make the following additional remarks. The Supreme Court's ruling in *KSR* states that prior-art elements "work[ing] together in an unexpected and fruitful manner" is an indicia of nonobviousness. *KSR* at 416. A skilled artisan would have understood at the time that pemetrexed disodium is a multitargeted antifolate having specific activity at three enzymes in the biosynthesis of nucleic acids. The enzymes are dihydrofolate reductase (DHFR), thymidine synthase (TS), and GAR formyltransferase (GARFT). (*Shih*, 1999 and *Shih*, 1997, attached.) All of these enzymes need a folate derivative to function. DHFR obviously has dihydrofolate as a substrate; TS needs N^5, N^{10}-methylenetetrahydrofolate as a methyl source (returning folate as dihydrofolate); and GARFT has N^{10}-formyltetrahydrofolate as a formyl source returning it as tetrahydrofolate. (*Kisliuk*, 1999 and *Kisliuk*, 1984, attached.) Pemetrexed disodium is, in simple terms, a folate analogue and acts by competing with folate at each of the enzymes' folate binding sites. If there is an excess of the natural ligand (the natural folate source) for the three enzymes then the effectiveness of pemetrexed disodium is reduced. This is shown for example in Table 1 of Worzalla. It can be seen that for the five cancer cell-lines reported, increasing the folic acid concentration from 1 μm to 10 μm gives up to a 14-fold decrease in efficacy of pemetrexed disodium (14-fold increase in IC_{50}). The skilled person, if they indeed had all of the knowledge of Taylor, Tsao, and Worzalla, would understand that by adding vitamin B12 they could be releasing the pool of N^5-methyltetrahydrofolate so causing an effective increase in the concentration of the natural folate substrate, thereby decreasing the efficacy of pemetrexed disodium. The skilled artisan would not have expected the reduction of severe toxicities associated with pemetrexed disodium, such as patient death, without the expected effect of reduction of pemetrexed disodium's efficacy.

At the time of the invention, the skilled artisan would have been aware it was standard of care to avoid vitamins in patients undergoing chemotherapy, because the usage of vitamins could decrease the effectiveness of the chemotherapy. See for example:

1. AstraZeneca's compound, Tomudex® (raltitrexed), is a TS inhibitor approved in 1995 in the United Kingdom and marketed in Europe for the treatment of colorectal cancer. The monograph as provided in Martindale's 1999, "The Complete Drug Reference" (attached) states that "Raltitrexed should not be given with folic or folinic acid which may impair its cytotoxic action." (page 560, Interactions.)

2. Methotrexate is a DHFR inhibitor that was approved in 1959 in the United States. The 1999 monograph as published by the "Physicians' Desk References" clearly states:

   "Vitamin preparations containing folic acid or its derivatives may decrease responses to systemically administered methotrexate. Preliminary animal and human studies have shown that small quantities of intravenously administered leucovorin enter the CSF primarily as 5-methyltetrahydrofolate and, in humans, remain 1-3 orders of magnitude lower than the usual methotrexate concentrations following intrathecal administration. However, high doses of leucovorin may reduce the efficacy of intrathecally-administered methotrexate. Folate deficiency states may increase methotrexate toxicity." (pages 1398-1399, *Drug Interactions*, attached.)

3. Fluorouracil (5-FU) is an inhibitor of TS. In the 1998 monograph as published by the "Physicians' Desk References" for 5-FU, there is a warning that the administration of folinic acid is associated with increased toxicity "Leucovorin calcium may enhance the toxicity of fluorouracil." (page 2463, *Drug Interactions*, attached.)

Leucovorin or folinic acid is a 5-formyl derivative of tetrahydrofolic acid. The 1999 monograph from the "Physicians' Desk References" describes leucovorin as "one of several active, chemically reduced derivatives of folic acid. It is useful as an antidote to drugs which act as folic acid antagonists," and "[a]dministration of leucovorin can counteract the therapeutic and toxic effects of folic acid antagonists such as methotrexate, which act by inhibiting dihydrofolate reductase. In contrast, leucovorin can enhance the therapeutic and toxic effects of fluoropyrimidines used in cancer therapy, such as 5-fluorouracil." (page 1389, *Drug Interactions*, attached.)

Applicants unexpectedly discovered administering vitamin B12 and folic acid as claimed reduces toxicity of pemetrexed disodium. (See Specification at pg 15, lines 21-25 and pg 16, lines 6-9.) This is clearly demonstrated by the examples in the specification wherein treatment toxicities were reduced in tumor bearing mice with or without the addition of folic acid. For example, the Specification at pg 15, lines 24-25 states, "Vitamin B12 supplementation with ALIMTA has a moderate effect on drug related toxicity, lowering drug related deaths to 3% and severe toxicities by about 25%." Page 15, lines 25-27 of the specification states, "The combination of vitamin B12 and folic acid with ALIMTA has lowered the drug related deaths to <1% in over 480 so treated." The specification also explains that pilot studies in humans established that vitamin B12 given to patients receiving ALIMTA experienced fewer side effects. Clinical studies sponsored by Eli Lilly (Lilly) confirmed less overall pemetrexed disodium-related

toxicity. Specifically, as is shown in the table below, reductions in Grade 3/4 hematologic and nonhematologic toxicities such as neutropenia, febrile neutropenia, and infection with Grade 3/4 neutropenia were reported when pretreatment with folic acid and vitamin B12 was administered.

Table 1

	Percent of occurrences prior to B12/folic acid treatment (N=246)	Percent of occurrences post B12/folic acid treatment (N=78)
Hematologic Toxicity/Non-Hematologic Toxicity	37%	6.4%
Neutropenia	32%	2.6%
Mucositis	5%	1.3%
Diarrhea	6%	2.6%
Neutropenia and Mucositis	3%	0%
Neutropenia and Diarrhea	3%	0%
Neutropenia and Infection	2%	0%

(See Specification, Table 1, page 16.)

Today, Lilly's pemetrexed disodium product, ALIMTA®, is an FDA approved product in the United States and its prescribing information (attached) includes the following information on the need to administer B12 and the effects of vitamin supplementation in reducing toxicity.

> Need for Folate and Vitamin B12 Supplementation Patients treated with ALIMTA must be instructed to take folic acid and vitamin B12 as a prophylactic measure to reduce treatment-related hematologic and GI toxicity *[see Dosage and Administration (2.3)]*. In clinical studies, less overall toxicity and reductions in Grade 3/4 hematologic and nonhematologic toxicities such as neutropenia, febrile neutropenia, and infection with Grade 3/4 neutropenia were reported when pretreatment with folic acid and vitamin B12 was administered.

(Approved Label for NDA 021462, lines 118-122.) The Approved Label goes on to instruct that "Patients must also receive one (1) intramuscular injection of vitamin B12 during the week preceding the first dose of ALIMTA and every 3 cycles thereafter." (Approved Label for NDA 021462, lines 33-34.) And that "Patients treated with ALIMTA must be instructed to take folic acid and vitamin B12 as a prophylactic measure to reduce treatment-related hematologic and gastrointestinal toxicity *[see Dosage and Administration (2.3)]*." (Approved Label for NDA 021462, lines 696-697.)

Table 8 of the Approved Label compares the incidence (percentage of patients) of CTC Grade 3/4 toxicities in patients who received vitamin supplementation with daily folic acid and vitamin B12 from the time of enrollment in the study (fully supplemented) with the incidence in

patients who never received vitamin supplementation (never supplemented) during the study in the ALIMTA plus cisplatin arm.

Table 8: Selected Grade 3/4 Adverse Events Comparing Fully Supplemented versus Never Supplemented Patients in the ALIMTA plus Cisplatin arm (% incidence)

Adverse Event[a] (%)	Fully Supplemented Patients (N=168)	Never Supplemented Patients (N=32)
Neutropenia/granulocytopenia	33	38
Thrombocytopenia	5	9
Vomiting	11	31
Febrile neutropenia	1	9
Infection with Grade 3/4 neutropenia	0	6
Diarrhea	4	9

[a] Refer to NCI CTC criteria for lab and non-laboratory values for each grade of toxicity (Version 2.0).

Clearly, Applicants have made a significant discovery not obvious in view of the references cited in the Office Action. A skilled artisan would not have expected the reduction of severe toxicities associated with pemetrexed disodium administration, such as patient death, without reduction of pemetrexed disodium's efficacy. (See Specification at pg 15, lines 21-25 and pg 16, lines 6-9.) Under the Supreme Court's decision in *KSR*, the combination of a methylmalonic acid lowering agent, particularly vitamin B12 or a pharmaceutical derivative, and pemetrexed disodium does more than yield predictable results, the combination works together in an unexpected and fruitful manner. Therefore, the rejection is clearly improper and should be withdrawn.

Rejection based upon Taylor in view of Tsao, Worzolla, Cleare, and general knowledge in the prior art

Because the combination of a methylmalonic acid lowering agent, folic acid, and pemetrexed disodium is not obvious to one of skill in the art under 35 U.S.C. 103(a), then the additional limitation introduced by the remaining dependent claims cannot be held obvious. (*See Callaway Golf Co. v. Acushnet Co.,* 576 F.3d 1331 at 1344, 91 U.S.P.Q.2d 1705 (Fed. Cir. 2009). Furthermore, the Examiner has misinterpreted the teaching of Worzalla. In addition to the arguments made *supra*, Worzolla et.al. discloses that the addition of folic acid may reduce the effectiveness of pemetrexed disodium. (See for example table 1 of Worzalla: for the 5 cancer cell-lines reported, increasing the folic acid concentration from 1 µm to 10 µm gives up to a 14-fold decrease in efficacy of pemetrexed disodium.) Worzolla provides no suggestion that lowering methylmalonic acid levels would further reduce associated toxicities while maintaining the therapeutic efficacy of pemetrexed disodium. Cleare does not disclose or provide rationale for the combination of platinum anti-tumor compounds with Applicant's claimed method of treating patients with pemetrexed disodium.

Additionally, the Examiner has presented no reason, as is required under *KSR* that the claimed dosing cycles and ranges are obvious. A *prima facie* case of obviousness requires supporting objective evidence to be sustained. An examiner must substantiate his or her "suspicions" or "hunches" on the basis of facts drawn from the prior art. Application of Lunsford, 53 C.C.P.A. 1011, 357 F.2d 385, 391, 148 U.S.P.Q. (BNA) 721, 725 (1966). Applicants respectfully assert that the Examiner's allegation that "readily optimized effective and concurrent administration dosage forms" are available in the art or are within "the ability of tasks routinely performed…without undue experimentation" does not rise to the level of "supporting objective evidence" under Application of Lunsford. Applicants respectfully submit that the Examiner could not arrive at the presently claimed invention, its dosing ranges and/or its cyclic administration.

Conclusion

Applicants respectfully contend that a *prima facie* case of obviousness has not been established, the Applicants' claimed invention is unobvious. A skilled artisan would not have expected the reduction of severe toxicities associated with pemetrexed disodium, such as patient death, without the expected effect of reduction of pemetrexed disodium's efficacy. The rejection is improper and should be withdrawn.

Entry of the amendments and allowance of the claims in view of the amendments and discussion *supra* are respectfully requested.

Respectfully submitted,

/Elizabeth A McGraw/

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Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

November 13, 2009

Amendments to the Claims

The following listing of claims will replace all prior versions, and listing, of claims in the application.

Listing of Claims:

Claims 1-39 (Cancelled)

40. (currently amended) A method for administering pemetrexed disodium to a patient in need thereof comprising administering an effective amount of folic acid and an effective amount of a methylmalonic acid lowering agent followed by administering an effective amount of pemetrexed disodium ~~in combination with a methylmalonic acid lowering agent~~, wherein~~:~~

the methylmalonic acid lowering agent is selected from the group consisting of vitamin B12, hydroxycob~~o~~alamin, cyano-10-chlorocob~~o~~alamin, aquocob~~o~~alamin perchlorate, aquo-10-cob~~o~~alamin perchlorate, azidocob~~o~~alamin, cobalamin, cyanocobalamin, or chlorocob~~o~~alamin~~;~~

~~the methylmalonic acid lowering agent is administered from about 1 week to about 3 weeks prior to the first administration of the pemetrexed disodium; and~~

~~the methylmalonic acid lowering agent administration is repeated about every 6 to about every 12 weeks until administration of the pemetrexed disodium is discontinued~~.

41. (currently amended) The method of claim 40, wherein the methylmalonic acid lowering agent is vitamin B12.

42. (previously presented) The method of claim 41, wherein the vitamin B12 is administered as an intramuscular injection of about 500 μg to about 1500 μg.

43. (previously presented) The method of claim 42, wherein the vitamin B12 is administered as an intramuscular injection of about 1000 μg.

44. (currently amended) The method of claim 41, 42 or 43, wherein the vitamin B12 administration is repeated about every ~~9 weeks~~ 6 to about every 12 weeks following the administration of vitamin B12 until the administration of the pemetrexed disodium is discontinued.

45 - 46. (cancelled)

47. (currently amended) The method of claim ~~46~~ 44 wherein the folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

48. (previously presented) The method of claim ~~47~~ 44 wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

49. (currently amended) The method according to any one of claims 40-43~~46-48~~, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

50. (previously presented) The method of claim 49 wherein about 350µg to about 1000 µg of folic acid is administered.

51. (previously presented) The method of claim 50 wherein 350 µg to 600 µg of folic acid is administered.

52. (currently amended) The method of claim 40 ~~or 45~~ further comprising the administration of cisplatin to the patient.

53. (new) An improved method for administering pemetrexed disodium to a patient in need of chemotherapeutic treatment, wherein the improvement comprises:

a) administration of between about 350 µg and about 1000 µg of folic acid prior to the first administration of pemetrexed disodium;

b) administration of about 500µg to about 1500µg of vitamin B12, prior to the first administration of pemetrexed disodium; and

c) administration of pemetrexed disodium.

54. (new) The method of claim 53 further comprising the administration of cisplatin to the patient.

55. (new) The method of claim 53, wherein vitamin B12 is administered as an intramuscular injection of about 500 µg to about 1500 µg.

56. (new) The method of claim 55, wherein vitamin B12 is administered as an intramuscular injection of about 1000 µg.

57. (new) The method of claim 56, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

58. (new) The method of claim 57 wherein about 350μg to about 1000 μg of folic acid is administered.

59. (new) The method of claim 58 wherein 350 μg to 600 μg of folic acid is administered.

60. (new) The method of claim 59 wherein folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

61. (new) The method of claim 59 wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

62. (new) The method of claim 53, 59, or 60, wherein the vitamin B12 administration is repeated about every 6 to about every 12 weeks following the administration of vitamin B12 until administration of pemetrexed disodium is discontinued.

63. (new) The method of claim 62 further comprising the administration of cisplatin to the patient.

PTO/SB/08a (07-09)
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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	11776329
Filing Date	2007-07-11
First Named Inventor	Clet Niyikiza
Art Unit	1614
Examiner Name	
Attorney Docket Number	X14173B

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2007-07-11
	First Named Inventor	Clet Niyikiza
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	Examiner Name	
	Attorney Docket Number	X14173B

	1	ALIMTA, NDA 021462, Approved Label of 07/02/2009.	☐
	2	"Clinical Chemistry: principle, procedures, correlations," 3rd edition, 1996, published by Lippincott: pp. 618-627.	☐
	3	Fluorouracil, Physicians Desk References, (c) 1998, pp 2463-2464.	☐
	4	HAMMOND, L., et al., "A phase I and pharmacokinetic (PK) study of the multitarget antifol (MTA) LY231514 with folic acid, " American Society of Clinical Oncology (ASCO) Meeting Abstract No. 866 (1998).	☐
	5	KISLIUK, RL., 1984. "The Biochemistry of Folates." In Sirotnak (Ed.), Folate Antagonists as Therapeutic Agents. pp. 2-68. Harcourt Brace Jovanovich, Publishers.	☐
	6	KISLIUK, RL., 1999. "Folate Biochemistry in RElation to Antifolate Selectivity." In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey.	☐
	7	Leucovorin, Physicians Desk Reference, (c) 1999. pp 1389-1391.	☐
	8	Methotrexate, Physicians Desk Reference, (c) 1999. pp. 1397-1413.	☐
	9	MORGAN, et al., "Folic acid supplementation prevent deficient blood folate levels and hyperhomocysteinemia during long-term, low dose methotrexate therapy for rheumatoid arthritis: implications for cardiovascular disease prevent," J. Rheumatol. 25:441-446. (1998).	☐
	10	NIYIKIZA, C., et al., "LY231514 (MTA): relationship of vitamin metabolite profile to toxicity," American Society of Clinical Oncology (ASCO) Meeting Abstract No. 2139 (1998).	☐
	11	Raltitrexed, The Complete Drug Reference, Martindale, 32nd Ed., Pharmaceutical Press, London, pp 560.	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11776329
	Filing Date	2007-07-11
	First Named Inventor	Clet Niyikiza
	Art Unit	1614
	Examiner Name	
	Attorney Docket Number	X14173B

	12	SHIH, C., et al., "LY231514, a Pyrrolo[2,3-d]pyrimidine-based Antifolate that Inhibits Multiple Folate-requiring Enzymes," Cancer Research. 57:1116-1123. 1997.	☐
	13	SHIH, C., et al., "Preclinical Pharmacology Studies and the Clinical Development of a Novel Multitargeted Antifolate, MTA (LY231514)," In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey.	☐
	14	VOLKOV, I., "The master key effect of vitamin B12 in treatment of malignancy - A potential therapy?", Medical Hypotheses. 70:324-328. 2008.	☐
	15		☐

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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.

☒ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ None

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	/Elizabeth A McGraw/	Date (YYYY-MM-DD)	2009-11-13
Name/Print	Elizabeth A. McGraw	Registration Number	44646

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removed by peritoneal dialysis. Pharmacokinetic studies have shown that total body clearance of AMICAR is markedly decreased in patients with severe renal failure.

DOSAGE AND ADMINISTRATION

INTRAVENOUS

AMICAR (aminocaproic acid) Injection is administered by infusion, utilizing the usual compatible intravenous vehicles (e.g., Sterile Water for Injection, Sodium Chloride for Injection, 5% Dextrose or Ringer's Injection). Although Sterile Water for Injection is compatible for intravenous injection, the resultant solution is hypo-osmolar. RAPID INJECTION OF AMICAR INJECTION UNDILUTED INTO A VEIN IS NOT RECOMMENDED.

For the treatment of acute bleeding syndromes due to elevated fibrinolytic activity, it is suggested that 16 to 20 mL (4 to 5 g) of AMICAR Injection in 250 mL of diluent be administered by infusion during the first hour of treatment, followed by a continuing infusion at the rate of 4 mL (1 g) per hour in 50 mL of diluent. This method of treatment would ordinarily be continued for about 8 hours or until the bleeding situation has been controlled.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

ORAL THERAPY

If the patient is able to take medication by mouth, an identical dosage regimen may be followed by administering AMICAR Tablets or AMICAR Syrup, 25% as follows: For the treatment of acute bleeding syndromes due to elevated fibrinolytic activity, it is suggested that 10 tablets (5 g) or 4 teaspoonfuls of syrup (5 g) of AMICAR be administered during the first hour of treatment, followed by a continuing rate of 2 tablets (1 g) or 1 teaspoonful of syrup (1.25 g) per hour. This method of treatment would ordinarily be continued for about 8 hours or until the bleeding situation has been controlled.

HOW SUPPLIED

AMICAR® (aminocaproic acid) Injection, supplied as follows:

Each 20 mL vial contains 5 g of aminocaproic acid (250 mg/mL) as an aqueous solution with benzyl alcohol 0.9% as preservative.

20 mL vial - NDC 58406-610-12

Each 96 mL single-use infusion vial contains 24 g of aminocaproic acid (250 mg/mL) as an aqueous solution with benzyl alcohol 0.9% as preservative.

96 mL vial - NDC 58406-610-13

STORE BETWEEN 15°-30°C (59°-86°F).

DO NOT FREEZE.

Manufactured for IMMUNEX CORPORATION, Seattle, WA 98101

by LEDERLE PARENTERALS, INC., Carolina, Puerto Rico 00987

AMICAR® (aminocaproic acid) Syrup, 25%, supplied as follows:

Each mL of raspberry-flavored syrup contains 250 mg of aminocaproic acid.

16 Fl. Oz. (473 mL) Bottle - NDC 58406-611-90

STORE BETWEEN 15°-30°C (59°-86°F).

Dispense in tight containers.

DO NOT FREEZE.

AMICAR® (aminocaproic acid) Tablets, supplied as follows: Each round, white tablet, engraved with LL on one side and scored on the other with A to the left of the score and 10 on the right, contains 500 mg of aminocaproic acid.

Bottle of 100 - NDC 58406-612-61

STORE BETWEEN 15°-30°C (59°-86°F).

Dispense in tight containers.

Manufactured for
IMMUNEX CORPORATION
Seattle, WA 98101
by LEDERLE LABORATORIES DIVISION
American Cyanamid Company, Pearl River, NY 10965

REFERENCES

1. Stefanini M, Dameshek W: The Hemorrhagic Disorders, Ed. 2, New York, Grune and Stratton. 1962; pp. 510-514.

REV 0197-02

CI 4565-2 Revised June 12, 1996 Printed in USA

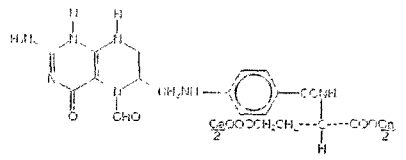
LEUCOVORIN CALCIUM FOR INJECTION ℞

DESCRIPTION

Leucovorin is one of several active, chemically reduced derivatives of folic acid. It is useful as an antidote to drugs which act as folic acid antagonists.

Also known as folinic acid, Citrovorum factor, or 5-formyl-5,6,7,8 tetrahydrofolic acid, this compound has the chemical designation of L-Glutamic acid, N-[4-[[(2-amino-5-formyl-1,4,5,6,7,8-hexahydro-4-oxo-6 pteridinyl)methyl]amino]benzoyl]-,calcium salt (1:1). The formula weight is 511.51 and the structural formula of leucovorin calcium is:

(See chemical structure at top of next column)



Leucovorin Calcium for Injection

Leucovorin Calcium for Injection is indicated for intravenous or intramuscular administration and is supplied as a sterile lyophilized powder. The 350 mg vial is preservative free. The inactive ingredient is sodium chloride 140 mg/vial for the 350 mg vial. Sodium hydroxide and/or hydrochloric acid are used to adjust the pH to approximately 8.1 during manufacture. One milligram of leucovorin calcium contains 0.002 mmol of leucovorin and 0.002 mmol of calcium.

CLINICAL PHARMACOLOGY

Leucovorin is a mixture of the diastereoisomers of the 5-formyl derivative of tetrahydrofolic acid (THF). The biologically active compound of the mixture is the (-)-*l*-isomer, known as Citrovorum factor or (-) folinic acid. Leucovorin does not require reduction by the enzyme dihydrofolate reductase in order to participate in reactions utilizing folates as a source of "one-carbon" moieties. *l*-Leucovorin (*l*-5 formyltetrahydrofolate) is rapidly metabolized (via 5,10 methenyltetrahydrofolate then 5,10-methylenetetrahydrofolate) to *l*-5 methyltetrahydrofolate. *l*-5-Methyltetrahydrofolate can in turn be metabolized via other pathways back to 5,10-methylenetetrahydrofolate, which is converted to 5-methyltetrahydrofolate by an irreversible, enzyme catalyzed reduction using the cofactors FADH2 and NADPH.

Administration of leucovorin can counteract the therapeutic and toxic effects of folic acid antagonists such as methotrexate, which act by inhibiting dihydrofolate reductase.

In contrast, leucovorin can enhance the therapeutic and toxic effects of fluoropyrimidines used in cancer therapy, such as 5-fluorouracil. Concurrent administration of leucovorin does not appear to alter the plasma pharmacokinetics of 5-fluorouracil. 5-Fluorouracil is metabolized to fluorodeoxyuridylic acid, which binds to and inhibits the enzyme thymidylate synthase (an enzyme important in DNA repair and replication).

Leucovorin is readily converted to another reduced folate, 5,10 methylenetetrahydrofolate, which acts to stabilize the binding of fluorodeoxyuridylic acid to thymidylate synthase and thereby enhances the inhibition of this enzyme.

The pharmacokinetics after intravenous, intramuscular, and oral administration of a 25 mg dose of leucovorin were studied in male volunteers. After intravenous administration, serum total reduced folates (as measured by *Lactobacillus casei* assay) reached a mean peak of 1259 ng/mL (range 897-1625). The mean time to peak was 10 minutes. This initial rise in total reduced folates was primarily due to the parent compound 5-formyl-THF (measured by *Streptococcus faecalis* assay) which rose to 1206 ng/mL at 10 minutes. A sharp drop in parent compound followed and coincided with the appearance of the active metabolite 5 methyl-THF which became the predominant circulating form of the drug.

The mean peak of 5-methyl-THF was 258 ng/mL and occurred at 1.3 hours. The terminal half-life for total reduced folates was 6.2 hours. The area under the concentration versus time curves (AUCs) for *l*-leucovorin, *d*-leucovorin and 5-methyltetrahydrofolate were 28.4 ± 3.5, 956 ± 97 and 129 ± 12 (mg.min/L ± S.E.). When a higher dose of *d,l*-leucovorin (200 mg/m^2) was used, similar results were obtained. The *d*-isomer persisted in plasma at concentrations greatly exceeding those of the *l*-isomer.

After intramuscular injection, the mean peak of serum total reduced folates was 436 ng/mL (range 240-725) and occurred at 52 minutes. Similar to IV administration, the initial sharp rise was due to the parent compound. The mean peak of 5-formyl-THF was 360 ng/mL and occurred at 28 minutes. The level of the metabolite 5-methyl-THF increased subsequently over time until at 1.5 hours it represented 50% of the circulating total folates. The mean peak of 5-methyl-THF was 226 ng/mL at 2.8 hours. The terminal half-life of total reduced folates was 6.2 hours. There was no difference of statistical significance between IM and IV administration in the AUC for total reduced folates, 5-formyl-THF, or 5 methyl-THF.

After oral administration of leucovorin reconstituted with aromatic elixir, the mean peak concentration of serum total reduced folates was 393 ng/mL (range 160-550). The mean time to peak was 2.3 hours and the terminal half-life was 5.7 hours. The major component was the metabolite 5-methyltetrahydrofolate to which leucovorin is primarily converted in the intestinal mucosa. The mean peak of 5-methyl-THF was 367 ng/mL at 2.4 hours. The peak level of the parent compound was 51 ng/mL at 1.2 hours. The AUC of total reduced folates after oral administration of the 25 mg dose was 92% of the AUC after intravenous administration.

Following oral administration, leucovorin is rapidly absorbed and expands the serum pool of reduced folates. At a dose of 25 mg, almost 100% of the *l*-isomer but only 20% of the *d*-isomer is absorbed. Oral absorption of leucovorin is saturable at doses above 25 mg. The apparent bioavailability of leucovorin was 97% for 25 mg, 75% for 50 mg, and 37% for 100 mg.

In a randomized clinical study conducted by the Mayo Clinic and the North Central Cancer Treatment Group (Mayo/NCCTG) in patients with advanced metastatic colorectal cancer three treatment regimens were compared: Leucovorin (LV) 200 mg/m^2 and 5-fluorouracil (5-FU) 370 mg/m2 versus LV 20 mg/m^2 and 5-FU 425 mg/m^2 versus 5-FU 500 mg/m^2. All drugs were administered by slow intravenous infusion daily for 5 days repeated every 28-35 days. Response rates were 26% (p = 0.04 versus 5-FU alone), 43% (p = 0.001 versus 5-FU alone) and 10% for the high dose leucovorin, low dose leucovorin and 5-FU alone groups respectively. Respective median survival times were 12.2 months (p = 0.037), 12 months (p = 0.050), and 7.7 months. The low dose LV regimen gave a statistically significant improvement in weight gain of more than 5%, relief of symptoms, and improvement in performance status. The high dose LV regimen gave a statistically significant improvement in performance status and trended toward improvement in weight gain and in relief of symptoms but these were not statistically significant.

In a second Mayo/NCCTG randomized clinical study the 5-FU alone arm was replaced by a regimen of sequentially administered methotrexate (MTX), 5-FU, and LV. Response rates with LV 200 mg/m^2 and 5-FU 370 mg/m2 versus LV 20 mg/m^2 and 5-FU 425 mg/m^2 versus sequential MTX and 5-FU and LV were respectively 31% (p = <.01), 42% (p = <.01), and 14%. Respective median survival times were 12.7 months (p = <.04), 12.7 months (p = <.01), and 8.4 months. No statistically significant difference in weight gain of more than 5% or in improvement in performance status was seen between the treatment arms.

INDICATIONS AND USAGE

Leucovorin calcium rescue is indicated after high-dose methotrexate therapy in osteosarcoma. Leucovorin calcium is also indicated to diminish the toxicity and counteract the effects of impaired methotrexate elimination and of inadvertent overdosages of folic acid antagonists.

Leucovorin calcium is indicated in the treatment of megaloblastic anemias due to folic acid deficiency when oral therapy is not feasible.

Leucovorin is also indicated for use in combination with 5-fluorouracil to prolong survival in the palliative treatment of patients with advanced colorectal cancer. Leucovorin should not be mixed in the same infusion as 5-fluorouracil because a precipitate may form.

CONTRAINDICATIONS

Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations continue to progress.

WARNINGS

In the treatment of accidental overdosages of folic acid antagonists, intravenous leucovorin should be administered as promptly as possible. As the time interval between antifolate administration [eg, methotrexate (MTX)] and leucovorin rescue increases, leucovorin's effectiveness in counteracting toxicity decreases. In the treatment of accidental overdosages of intrathecally administered folic acid antagonists, do not administer leucovorin intrathecally. LEUCOVORIN MAY BE HARMFUL OR FATAL IF GIVEN INTRATHECALLY.

Monitoring of the serum MTX concentration is essential in determining the optimal dose and duration of treatment with leucovorin.

Delayed MTX excretion may be caused by a third space fluid accumulation (ie, ascites, pleural effusion), renal insufficiency, or inadequate hydration. Under such circumstances, higher doses of leucovorin or prolonged administration may be indicated. Doses higher than those recommended for oral use must be given intravenously.

Because of the benzyl alcohol contained in certain diluents used for Leucovorin Calcium for Injection, when doses greater than 10 mg/m2 are administered, Leucovorin Calcium for Injection should be reconstituted with Sterile Water for Injection, USP, and used immediately. (See DOSAGE AND ADMINISTRATION.)

Because of the calcium content of the leucovorin solution, no more than 160 mg of leucovorin should be injected intravenously per minute (16 mL of a 10 mg/mL, or 8 mL of a 20 mg/mL solution per minute).

Leucovorin enhances the toxicity of 5-fluorouracil. When these drugs are administered concurrently in the palliative therapy of advanced colorectal cancer, the dosage of 5-fluorouracil must be lower than usually administered. Although the toxicities observed in patients treated with the combination of leucovorin plus 5-fluorouracil are qualitatively similar to those observed in patients treated with 5-fluoro-

Continued on next page

Leucovorin Calcium for Inj.—Cont.

uracil alone, gastrointestinal toxicities (particularly stomatitis and diarrhea) are observed more commonly and may be more severe and of prolonged duration in patients treated with the combination.

In the first Mayo/NCCTG controlled trial, toxicity, primarily gastrointestinal, resulted in 7% of patients requiring hospitalization when treated with 5-fluorouracil alone or 5-fluorouracil in combination with 200 mg/m^2 of leucovorin, and 20% when treated with 5-fluorouracil in combination with 20 mg/m^2 of leucovorin. In the second Mayo/NCCTG trial, hospitalizations related to treatment toxicity also appeared to occur more often in patients treated with the low dose leucovorin/5-fluorouracil combination than in patients treated with the high dose combination — 11% versus 3%.

Therapy with leucovorin/5-fluorouracil must not be initiated or continued in patients who have symptoms of gastrointestinal toxicity of any severity, until those symptoms have completely resolved. Patients with diarrhea must be monitored with particular care until the diarrhea has resolved, as rapid clinical deterioration leading to death can occur. In an additional study utilizing higher weekly doses of 5-FU and leucovorin, elderly and/or debilitated patients were found to be at greater risk for severe gastrointestinal toxicity.

Seizures and/or syncope have been reported rarely in cancer patients receiving leucovorin, usually in association with fluoropyrimidine administration, and most commonly in those with CNS metastases or other predisposing factors, however, a causal relationship has not been established.

The concomitant use of leucovorin with trimethoprim-sulfamethoxazole for the acute treatment of Pneumocystis carinii pneumonia in patients with HIV infection was associated with increased rates of treatment failure and morbidity in a placebo-controlled study.

PRECAUTIONS

General

Parenteral administration is preferable to oral dosing if there is a possibility that the patient may vomit or not absorb the leucovorin. Leucovorin has no effect on non-hematologic toxicities of MTX such as the nephrotoxicity resulting from drug and/or metabolite precipitation in the kidney. **Since leucovorin enhances the toxicity of fluorouracil, leucovorin/5 fluorouracil combination therapy for advanced colorectal cancer should be administered under the supervision of a physician experienced in the use of antimetabolite cancer chemotherapy. Particular care should be taken in the treatment of elderly or debilitated colorectal cancer patients, as these patients may be at increased risk of severe toxicity.**

Laboratory Tests

Patients being treated with the leucovorin/5-fluorouracil combination should have a CBC with differential and platelets prior to each treatment. During the first two courses a CBC with differential and platelets has to be repeated weekly and thereafter once each cycle at the time of anticipated WBC nadir. Electrolytes and liver function tests should be performed prior to each treatment for the first three cycles then prior to every other cycle. Dosage modifications of fluorouracil should be instituted as follows, based on the most severe toxicities:

Diarrhea and/or Stomatitis	WBC/mm^3 Nadir	Platelets/mm^3 Nadir	5-FU Dose
Moderate	1,000-1,900	25-75,000	decrease 20%
Severe	<1,000	<25,000	decrease 30%

If no toxicity occurs, the 5-fluorouracil dose may increase 10%. Treatment should be deferred until WBCs are 4,000/mm^3 and platelets 130,000/mm^3. If blood counts do not reach these levels within two weeks, treatment should be discontinued. Patients should be followed up with physical examination prior to each treatment course and appropriate radiological examination as needed. Treatment should be discontinued when there is clear evidence of tumor progression.

Drug Interactions

Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible pediatric patients.

Preliminary animal and human studies have shown that small quantities of systemically administered leucovorin enter the CSF primarily as 5 methyltetrahydrofolate and, in humans, remain 1–3 orders of magnitude lower than the usual methotrexate concentrations following intrathecal administration. However, high doses of leucovorin may reduce the efficacy of intrathecally administered methotrexate.

Leucovorin may enhance the toxicity of 5-fluorouracil. (See WARNINGS.)

Pregnancy: Teratogenic Effects:

"Pregnancy Category C." Adequate animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use: See Drug Interactions.

ADVERSE REACTIONS

Allergic sensitization, including anaphylactoid reactions and urticaria, has been reported following administration of both oral and parenteral leucovorin. No other adverse reactions have been attributed to the use of leucovorin per se. The following table summarizes significant adverse events occurring in 316 patients treated with the leucovorin-5-fluorouracil combinations compared against 70 patients treated with 5-fluorouracil alone for advanced colorectal carcinoma. These data are taken from the Mayo/NCCTG large multicenter prospective trial evaluating the efficacy and safety of the combination regimen.

[See first table below]

OVERDOSAGE

Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION

Advanced Colorectal Cancer: Either of the following two regimens is recommended:

1. Leucovorin is administered at 200 mg/m^2 by slow intravenous injection over a minimum of 3 minutes, followed by 5-fluorouracil at 370 mg/m^2 by intravenous injection.
2. Leucovorin is administered at 20 mg/m^2 by intravenous injection followed by 5-fluorouracil at 425 mg/m^2 by intravenous injection.

5-Fluorouracil and leucovorin should be administered separately to avoid the formation of a precipitate.

Treatment is repeated daily for five days. This five-day treatment course may be repeated at 4 week (28-day) intervals, for 2 courses and then repeated at 4–5 week (28–35 day) intervals provided that the patient has completely recovered from the toxic effects of the prior treatment course. In subsequent treatment courses, the dosage of 5-fluorouracil should be adjusted based on patient tolerance of the prior treatment course. The daily dosage of 5-fluorouracil should be reduced by 20% for patients who experienced moderate hematologic or gastrointestinal toxicity in the prior treatment course, and by 30% for patients who experienced severe toxicity. (see PRECAUTIONS: Laboratory Tests). For patients who experienced no toxicity in the prior treatment course, 5-fluorouracil dosage may be increased by 10%. Leucovorin dosages are not adjusted for toxicity.

Several other doses and schedules of leucovorin/5-fluorouracil therapy have also been evaluated in patients with advanced colorectal cancer; some of these alternative regimens may also have efficacy in the treatment of this disease. However, further clinical research will be required to confirm the safety and effectiveness of these alternative leucovorin/5-fluorouracil treatment regimens.

Leucovorin Rescue After High-Dose Methotrexate Therapy.

The recommendations for leucovorin rescue are based on a methotrexate dose of 12–15 grams/m^2 administered by intravenous infusion over 4 hours (see methotrexate package insert for full prescribing information).

Leucovorin rescue at a dose of 15 mg (approximately 10 mg/m^2) every 6 hours for 10 doses starts 24 hours after the beginning of the methotrexate infusion. In the presence of gastrointestinal toxicity, nausea or vomiting, leucovorin should be administered parenterally. Do not administer leucovorin intrathecally.

Serum creatinine and methotrexate levels should be determined at least once daily. Leucovorin administration, hydration, and urinary alkalinization (pH of 7.0 or greater) should be continued until the methotrexate level is below 5 x 10-8 M (0.05 micromolar). The leucovorin dose should be adjusted or leucovorin rescue extended based on the above guidelines.

[See second table below]

Patients who experience delayed early methotrexate elimination are likely to develop reversible renal failure. In addition to appropriate leucovorin therapy, these patients require continuing hydration and urinary alkalinization, and close monitoring of fluid and electrolyte status, until the serum methotrexate level has fallen to below 0.05 micromolar and the renal failure has resolved.

Some patients will have abnormalities in methotrexate elimination or renal function following methotrexate administration, which are significant but less severe than the abnormalities described in the table above. These abnormalities may or may not be associated with significant clinical toxicity. If significant clinical toxicity is observed, leucovorin rescue should be extended for an additional 24 hours (total of 14 doses over 84 hours) in subsequent courses of

PERCENTAGE OF PATIENTS TREATED WITH LEUCOVORIN/FLUOROURACIL FOR ADVANCED COLORECTAL CARCINOMA REPORTING ADVERSE EXPERIENCES OR HOSPITALIZED FOR TOXICITY

	(High LV)/5-FU (N=155)		(Low LV)/5-FU (N=161)		5-FU Alone (N=70)	
	Any (%)	Grade 3+ (%)	Any (%)	Grade 3+ (%)	Any (%)	Grade 3+ (%)
Leukopenia	69	14	83	23	93	48
Thrombocytopenia	8	2	8	1	18	3
Infection	8	1	3	1	7	2
Nausea	74	10	80	9	60	6
Vomiting	46	8	44	9	40	7
Diarrhea	66	18	67	14	43	11
Stomatitis	75	27	84	29	59	16
Constipation	3	0	4	0	1	–
Lethargy/Malaise/Fatigue	13	3	12	2	6	3
Alopecia	42	5	43	6	37	7
Dermatitis	21	2	25	1	13	–
Anorexia	14	1	22	4	14	–
Hospitalization for Toxicity	5%		15%		7%	

High LV = Leucovorin 200 mg/m^2, Low LV = Leucovorin 20 mg/m^2
Any = percentage of patients reporting toxicity of any severity
Grade 3+ = percentage of patients reporting toxicity of Grade 3 or higher

GUIDELINES FOR LEUCOVORIN DOSAGE AND ADMINISTRATION
DO NOT ADMINISTER LEUCOVORIN INTRATHECALLY

Clinical Situation	Laboratory Findings	Leucovorin Dosage and Duration
Normal Methotrexate Elimination	Serum methotrexate level approximately 10 micromolar at 24 hours after administration, 1 micromolar at 48 hours, and less than 0.2 micromolar at 72 hours.	15 mg PO, IM, or IV q 6 hours for 60 hours (10 doses starting at 24 hours after start of methotrexate infusion)
Delayed Late Methotrexate Elimination	Serum methotrexate level remaining above 0.2 micromolar at 72 hours, and more than 0.05 micromolar at 96 hours after administration.	Continue 15 mg PO, IM, or IV q 6 hours, until methotrexate level is less than 0.05 micromolar.
Delayed Early Methotrexate Elimination and/or Evidence of Acute Renal Injury	Serum methotrexate level of 50 micromolar or more at 24 hours, or 5 micromolar or more at 48 hours after administration, OR; a 100% or greater increase in serum creatinine level at 24 hours after methotrexate administration (eg, an increase from 0.5 mg/dL to a level of 1 mg/dL or more)	150 mg IV q 3 hours, until methotrexate level is less than 1 micromolar; then 15 mg IV q 3 hours until methotrexate level is less than 0.05 micromolar.

therapy. The possibility that the patient is taking other medications which interact with methotrexate (eg, medications which may interfere with methotrexate elimination or binding to serum albumin) should always be reconsidered when laboratory abnormalities or clinical toxicities are observed.

Impaired Methotrexate Elimination or Inadvertent Overdosage. Leucovorin rescue should begin as soon as possible after an inadvertent overdosage and within 24 hours of methotrexate administration when there is delayed excretion (see WARNINGS). Leucovorin 10 mg/m^2 should be administered IV, IM, or PO every 6 hours until the serum methotrexate level is less than 10^{-8} M. In the presence of gastrointestinal toxicity, nausea, or vomiting, leucovorin should be administered parenterally. Do not administer leucovorin intrathecally.

Serum creatinine and methotrexate levels should be determined at 24 hour intervals. If the 24 hour serum creatinine has increased 50% over baseline or if the 24 hour methotrexate level is greater than 5×10^{-6} M or the 48 hour level is greater than 9×10^{-7} M, the dose of leucovorin should be increased to 100 mg/m^2 IV every 3 hours until the methotrexate level is less than 10^{-8} M.

Hydration (3 L/d) and urinary alkalinization with sodium bicarbonate solution should be employed concomitantly. The bicarbonate dose should be adjusted to maintain the urine pH at 7.0 or greater.

Megaloblastic Anemia Due to Folic Acid Deficiency: Up to 1 mg daily. There is no evidence that doses greater than 1 mg/day have greater efficacy than those of 1 mg; additionally, loss of folate in urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

Each 350 mg vial of Leucovorin Calcium for Injection when reconstituted with 17 mL of sterile diluent yields a leucovorin concentration of 20 mg leucovorin per mL. Leucovorin Calcium for Injection contains no preservative. Reconstitute with Bacteriostatic Water for Injection, USP, which contains benzyl alcohol, or with Sterile Water for Injection, USP. When reconstituted with Bacteriostatic Water for Injection, USP, the resulting solution must be used within 7 days. If the product is reconstituted with Sterile Water for Injection, USP, it must be used immediately.

Because of the benzyl alcohol contained in Bacteriostatic Water for Injection, USP, when doses greater than 10 mg/m^2 are administered Leucovorin Calcium for Injection should be reconstituted with Sterile Water for Injection, USP, and used immediately. (See WARNINGS.) Because of the calcium content of the leucovorin solution, no more than 160 mg of leucovorin should be injected intravenously per minute (16 mL of a 10 mg/mL, or 8 mL of a 20 mg/mL solution per minute).

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Leucovorin should not be mixed in the same infusion as 5-fluorouracil, since this may lead to the formation of a precipitate.

HOW SUPPLIED

Leucovorin Calcium for Injection is supplied in sterile, single-use vials

NDC 58406-623-07 - 350 mg Vial

STORE AT 25°C (77°F); EXCURSIONS PERMITTED TO 15–30°C (59°–86°F).

PROTECT FROM LIGHT.

Manufactured for
IMMUNEX CORPORATION,
Seattle, WA 98101
by LEDERLE PARENTERALS, INC.,
Carolina, Puerto Rico 00987

CI 4820-4
Rev 0163-04 Issued 12/97

LEUCOVORIN CALCIUM TABLETS ℞

[lu-co-vor-in cal-sēē-um]

DESCRIPTION

Leucovorin is one of several active, chemically reduced derivatives of folic acid. It is useful as an antidote to drugs which act as folic acid antagonists. Also known as folinic acid, Citrovorum factor, or 5-formyl-5,6,7,8-tetrahydrofolic acid, this compound has the chemical designation of L-Glutamic acid, *N*-[4-[[(2-amino-5-formyl-1,4,5,6,7,8-hexa hydro-4-oxo-6-pteridinyl) methyl] amino] benzoyl] -, calcium salt (1:1). The formula weight is 511.51 and the structural formula of leucovorin calcium is:

[See chemical structure at top of next column]

Leucovorin Calcium Tablets, 5 mg, contain 5 mg of leucovorin (equivalent to 5.40 mg of anhydrous leucovorin calcium) and the following inactive ingredients: Corn Starch, Dibasic Calcium Phosphate, Magnesium Stearate, and Pregelatinized Starch.

Leucovorin Calcium Tablets, 15 mg, contain 15 mg of leucovorin (equivalent to 16.20 mg of anhydrous leucovorin calcium) and the following inactive ingredients: Lactose, Magnesium Stearate, Microcrystalline Cellulose, Pregelatinized Starch, and Sodium Starch Glycolate.

Leucovorin Calcium Tablets are indicated for oral administration only.

CLINICAL PHARMACOLOGY

Leucovorin is a mixture of the diastereoisomers of the 5-formyl derivative of tetrahydrofolic acid. The biologically active component of the mixture is the (-)-L-isomer, known as Citrovorum factor, or (-)-folinic acid. Leucovorin does not require reduction by the enzyme dihydrofolate reductase in order to participate in reactions utilizing folates as a source of "one-carbon" moieties. Following oral administration, leucovorin is rapidly absorbed and enters the general body pool of reduced folates.

The increase in plasma and serum reduced folate activity (determined microbiologically with *Lactobacillus casei*) seen after oral administration of leucovorin is predominantly due to 5-methyltetrahydrofolate.

Following a 20 mg dose of leucovorin calcium, the mean maximum serum total reduced folate concentrations were:

Tablet	364 ± 12.1 ng/mL at 2.0	± 0.07 hours
Oral Solution	375 ± 12.8 ng/mL at 2.1	± 0.11 hours
Parenteral	355 ± 17.2 ng/mL at 0.96	± 0.10 hours

The half-life of plasma 5-formyltetrahydrofolate was 1.5 ± 0.08 hours and that of the 5-methyltetrahydrofolate was 3.0 ± 0.09 hours.

Oral tablets produced equivalent bioavailability (8% difference) when compared to the parenteral administration. The parenteral solution also provided equal bioavailability to the tablets when administered orally (2% difference). Oral absorption of leucovorin is saturable at doses above 25 mg. The apparent bioavailability of leucovorin was 97% for 25 mg, 75% for 50 mg and 37% for 100 mg.

INDICATIONS

Leucovorin calcium rescue is indicated after high-dose methotrexate therapy in osteosarcoma. Leucovorin is also indicated to diminish the toxicity and counteract the effects of impaired methotrexate elimination and of inadvertent overdosages of folic acid antagonists.

CONTRAINDICATIONS

Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS

In the treatment of accidental overdosages of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration [eg, methotrexate (MTX)] and leucovorin rescue increases, leucovorin's effectiveness in counteracting toxicity diminishes.

Monitoring of serum MTX concentration is essential in determining the optimal dose and duration of treatment with leucovorin.

Delayed MTX excretion may be caused by a third space fluid accumulation (ie, ascites, pleural effusion), renal insufficiency, or inadequate hydration. Under such circumstances, higher doses of leucovorin or prolonged administration may be indicated. Doses higher than those recommended for oral use must be given intravenously.

Leucovorin may enhance the toxicity of fluorouracil. Deaths from severe enterocolitis, diarrhea, and dehydration have been reported in elderly patients receiving weekly leucovorin and fluorouracil.[1] Concomitant granulocytopenia and fever were present in some but not all of the patients.

Seizures and/or syncope have been reported rarely in cancer patients receiving leucovorin, usually in association with fluoropyrimidine administration, and most commonly in those with CNS metastases or other predisposing factors, however, a causal relationship has not been established.[2]

PRECAUTIONS

General

Parenteral administration is preferable to oral dosing if there is a possibility that the patient may vomit or not absorb the leucovorin. Leucovorin has no effect on other established toxicities of MTX such as the nephrotoxicity resulting from drug and/or metabolite precipitation in the kidney.

Drug Interactions

Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Preliminary animal and human studies have shown that small quantities of systemically administered leucovorin enter the CSF primarily as 5-methyltetrahydrofolate and, in humans, remain 1–3 orders of magnitude lower than the usual methotrexate concentrations following intrathecal administration. However, high doses of leucovorin may reduce the efficacy of intrathecally administered methotrexate.

Leucovorin may enhance the toxicity of fluorouracil (see WARNINGS).

Pregnancy: Teratogenic Effects

"Pregnancy Category C." Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use: see **Drug Interactions.**

ADVERSE REACTIONS

Allergic sensitization, including anaphylactoid reactions and urticaria, has been reported following the administration of both oral and parenteral leucovorin.

OVERDOSAGE

Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION

Leucovorin Calcium Tablets are intended for oral administration. Because absorption is saturable, oral administration of doses greater than 25 mg is not recommended.

Leucovorin Rescue after High-Dose Methotrexate Therapy: The recommendations for leucovorin rescue are based on a methotrexate dose of 12–15 grams/m^2 administered by intravenous infusion over 4 hours (see methotrexate package insert for full prescribing information).[3] Leuco-

Continued on next page

GUIDELINES FOR LEUCOVORIN DOSAGE AND ADMINISTRATION
DO NOT ADMINISTER LEUCOVORIN INTRATHECALLY

Clinical Situation	Laboratory Findings	Leucovorin Dosage and Duration
Normal Methotrexate Elimination	Serum methotrexate level approximately 10 micromolar at 24 hours after administration, 1 micromolar at 48 hours, and less than 0.2 micromolar at 72 hours.	15 mg PO, IM, or IV q 6 hours for 60 hours (10 doses starting at 24 hours after start of methotrexate infusion).
Delayed Late Methotrexate Elimination	Serum methotrexate level remaining above 0.2 micromolar at 72 hours, and more than 0.05 micromolar at 96 hours after administration.	Continue 15 mg PO, IM, or IV q 6 hours, until methotrexate level is less than 0.05 micromolar.
Delayed Early Methotrexate Elimination and/or Evidence of Acute Renal Injury	Serum methotrexate level of 50 micromolar or more at 24 hours, or 5 micromolar or more at 48 hours after administration, OR; a 100% or greater increase in serum creatinine level at 24 hours after methotrexate administration (eg, an increase from 0.5 mg/dL to a level of 1 mg/dL or more).	150 mg IV q 3 hours, until methotrexate level is less than 1 micromolar; then 15 mg IV q 3 hours until methotrexate level is less than 0.05 micromolar.

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15. Richter BR, Haddenann W, Ko-nigsmann M, *et al.* Recombinant human granylocyte-macrophage colony stimulating factor after chemotherapy in patients with acute myeloid leukemia at higher age or after relapse. Bloodd 1991; 78(5):1190–1197.
16. Blazar BR, Kersey JH, McGlave, *et al. In vivo* administration of recombinant human granylocyte/macrophage colony-stimulating factor in acute lymphoblastic patients receiving purged autographs. Blood 1989; 73(3): 849–857.

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Issued 02/98

METHOTREXATE SODIUM TABLETS ℞
METHOTREXATE SODIUM FOR INJECTION ℞
METHOTREXATE LPF® SODIUM (METHOTREXATE Sodium Injection) and METHOTREXATE SODIUM INJECTION ℞

WARNINGS

METHOTREXATE SHOULD BE USED ONLY BY PHYSICIANS WHOSE KNOWLEDGE AND EXPERIENCE INCLUDE THE USE OF ANTIMETABOLITE THERAPY.

BECAUSE OF THE POSSIBILITY OF SERIOUS TOXIC REACTIONS (WHICH CAN BE FATAL):

METHOTREXATE SHOULD BE USED ONLY IN LIFE THREATENING NEOPLASTIC DISEASES, OR IN PATIENTS WITH PSORIASIS OR RHEUMATOID ARTHRITIS WITH SEVERE, RECALCITRANT, DISABLING DISEASE WHICH IS NOT ADEQUATELY RESPONSIVE TO OTHER FORMS OF THERAPY.

DEATHS HAVE BEEN REPORTED WITH THE USE OF METHOTREXATE IN THE TREATMENT OF MALIGNANCY, PSORIASIS, AND RHEUMATOID ARTHRITIS.

PATIENTS SHOULD BE CLOSELY MONITORED FOR BONE MARROW, LIVER, LUNG AND KIDNEY TOXICITIES. (See **PRECAUTIONS.**)

PATIENTS SHOULD BE INFORMED BY THEIR PHYSICIAN OF THE RISKS INVOLVED AND BE UNDER A PHYSICIAN'S CARE THROUGHOUT THERAPY.

THE USE OF METHOTREXATE HIGH DOSE REGIMENS RECOMMENDED FOR OSTEOSARCOMA REQUIRES METICULOUS CARE. (See **DOSAGE AND ADMINISTRATION.**) HIGH DOSE REGIMENS FOR OTHER NEOPLASTIC DISEASES ARE INVESTIGATIONAL AND A THERAPEUTIC ADVANTAGE HAS NOT BEEN ESTABLISHED.

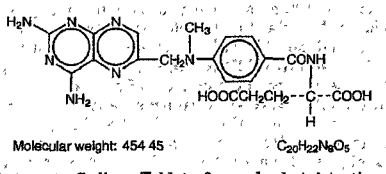
METHOTREXATE FORMULATIONS AND DILUENTS CONTAINING PRESERVATIVES MUST NOT BE USED FOR INTRATHECAL OR HIGH DOSE METHOTREXATE THERAPY.

1. Methotrexate has been reported to cause fetal death and/or congenital anomalies. Therefore, it is not recommended for women of childbearing potential unless there is clear medical evidence that the benefits can be expected to outweigh the considered risks. Pregnant women with psoriasis or rheumatoid arthritis should not receive methotrexate. (See **CONTRAINDICATIONS.**)
2. Methotrexate elimination is reduced in patients with impaired renal function, ascites, or pleural effusions. Such patients require especially careful monitoring for toxicity, and require dose reduction or, in some cases, discontinuation of methotrexate administration.
3. Unexpectedly severe (sometimes fatal) bone marrow suppression and gastrointestinal toxicity have been reported with concomitant administration of methotrexate (usually in high dosage) along with some nonsteroidal anti-inflammatory drugs (NSAIDs). (See **PRECAUTIONS, Drug Interactions.**)
4. Methotrexate causes hepatotoxicity, fibrosis and cirrhosis, but generally only after prolonged use. Acutely, liver enzyme elevations are frequently seen. These are usually transient and asymptomatic, and also do not appear predictive of subsequent hepatic disease. *Liver biopsy after sustained use often* shows histologic changes, and fibrosis and cirrhosis have been reported; these latter lesions may not be preceded by symptoms or abnormal liver function tests in the psoriasis population. For this reason, periodic liver biopsies are usually recommended for psoriatic patients who are under long-term treatment. Persistent abnormalities in liver function tests may precede appearance of fibrosis or cirrhosis in the rheumatoid arthritis population. (See **PRECAUTIONS, Organ System Toxicity,** *Hepatic.*)
5. Methotrexate-induced lung disease is a potentially dangerous lesion, which may occur acutely at any time during therapy and which has been reported at doses as low as 7.5 mg/week. It is not always fully reversible. Pulmonary symptoms (especially a dry, nonproductive cough) may require interruption of treatment and careful investigation.
6. Diarrhea and ulcerative stomatitis require interruption of therapy; otherwise, hemorrhagic enteritis and death from intestinal perforation may occur.
7. Malignant lymphomas, which may regress following withdrawal of methotrexate, may occur in patients receiving low-dose methotrexate and, thus, may not require cytotoxic treatment. Discontinue methotrexate first and, if the lymphoma does not regress, appropriate treatment should be instituted.
8. Like other cytotoxic drugs, methotrexate may induce "tumor lysis syndrome" in patients with rapidly growing tumors. Appropriate supportive and pharmacologic measures may prevent or alleviate this complication.
9. Severe, occasionally fatal, skin reactions have been reported following single or multiple doses of methotrexate. Reactions have occurred within days of oral, intramuscular, intravenous, or intrathecal methotrexate administration. Recovery has been reported with discontinuation of therapy. (See **PRECAUTIONS, Organ System Toxicity,** *Skin*)
10. Potentially fatal opportunistic infections, especially *Pneumocystis carinii* pneumonia, may occur with methotrexate therapy.

DESCRIPTION

Methotrexate (formerly Amethopterine) is an antimetabolite used in the treatment of certain neoplastic diseases, sever psoriasis, and adult rheumatoid arthritis.

Chemically methotrexate is *N*-[4[[(2,4-diamino-6-pteridinyl) methyl]methylamino]benzoyl]-L-glutamic acid. The structural formula is:



Molecular weight: 454.45 $C_{20}H_{22}N_8O_5$

Methotrexate Sodium Tablets for oral administration are available in bottles of 100 and in a packaging system designated as the RHEUMATREX® Methotrexate Sodium Dose Pack for therapy with a weekly dosing schedule of 5 mg, 7.5 mg, 10 mg, 12.5 mg and 15 mg. Methotrexate Sodium Tablets contain an amount of methotrexate sodium equivalent to 2.5 mg of methotrexate and the following inactive ingredients: Lactose, Magnesium Stearate and Pregelatinized Starch. May also contain Corn Starch.

Methotrexate Sodium Injection and for injection products are sterile and non-pyrogenic and may be given by the intramuscular, intravenous, intra-arterial or intrathecal route. (See **DOSAGE AND ADMINISTRATION.**) However, the preservative formulation contains Benzyl Alcohol and must not be used for intrathecal or high dose therapy.

Methotrexate Sodium Injection, Isotonic Liquid, Contains Preservative is available in 25 mg/mL, 2 mL (50 mg) and 10 mL (250 mg) vials.

Each 25 mg/mL, 2 mL and 10 mL vial contains methotrexate sodium equivalent to 50 mg and 250 mg methotrexate respectively, 0.90% w/v of Benzyl Alcohol as a preservative, and the following inactive ingredients: Sodium Chloride 0.260% w/v and Water for Injection qs ad 100% v. Sodium Hydroxide and, if necessary, Hydrochloric Acid are added to adjust the pH to approximately 8.5.

Methotrexate LPF® Sodium (methotrexate sodium injection), Isotonic Liquid, Preservative Free, for single use only, is available in 25 mg/mL, 2 mL (50 mg), 4 mL (100 mg), 8 mL (200 mg) and 10 mL (250 mg) vials.

Each 25 mg/mL, 2 mL, 4 mL, 8 mL and 10 mL vial contains methotrexate sodium equivalent to 50 mg, 100 mg, 200 mg and 250 mg methotrexate respectively, and the following inactive ingredients: Sodium Chloride 0.490% w/v and Water for Injection qs ad 100% v. Sodium Hydroxide and, if necessary, Hydrochloric Acid are added to adjust the pH to approximately 8.5. The 2 mL, 4 mL, 8 mL and 10 mL solutions contain approximately 0.43 mEq, 0.86 mEq, 1.72 mEq and 2.15 mEq of Sodium per vial, respectively, and are isotonic solutions.

Methotrexate Sodium for Injection, Lyophilized, Preservative Free, for single use only, is available in 20 mg and 1 gram vials.

Each 20 mg and 1 g vial of lyophilized powder contains methotrexate sodium equivalent to 20 mg and 1 g methotrexate respectively. Contains no preservative. Sodium Hydroxide and, if necessary, Hydrochloric Acid are added during manufacture to adjust the pH. The 20 mg vial contains approximately 0.14 mEq of Sodium and the 1 g vial contains approximately 7 mEq Sodium.

CLINICAL PHARMACOLOGY

Methotrexate inhibits dihydrofolic acid reductase. Dihydrololates must be reduced to tetrahydrofolates by this enzyme before they can be utilized as carriers of one-carbon groups in the synthesis of purine nucleotides and thymidylate. Therefore, methotrexate interferes with DNA synthesis, repair, and cellular replication. Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, and cells of the urinary bladder are in general more sensitive to this effect of methotrexate. When cellular proliferation in malignant tissues is greater than in most normal tissues, methotrexate may impair malignant growth without irreversible damage to normal tissues.

The mechanism of action in rheumatoid arthritis is unknown, it may affect immune function. Two reports describe *in vitro* methotrexate inhibition of DNA precursor uptake by stimulated mononuclear cells, and another describes in animal polyarthritis partial correction by methotrexate of spleen cell hyporesponsiveness and suppressed IL 2 production. Other laboratories, however, have been unable to demonstrate similar effects. Clarification of methotrexate's effect on immune activity and its relation to rheumatoid *immunopathogenesis await further studies.*

In patients with rheumatoid arthritis, effects of methotrexate on articular swelling and tenderness can be seen as early as 3 to 6 weeks. Although methotrexate clearly ameliorates symptoms of inflammation (pain, swelling, stiffness), there is no evidence that it induces remission of rheumatoid arthritis nor has a beneficial effect been demonstrated on bone erosions and other radiologic changes which result in impaired joint use, functional disability, and deformity.

Most studies of methotrexate in patients with rheumatoid arthritis are relatively short term (3 to 6 months). Limited data from long-term studies indicate that an initial clinical improvement is maintained for at least two years with continued therapy.

In psoriasis, the rate of production of epithelial cells in the skin is greatly increased over normal skin. This differential in proliferation rates is the basis for the use of methotrexate to control the psoriatic process.

Methotrexate in high doses, followed by leucovorin rescue, is used as a part of the treatment of patients with non-metastatic osteosarcoma. The original rationale for high dose methotrexate therapy was based on the concept of selective rescue of normal tissues by leucovorin. More recent evidence suggests that high dose methotrexate may also overcome methotrexate resistance caused by impaired active transport, decreased affinity of dihydrofolic acid reductase for methotrexate, increased levels of dihydrofolic acid reductase resulting from gene amplification, or decreased polyglutamation of methotrexate. The actual mechanism of action is unknown.

Two Pediatric Oncology Group studies (one randomized and one non-randomized) demonstrated a significant improvement in relapse-free survival in patients with non-metastatic osteosarcoma when high dose methotrexate with leucovorin rescue was used in combination with other chemotherapeutic agents following surgical resection of the primary tumor. These studies were not designed to demonstrate the specific contribution of high dose methotrexate/leucovorin rescue therapy to the efficacy of the combination. However, a contribution can be inferred from the reports of objective responses to this therapy in patients with metastatic osteosarcoma, and from reports to extensive tumor necrosis following preoperative administration of this therapy to patients with non-metastatic osteosarcoma.

Pharmacokinetics

Absorption — In adults, oral absorption appears to be dose dependent. Peak serum levels are reached within one to two hours. At doses of 30 mg/m^2 or less, methotrexate is generally well absorbed with a mean bioavailability of about 60%. The absorption of doses greater than 80 mg/m^2 is significantly less, possibly due to a saturation effect.

In leukemic pediatric patients, oral absorption has been reported to vary widely (23% to 95%). A twenty fold difference between highest and lowest peak levels (C_{max}: 0.11 to 2.3 micromolar after a 20 mg/m^2 dose) has been reported. Significant interindividual variability has also been noted in time to peak concentration (T_{max}: 0.67 to 4 hrs after a 15 mg/m^2 dose) and fraction of dose absorbed. Food has been shown to delay absorption and reduce peak concentration.

Continued on next page

Methotrexate Sodium—Cont.

Methotrexate is generally completely absorbed from parenteral routes of injection. After intramuscular injection, peak serum concentrations occur in 30 to 60 minutes.

Distribution — After intravenous administration, the initial volume of distribution is approximately 0.18 L/kg (18% of body weight) and steady-state volume of distribution is approximately 0.4 to 0.8 L/kg (40% to 80% of body weight). Methotrexate competes with reduced folates for active transport across cell membranes by means of a single carrier-mediated active transport process. At serum concentrations greater than 100 micromolar, passive diffusion becomes a major pathway by which effective intracellular concentrations can be achieved. Methotrexate in serum is approximately 50% protein bound. Laboratory studies demonstrate that it may be displaced from plasma albumin by various compounds including sulfonamides, salicylates, tetracyclines, chloramphenicol, and phenytoin.

Methotrexate does not penetrate the blood-cerebrospinal fluid barrier in therapeutic amounts when given orally or parenterally. High CSF concentrations of the drug may be attained by intrathecal administration.

In dogs, synovial fluid concentrations after oral dosing were higher in inflamed than uninflamed joints. Although salicylates did not interfere with this penetration, prior prednisone treatment reduced penetration into inflamed joints to the level of normal joints.

Metabolism — After absorption, methotrexate undergoes hepatic and intracellular metabolism to polyglutamated forms which can be converted back to methotrexate by hydrolase enzymes. These polyglutamates act as inhibitors of dihydrolase reductase and thymidylate synthetase. Small amounts of methotrexate polyglutamates may remain in tissues for extended periods. The retention and prolonged drug action of these active metabolites vary among different cells, tissues and tumors. A small amount of metabolism to 7-hydroxymethotrexate may occur at doses commonly prescribed. Accumulation of this metabolite may become significant at the high doses used in osteogenic sarcoma. The aqueous solubility of 7-hydroxymethotrexate is 3 to 5 fold lower than the parent compound. Methotrexate is partially metabolized by intestinal flora after oral administration.

Half Life — The terminal half life reported for methotrexate is approximately three to ten hours for patients receiving treatment for psoriasis, or rheumatoid arthritis or low dose antineoplastic therapy (less than 30 mg/m^2). For patients receiving high doses of methotrexate, the terminal half-life is eight to 15 hours.

Excretion — Renal excretion is the primary route of elimination and is dependent upon dosage and route of administration. With IV administration, 80% to 90% of the administered dose is excreted unchanged in the urine within 24 hours. There is limited biliary excretion, amounting to 10% or less of the administered dose. Enterohepatic recirculation of methotrexate has been proposed.

Renal excretion occurs by glomerular filtration and active tubular secretion. Nonlinear elimination due to saturation of renal tubular reabsorption has been observed in psoriatic patients at doses between 7.5 and 30 mg. Impaired renal function, as well as concurrent use of drugs such as weak organic acids that also undergo tubular secretion, can markedly increase methotrexate serum levels. Excellent correlation has been reported between methotrexate clearance and endogenous clearance.

Methotrexate clearance rates vary widely and are generally decreased at higher doses. Delayed drug clearance has been identified as one of the major factors responsible for methotrexate toxicity. It has been postulated that the toxicity of methotrexate for normal tissues is more dependent upon the duration of exposure to the drug rather than the peak level achieved. When a patient has delayed drug elimination due to compromised renal function, a third space effusion, or other causes, methotrexate serum concentrations may remain elevated for prolonged periods.

The potential for toxicity from high dose regimens or delayed excretion is reduced by the administration of leucovorin calcium during the final phase of methotrexate plasma elimination. Pharmacokinetic monitoring of methotrexate serum concentrations may help identify those patients at high risk for methotrexate toxicity and aid in proper adjustment of leucovorin dosing. Guidelines for monitoring serum methotrexate levels, and for adjustment of leucovorin dosing to reduce the risk of methotrexate toxicity, are provided below in **DOSAGE AND ADMINISTRATION.**

Methotrexate has been detected in human breast milk. The highest breast milk to plasma concentration ratio reached 0.08:1.

INDICATIONS AND USAGE

Neoplastic Diseases

Methotrexate is indicated in the treatment of gestational choriocarcinoma, chorioadenoma destruens and hydatidiform mole.

In acute lymphocytic leukemia, methotrexate is indicated in the prophylaxis of meningeal leukemia and is used in maintenance therapy in combination with other chemotherapeutic agents. Methotrexate is also indicated in the treatment of meningeal leukemia.

Methotrexate is used alone or in combination with other anticancer agents in the treatment of breast cancer, epidermoid cancers of the head and neck, advanced mycosis fungoides, and lung cancer, particularly squamous cell and small cell types. Methotrexate is also used in combination with other chemotherapeutic agents in the treatment of advanced stage non-Hodgkin's lymphomas.

Methotrexate in high doses followed by leucovorin rescue in combination with other chemotherapeutic agents is effective in prolonging relapse-free survival in patients with nonmetastatic osteosarcoma who have undergone surgical resection or amputation for the primary tumor.

Psoriasis

Methotrexate is indicated in the symptomatic control of severe, recalcitrant, disabling psoriasis that is not adequately responsive to other forms of therapy, *but only when the diagnosis has been established, as by biopsy and/or after dermatologic consultation.* It is important to ensure that a psoriasis "flare" is not due to an undiagnosed concomitant disease affecting immune responses.

Rheumatoid Arthritis

Methotrexate is indicated in the management of selected adults with severe, active, classical or definite rheumatoid arthritis (ARA criteria) who have had an insufficient therapeutic response to, or are intolerant of, an adequate trial of first-line therapy including full dose NSAIDs and usually a trial of at least one or more disease-modifying antirheumatic drugs.

Aspirin, nonsteroidal anti-inflammatory agents, and/or low dose steroids may be continued, although the possibility of increased toxicity with concomitant use of NSAIDs including salicylates has not been fully explored. (See **PRECAUTIONS, Drug Interactions.**) Steroids may be reduced gradually in patients who respond to methotrexate. Combined use of methotrexate with gold; penicillamine, hydroxychloroquine, sulfasalazine, or cytotoxic agents; has not been studied and may increase the incidence of adverse effects. Rest and physiotherapy as indicated should be continued.

CONTRAINDICATIONS

Methotrexate can cause fetal death or teratogenic effects when administered to a pregnant woman. Methotrexate is contraindicated in pregnant women with psoriasis or rheumatoid arthritis and should be used in the treatment of neoplastic diseases only when the potential benefit outweighs the risk to the fetus. Women of childbearing potential should not be started on methotrexate until pregnancy is excluded and should be fully counseled on the serious risk to the fetus (see **PRECAUTIONS**) should they become pregnant while undergoing treatment. Pregnancy should be avoided if either partner is receiving methotrexate; during and for a minimum of three months after therapy for male patients, and during and for at least one ovulatory cycle after therapy for female patients. (See Boxed **WARNINGS.**)

Because of the potential for serious adverse reactions from methotrexate in breast fed infants, it is contraindicated in nursing mothers.

Patients with psoriasis or rheumatoid arthritis with alcoholism, alcoholic liver disease or other chronic liver disease should not receive methotrexate.

Patients with psoriasis or rheumatoid arthritis who have overt or laboratory evidence of immunodeficiency syndromes should not receive methotrexate.

Patients with psoriasis or rheumatoid arthritis who have preexisting blood dyscrasias, such as bone marrow hypoplasia, leukopenia, thrombocytopenia or significant anemia, should not receive methotrexate.

Patients with a known hypersensitivity to methotrexate should not receive the drug.

WARNINGS—SEE BOXED WARNINGS.

PRECAUTIONS

General

Methotrexate has the potential for serious toxicity. (See Boxed **WARNINGS.**) Toxic effects may be related in frequency and severity to dose or frequency of administration but have been seen at all doses. Because they can occur at any time during therapy, it is necessary to follow patients on methotrexate closely. Most adverse reactions are reversible if detected early. When such reactions do occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken. If necessary, this could include the use of leucovorin calcium. (See **OVERDOSAGE.**) If methotrexate therapy is reinstituted, it should be carried out with caution, with adequate consideration of further need for the drug and with increased alertness as to possible recurrence of toxicity.

The clinical pharmacology of methotrexate has not been well studied in older individuals. Due to diminished hepatic and renal function as well as decreased folate stores in this population, relatively low doses should be considered, and these patients should be closely monitored for early signs of toxicity.

Information for Patients

Patients should be informed of the early signs and symptoms of toxicity, of the need to see their physician promptly if they occur, and the need for close follow-up, including periodic laboratory tests to monitor toxicity.

Both the physician and pharmacist should emphasize to the patient that the recommended dose is taken weekly in rheumatoid arthritis and psoriasis, and that mistaken daily use of the recommended dose has led to fatal toxicity. Patients should be encouraged to read the Patients Instructions sheet within the Dose Pack. Prescriptions should not be written or refilled on a PRN basis.

Patients should be informed of the potential benefit and risk in the use of methotrexate. The risk of effects on reproduction should be discussed with both male and female patients taking methotrexate.

Laboratory Tests

Patients undergoing methotrexate therapy should be closely monitored so that toxic effects are detected promptly. Baseline assessment should include a complete blood count with differential and platelet counts, hepatic enzymes, renal function tests, and a chest X-ray. During therapy of rheumatoid arthritis and psoriasis, monitoring of these parameters is recommended: hematology at least monthly, renal function and liver function every 1 to 2 months. More frequent monitoring is usually indicated during antineoplastic therapy. *During initial or changing doses,* or during periods of increased risk of elevated methotrexate blood levels (eg, dehydration), more frequent monitoring may also be indicated.

Transient liver function test abnormalities are observed frequently after methotrexate administration and are usually not cause for modification of methotrexate therapy. Persistent liver function test abnormalities, and/or depression of serum albumin may be indicators of serious liver toxicity and require evaluation. (See **PRECAUTIONS, Organ System Toxicity,** *Hepatic*

A relationship between abnormal liver function tests and fibrosis or cirrhosis of the liver has not been established for patients with psoriasis. Persistent abnormalities in liver function tests may precede appearance of fibrosis or cirrhosis in the rheumatoid arthritis population.

Pulmonary function tests may be useful if methotrexate induced lung disease is suspected, especially if baseline measurements are available.

Drug Interactions

Nonsteroidal anti-inflammatory drugs should not be administered prior to or concomitantly with the high doses of methotrexate used in the treatment of osteosarcoma. Concomitant administration of some NSAIDs with high dose methotrexate therapy has been reported to elevate and prolong serum methotrexate levels, resulting in deaths from severe hematologic and gastrointestinal toxicity.

Caution should be used when NSAIDs and salicylates are administered concomitantly with lower doses of methotrexate. These drugs have been reported to reduce the tubular secretion of methotrexate in an animal model and may enhance its toxicity.

Despite the potential interactions, studies of methotrexate in patients with rheumatoid arthritis have usually included concurrent use of constant dosage regimens of NSAIDs without apparent problems. It should be appreciated, however, that the doses used in rheumatoid arthritis (7.5 to 15 mg/week) are somewhat lower than those used in psoriasis and that larger doses could lead to unexpected toxicity.

Methotrexate is partially bound to serum albumin, and toxicity may be increased because of displacement by certain drugs, such as salicylates, phenylbutazone, phenytoin, and sulfonamides. Renal tubular transport is also diminished by probenecid; use of methotrexate with this drug should be carefully monitored.

In the treatment of patients with osteosarcoma, caution must be exercised if high-dose methotrexate is administered in combination with a potentially nephrotoxic chemotherapeutic agent (eg, cisplatin).

Oral antibiotics such as tetracycline, chloramphenicol, and nonabsorbable broad spectrum antibiotics, may decrease intestinal absorption of methotrexate or interfere with the enterohepatic circulation by inhibiting bowel flora and suppressing metabolism of the drug by bacteria.

Penicillins may reduce the renal clearance of methotrexate, increased serum concentrations of methotrexate with concomitant hematologic and gastrointestinal toxicity have been observed with high and low dose methotrexate. Use of methotrexate with penicillins should be carefully monitored.

Patients receiving concomitant therapy with methotrexate and etretinate or other retinoids should be monitored closely for possible increased risk of hepatotoxicity.

Methotrexate may decrease the clearance of theophylline; theophylline levels should be monitored when used concurrently with methotrexate.

Vitamin preparations containing folic acid or its derivatives may decrease responses to systemically administered methotrexate. Preliminary animal and human studies have shown that small quantities of intravenously administered

leucovorin enter the CSF primarily as 5-methyltetrahydrofolate acid, in humans, remain 1-3 orders of magnitude lower than the usual methotrexate concentrations following intrathecal administration. However, high doses of leucovorin may reduce the efficacy of intrathecally administered methotrexate.

Folate deficiency states may increase methotrexate toxicity. Trimethoprim/sulfamethoxazole has been reported rarely to increase bone marrow suppression in patients receiving methotrexate, probably by an additive antifolate effect.

Carcinogenesis, Mutagenesis, and Impairment of Fertility

No controlled human data exist regarding the risk of neoplasia with methotrexate. Methotrexate has been evaluated in a number of animal studies for carcinogenic potential with inconclusive results. Although there is evidence that methotrexate causes chromosomal damage to animal somatic cells and human bone marrow cells, the clinical significance remains uncertain. Non-Hodgkin's lymphoma and other tumors have been reported in patients receiving low-dose oral methotrexate. However, there have been instances of malignant lymphoma arising during treatment with low dose oral methotrexate, which have regressed completely following withdrawal of methotrexate, without requiring active anti-lymphoma treatment. Benefits should be weighed against the potential risks before using methotrexate alone or in combination with other drugs, especially in pediatric patients or young adults. Methotrexate causes embryotoxicity, abortion, and fetal defects in humans. It has also been reported to cause impairment of fertility, oligospermia and menstrual dysfunction in humans, during and for a short period after cessation of therapy.

Pregnancy

Psoriasis and rheumatoid arthritis: Methotrexate is in Pregnancy Category X. See **CONTRAINDICATIONS.**

Nursing Mothers

See **CONTRAINDICATIONS**

Pediatric Use

Safety and effectiveness in pediatric patients have not been established, other than in cancer chemotherapy.

Organ System Toxicity

Gastrointestinal If vomiting, diarrhea, or stomatitis occur, which may result in dehydration, methotrexate should be discontinued until recovery occurs. Methotrexate should be used with extreme caution in the presence of peptic ulcer disease or ulcerative colitis.

Hematologic: Methotrexate can suppress hematopoiesis and cause anemia, leukopenia, and/or thrombocytopenia. In patients with malignancy and preexisting hematopoietic impairment, the drug should be used with caution, if at all. In controlled clinical trials in rheumatoid arthritis (n=128), leukopenia (WBC <3000/mm^3) was seen in 2 patients, thrombocytopenia (platelets <100,000/mm^3) in 6 patients, and pancytopenia in 2 patients.

In psoriasis and rheumatoid arthritis, methotrexate should be stopped immediately if there is a significant drop in blood counts. In the treatment of neoplastic diseases, methotrexate should be continued only if the potential benefit warrants the risk of severe myelosuppression. Patients with profound granulocytopenia and fever should be evaluated immediately and usually require parenteral broad-spectrum antibiotic therapy.

Hepatic: Methotrexate has the potential for acute (elevated transaminases) and chronic (fibrosis and cirrhosis) hepatotoxicity. Chronic toxicity is potentially fatal, it generally has occurred after prolonged use (generally two years or more) and after a total dose of at least 1.5 grams. In studies in psoriatic patients, hepatotoxicity appeared to be a function of total cumulative dose and appeared to be enhanced by alcoholism, obesity, diabetes and advanced age. An accurate incidence rate has not been determined; the rate of progression and reversibility of lesions is not known. Special caution is indicated in the presence of preexisting liver damage or impaired hepatic function.

In psoriasis, liver function tests, including serum albumin, should be performed periodically prior to dosing but are often normal in the face of developing fibrosis or cirrhosis. These lesions may be detectable only by biopsy. The usual recommendation is to obtain a liver biopsy at 1) pretherapy or shortly after initiation of therapy (2–4 months), 2) a total cumulative dose of 1.5 grams, and 3) after each additional 1.0 to 1.5 grams.[1] Moderate fibrosis or any cirrhosis normally leads to discontinuation of the drug; mild fibrosis normally suggests a repeat biopsy in 6 months. Milder histologic findings such as fatty change and low grade portal inflammation are relatively common pretherapy. Although these mild changes are usually not a reason to avoid or discontinue methotrexate therapy, the drug should be used with caution.

In rheumatoid arthritis, age at first use of methotrexate and duration of therapy have been reported as risk factors for hepatotoxicity; other risk factors, similar to those observed in psoriasis, may be present in rheumatoid arthritis but have not been confirmed to date. Persistent abnormalities in liver function tests may precede appearance of fibrosis or cirrhosis in this population. There is a combined reported experience in 217 rheumatoid arthritis patients with liver biopsies both before and during treatment (after a cumulative dose of at least 1.5 g) and in 714 patients with a biopsy only during treatment. There are 64 (7%) cases of fibrosis and 1 (0.1%) case of cirrhosis. Of the 64 cases of fibrosis 59 were deemed mild. The reticulin stain is more sensitive for early fibrosis and its use may increase these figures. It is unknown whether even longer use will increase these risks.

Liver function tests should be performed at baseline and at 4–8 week intervals in patients receiving methotrexate for rheumatoid arthritis. Pretreatment liver biopsy should be performed for patients with a history of excessive alcohol consumption, persistently abnormal baseline liver function test values or chronic hepatitis B or C infection. During therapy, liver biopsy should be performed if there are persistent liver function test abnormalities or there is a decrease in serum albumin below the normal range (in the setting of well controlled rheumatoid arthritis).

If the results of a liver biopsy show mild changes (Roenigk grades I, II, IIIa), methotrexate may be continued and the patient monitored as per recommendations listed above. Methotrexate should be discontinued in any patient who displays persistently abnormal liver function tests and refuses liver biopsy or in any patient whose liver biopsy shows moderate to severe changes (Roenigk grade IIIb or IV).[2]

Infection or Immunologic States: Methotrexate should be used with extreme caution in the presence of active infection, and is usually contraindicated in patients with overt or laboratory evidence of immunodeficiency syndromes. Immunization may be ineffective when given during methotrexate therapy. Immunization with live virus vaccines is generally not recommended. There have been reports of disseminated vaccinia infections after smallpox immunization in patients receiving methotrexate therapy. Hypogammaglobulinemia has been reported rarely.

Potentially fatal opportunistic infections, especially *Pneumocystis carinii* pneumonia, may occur with methotrexate therapy. When a patient presents with pulmonary symptoms, the possibility of *Pneumocystis carinii* pneumonia should be considered.

Neurologic: There have been reports of leukoencephalopathy following intravenous administration of methotrexate to patients who have had craniospinal irradiation. Serious neurotoxicity, frequently manifested as generalized or local seizures, has been reported with unexpectedly increased frequency among pediatric patients with acute lymphoblastic leukemia who were treated with intermediate-dose intravenous methotrexate (1 gm/m^2). Symptomatic patients were commonly noted to have leukoencephalopathy and/or microangiopathic calcifications on diagnostic imaging studies. Chronic leukoencephalopathy has also been reported in patients who received repeated doses of high-dose methotrexate with leucovorin rescue even without cranial irradiation. Discontinuation of methotrexate does not always result in complete recovery.

A transient acute neurologic syndrome has been observed in patients treated with high dosage regimens. Manifestations of this stroke-like encephalopathy may include confusion, hemiparesis, seizures and coma. The exact cause is unknown.

After the intrathecal use of methotrexate, the central nervous system toxicity which may occur can be classified as follows: acute chemical arachnoiditis manifested by such symptoms as headache, back pain, nuchal rigidity, and fever; sub-acute myelopathy characterized by paraparesis/paraplegia associated with involvement with one or more spinal nerve roots; chronic leukoencephalopathy manifested by confusion, irritability, somnolence, ataxia, dementia, seizures and coma. This condition can be progressive and even fatal.

Pulmonary: Pulmonary symptoms (especially a dry nonproductive cough) or a nonspecific pneumonitis occurring during methotrexate therapy may be indicative of a potentially dangerous lesion and require interruption of treatment and careful investigation. Although clinically variable, the typical patient with methotrexate induced lung disease presents with fever, cough, dyspnea, hypoxemia, and an infiltrate on chest X-ray; infection needs to be excluded. This lesion can occur at all dosages.

Renal: High doses of methotrexate used in the treatment of osteosarcoma may cause renal damage leading to acute renal failure. Nephrotoxicity is due primarily to the precipitation of methotrexate and 7-hydroxymethotrexate in the renal tubules. Close attention to renal function including adequate hydration, urine alkalinization and measurement of serum methotrexate and creatinine levels are essential for safe administration.

Skin: Severe, occasionally fatal, dermatologic reactions, including toxic epidermal necrolysis, Stevens-Johnson syndrome, exfoliative dermatitis, skin necrosis, and erythema multiforme, have been reported in children and adults, within days of oral, intramuscular, intravenous, or intrathecal methotrexate administration. Reactions were noted after single or multiple, low, intermediate or high doses of methotrexate in patients with neoplastic and non-neoplastic diseases.

Other Processes: Methotrexate should be used with extreme caution in the presence of debility.

Methotrexate exits slowly from third space compartments (eg, pleural effusions or ascites). This results in a prolonged terminal plasma half-life and unexpected toxicity. In patients with significant third space accumulations, it is advisable to evacuate the third before treatment and to monitor plasma methotrexate levels.

Lesions of psoriasis may be aggravated by concomitant exposure to ultraviolet radiation. Radiation dermatitis and sunburn may be "recalled" by the use of methotrexate.

ADVERSE REACTIONS

IN GENERAL, THE INCIDENCE AND SEVERITY OF ACUTE SIDE EFFECTS ARE RELATED TO DOSE AND FREQUENCY OF ADMINISTRATION. THE MOST SERIOUS REACTIONS ARE DISCUSSED ABOVE UNDER ORGAN SYSTEM TOXICITY IN THE PRECAUTION SECTION. THAT SECTION SHOULD ALSO BE CONSULTED WHEN LOOKING FOR INFORMATION ABOUT ADVERSE REACTIONS WITH METHOTREXATE.

The most frequently reported adverse reactions include ulcerative stomatitis, leukopenia, nausea, and abdominal distress. Other frequently reported adverse effects are malaise, undue fatigue, chills and fever, dizziness and decreased resistance to infection.

Other adverse reactions that have been reported with methotrexate are listed below by organ system. In the oncology setting, concomitant treatment and the underlying disease make specific attribution of a reaction to methotrexate difficult.

Alimentary System: gingivitis, pharyngitis, stomatitis, anorexia, nausea, vomiting, diarrhea, hematemesis, melena, gastrointestinal ulceration and bleeding, enteritis, pancreatitis.

Cardiovascular: pericarditis, pericardial effusion, hypotension, and thromboembolic events (including arterial thrombosis, cerebral thrombosis, deep vein thrombosis, retinal vein thrombosis, thrombophlebitis, and pulmonary embolus).

Central Nervous System: headaches, drowsiness, blurred vision. Aphasia, hemiparesis, paresis and convulsions have also occurred following administration of methotrexate. Following low doses, there have been occasional reports of transient subtle cognitive dysfunction, mood alteration, unusual cranial sensation, leukoencephalopathy, or encephalopathy.

Infection: There have been case reports of sometimes fatal opportunistic infections in patients receiving methotrexate therapy for neoplastic and non-neoplastic diseases. *Pneumocystis carinii* pneumonia was the most common infection. Other reported infections included nocardiosis; histoplasmosis, cryptococcosis, *Herpes zoster, H. simplex* hepatitis, and disseminated *H. simplex.*

Ophthalmic: conjunctivitis, serious visual changes of unknown etiology.

Pulmonary System: intestinal pneumonitis deaths have been reported, and chronic interstitial obstructive pulmonary disease has occasionally occurred.

Skin: erythematous rashes, pruritus, urticaria, photosensitivity, pigmentary changes, alopecia, ecchymosis, telangiectasia, acne furunculosis, erythema multiforme, toxic epidermal necrolysis, Stevens-Johnson syndrome, skin necrosis, and exfoliative dermatitis.

Urogenital System: severe nephropathy or renal failure, azotemia, cystitis, hematuria; defective oogenesis or spermatogenesis, transient oligospermia, menstrual dysfunction, vaginal discharge, and gynecomastia; infertility, abortion, fetal defects.

Other rarer reactions related to or attributed to the use of methotrexate such as nodulosis, vasculitis, arthralgia/myalgia, loss of libido/impotence, diabetes, osteoporosis, sudden death, reversible lymphomas, and tumor lysis syndrome. Anaphylactoid reactions have been reported.

Adverse Reactions in Double-Blind Rheumatoid Arthritis Studies

The approximate incidences of methotrexate-attributed (ie, placebo rate subtracted) adverse reactions in 12 to 18 week double-blind studies of patients (n=128) with rheumatoid arthritis treated with low-dose oral (7.5 to 15 mg/week) pulse methotrexate are listed below. Virtually all of these patients were on concomitant nonsteroidal anti-inflammatory drugs and some were also taking low dosages of corticosteroids.

Incidence greater than 10%: Elevated liver function tests 15%, nausea/vomiting 10%.3.

Incidence 3% to 10%. Stomatitis, thrombocytopenia (platelet count less than 100,000/mm^3).

Incidence 1% to 3%: Rash/pruritus/dermatitis, diarrhea, alopecia, leukopenia (WBC less than 3000/mm^3), pancytopenia, dizziness.

No pulmonary toxicity was seen in these two trials. Thus, the incidence is probably less than 2.5% (95% C.L.). Hepatic histology was not examined in these short-term studies. (See **PRECAUTIONS.**)

Continued on next page

Methotrexate Sodium—Cont.

Other less common reactions included decreased hematocrit, headache, upper respiratory infection, anorexia, arthralgias, chest pain, coughing, dysuria, eye discomfort, epistaxis, fever, infection, sweating, tinnitus, and vaginal discharge.

Adverse Reactions in Psoriasis

There are no recent placebo-controlled trials in patients with psoriasis. There are two literature reports (Roenigk, 1969 and Nylors, 1978) describing large series (n=204, 248) of psoriasis patients treated with methotrexate. Dosages ranged up to 25 mg per week and treatment was administered for up to four years. With the exception of alopecia, photosensitivity, and "burning of skin lesions" (each 3% to 10%), the adverse reaction rates in these reports were very similar to those in the rheumatoid arthritis studies.

OVERDOSAGE

Leucovorin is indicated to diminish the toxicity and counteract the effect of inadvertently administered overdosages of methotrexate. Leucovorin administration should begin as promptly as possible. As the time interval between methotrexate administration and leucovorin initiation increases, the effectiveness of leucovorin in counteracting toxicity decreases. Monitoring of the serum methotrexate concentration is essential in determining the optimal dose and duration of treatment with leucovorin.

In cases of massive overdosage, hydration and urinary alkalinization may be necessary to prevent the precipitation of methotrexate and/or its metabolites in the renal tubules. Neither hemodialysis nor peritoneal dialysis have been shown to improve methotrexate elimination.

Accidental intrathecal overdosage may require intensive systemic support, high-dose systemic leucovorin, alkaline diuretics and rapid CSF drainage and ventriculolumbar perfusion.

DOSAGE AND ADMINISTRATION

Neoplastic Diseases

Oral administration in tablet form is often preferred when low doses are being administered since absorption is rapid and effective serum levels are obtained. Methotrexate sodium injection and for injection may be given by the intramuscular, intravenous, intra-arterial or intrathecal route. However, the preserved formulation contains Benzyl Alcohol and must not be used for intrathecal or high dose therapy. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Choriocarcinoma and similar trophoblastic diseases: Methotrexate is administered orally or intramuscularly in doses of 15 to 30 mg daily for a five-day course. Such courses are usually repeated for 3 to 5 times as required, with rest periods of one or more weeks interposed between courses, until any manifesting toxic symptoms subside. The effectiveness of therapy is ordinarily evaluated by 24 hour quantitative analysis of urinary chorionic gonadrotropin (hCG), which should return to normal or less than 50 IU/24 hr usually after the third or fourth course and usually be followed by a complete resolution of measurable lesions in 4 to 6 weeks. One to two courses of methotrexate after normalization of hCG is usually recommended. Before each course of the drug careful clinical assessment is essential. Cyclic combination therapy of methotrexate with other antitumor drugs has been reported as being useful.

Since hydatidiforme mole may precede choriocarcinoma, prophylactic chemotherapy with methotrexate has been recommended.

Chorioadenoma destruens is considered to be an invasive form of hydatidiform mole. Methotrexate is administered in these disease states in doses similar to those recommended for choriocarcinoma.

Leukemia: Acute lymphoblastic leukemia in pediatric patients and young adolescents is the most responsive to present day chemotherapy. In young adults and older patients, clinical remission is more difficult to obtain and early relapse is more common.

Methotrexate alone or in combination with steroids was used initially for induction of remission in acute lymphoblastic leukemias. More recently corticosteroid therapy, in combination with other antileukemic drugs or in cyclic combinations with methotrexate included, has appeared to produce rapid and effective remissions. When used for induction, methotrexate in doses of 3.3 mg/m^2 in combination with 60 mg/m^2 of prednisone, given daily, produced remissions in 50% of patients treated, usually within a period of 4 to 6 weeks. Methotrexate in combination with other agents appears to be the drug of choice for securing maintenance of drug-induced remissions. When remission is achieved and supportive care has produced general clinical improvement, maintenance therapy is initiated, as follows: Methotrexate is administered 2 times weekly either by mouth or intramuscularly in total weekly doses of 30 mg/m^2. It has also been given in doses of 2.5 mg/kg intravenously every 14 days. If and when relapse does occur, reinduction of remission can again usually be obtained by repeating the initial induction regimen.

A variety of combination chemotherapy regimens have been used for both induction and maintenance therapy in acute lymphoblastic leukemia. The physician should be familiar with the new advances in antileukemic therapy.

Meningeal Leukemia: In the treatment of prophylaxis of meningeal leukemia, methotrexate must be administered intrathecally. Preservative free methotrexate is diluted to a concentration of 1 mg/mL in an appropriate sterile, preservative free medium such as 0.9% Sodium Chloride Injection, USP.

The cerebrospinal fluid volume is dependent on age and not on body surface area. The CSF is at 40% of the adult volume at birth and reaches the adult volume in several years. Intrathecal methotrexate administration at a dose of 12 mg/m^2 (maximum 15 mg) has been reported to result in low CSF methotrexate concentrations and reduced efficacy in pediatric patients and high concentrations and neurotoxicity in adults. The following dosage regimen is based on age instead of body surface area:

Age (years)	Dose (mg)
<1	6
1	8
2	10
3 or older	12

In one study in patients under the age of 40, this dosage regimen appeared to result in more consistent CSF methotrexate concentrations and less neurotoxicity. Another study in pediatric patients with acute lymphocytic leukemia compared this regimen to a dose of 12 mg/m^2 (maximum 15 mg), a significant reduction in the rate of CNS relapse was observed in the group whose dose was based on age.

Because the CSF volume and turnover may decrease with age, a dose reduction may be indicated in elderly patients. For the treatment of meningeal leukemia, intrathecal methotrexate may be given at intervals of 2 to 5 days. However, administration at intervals of less than 1 week may result in increased subacute toxicity. Methotrexate is administered until the cell count of the cerebrospinal fluid returns to normal. At this point one additional dose is advisable. For prophylaxis against meningeal leukemia, the dosage is the same as for treatment except for the intervals of administration. On this subject, it is advisable for the physician to consult the medical literature.

Untoward side effects may occur with any given intrathecal injection and are commonly neurological in character. Large doses may cause convulsions. Methotrexate given by the intrathecal route appears significantly in the systemic circulation and may cause systemic methotrexate toxicity. Therefore, systemic antileukemic therapy with the drug should be appropriately adjusted reduced, or discontinued. Focal leukemic involvement of the central nervous system may not respond to intrathecal chemotherapy and is best treated with radiotherapy.

Lymphomas: In Burkitt's tumor, Stages I–II, methotrexate has produced prolonged remissions in some cases. Recommended dosage is 10 to 25 mg/day orally for 4 to 8 days. In Stage III, methotrexate is commonly given concomitantly with other antitumor agents. Treatment in all stages usually consists of several courses of the drug interposed with 7 to 10 day rest periods. Lymphosarcomas in Stage III may respond to combined drug therapy with methotrexate given in doses of 0.625 to 2.5 mg/kg daily.

Mycosis Fungoides: Therapy with methotrexate appears to produce clinical remissions in one half of the cases treated. Dosage is usually 2.5 to 10 mg daily by mouth for weeks or months. Dose levels of drug and adjustment of dose regimen by reduction or cessation of drug are guided by patient response and hematologic monitoring. Methotrexate has also been given intramuscularly in doses of 50 mg once weekly or 25 mg 2 times weekly.

Osteosarcoma: An effective adjuvant chemotherapy regimen requires the administration of several cytotoxic chemotherapeutic agents. In addition to high-dose methotrexate with leucovorin rescue, these agents may include doxorubicin, cisplatin, and the combination of bleomycin, cyclophosphamide and dactinomycin (BCD) in the doses and schedule shown in the table below. The starting dose for high dose methotrexate treatment is 12 grams/m^2. If this dose is not sufficient to produce a peak serum methotrexate concentration of 1,000 micromolar (10^{-3} mol/L) at the end of the methotrexate infusion, the dose may be escalated to 15 grams/m^2 in subsequent treatments. If the patient is vomiting or is unable to tolerate oral medication, leucovorin is given IV or IM at the same dose and schedule.

Drug*	Dose*	Treatment Week After Surgery
Methotrexate	12 g/m^2 IV as 4 hour infusion (starting dose)	4,5,6,7,11,12,15, 16,29,30,44,45
Leucovorin	15 mg orally every six hours for 10 doses starting at 24 hours after start of methotrexate infusion.	
Doxorubicin† as a single drug	30 mg/m^2/day IV × 3 days	8,17
Doxorubicin†	50 mg/m^2 IV	20,23,33,36
Cisplatin†	100 mg/m^2 IV	20,23,33,36
Bleomycin†	15 units/m^2 IV × 2 days	2,13,26,39,42
Cyclophosphamide†	600 mg/m^2 IV × 2 days	2,13,26,39,42
Dactinomycin†	0.6 mg/m^2 IV × 2 days	2,13,26,39,42

* Link MP, Goorin AM, Miser AW, et al: The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. *N Engl J of Med* 1986; 314(No.25):1600–1606.

† See each respective package insert for full prescribing information. Dosage modifications may be necessary because of drug-induced toxicity.

When these higher doses of methotrexate are to be administered, the following safety guidelines should be closely observed.

GUIDELINES FOR METHOTREXATE THERAPY WITH LEUCOVORIN RESCUE

1. Administration of methotrexate should be delayed until recovery if
   - the WBC count is less than 1500/microliter
   - the neutrophil count is less than 200/microliter
   - the platelet count is less than 75,000/microliter
   - the serum bilirubin level is greater than 1.2 mg/dL
   - the SGPT level is greater than 450 U
   - mucositis is present, until there is evidence of healing
   - persistent pleural effusion is present; this should be drained dry prior to infusion.
2. Adequate renal function must be documented.
   a. Serum creatinine must be normal, and creatinine clearance must be greater than 60 mL/min, before initiation of therapy.
   b. Serum creatinine must be measured prior to each subsequent course of therapy. If serum creatinine has increased by 50% or more compared to a prior value, the creatinine clearance must be measured and documented to be greater than 60 mL/min (even if the serum creatinine is still within the normal range).
3. Patients must be well hydrated, and must be treated with sodium bicarbonate for urinary alkalinization.
   a. Administer 1,000 mL/m^2 of intravenous fluid over 6 hours prior to initiation of the methotrexate infusion. Continue hydration at 125 mL/m^2/hr (3 liters/m^2/day) during the methotrexate infusion, and for 2 days after the infusion has been completed.
   b. Alkalinize urine to maintain pH above 7.0 during methotrexate infusion and leucovorin calcium therapy. This can be accomplished by the administration of sodium bicarbonate orally or by incorporation into a separate intravenous solution.
4. Repeat serum creatinine and serum methotrexate 24 hours after starting methotrexate and at least once daily until the methotrexate level is below (0.05 micromolar).
5. The table below provides guidelines for leucovorin calcium dosage based upon serum methotrexate levels. (See table below.‡)
   Patients who experience delayed early methotrexate elimination are likely to develop nonreversible oliguric renal failure. In addition to appropriate leucovorin therapy, these patients require continuing hydration and urinary alkalinization, and close monitoring of fluid and electrolyte status, until the serum methotrexate level has fallen to below 0.05 micromolar and the renal failure has resolved.
6. Some patients will have abnormalities in methotrexate elimination, or abnormalities in renal function following methotrexate administration, which are significant but less severe than the abnormalities described in the table below. These abnormalities may or may not be associated with significant clinical toxicity. If significant clinical toxicity is observed leucovorin rescue should be extended for an additional 24 hours (total 14 doses over 84 hours) in subsequent courses of therapy. The possibility that the

patient is taking other medications which interact with methotrexate (eg, medications which may interfere with methotrexate binding to serum albumin, or elimination) should always be reconsidered when laboratory abnormalities or clinical toxicities are observed.

CAUTION: DO NOT ADMINISTER LEUCOVORIN INTRATHECALLY.

Psoriasis and Rheumatoid Arthritis

The patient should be fully informed of the risks involved and should be under constant supervision of the physician. (See **Information for Patients** Under **PRECAUTIONS**.) Assessment of hematologic, hepatic, renal, and pulmonary function should be made by history, physical examination, and laboratory tests before beginning, periodically during, and before reinstituting methotrexate therapy. (See **PRECAUTIONS**.) Appropriate steps should be taken to avoid conception during methotrexate therapy. (See **PRECAUTIONS** and **CONTRAINDICATIONS**.)

Weekly therapy may be instituted with the RHEUMATREX® Methotrexate Sodium 2.5 mg Tablet Dose Packs which are designed to provide doses over a range of 5 mg to 15 mg administered as a single weekly dose. The dose packs are not recommended for administration of methotrexate in weekly doses greater than 15 mg. All schedules should be continually tailored to the individual patient. An initial test dose may be given prior to the regular dosing schedule to detect any extreme sensitivity to adverse effects. (See **ADVERSE REACTIONS**.) Maximal myelosuppression usually occurs in seven to ten days.

Psoriasis: Recommended Starting Dose Schedules

1. Weekly single oral, IM or IV dose schedule: 10 to 25 mg per week until adequate response is achieved.
2. Divide oral dose schedule: 2.5 mg at 12-hour intervals for three doses.

Dosages in each schedule may be gradually adjusted to achieve optimal clinical response. 30 mg/week should not ordinarily be exceeded.

Once optimal clinical response has been achieved, each dosage schedule should be reduced to the lowest possible amount of drug and to the longest possible rest period. The use of methotrexate may permit the return to conventional topical therapy, which should be encouraged.

Rheumatoid Arthritis: Recommended Starting Dosage Schedules

1. Single oral doses of 7.5 mg once weekly.
2. Divide oral dosages of 2.5 mg at 12 hour intervals for 3 doses given as a course once weekly.

Dosages in each schedule may be adjusted gradually to achieve an optimal response, but not ordinarily to exceed a total weekly dose of 20 mg. Limited experience shows a significant increase in the incidence and severity of serious toxic reactions, especially bone marrow suppression, at doses greater than 20 mg/wk.

Once response has been achieved, each schedule should be reduced, if possible, to the lowest possible effective dose. Therapeutic response usually begins within 3 to 6 weeks and the patient may continue to improve for another 12 weeks or more.

The optimal duration of therapy is unknown. Limited data available from long-term studies indicate that the initial clinical improvement is maintained for at least two years with continued therapy. When methotrexate is discontinued, the arthritis usually worsens within 3 to 6 weeks.

HANDLING AND DISPOSAL

Procedures for proper handling and disposal of anticancer drugs should be considered. Several guidelines on this subject have been published.[3-8] There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

RECONSTITUTION OF LYOPHILIZED POWDERS

Reconstitute immediately prior to use.

Methotrexate Sodium for Injection should be reconstituted with an appropriate sterile, preservative free medium such as 5% Dextrose Solution, USP, or Sodium Chloride Injection, USP. Reconstitute the 20 mg vial to a concentration no greater than 25 mg/mL. **The 1 gram vial should be reconstituted with 19.4 mL to a concentration of 50 mg/mL.** When high doses of methotrexate are administered by IV infusion, the total dose is diluted in 5% Dextrose Solution. For intrathecal injection, reconstitute to a concentration of 1 mg/mL with an appropriate sterile, preservative free medium such as Sodium Chloride Injection, USP.

DILUTION INSTRUCTIONS FOR LIQUID METHOTREXATE SODIUM INJECTION PRODUCTS

Methotrexate Sodium Injection, Isotonic Liquid, Contains Preservatives

If desired, the solution may be further diluted with a compatible medium such as Sodium Chloride Injection, USP. Storage for 24 hours at a temperature of 21 to 25°C results in a product which is within 90% of label potency.

Methotrexate LPF® Sodium (methotrexate sodium injection), Isotonic Liquid, Preservative Free, for Single Use Only

If desired, the solution may be further diluted immediately prior to use with an appropriate sterile, preservative free medium such as 5% Dextrose Solution, USP or Sodium Chloride Injection, USP.

LEUCOVORIN RESCUE SCHEDULES FOLLOWING TREATMENT WITH HIGHER DOSES OF METHOTREXATE

Clinical Situation	Laboratory Findings	Leucovorin Dosage and Duration
Normal Methotrexate Elimination	Serum methotrexate level approximately 10 micromolar at 24 hours after administration, 1 micromolar at 48 hours, and less than 0.2 micromolar at 72 hours.	15 mg PO, IM or IV q 6 hours for 60 hours (10 doses starting at 24 hours after start of methotrexate infusion)
Delayed Late Methotrexate Elimination	Serum methotrexate level remaining above 0.2 micromolar at 72 hours, and more than 0.05 micromolar at 96 hours after administration.	Continue 15 mg PO, IM or IV q six hours, until methotrexate level is less than 0.05 micromolar.
Delayed Early Methotrexate Elimination and/or Evidence of Acute Renal Injury	Serum methotrexate level of 50 micromolar or more at 24 hours, or 5 micromolar or more at 48 hours after administration, OR; a 100% or greater increase in serum creatinine level at 24 hours after methotrexate administration (eg, an increase from 0.5 mg/dL to a level of 1 mg/dL or more).	150 mg IV q three hours, until methotrexate level is less than 1 micromolar; then 15 mg IV q three hours, until methotrexate level is less than 0.05 micromolar.

HOW SUPPLIED

Parenteral:

Methotrexate Sodium for Injection, Lyophilized, Preservative Free, for Single Use Only. Each 20 mg and 1 g vial of lyophilized powder contains methotrexate sodium equivalent to 20 mg and 1 g methotrexate respectively.

20 mg Vial — NDC 58406-673-01 (Dark Blue Cap)
1 g Vial — NDC 58406-671-05 (Red Cap)

Methotrexate LPF® Sodium (methotrexate sodium injection), Isotonic Liquid, Preservative Free, for Single Use Only. Each 25 mg/mL, 2 mL, 4 mL, 8 mL and 10 mL vial contains methotrexate sodium equivalent to 50 mg, 100 mg, 200 mg and 250 mg methotrexate respectively.

50 mg — 2 mL Vial— NDC 58406-683-15 (Brown Cap)
100 mg — 4 mL Vial— NDC 58406-683-18 (Light Blue Cap)
200 mg — 8 mL Vial— NDC 58406-683-12 (Orange Cap)
250 mg —10 mL Vial— NDC 58406-683-16 (Violet Cap)

Methotrexate Sodium Injection, Isotonic Liquid, Contains Preservative. Each 25 mg/mL, 2 mL and 10 mL vial contains methotrexate sodium equivalent to 50 mg and 250 mg methotrexate respectively.

50 mg — 2 mL Vial — NDC 58406-681-14 (Red Cap)
250 mg — 10 mL Vial — NDC 58406-681-17 (Brown Cap)

Store at 25°C (77°F); excursions permitted to 15°–30°C (59°–86°F) [see USP Controlled Room Temperature]. Protect from light.

IMMUNEX®
Manufactured for
IMMUNEX CORPORATION, Seattle, WA 98101
by
LEDERLE PARENTERALS, INC., Carolina, Puerto Rico 00987

Oral:

Description

Methotrexate Sodium Tablets contain an amount of methotrexate sodium equivalent to 2.5 mg of methotrexate and are round, convex, yellow tablets, engraved with LL on one side, scored in half on the other side, and engraved with M above the score, and 1 below.

NDC 0005-4507-23 — Bottle of 100

RHEUMATREX® Methotrexate Sodium Tablet 2.5 mg Dose Packs — (each tablet equivalent to 2.5 mg of methotrexate)

NDC 0005-4507-04—RHEUMATREX® Methotrexate Sodium Tablets Dose Pack—4 cards each containing two 2.5 mg tablets, ie, 5 mg per week.
NDC 0005-4507-05—RHEUMATREX® Methotrexate Sodium Tablets Dose Pack—4 cards each containing three 2.5 mg tablets, ie, 7.5 mg per week.
NDC 0005-4507-07—RHEUMATREX® Methotrexate Sodium Tablets Dose Pack—4 cards each containing four 2.5 mg tablets, ie, 10 mg per week.
NDC 0005-4507-09—RHEUMATREX® Methotrexate Sodium Tablets Dose Pack—4 cards each containing five 2.5 mg tablets, ie, 12.5 mg per week.
NDC 0005-4507-91—RHEUMATREX® Methotrexate Sodium Tablets Dose Pack—4 cards each containing six 2.5 mg tablets, ie, 15 mg per week.

Store at 25°C (77°F); excursions permitted to 15°–30°C (59°–86°F) [see USP Controlled Room Temperature]. Protect from light.

LEDERLE PHARMACEUTICAL DIVISION
of American Cyanamid Company
Pearl River, NY 10965
CI 4814-3 Revised 11/97
REV 0163-04

REFERENCES

1. Roenigk HH, Auerbach R, Maibach HI, et al. Methotrexate in Psoriasis: Revised Guidelines. *J Am Acad Dermatol* 1988; 19:145–156.
2. Kremer JM, et al. Methotrexate for Rheumatoid Arthritis: Suggested Guidelines for Monitoring Liver Toxicity. *Arth Rheum* 1994; 37:316–328.
3. Recommendations for the Safe Handling of Parenteral Antineoplastic Drugs. NIH Publication No. 83-2621. For sale by the Superintendent of Documents, US Government Printing Office, Washington, DC 20402.
4. AMA Council Report. Guidelines for Handling Parenteral Antineoplastics. *JAMA*, March 15, 1985.
5. National Study Commission on Cytotoxic Exposure - Recommendations for Handling Cytotoxic Agents. Available from Louis P. Jeffrey, ScD, Chairman, National Study Commission on Cytotoxic Exposure, Massachusetts College of Pharmacy and Allied Health Sciences, 179 Longwood Avenue, Boston, Massachusetts 02115.
6. Clinical Oncological Society of Australia: Guidelines and Recommendations for Safe Handling of Antineoplastic Agents. *Med J Australia* 1983; 1:426–428.
7. Jones RB, et al. Safe Handling of Chemotherapeutic Agents: A Report from the Mount Sinai Medical Center. *Ca - A Cancer Journal for Clinicians* Sept/Oct 1983; 258-263.
8. American Society of Hospital Pharmacists Technical Assistance Bulletin on Handling Cytotoxic and Hazardous Drugs. *Am J Hosp Pharm* 1990; 47:1033–1049.

[See table above]

NOVANTRONE®

mitoxantrone for injection concentrate

WARNING

NOVANTRONE® (mitoxantrone for injection concentrate) should be administered under the supervision of a physician experienced in the use of cancer chemotherapeutic agents.

Except for the treatment of acute nonlymphocytic leukemia, NOVANTRONE® therapy generally should not be given to patients with baseline neutrophil counts of less than 1,500 cells/mm^3. In order to monitor the occurrence of bone marrow suppression, primarily neutropenia, which may be severe and result in infection, it is recommended that frequent peripheral blood cell counts be performed on all patients receiving NOVANTRONE®.

DESCRIPTION

NOVANTRONE® (mitoxantrone hydrochloride) is a synthetic antineoplastic anthracenedione for intravenous use. The molecular formula is $C_{22}H_{28}N_4O_6 \cdot 2HCl$ and the molecular weight is 517.41. It is supplied as a concentrate which MUST BE DILUTED PRIOR TO INJECTION. The concentrate is a sterile, nonpyrogenic, dark blue aqueous solution containing mitoxantrone hydrochloride equivalent to 2 mg/mL mitoxantrone free base, with sodium chloride (0.80% w/v), sodium acetate (0.005% w/v), and acetic acid (0.046% w/v) as inactive ingredients. The solution has a pH of 3.0 to 4.5 and contains 0.14 mEq of sodium per mL. The product does not contain preservatives. The chemical name is 1,4-

Continued on next page

Folic Acid Supplementation Prevents Deficient Blood Folate Levels and Hyperhomocysteinemia During Longterm, Low Dose Methotrexate Therapy for Rheumatoid Arthritis: Implications for Cardiovascular Disease Prevention

SARAH L. MORGAN, JOSEPH E. BAGGOTT, JEANNETTE Y. LEE, and GRACIELA S. ALARCÓN

ABSTRACT. Objective. To determine the effect of longterm methotrexate (MTX) therapy and folic acid supplementation on folate nutriture and homocysteine levels in patients with rheumatoid arthritis.

Methods. A double blind, placebo controlled trial lasting one year was conducted at one academic medical center. A total of 79 patients taking low dose MTX were followed up to one year. The patients were randomized to receive placebo or 5 or 27.5 mg folic acid supplementation per week.

Results. Plasma and erythrocyte folate levels and plasma homocysteine levels were determined. The folate nutriture of patients taking low dose MTX declined without folic acid supplementation. Plasma homocysteine levels increased significantly over a one year period in the placebo group. Low folate nutriture and hyperhomocysteinemia occurred with greater frequency in the placebo group than in the folic acid supplemented groups.

Conclusion. For longterm, low dose MTX therapy, there are now at least 3 reasons to consider supplementation with folic acid (a low cost prescription): (1) to prevent MTX toxicity, (2) to prevent or treat folate deficiency, and (3) to prevent hyperhomocysteinemia, considered by many investigators to be a risk factor for cardiovascular disease. (J Rheumatol 1998;25:441–6)

Key Indexing Terms:
HOMOCYSTEINE METHOTREXATE FOLIC ACID RHEUMATOID ARTHRITIS

Methotrexate (MTX) is an antifolate widely used in low doses in the therapy of autoimmune diseases, psoriasis, inflammatory bowel disease, and asthma[1–6]. It is now the leading disease modifying antirheumatic drug for the treatment of rheumatoid arthritis (RA), a disease that affects roughly 1% of the adult population around the world[7–1]. About 184,000 patients with RA were treated with MTX in 1995 (Wyeth-Ayerst, personal communication) in the United States alone. We have shown that the resulting interference with folate metabolism is correlated with toxicity and that folic acid (pteroylglutamic acid), in doses of 5–27.5 mg/week, lowers the toxicity of low dose MTX therapy for RA[7,8]. We also postulate that folate mediated processes are involved in mechanisms of efficacy and toxicity of MTX[9]. Homocysteine is a sulfur-containing amino acid that may be remethylated via a folate dependent reaction to form methionine. Folate nutriture is a major determinant of plasma homocysteine levels and blood folate levels are generally inversely correlated to homocysteine levels[10–16]. Plasma homocysteine levels > 15 µmol/l have been implicated as an independent risk factor for cardiovascular disease[17–27].

Because of these relationships, monitoring of blood folates (plasma and erythrocyte) and homocysteine levels during longterm MTX therapy may have important clinical implications. We report plasma and erythrocyte (red blood cells, RBC) folates and plasma homocysteine levels during a one year randomized, double blind, placebo controlled trial to study the effect of folic acid supplementation during low dose MTX therapy for RA[8]. We hypothesized that chronically low folate nutriture and chronic hyperhomocysteinemia would be more frequent in the non-folic acid supplemented group.

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MATERIALS AND METHODS

Participants and study design. The study was approved by the Institutional Review Board of the University of Alabama. As described[4], the participants were 79 patients aged 19–78 years of age who fulfilled the American College of Rheumatology revised criteria for RA[28]. The primary outcomes of that study were the effect of folic acid supplementation on measures of toxicity and efficacy[4]. Patients were enrolled in a one year, double blind, placebo controlled trial to evaluate the effects of 5 mg (low folic acid (FA) group) or 27.5 mg (high FA group) folic acid supplementation per week during low dose MTX therapy[4]. The patients took folic acid or identical placebo capsules on the 5 days of the week when MTX was not ingested. MTX was taken in a single dose on one day of the week. There were no significant differences between the 3 groups regarding mean age, sex, racial distribution, mean disease duration, previous use of folic acid containing vitamins, IgM rheumatoid factor positivity defined as > 30 IU/ml or > 1:160 titer, concurrent use of aspirin or nonsteroidal antiinflammatory drugs, mean prednisone dose, or cumulative MTX dose at the end of the trial. The mean (± standard deviation) age of the entire population was 53.2 (± 13.6) years. Seventy-six percent of the patients were women. Seventy-six percent of the patients were Caucasian. Sixty-two percent were concurrently taking prednisone and 80% were IgM rheumatoid factor positive. If patients had abnormal values on a vitamin panel min A, plasma and RBC folate, vitamin B_{12}, vitamin B_6, thiamine/riboflavin, and vitamin C) other than folates, the abnormality was treated with appropriate single vitamin supplementation. No other vitamin supplements were permitted except as noted.

Patients were examined immediately before MTX initiation (Visit 1) and after a mean of 13, 26, 39, and 53 weeks of therapy (Visits 2–5, respectively) for clinical evaluation and venipuncture. A one day dietary recall using the Minnesota Nutrition Data System software, Food Database version 6A, Nutrient Database version F21, was performed at each visit to assess nutrient intakes[29].

Vitamin and homocysteine assays. At Visit 1, blood was drawn for the assessment of vitamins B_6 and vitamin B_{12} nutriture[30,31]. Blood for plasma and RBC folate levels was drawn 5–7 days after MTX dosing in a tube containing EDTA and assayed at all visits using a MTX resistant *Lactobacillus casei* microbiological assay[32,33]. The blood was drawn 5–7 days after MTX dosing. Criteria for adequacy of folate status were based on the categories of folate adequacy established by Selhub and Rosenberg[34]. Serum folate levels were considered to be low when values were < 6.7 nmol/l and RBC folate levels were considered low when values were < 315 nmol/l[34]. Homocysteine levels were assayed using high performance liquid chromatography[35]. Values > 15 µmol/l were considered to be elevated[34]. Blood was generally processed within 30 min of phlebotomy and plasma frozen at -70°C until time of analysis.

Statistical analysis. Two way repeated measures analyses of variance were used to evaluate the effects of treatment group, time, and its interaction on plasma and RBC folate levels, and on plasma homocysteine levels after baseline. If a significant treatment effect was detected, Tukey's studentized range test was used to evaluate pairwise comparisons between treatment groups[37].

Fisher's exact test was used to compare the 3 treatment groups with respect to the proportions of patients who had 0, 1, or more than 1 occurrence of deficient plasma or RBC folate levels or elevated plasma homocysteine levels (see Tables 1 and 2).

Linear regression analyses were performed to evaluate the change in homocysteine levels over time after baseline in each treatment group.

Pearson correlation analyses were done to evaluate the baseline relationship of RBC and plasma folate levels with plasma homocysteine levels and the correlation of dietary folate, vitamin B_{12}, and vitamin B_6 intakes with plasma homocysteine levels.

RESULTS

Plasma and RBC folate levels during the trial. Figures 1 and 2 show plasma and RBC folate levels at each visit. There were no significant differences between plasma and RBC folate levels at baseline ($p > 0.05$). There were striking differences between treatment groups with respect to plasma and RBC folate levels across followup visits. For plasma and RBC folate levels, significant differences ($p < 0.001$) were found for all 3 pairwise comparisons (low FA vs high FA, high FA vs placebo, and low FA vs placebo) at followup visits. Significant treatment–visit interactions were observed for plasma folate ($p < 0.001$) and RBC folate ($p = 0.003$).

Multiple instances of a low blood folate level are likely to be more clinically significant than an isolated low blood folate level. The number of patients with 0, 1, or more than

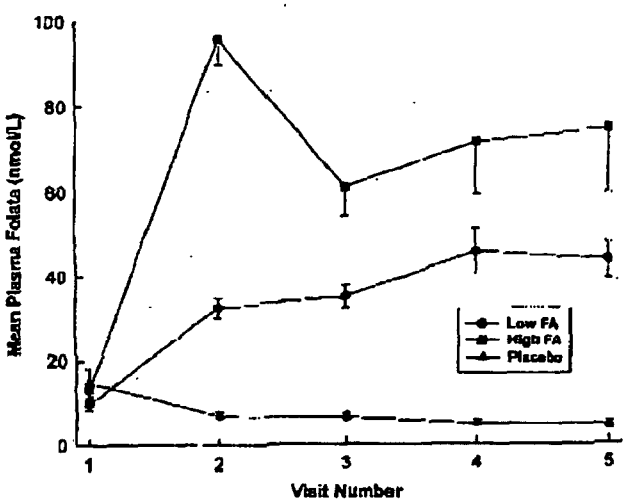

Figure 1. Plasma folate levels (nmol/l) in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

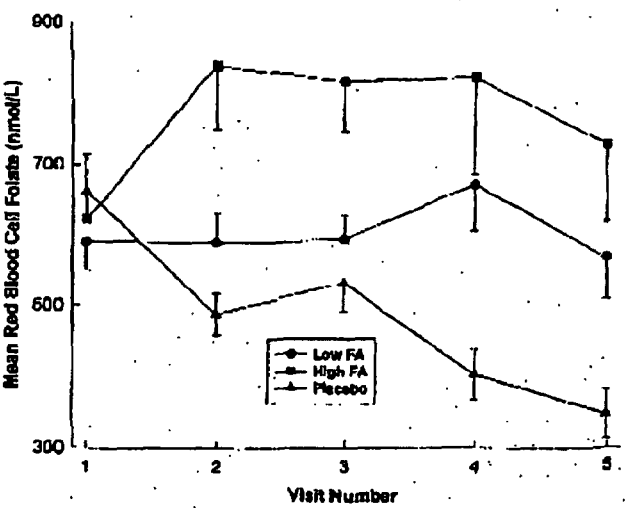

Figure 2. RBC folate levels (nmol/l) in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

l low blood folate level during Visits 2–5 is shown in Table 1. A clear majority of the patients in the placebo group had 2 or more deficient levels, while the vast majority in the folic acid supplemented groups maintained normal blood folate levels throughout the study ($p < 0.001$).

Plasma homocysteine levels during the trial. There were no significant differences in baseline plasma homocysteine levels between groups at Visit 1 ($p > 0.05$). Normal mean homocysteine levels in 40–70-year-old women and men range from 8.0 to 10.3 µmol/l[20,38]. The mean values in the placebo group were in the range of 13.6–21.7 µmol/l at Visits 2–5 (Figure 3), substantially above the range of normal means. After baseline, for Visits 2–5, the mean overall plasma homocysteine level was 17.4 µmol/l in the placebo group. The means in the folic acid supplemented groups at almost all visits were comparable with the above population norms[20,38].

Figure 3 shows plasma homocysteine levels over time. At Visit 1, significant treatment differences were observed with respect to homocysteine levels. The low FA and high FA groups differed significantly from the placebo groups across Visits 2–5 ($p < 0.001$).

Multiple instances of hyperhomocysteinemia are more likely to be significant than an isolated elevated homocysteine level. The percentage of patients with more than one elevated plasma homocysteine level, assayed every 3 months, is shown in Table 2. About 40% of the placebo group had 2 or more elevated plasma homocysteine levels during the course of the trial. In contrast, chronic hyperho-

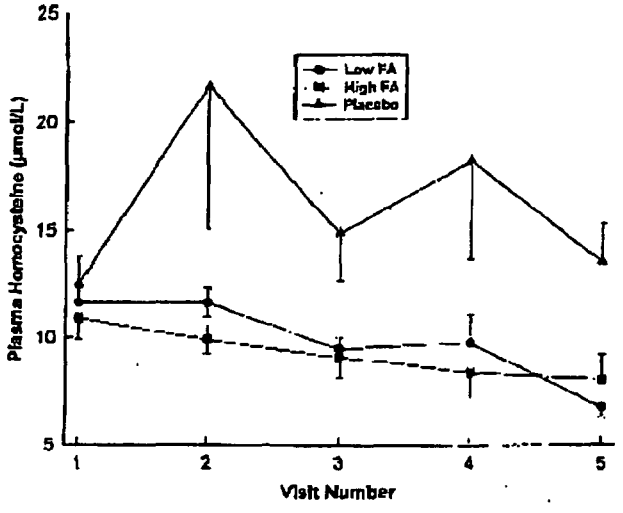

Figure 3. Plasma homocysteine (µmol/l) levels in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

Table 1. Chronic deficient folate nutriture in MTX treated patients with RA receiving high and low dose folic acid supplements and placebo.

	Number (%) of Patients with 0, 1, 2, 3, or 4 Deficient Plasma or RBC Folate Assays During Followup Visits				
	0	1	2	3	4
Group					
Low FA	19 (76)	5 (20)	1 (4)	0 (0)	0 (0)
High FA	20 (77)	6 (23)	0 (0)	0 (0)	0 (0)
Placebo	7 (25)	2 (7)	5 (18)	7 (25)	7 (25)

The larger number of deficient plasma and/or deficient RBC folate assays was used; plasma folate < 6.7 nmol/l and RBC folate < 315 nmol/l were considered deficient. Fisher's exact test, $p < 0.001$, was used to compare the 3 treatment groups with respect to the proportions of 0, 1, or more than 1 occurrence of deficient folate levels.

Table 2. Chronic hyperhomocysteinemia in MTX treated patients with RA receiving low and high dose folic acid supplements and placebo.

	Number (%) of Patients with 0, 1, 2, 3, or 4 Elevated Homocysteine Assays During Followup Visit				
	0	1	2	3	4
Group					
Low FA	20 (80)	4 (16)	1 (4)	0	0
High FA	25 (96)	0	0	0	1 (4)
Placebo	15 (53)	2 (7)	5 (18)	3 (11)	3 (11)

Homocysteine levels > 15 µmol/l are considered elevated. Fisher's exact test, $p < 0.001$, was used to compare the 3 treatment groups with respect to the proportions of patients who had 0, 1, or more than 1 occurrence of elevated plasma homocysteine levels.

mocysteinemia was found in less than 5% of the subjects in the folic acid supplemented groups ($p < 0.001$).

Figure 3 shows that homocysteine levels decreased with time for the folic acid supplemented groups ($p < 0.001$ low FA; $p = 0.023$ high FA), but not in the placebo group. The rate of decline was -4.64 µmol/l/year for the low FA, -2.88 µmol/l/year for the high FA group, and 0.20 µmol/l/year for the placebo group.

Dietary intake and correlations with biochemical indices. Mean dietary folate, vitamin B_{12}, and vitamin B_6 intakes were not significantly different within groups at any visit or between groups. Dietary folate, vitamin B_{12}, and vitamin B_6 intakes were not significantly correlated with homocysteine levels across groups or for any individual treatment group.

Correlations of vitamin levels with homocysteine. At Visit 1 (baseline), plasma folate ($N = 79$; $r = -0.334$, $p = 0.002$), RBC folate ($N = 79$; $r = -0.344$, $p = 0.003$) were significantly correlated with plasma homocysteine levels. At Visit 1 vitamin B_6 and vitamin B_{12} status was not correlated with baseline plasma homocysteine levels. In the placebo group, there was a relatively weak negative correlation between plasma folate and plasma homocysteine levels at Visits 2–5 ($r = -0.23$, $p = 0.025$).

DISCUSSION

MTX is increasingly being used for treatment of different chronic disorders, including RA; therefore, the metabolic consequences of chronic administration are important[1–6]. Low dose MTX therapy, given over a one year period, adversely affects both plasma and RBC folate levels. This effect was most pronounced in the placebo treated group and produced chronic deficient blood folate levels. We have shown that the C_1 index, a direct measure of the folate dependent formation of serine from formate and glycine in leukocytes, is lower in patients with RA treated with MTX compared to patients with RA not receiving MTX[39]. Folic acid supplementation (both 5 and 27.5 mg per week) prevents the decrement in folate status[8].

Our data indicate that both plasma and RBC folate are primary determinants of homocysteine levels in patients with RA taking MTX; this agrees with the observations in populations not treated with antifolates[11–16]. There were no significant relationships between vitamin B_6 and vitamin B_{12} levels and homocysteine levels at Visit 1. This indicates that folate was the predominant vitamin factor regulating the plasma homocysteine levels in our group of patients with RA, before the initiation of MTX therapy. The finding of no association between vitamin B_6 and homocysteine levels differs from Roubenoff, *et al*, who found low pyridoxal phosphate levels in patients with RA[40]. We did not have a control group for comparison in this trial and methionine loading tests were not performed during this protocol, which may explain differences. In addition, there were no significant relationships between dietary folate intakes and plasma homocysteine levels in any of the groups at Visits 2–5. This may reflect that naturally occurring food folate has been shown to be relatively ineffective at increasing folate status and perhaps altering homocysteine levels[41]. On the other hand, our previous findings suggest that food folate intakes of > 400 µg/day are effective in lowering the probability of MTX toxicity[8].

A substantial number of patients in the placebo group developed MTX induced chronic hyperhomocysteinemia, a condition largely prevented by folic acid supplementation in the other treatment arms. There is evidence suggesting that hyperhomocysteinemia is directly involved in the etiology of vascular atherosclerotic disease[17–20], early onset venous and arterial occlusion[21–23], coronary artery disease[24–26], and carotid artery stenosis[27]. It follows that MTX treated patients with RA should also have an increased risk for coronary artery disease, peripheral vascular disease, and cerebrovascular disease. There are data supporting the relationship between hyperhomocysteinemia and thrombosis in patients with systemic lupus erythematosus[42]. Hyperhomocysteinemia has also been observed in a small group of patients with RA in Sweden[43]. Specific epidemiological data for supporting the above assertion are, with one exception[44], lacking in MTX treated patients. In the one albeit small study specifically addressing MTX as a risk factor for cardiovascular disease, MTX treated patients with RA were compared to the healthy population[44]. Standardized mortality rates were 2.9 and 1.4, respectively, but the confidence intervals encompassed the unity (0.6–8.6 and 0.6–2.6, respectively) and by definition cannot be regarded as statistically significant, which likely relates to the relatively small size of the cohort studied.

Hyperhomocysteinemia in the low dose MTX treated population may be due to the interference in folate metabolism by the drug itself or to drug induced folate deficiency, or a combination of both[45]. It is intriguing that low serum folate levels, per se, were found to be associated with increased risk of fatal heart disease in the Nutrition Canada survey[26].

It is known that folic acid supplementation lowers homocysteine levels in an "at risk" population, such as in patients during the post-myocardial infarction period[46]. Based on our findings, low dose MTX treated patients should be added to the "at risk" population for increased cardiovascular risk due to hyperhomocysteinemia. The cereal grain fortification mandated by the Food and Drug Administration, in large part to prevent neural tube defects, also has the possibility of benefiting the population taking antifolate drugs by preventing toxicity, increasing folate levels, and decreasing homocysteine levels[7,8,47,48]. Folic acid supplements rather than food folates may have a more predictable effect in increasing blood folate levels[41]. It would still seem prudent to evaluate vitamin B_{12} status before prescribing longterm folic acid supplementation because of a high prevalence of

vitamin B_{12} deficiency in the RA population[49] and concerns of masking the nutritional anemia of B_{12} deficiency[47,50]. The prevalence of the thermolabile mutation in methylenetetrahydrofolate reductase or heterozygosity for cystathionine-β synthase deficiency in the population taking low dose MTX may merit further investigation, since these conditions produce hyperhomocysteinemia and may have an effect on recommended folic acid supplement doses[51–54].

We acknowledge that problems in sample handling can produce spurious increases in the homocysteine levels[55–57]. It has been shown that samples for homocysteine levels should be placed immediately on ice to prevent homocysteine from leaching from RBC into plasma and falsely elevating plasma homocysteine levels. In our study, samples were generally centrifuged and frozen at −70°C within 30 min so that artefactual hyperhomocysteinemia should be equal in all groups. All patient groups were enrolled simultaneously; therefore the blood was stored about the same length of time in all groups before analysis. It is also very unlikely that sample handling could account for the observation of chronic hyperhomocysteinemia found only in the placebo group.

These observations regarding homocysteine levels during low dose MTX therapy do not agree with our previous observations that homocysteine levels did not become elevated after 6 months of low dose MTX therapy[58]. The timing of blood sampling may have been a factor in the previous study; most samples were drawn 5–7 days after MTX administration, when homocysteine levels have been shown to return to normal after a low dose of MTX[45]. The failure to detect MTX induced hyperhomocysteinemia in our previous study may also be due to a shorter trial of MTX in the previous study (6 months vs one year) and a smaller number of patients in that study.

In summary, there are now 3 reasons to recommend low cost folic acid supplements in patients receiving longterm, low dose MTX therapy: (1) folic acid supplementation lessens MTX toxicity[7,8]; (2) folic acid supplementation should prevent chronic blood folate deficiency during therapy; and (3) folic acid supplementation lowers the prevalence of chronic hyperhomocysteinemia, which has been linked to cardiovascular disease risk in the general population.

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REFERENCES

1. Alarcón GS. Methotrexate: Its use for the treatment of rheumatoid arthritis and other rheumatic disorders. In: Koopman WJ, editor. Arthritis and allied conditions textbook of rheumatology, 13th ed. Baltimore: Williams and Wilkins; 1997:679–707.
2. Tugwell P, Bennett K, Gent M. Methotrexate in rheumatoid arthritis. Indications, contraindications, efficacy, and safety. Ann Intern Med 1987;107:358–66.
3. Weinblatt ME, Weissman BN, Holdsworth DE, et al. Long-term prospective study of methotrexate in the treatment of rheumatoid arthritis. 84-month update. Arthritis Rheum 1992;35:129–37.
4. Kremer J, Phelps CT. Long-term prospective study of the use of methotrexate in the treatment of rheumatoid arthritis. Update after a mean of 90 months. Arthritis Rheum 1992;35:138–45.
5. Mullarkey MF, Blumenstein BA, Andrade WT, Bailey OA, Olason I, Wetzel CE. Methotrexate in the treatment of corticosteroid-dependent asthma. A double-blind crossover study. N Engl J Med 1988;318:603–7.
6. Kozarek RA, Patterson DJ, Gelfand MD, Botoman VA, Ball TJ, Wilske KR. Methotrexate induces clinical and histological remission in patients with refractory inflammatory bowel disease. Ann Intern Med 1989;110:353–56.
7. Morgan SL, Baggott JE, Vaughn WH, et al. The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 1990;33:9–18.
8. Morgan SL, Baggott JE, Vaughn WH, et al. Supplementation with folic acid during methotrexate therapy for rheumatoid arthritis. A double-blind, placebo-controlled trial. Ann Intern Med 1994;121:833–41.
9. Baggott JE, Morgan SL, Ha T-S, Alarcón GS, Koopman WJ, Krumdieck CL. Antifolates in rheumatoid arthritis: a hypothetical mechanism of action. Clin Exp Rheumatol 1993;11:S101–5.
10. Kang S-S, Wong PWK, Norusis M. Homocysteinemia due to folate deficiency. Metabolism 1987;36:458–62.
11. Chu RC, Hall CA. The total serum homocysteine as an indicator of vitamin B_{12} and folate status. Am J Clin Pathol 1988;90:446–9.
12. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. Am J Clin Nutr 1993;57:47–53.
13. Lewis CA, Pancharuniti N, Sauberlich HE. Plasma folate adequacy as determined by homocysteine level. Ann NY Acad Sci 1992;669:360–2.
14. Pancharuniti N, Lewis CA, Sauberlich HE, et al. Plasma homocyst(e)ine, folate and vitamin B-12 concentrations and risk for early-onset coronary artery disease. Am J Clin Nutr 1994;54:940–8.
15. Mason JB, Miller JW. The effects of vitamins B_{12}, B_6, and folate on blood homocysteine levels. Ann NY Acad Sci 1992;669:197–204.
16. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693–8.
17. Clarke R, Daly L, Robinson K, et al. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149–55.
18. Graham IM, Daly LE, Refsum HM, et al. Plasma homocysteine as a risk factor for vascular diseases. The European concerted action project. J Am Med Assoc 1997;277:1775–81.
19. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;274:1049–57.
20. Nygård O, Vollset SE, Refsum H, et al. Total plasma homocysteine and cardiovascular risk profile. JAMA 1995;274:1526–33.
21. Aronson DC, Oukenhorst W, Ruben AMTJ, Oudenhoven LFD, Brommer EJP, van Bockel JH. Impaired homocysteine metabolism: a risk factor in young adults with atherosclerotic arterial occlusive disease of the leg. Br J Surg 1994;81:1114–8.
22. Fermo IL, Vigano D'Angelo S, Paroni R, Mazzola G, Caluri G, D'Angelo A. Prevalence of moderate hyperhomocysteinemia in patients with early-onset venous and arterial occlusive disease. Ann Intern Med 1995;123:747–53.
23. Mayer EL, Jacobsen DW, Robinson K. Homocysteine and coronary

atherosclerosis. J Am Coll Cardiol 1996;27:517–27.
24. Stampfer MJ, Malinow R, Willett WC, et al. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877–81.
25. Verhoef P, Stampfer MJ, Buring JE, et al. Homocysteine metabolism and risk of myocardial infarction: Relation with vitamins B_6, B_{12}, and folate. Am J Epidemiol 1996;143:845–59.
26. Morrison HI, Schaubel D, Desmeules M, Wigle DT. Serum folate and risk of fatal coronary heart disease. JAMA 1996;275:1893–6.
27. Selhub J, Jacques PF, Bostom AG, et al. Association between plasma homocysteine and extracranial carotid artery stenosis. N Engl J Med 1995;332:286–91.
28. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
29. Schakel SF, Sievert YA, Buzzard IM. Sources of data for developing and maintaining a nutrient database. J Am Diet Assoc 1988;88:1268–71.
30. Lau KS, Gottlieb C, Wasserman LR, Herbert V. Measurement of serum vitamin B_{12} level using radioisotope dilution and coated charcoal. Blood 1965;26:202–14.
31. Dayoumi RA, Rosalki SB. Evaluation of methods of coenzyme activation of erythrocyte enzymes for detection of deficiency of vitamins B_1, B_2, and B_6. Clin Chem 1976;22:327–35.
32. Tamura T. Microbiological assay of folates. In: Picciano MF, Stokstad EL, Gregory JF, editors. Folic acid metabolism in health and disease. New York, NY: Wiley-Liss, 1990:121–37.
33. Tamura T, Freeberg LE, Cornwell PE. Inhibition by EDTA of growth of *Lactobacillus casei* in the folate microbiological assay and its reversal by added manganese or iron. Clin Chem 1990;36:1993.
34. Selhub J, Rosenberg IH. Folic acid. In: Ziegler EE, Filer LJ, editors. Present knowledge in nutrition, 7th ed. Washington, DC: ILSI Press, 1996:206–19.
35. Cornwell PE, Morgan SL, Vaughn WH. Modification of a high-performance liquid chromatographic method for assay of homocysteine in human plasma. J Chromatography 1993;617:136–9.
36. Guttormsen AB, Ueland PM, Nesthus I, et al. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (> 40 μmol/liter). J Clin Invest 1996;98:2174–83.
37. Sokal RR, Rohlf FG. Biometry. San Francisco: WH Freeman; 1981.
38. Rasmussen K, Møller J, Lyngbak M, Holm Pedersen A-M, Dybkjær L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. Clin Chem 1996;42:630–6.
39. Morgan SL, Baggott JE, Alfz-Smith M. Folate status of rheumatoid arthritis patients receiving long-term, low-dose methotrexate therapy. Arthritis Rheum 1987;30:1348–56.
40. Roubenoff R, Dellaripa P, Nadeau M, et al. Abnormal homocysteine metabolism in rheumatoid arthritis. Arthritis Rheum 1997;40:718–22.
41. Cuskelly GJ, McNulty H, Scott JM. Effect of increasing dietary folate on red cell folate: implications for prevention of neural tube defects. Lancet 1996;347:657–9.
42. Petri M, Roubenoff R, Dallal GE, Nadeau MR, Selhub J, Rosenberg IH. Plasma homocysteine as risk factor for atherothrombotic events in systemic lupus erythematosus. Lancet 1996;348:1120–4.
43. Pettersson T, Friman C, Nilsson B, Norberg B. Hyperhomocysteinemia in rheumatoid arthritis [abstract]. Arthritis Rheum 1995;38 Suppl: S366.
44. Alarcón GS, Tracy IC, Strand GM, Singh K, Macaluso M. Survival and drug discontinuation analyses in a large cohort of methotrexate treated rheumatoid arthritis patients. Ann Rheum Dis 1995;54:708–12.
45. Refsum H, Helland S, Ueland PM. Fasting plasma homocysteine as a sensitive parameter of antifolate effect: a study of psoriasis patients receiving low-dose methotrexate. Clin Pharm Ther 1989;46:510–20.
46. Landgren F, Israelsson B, Lindgren A, Hultberg B, Andersson A, Brattström L. Plasma homocysteine in acute myocardial infarction: homocysteine-lowering effect of folic acid. J Int Med 1995;237:381–8.
47. Tucker KL, Mahnken B, Wilson PWF, Jacques P, Selhub J. Folic acid fortification of the food supply. Potential benefits and risks for the elderly population. JAMA 1996;276:1879–85.
48. Yetley EA, Rader JI. Folate fortification of cereal-grain products: FDA policies and actions. Cereal Foods World 1995;40:67–72.
49. Alfz-Smith M, Miller RK, Cornwell PE, Butterworth CE Jr, Herbert V. Low serum vitamin B_{12} levels in rheumatoid arthritis [abstract]. Arthritis Rheum 1982;25 Suppl:S116.
50. Scott JM, Weir DG. The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anemia. Lancet 1981;2:337–40.
51. Jacques PF, Bostom AG, Williams RR, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase and plasma homocysteine concentrations. Circulation 1996;93:7–9.
52. Kang S-S, Zhou J, Wong PWK, Kuwajisyn J, Strokosch G. Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 1988;43:414–21.
53. Kang S-S, Wong PWK, Zhou J, et al. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. Metabolism 1988;37:611–3.
54. Rozen R. Molecular genetic aspects of hyperhomocysteinemia and its relation to folic acid. Clin Invest Med 1996;19:171–8.
55. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. The effect of blood sample aging and food consumption on plasma total homocysteine levels. Clinica Chimica Acta 1992;207:119–28.
56. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 1993;39:1764–79.
57. Andersson A, Isaksson A, Hultberg B. Homocysteine export from erythrocytes and its implication for plasma sampling. Clin Chem 1992;38:1311–5.
58. Morgan SL, Baggott JE, Refsum H, Ueland PM. Homocysteine levels in patients with rheumatoid arthritis treated with low-dose methotrexate. Clin Pharmacol Ther 1991;50:547–56.

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PROPHYLACTIC MASTECTOMY (PM) AND OOPHORECTOMY (PO) IN WOMEN UNDERGOING BRCA1/2 TESTING. *D. Schrag, K.J. Kalkbrenner, T.L. Light, K.A. Schneider, J.E. Garber. Dana Farber Cancer Institute, Boston, MA.*

Women tested for BRCA1/2 mutations may consider PM and/or PO based on the results of genetic testing for predisposing mutations. A cohort of 88 women with at least 10% risk of inherited breast/ovarian cancer provided information about attitudes towards PM and PO before testing and again at mean 5.5 months following results disclosure. 46 women had prior breast/ovarian cancer (CA); 42 women had not had cancer (NC). Before genetic testing, 8 women had had PM, 12 PO and 5 therapeutic oophorectomy. At baseline, 37/80 had discussed PM with a physician and 33/71 had discussed PO 8/80 were considering PM and 24/71 PO. Following BRCA disclosure, 6 women underwent PM (3 CA, 3 NC) and 5 had PO (2 CA, 3 NC); one woman (NC) had both procedures. Mutations were identified in all women having prophylactic surgery following results disclosure except for 2 who had PM with indeterminate results but abnormal breast biopsies. In addition, 13 were still considering PM (8+, 4?) and 19 were considering PO (12+, 6?, 1−) For the entire cohort, no cancers have been detected at PM; one borderline ovarian cancer was found at PO. PM and PO are often considered by women who have BRCA1/2 mutation testing even with indeterminate test results.

*2139

LY231514 (MTA): RELATIONSHIP OF VITAMIN METABOLITE PROFILE TO TOXICITY. *C. Niyikiza, J. Walling, D. Thornton, D. Seitz, and R. Allen. Eli Lilly and Company, Indianapolis, IN, and Univ of Colorado Health Science Center, Denver, CO.*

LY231514 (MTA) is a new generation multitargeted antifolate antimetabolite with inhibitory activity against thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyl transferase. Of a total of 246 patients (pts) in phase II trials treated with MTA (600 mg/m² IV over 10 minutes once every 21 days) 118 pts also had vitamin metabolites measured. Because earlier studies with other antifolates had suggested that nutritional status may play a role in the likelihood that a patient will experience severe toxicity, levels of the vitamin metabolites homocysteine, cystathionine and methylmalonic acid were measured at baseline and once each cycle thereafter. A multivariate statistical analysis of the data was conducted in order to determine which among a set of pre-specified predictors (creatinine clearance, albumin levels, liver enzyme levels, and vitamin metabolites) might correlate with toxicity. There was a strong correlation between baseline homocysteine levels and the development of the following toxicities at any time during the study: CTC Grade 4 neutropenia (57 pts, $p < 0.0001$), Grade 4 thrombocytopenia (13 pts, $p < 0.0001$), Grade 3 or 4 mucositis (8 pts, $p < 0.0003$), and Grade 3 or 4 diarrhea (8 pts, $p < 0.004$). Cystathionine levels did not correlate with hematologic toxicity or mucositis but were moderately correlated with fatigue ($p < 0.04$). Maximum cystathionine levels doubled from baseline during treatment with MTA. No correlation between toxicity (CTC Grades as defined above) and the remaining pre-specified predictors was seen. Toxicity was seen in all patients with homocysteine levels above a threshold concentration of 10 μM. A correlation over time between homocysteine levels and CTC Grade 4 neutropenia and thrombocytopenia and CTC Grade 3 or 4 mucositis was also observed, but only in the first two cycles of treatment. Maximum homocysteine levels did not appear to change from baseline during treatment with MTA.

*2140

PHASE I CHEMOPREVENTION CLINICAL TRIAL OF CURCUMIN *A.L. Cheng, J.K. Lin, M.M. Hsu, T.S. Shen, J.Y. Ko, J.T. Lin, B.J. Lin, M.S. Wu, H.S. Yu, S.H. Jee, G.S. Chen, T.M. Chen, C.A. Chen, M.K. Lai, Y.S. Pu, M.H. Pan, Y.J. Wang, C.C. Tsai, C.Y. Hsieh. National Taiwan University College of Medicine, Taipei, Taiwan; and Kaohsiung Medical College, Kaohsiung, Taiwan.*

Curcumin (diferuloylmethane), a yellow substance from the root of the plant Curcuma longa Linn., has been demonstrated to inhibit murine carcinogenesis of skin, stomach, intestine and oral cavity. A phase-I clinical trial was conducted to examine the toxicology, the pharmacokinetics and the biologically effective dose of curcumin in humans. Five types of high-risk individual were eligible: 1. recently-resected urinary bladder cancer (BC), 2. arsenic Bowen's disease (BD), 3. uterine cervical intraepithelial neoplasia (CIN), 4. oral leukoplakia (OL), and 5. intestinal metaplasia of gastric mucosa (IM). The starting dose was 500 mg/day, taken orally for 3 months. If no any ≥ Grade II toxicity was noted in at least 3 p'ts, the dose was escalated successively to 1000 (level II), 2000 (level III), 4000 (level IV), and 8000 mg/day (level V). Lesion sites were biopsied before and 3 months after taking curcumin. Serum curcumin was quantitated by HPLC method. In a total of 25 p'ts enrolled, no treatment-related toxicity was noted up to 8000 mg/day (level V). Serum concentration usually peaked at 1 to 2 hours after oral intake, and gradually declined within 12 hours. The average peak serum concentrations after taking 4000 mg, 6000 mg and 8000 mg of curcumin were 0.41 ± 0.07 μM, 0.57 ± 0.05 μM, and 1.75 ± 0.80 μM, respectively. Although 3 of 25 p'ts proceeded to develop frank malignancies, histological improvement of the precancerous lesions was seen in 1 (level III) of the 2 p'ts with BC, 2 (both level IV) of 7 p'ts with OL, 1 (level II) of 6 p'ts with IM, 1 (level I) of 4 p'ts with CIN, and 2 (level I and III) of 6 p'ts with BD. Although curcumin is probably non-toxic even up to more than 8000 mg/day, the bulky volume of drug tablets became a limiting factor itself. Therefore, for future phase II studies, doses close to 8000 mg/day may be recommended.

*2141

FACTORS INFLUENCING THE DECISION TO UNDERGO BRCA1/2 GENE TESTING: A STUDY OF ASHKENAZI JEWISH WOMEN WITH A PERSONAL HISTORY OF BREAST CANCER (BC), ENROLLED IN AN ONTARIO CANCER GENETICS NETWORK PROTOCOL. *K.A. Phillips, J. Hunter, E. Warner, W. Meschino, G. Glendon, I.L. Andrulis and P.J. Goodwin. Mt Sinai Hospital, Princess Margaret Hospital, Toronto-Sunnybrook Regional Cancer Center, North York General Hospital, Toronto, Ontario, Canada.*

The purpose of this study was to examine the contribution of demographic, medical, psychosocial, and cultural/religious factors in decision making regarding testing for BRCA1 and BRCA2 mutations, in Canadian Jewish women with BC, unselected for family history. A self-administered questionnaire was developed and distributed, (after genetic counseling), to 134 individuals enrolled in a research-based testing program for Ashkenazi women. Data for the first 52 participants are presented. The response rate was 40 (77%). Respondents had the following demographic features: age 40–75 years (median = 59), married 83%, had children 92%, post-secondary education 55%, practicing Jew 88%, extra health insurance 77%, median age of BC diagnosis = 50. No patient had ovarian cancer (OC). 45% had at least one 1st degree relative with BC or OC (median perceived risk for being a gene carrier 50%). 35% had no affected relatives (median perceived risk for being a carrier = 15%). The 5 factors most frequently identified as "definitely an important factor in my decision making" were, desire to contribute to research (90%), curiosity (77%), potential benefit to other family members (64%), potential for personal cancer prevention (59%), and impact on ovarian cancer screening practice (41%). 53% and 38% of women respectively, identified a potential change in their perspective on prophylactic oophorectomy and mastectomy as at least "somewhat important." Main concerns related to insurance discrimination (35%); confidentiality (30%), accuracy and interpretability of results (33%), potential impact on marriage prospects for family members (20%), and focus on the Jewish community (15%). Potential employer discrimination and impact on life planning were "not a factor" for most (90%,82%). The focus on factors unrelated to personal physical health is notable. The generalisability of these results to women not affected by BC requires further study. Final results for the 134 patients will be presented.

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introduction to this chapter as indicated by the cross-references given below.

Doses of procarbazine hydrochloride are calculated in terms of procarbazine. In many of the combination regimens it has been given to adults and children in doses of the equivalent of procarbazine 100 mg per m^2 body-surface on days 1 to 14 of each 4- or 6-week cycle. If used as a single agent a dose equivalent to 50 mg of procarbazine by mouth daily, increased by 50 mg daily to 250 to 300 mg daily in divided doses has been suggested in the UK, while in the USA the recommended regimen is 2 to 4 mg per kg body-weight daily for the first week, subsequently increased to 4 to 6 mg per kg, doses being given to the nearest 50 mg. These doses are continued until maximum response is achieved or leucopenia, thrombocytopenia, or other signs of toxicity ensue. Maintenance doses are usually 50 to 150 mg, or 1 to 2 mg per kg, daily, until a cumulative dose of at least 6 g has been given. In children, initial daily doses of the equivalent of 50 mg per m^2 have been suggested (some sources simply suggest a dose of 50 mg), increased to 100 mg per m^2 and then adjusted according to response.

The haematological status of the patient should be determined at least every 3 or 4 days and hepatic and renal function determined weekly.

Blood disorders, non-malignant. Combination chemotherapy with regimens including procarbazine has been employed in a few patients with refractory idiopathic thrombocytopenic purpura (p.1023), and has produced prolonged remission although in most cases of the disease such aggressive therapy is difficult to justify.

Malignant neoplasms. Procarbazine is a component of the MOPP regimen which was the first combination regimen to be widely and successfully employed to treat Hodgkin's disease (see p.481). Procarbazine has also been used in the treatment of mycosis fungoides, other non-Hodgkin's lymphomas, and tumours of the brain, as discussed under Choice of Antineoplastic, p.482, p 482, and p.485.

Preparations

USP 23: Procarbazine Hydrochloride Capsules.

Proprietary Preparations (details are given in Part 3)
Aust.: Natulan; *Austral.:* Natulan; *Belg.:* Natulan; *Canad.:* Natulan; *Fr.:* Natulan; *Ger.:* Natulan; *Ital.:* Natulan; *Neth.:* Natulan; *Norw.:* Natulan; *S.Afr.:* Natulan, *Spain:* Natulan; *Swed.:* Natulanar†; *Switz.:* Natulan; *UK:* Natulan; *USA:* Matulane.

Raltitrexed (11782-z)

Raltitrexed *(BAN, USAN, rINN)*

ICI-D1694; ZD-1694 *N*-{5-[3,4-Dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl(methyl)amino]-2-thenoyl}-L-glutamic acid.

$C_{21}H_{22}N_4O_6S$ = 458.5.
CAS — 112887-68-0.

Adverse Effects, Treatment, and Precautions

Raltitrexed produces bone marrow depression, usually mild to moderate, with leucopenia, anaemia, and, less frequently, thrombocytopenia. The nadir of the white cell count usually occurs 7 to 14 days after a dose, with recovery by the third week. Gastro-intestinal toxicity is also common, with nausea and vomiting, diarrhoea, and anorexia: mucositis may occur. Reversible increases in liver enzyme values have occurred. Other adverse effects include weakness and malaise, fever, pain, headache, skin rashes, arthralgia, muscle cramps, weight loss, peripheral oedema, alopecia, and conjunctivitis. The use of folinic acid 25 mg per m^2 every 6 hours intravenously has been suggested in patients who develop very severe toxicity.

Raltitrexed should be given with care to patients with impaired hepatic function and should be avoided if impairment is severe. It should also be avoided in severe renal impairment and be given in reduced doses in moderate impairment of renal function. Care is also advisable in debilitated or elderly patients.

Raltitrexed is teratogenic: pregnancy should be avoided if either partner is receiving the drug. It may impair male fertility.

Interactions

Raltitrexed should not be given with folic or folinic acid which may impair its cytotoxic action. (For the deliberate use of folinic acid to counteract the effects of raltitrexed in patients with severe toxicity see above.)

Pharmacokinetics

Following intravenous administration raltitrexed exhibits triphasic pharmacokinetics, with an initial rapid decline from peak plasma concentrations followed by a slow terminal elimination phase. Raltitrexed is actively transported into cells and metabolised to active polyglutamate forms. The remainder of a dose is excreted unchanged, about 50% of a dose appearing in the urine, and about 15% in the faeces. The terminal elimination half-life is stated to be about 8 days. Clearance is markedly reduced in renal impairment.

Uses and Administration

Raltitrexed is a folate analogue that is a potent and specific inhibitor of the enzyme thymidylate synthase, which is involved in the synthesis of DNA. It is used in the treatment of advanced colorectal cancer and has also been tried in breast cancer.

The recommended initial dose is 3 mg of raltitrexed per m^2 body-surface, given by intravenous infusion over 15 minutes. Subsequent doses, which should be reduced by up to 50% depending on the severity of initial toxicity, may be given at intervals of 3 weeks provided toxicity has resolved. Doses should be reduced by 50% and repeated at 4-week intervals in patients with creatinine clearance less than 65 mL per minute; the drug should not be given if creatinine clearance is below 25 mL per minute.

A full blood count should be performed before each dose and treatment withheld if the white cell count is below 4000 per mm^3 or the platelet count less than 100 000 per mm^3. Hepatic and renal function should also be tested.

References.[1-6] Although clearly active[1-3] against colorectal cancer (p.487) raltitrexed does not seem to provide much improvement in survival-time for patients with metastatic disease.[4,5] However, it may well improve quality of life for these patients, since it is associated with less gastro-intestinal and haematological toxicity than some conventional fluorouracil regimens,[2] and is more convenient to administer.[4] Raltitrexed is also active against some other malignancies, notably of the breast[3] (p.485).

1 Jackman AL, *et al.* ZD1694 (Tomudex): a new thymidylate synthase inhibitor with activity in colorectal cancer. *Eur J Cancer* 1995; **31A:** 1277–82.
2. Cunningham D, *et al.* Tomudex (ZD 1694): results of a randomised trial in advanced colorectal cancer demonstrate efficacy and reduced mucositis and leucopenia. *Eur J Cancer* 1995; **31A:** 1945–54
3. Cunningham D, *et al.* Tomudex (ZD1694) a novel thymidylate synthase inhibitor with clinical antitumour activity in a range of solid tumours. *Ann Oncol* 1996; **7:** 179–82.
4. Mead GM. Raltitrexed, a new drug for advanced colorectal cancer *Lancet* 1996, **347:** 1568–9.
5. Anonymous. Raltitrexed in colorectal cancer. *Drug Ther Bull* 1996; **34:** 78–80.
6. Gunasekara NS, Faulds D. Raltitrexed: a review of its pharmacological properties and clinical efficacy in the management of advanced colorectal cancer. *Drugs* 1998; **55:** 423–35

Preparations

Proprietary Preparations (details are given in Part 3)
Austral.: Tomudex; *Fr.:* Tomudex; *Irl.:* Tomudex; *Ital.:* Tomudex; *Neth.:* Tomudex; *UK:* Tomudex.

Ranimustine (3170-w)

Ranimustine *(rINN).*

MCNU; NSC-0270516; Ranomustine. Methyl 6-[3-(2-chloroethyl)-3-nitrosoureido]-6-deoxy-α-D-glucopyranoside.

$C_{10}H_{18}ClN_3O_7$ = 327.7.
CAS — 58994-96-0.

Ranimustine is a nitrosourea derivative with general properties similar to those of carmustine (see p.511). It is used intravenously in the treatment of malignant neoplasms in usual doses of 50 to 90 mg per m^2 body-surface every 6 to 8 weeks according to haematological response.

Preparations

Proprietary Preparations (details are given in Part 3)
Jpn: Cymerin.

Razoxane (1861-r)

Razoxane *(BAN, rINN).*

ICI-59118; ICRF-159; NSC-129943 (±)-4,4′-Propylenebis(piperazine-2,6-dione).

$C_{11}H_{16}N_4O_4$ = 268.3.
CAS — 21416-87-5.

Razoxane is an antineoplastic with inhibitory activity during the pre-mitotic and early mitotic phases of cell growth (G_2-M). It enhances the effects of radiotherapy. It has been used in association with radiotherapy in the treatment of sarcomas, including Kaposi's sarcoma. Razoxane has also been tried in other malignant diseases including acute leukaemias and non-Hodgkin's lymphomas. However, it is no longer widely employed. Razoxane was formerly used in psoriasis, but its carcinogenic properties militate against such use, as discussed below.

In the treatment of sarcomas it has generally been given by mouth in doses of 125 mg twice daily; higher doses have been given in the management of acute leukaemias and Kaposi's sarcoma. The peripheral blood count should be monitored during treatment.

The principal adverse effects of razoxane include bone-marrow depression, gastro-intestinal disturbances, skin reactions, and alopecia. It may enhance the adverse effects of radiotherapy. Razoxane therapy has been associated with the development of secondary malignancies: it is contra-indicated in the treatment of non-malignant conditions.

Cardiotoxicity. Dexrazoxane (p.978), the (+)-enantiomorph of razoxane, is reportedly effective in preventing doxorubicin-induced cardiotoxicity when given concomitantly.[1,2] It has been suggested that the beneficial effect may derive from chelation of body iron by dexrazoxane and minimising the formation of reactive hydroxyl radicals.[3]

1 Speyer JL, *et al.* Protective effect of the bispiperazinedione ICRF-187 against doxorubicin-induced cardiotoxicity in women with advanced breast cancer. *N Engl J Med* 1988, **319:** 745–52.
2. Kolaric K, *et al.* A phase II trial of cardioprotection with Cardioxane (ICRF-187) in patients with advanced breast cancer receiving 5-fluorouracil, doxorubicin and cyclophosphamide. *Oncology* 1995, **52:** 251–5.
3 Seifert CF, *et al.* Dexrazoxane in the prevention of doxorubicin-induced cardiotoxicity. *Ann Pharmacother* 1994; **28:** 1063–72

Malignant neoplasms. Razoxane in association with radiotherapy produced partial response in 12 of 25 patients with liver metastases from gastro-intestinal or lung cancers;[1] 4 further patients had minor responses (less than 50% reduction in hepatic tumour load) and 2 had disease progression. Therapy consisted of razoxane 125 mg three times daily and then 125 mg daily for 1 month with radiotherapy on days 4 to 8. It was considered unlikely that such results could have been achieved with this dose of radiotherapy (4 Gray daily) or razoxane if either was given alone, but it remained to be seen whether responses would translate into improved long-term survival. Quality of life was excellent in most patients although 2 showed evidence of radiation hepatitis.

1 Hellmann K, *et al.* Responses of liver metastases to radiotherapy and razoxane. *J R Soc Med* 1992, **85:** 136–8.

Skin disorders, non-malignant. Razoxane was formerly used in the systemic treatment of psoriasis, and has been found to be extremely effective, with an initial response rate of 97% overall. It was found to be of use in all forms of cutaneous psoriasis and psoriatic arthropathy.[1] However, the development of acute myeloid leukaemias and other malignancies in patients given razoxane[2-5] has led to its being contra-indicated in non-malignant conditions.

For a discussion of psoriasis and its management, see p.1075.

1 Horton JJ, Wells RS. Razoxane: a review of 6 years' therapy in psoriasis. *Br J Dermatol* 1983; **109:** 669–73.
2 Horton JJ, *et al* Epitheliomas in patients receiving razoxane therapy for psoriasis. *Br J Dermatol* 1983; **109:** 675–8.
3. Lakham S, *et al.* Razoxane and leukaemia. *Lancet* 1984; **ii:** 288–9.
4. Caffrey EA, *et al.* Acute myeloid leukaemia after treatment with razoxane. *Br J Dermatol* 1985, **113:** 131–4.
5. Zuiable AG, *et al.* Razoxane and T-cell lymphoma. *Br J Dermatol* 1989; **121:** 149.

Preparations

Proprietary Preparations (details are given in Part 3)
UK: Razoxin.

9-*cis*-Retinoic Acid (15789-p)

Alitretinoin.

9-*cis*-Retinoic acid is a retinoid related to tretinoin (p.1093) that is under investigation by mouth in acute promyelocytic leukaemia and some other malignancies, and topically, as a 0.1% gel, in the management of Kaposi's sarcoma and other cutaneous neoplasms.

References.

1. Miller WH, *et al* 9-cis Retinoic acid induces complete remission but does not reverse clinically acquired retinoid resistance in acute promyelocytic leukemia. *Blood* 1995; **85:** 3021–7.
2 Weber C, Dumont E. Pharmacokinetics and pharmacodynamics of 9-cis-retinoic acid in healthy men. *J Clin Pharmacol* 1997: **37:** 566–74.

Rituximab (17534-n)

Rituximab *(BAN, USAN, rINN).*

IDEC-102; IDEC-C2B8.

CAS — 174722-31-7.

Rituximab is a chimeric monoclonal antibody to CD20 antigen used in the treatment of non-Hodgkin's lymphoma (p.482). Recommended doses are 375 mg per m^2 body-surface by intravenous infusion once weekly, for 4 doses. The first infusion is given initially at a rate of 50 mg per hour; subsequently this may be increased in increments of 50 mg per hour every 30 minutes to a maximum of 400 mg per hour. If well tolerated. Subsequent doses may be begun at a rate of 100 mg per hour, and incremented to a maximum of 400 mg

8 Preclinical Pharmacology Studies and the Clinical Development of a Novel Multitargeted Antifolate, MTA (LY231514)

Chuan Shih and Donald E. Thornton

CONTENTS

1. INTRODUCTION

Since the early 1950s, extensive research efforts have been devoted to the discovery and development of antifolate antimetabolites as chemotherapeutic agents for the management of neoplastic diseases. However, it was only in the last 10–15 yr, because of the rapid advances of medicinal chemistry, X-ray protein crystallography, molecular biology, pharmacology, and clinical medicine, that a significant number of new generation antifolates were brought forward for clinical development. Several folate-based antimetabolites are currently being investigated in clinical trials. These include lometrexol (6R-5,10-dideazatetrahydrofolic acid) *(1–3)*, LY309887 *(4)*, and AG2034 *(5)*, which are potent and selective inhibitors of glycinamide ribonucleotide formyltransferase (GARFT), an enzyme in the purine *de novo* biosynthetic pathway; trimetrexate *(6)*, edatrexate *(7,8)*, and PT523 *(9)* which act on dihydrofolate reductase (DHFR); raltitrexed *(10,11)*, AG337 *(12)*, BW1843U89 *(13)*, and ZD933 *(14)* which specifically target the enzyme thymidylate synthase (TS) involved in pyrimidine biosynthesis.

N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5 -yl)ethyl]-benzoyl]-L-glutamic acid, LY231514, is a structurally novel antifolate that possesses a unique 6-5 fused pyrrolo[2,3-d]pyrimidine nucleus instead of the more common 6-6 fused pteridine or quinazoline ring structure. LY231514 was discovered through struc-

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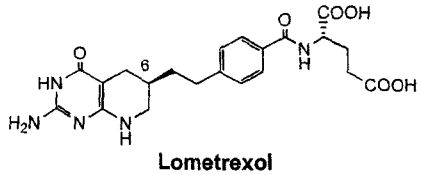
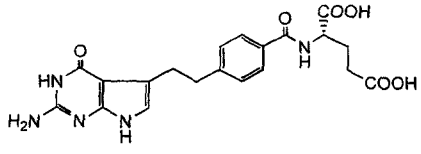
Edited by: A.L. Jackman © Humana Press Inc., Totowa, NJ



Lometrexol (6R-DDATHF)



MTA (LY231514)

Fig. 1. The structures of lometrexol (6R-5,10-dideazatetrahydrofolic acid, DDATHF) and MTA (N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid).

ture activity relationship (SAR) studies of the novel antipurine antifolate lometrexol series, by eliminating the C5 methylene of lometrexol and converting the sp3 center at C6 to sp2 geometry (Fig. 1) *(15,16)*. These modifications give rise to a very potent cytotoxic agent (IC_{50} = 15 n*M*) against human CCRF-CEM leukemia cells in culture. However, the end-product reversal pattern of this new pyrrolopyrimidine-based antifolate was completely different to the GARFT inhibitor lometrexol. The purine precusor hypoxanthine (100 μ*M*) or aminoimidazole carboxamide (AICA) (300 μ*M*) was incapable of protecting the cells from the cytotoxicity of LY231514. In contrast, thymidine (5 μ*M*) was able to provide partial protection to the cells up to 10X IC_{50} concentrations of LY231514. The replacement of the tetrahydropyridine ring of lometrexol with a pyrrole moiety caused a major loss of activity in the inhibition of purine biosynthesis and shifted the major site of action of LY231514 to the inhibition of pyrimidine biosynthesis (thymidylate cycle). As a "classical" antifolate, LY231514 was found to be one of the best known substrates for mammalian folylpolyglutamate synthetase (FPGS) *(17)* and it is believed that polyglutamation and the polyglutamated metabolites of LY231514 play profound roles in determining both the selectivity and antitumor activity of this novel agent. Recent studies have shown that the polyglutamates of LY231514, (e.g., the triglutamate glu_3 and the pentaglutamate glu_5) potently inhibit several key enzymes of the folate metabolism, including TS, DHFR, GARFT, and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT) *(18)*. As a result of this activity against several enzymes, LY231514 has become known as MTA, multitargeted antifolate.

The phase I clinical evaluation of MTA began in late 1992. Objective tumor responses were observed in patients with colorectal cancer and pancreatic cancer, some of whom had failed treatment with other TS inhibitors such as 5FU and raltitrexed *(19–21)*. Phase II studies have shown activity in a range of solid tumors, including colorectal, breast and nonsmall-cell lung cancers *(22–27)*. The purpose of this chapter is to comprehensively review the unique biochemical and pharmacological modes of action, and the recent phase I and II clinical findings of this novel multitargeted antifolate, MTA.

Inhibitor

Compound
MTA
MTA-glu_3
MTA-glu_5
MTX
MTX-glu_5

2

The inh
(rm)GARF
summarize
± 9 n*M*. It
polyglutam
significant
residues (g
ther extens
ward rhTS
DHFR (K_i
to MTA ha
identical *K*
glu_n inhibi
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MTA had
380 n*M* (2
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tion for M
pathway

Table 1
Inhibitory Activity of MTA, Methotrexate and Their Polyglutamates Against rhTS, rhDHFR, rmGARFT, and rhAICARFT (Ki [mean ± SE, n*M*])

Compound	*rhTS*	*rhDHFR*	*rmGARFT*	*rhAICARFT*
MTA	109 ± 9	7.0 ± 1.9	9300 ± 690	3580
MTA-glu_3	1.6 ± 0.1	7.1 ± 1.6	380 ± 92	480
MTA-glu_5	1.3 ± 0.3	7.2 ± 0.4	65 ± 16	265
MTX	13,000	0.004	80,000	143,000
MTX-glu_5	47	0.004	2500	56

2. PRECLINICAL PHARMACOLOGY STUDIES OF MTA

2.1. Folate Enzyme Inhibition Studies

The inhibition of recombinant human (rh)TS, rhDHFR, recombinant mouse (rm)GARFT, and rhAICARFT by MTA and its polyglutamates (glu_3 and glu_5) *(18)* is summarized in Table 1. The parent monoglutamate MTA inhibited rhTS with a K_i of 109 ± 9 n*M*. It has been well documented that mammalian TS shows a strong preference for polyglutamated folate substrates. The longer chain γ-glutamyl derivatives of MTA had significantly enhanced affinity toward rhTS. The addition of two extra γ-glutamyl residues (glu_3) to MTA resulted in 68-fold reduction of the K_i value (K_i = 1.6 n*M*). Further extension of the glutamate tail (MTA-glu_5) only slightly increased the affinity toward rhTS (K_i = 1.3 n*M*). MTA was also found to be a very potent inhibitor of human DHFR (K_i = 7.0 n*M*). In contrast to rhTS, attachment of additional γ-glutamyl residues to MTA had little effect on the inhibition of DHFR; MTA-glu_3 and MTA-glu_5 exhibited identical K_i values against rhDHFR, 7.1 n*M*. Tight-binding analysis showed that MTA-glu_n inhibited both TS and DHFR competitively. When MTA was tested against the enzymes along the purine *de novo* biosynthetic pathway, it only demonstrated moderate inhibition toward rmGARFT (K_i = 9.3 μ*M*). The triglutamate and pentaglutamate of MTA had significantly enhanced inhibitory activity against GARFT, with K_i values of 380 n*M* (24-fold) and 65 n*M* (144-fold), respectively. The pentaglutamate of MTA also inhibited human AICARFT with a K_i of 265 n*M*. Kinetic analysis confirmed the competitive inhibition pattern of MTA polyglutamates against both GARFT and AICARFT. Finally, MTA and its polyglutamates were competitive inhibitors of both the dehydrogenase and synthetase domains of C1 tetrahydrofolate synthase. The K_i values for the mono-, tri- and pentaglutamyl derivatives of MTA were 9.9, 3.9, and 4.7 μM, respectively, for dehydrogenase and 329,25,4 and 1.6 μM for synthetase. MTA was a relatively less potent inhibitor of C1 tetrahydrofolate synthase than other enzyme targets such as TS, DHFR, and GARFT. However, cell-culture experiments have suggested that the intracellular drug concentration of MTA can reach levels of 50 μ*M* (RM Schultz, unpublished observation), and at these concentrations the activity of C1 tetrahydrofolate synthase can also be greatly suppressed by MTA polyglutamates. The important role of TS in serving as a rate-limiting enzyme in folate metabolism, as well as the relative order of inhibitory potency toward TS by MTA-glu_n indicate that TS is a major site of action for MTA. Inhibition of DHFR and other enzymes in the *de novo* purine biosynthetic pathway may also contribute significantly to the overall antiproliferative effect of MTA

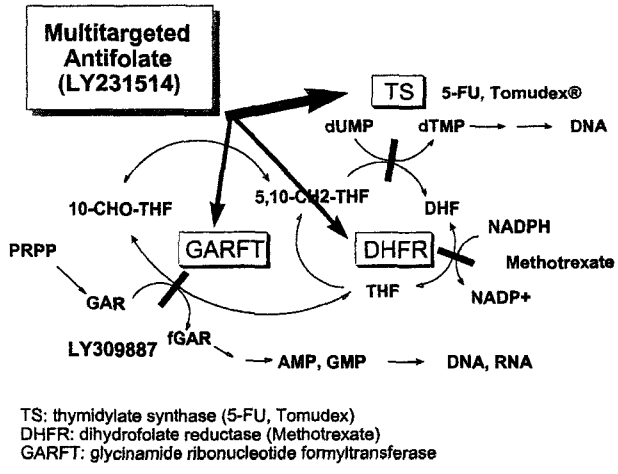

Fig. 2. Inhibition of multiple folate enzymes (TS, DHFR, and GARFT) by MTA and its polyglutamated metabolites.

(Fig. 2). This unique mode of action was further supported by additional cell-based studies (*vide infra*).

As a reference for comparison, the polyglutamates of methotrexate (MTX) also inhibit multiple folate-dependent enzymes. Chabner et al *(28)* reported that the pentaglutamate of MTX (MTX-glu_5) demonstrated a significant increase in affinity toward rhTS (K_i = 47 n*M*) and AICARFT (K_i = 56 n*M*) when compared with the parent monoglutamate. However, the affinity of MTX and its polyglutamates for DHFR (K_i = 4 p*M*) was several orders of magnitude (>12,000-fold) higher than its affinity for TS and AICARFT, suggesting that the primary intracellular target of MTX may still be DHFR.

2.2. Cell-Based End-Product Reversal Studies

MTA is very cytotoxic against CCRF-CEM leukemia cells in culture. This potent antiproliferative effect of MTA can be prevented by leucovorin, whereas only partial protection was observed with thymidine. In the presence of 5 μ*M* thymidine, the IC_{50} of MTA increased only 6–10-fold and this was significantly less than that of a pure TS inhibitor such as raltitrexed. This reversal pattern of MTA was further characterized in various human tumor cell lines such as GC3/C1 colon carcinoma and HCT-8 ileocecal carcinoma (Table 2). It was observed that 5 μ*M* thymidine fully protected the cells from cytotoxicity with raltitrexed, whereas similar treatment with thymidine only increased the IC_{50} of MTA by 18.7-fold (GC3/C1), and by 15-fold (HCT-8). Hypoxanthine (100 μ*M*) alone did not markedly influence the cytotoxicity of MTA. Similarly, AICA (300 μ*M*) did not modulate cytotoxicity. However, the combination of thymidine plus hypoxanthine completely reversed the cytotoxicity of MTA in all cell lines (IC_{50}s > 20 μ*M*). The reversal pattern of MTA was also significantly different from that of MTX. Neither thymidine nor hypoxanthine could protect the cells from the cytotoxic actions of MTX at all drug concentrations. The unusual reversal pattern observed for MTA suggests that in addition to TS, other important inhibitory sites may exist for this agent. The higher degree of protection by thymidine at low drug concentrations indicates that TS is a major target for MTA. Addition of hypoxanthine together with thymidine fully re-

Table 2
End-Product Reversal Studies with MTA[a] (IC_{50} (n*M*))

Cell line	*MTA alone*	*dThd[b] (5 μM)*	*Hypoxanthine[c] (100 μM)*	*dThd and Hypoxanthine*
CCRF-CEM	25	138	32	>40,000
GC3/C1	34	637	34	>40,000
HCT	220	3104	1077	>40,000

[a]Cytotoxicity determined by MTT analysis after 72 h exposure to drug, SE of triplicate determinations did not exceed 10% of mean.
[b]With the addition of 5 μ*M* of thymidine.
[c]With the addition of 100 μ*M* of hypoxanthine.

Table 3
Substrate Activity of MTA and Other Antifolates for Mouse and Hog Liver FPGS

Compound	K_m (μM)[a]	*rel.* V_{max}[b]	*rel.* V_{max}/K_m[c]
Mouse Liver FPGS			
Lometrexol	9.3 ± 1.6	1.0	1.0
MTA	0.80 ± 0.11	0.63 ± 0.18	13.7
Methotrexate	166.0 ± 14	0.50 ± 0.09	0.031
Hog Liver FPGS			
Lometrexol	16.4 ± 1.0	1.0	1.0
MTA	1.9 ± 0.5	0.74 ± 0.10	6.40
Methotrexate	116.0 ± 14	0.51 ± 0.08	0.07

[a]Values listed are mean ± standard error for $n \geq 3$ or ± 1/2 range for $n = 2$ replicate experiments.
[b]The ratio of V_{max} for a substrate to the V_{max} of lometrexol with either mouse or hog liver FPGS.
[c]The V_{max} of a substrate relative to lometrexol divided by the K_m of a substrate relative to lometrexol, the kinetics of a standard compound was measured in each experiment to allow accurate comparisons among substrate.

versed the cytotoxicity of MTA, suggesting that at higher concentrations, inhibition of DHFR and/or purine *de novo* biosynthetic enzymes were responsible for other secondary cytotoxic actions of the drug, a conclusion that is consistent with results from enzymatic studies. Recent finding that H630-R10 cells *(29)* (resistant to 5FU with a 39-fold amplification of TS protein) demonstrated a significantly reduced resistance to MTA (fivefold vs 6900-fold for raltitrexed) further support the conclusion that TS is not the sole molecular target for this novel agent.

2.3. The Role of Polyglutamation and Folate Transport

Polyglutamation plays an essential role in determining the overall biochemical and pharmacological properties of the classical antifolates. The formation of polyglutamates leads to the accumulation of polyglutamated metabolites to levels that are significantly higher than could otherwise be achieved at steady state by the parent monoglutamates, and thus serves as an important cellular retention mechanism for folates and antifolates. Studies have shown that MTA is an excellent substrate for mammalian FPGS. The substrate activity of MTA and several other antifolates for mouse and hog liver FPGS *(15,17)* are listed in Table 3. Both the substrate constants (K_m) and the relative first-order

Table 4
Antiproliferative Activity MTA Against ZR-75-1 Human Breast Carcinoma Cell Lines with Differing Transport Characteristics[a]

Cell Line	*Transport*[b]	*MTA, IC_{50} (nM)*
WT-AA6-FR+	RFC+, FBP+	22.7
Wild Type (WT)	RFC+, FBP−	110.2
MTX^R-BB3-FR+	RFC−, FBP+	1190.6

[a]Cytotoxicity determined after 72-h drug exposure by MTT assay. Assay medium contained 2 n*M* folinic acid as the sole folate source. SE of triplicate determinations did not exceed 10% of mean.

[b]Reduced folate carrier (RFC); folate binding protein-α (FBP).

rate constants (k′, V_{max}/K_m) have revealed the superior propensity of MTA for polyglutamation by mouse and hog liver FPGS. Recent studies indicate that MTA is an even better substrate for recombinant human FPGS (R.G. Moran, personal communication) and this makes MTA one of most efficient substrates for the enzyme FPGS studied to date.

To evaluate the role of FPGS in the cytotoxicity of MTA in cells, the CR15 line, a lometrexol-resistant CCRF-CEM subline, was utilized. Impaired polyglutamation in CR15 cells was identified as the primary mechanism of resistance to lometrexol *(30)*. It was estimated that CR15 cells had approx 10% of the FPGS activity of the wild-type cells and were markedly cross-resistant to MTA (>7800-fold increase in IC_{50} vs the parent CEM line), suggesting that polyglutamation is a major determinant in the cytotoxicity of MTA.

To investigate the mechanism for MTA transport, a panel of ZR-75-1 human breast carcinoma sublines, prepared by Dixon*(31)* and colleagues, with different transport characteristics was utilized (Table 4). The MTX-resistant cells (RFC+, FBP−), deficient in folate binding protein (FBP) activity, demonstrated only a 4.8-fold decrease in sensitivity to MTA, when compared to wild-type AA6-FR+ cells that expressed both reduced folate carrier (RFC) and FBP. Resistant cells (MTXR-BB3-FR+) deficient in RFC exhibited a much higher resistance to MTA (52-fold) than the wild-type cells. These data, plus the finding that MTA had rather poor affinity toward folate binding protein(s) in general (K_i = 99.7 n*M* vs K_i = 0.29 n*M* for lometrexol), suggest that RFC plays a predominant role in the transport and internalization of MTA.

2.4. Effects of MTA on Folate and Nucleoside Triphosphate Pools

The effects of MTA and several other antifolates (MTX, LY309887) on cellular folate and nucleotide metabolism have been examined in CCRF-CEM cells*(32)*. Exposure of cells labeled with 100 n*M* 5-formylTHF to 0.1 μ*M* MTX caused the loss of 10-formylTHF, THF, and 5-methylTHF and a concomitant accumulation of dihydrofolate (DHF), metabolic responses consistent with the blockade of DHFR by MTX. The GARFT inhibitor LY309887, on the other hand, caused accumulation of 10-formylTHF, a direct consequence of the inhibition of GARFT, which utilizes 10-formylTHF as the one-carbon donor for its enzymatic reactions. These effects are fully consistent with the known mechanisms of action of MTX and LY309887.

Because of the assay's inability to distinguish THF and methyleneTHF, the effects of MTA on cells could not be readily studied. However, exposure of cells to MTA (300 n*M*, also 10 times the IC_{50}) can trigger a slight decrease in THF and a compensatory increase in the level of 10-formylTHF. In light of the observed accumulation of 10-formylTHF by the specific GARFT inhibitor LY309887, it is tempting to attribute this small, yet significant, increase of 10-formylTHF to the inhibition of GARFT and AICARFT (antipurine effect) by MTA-glu_n, a hypothesis that can be verified by direct measurement of the effect of MTA on the metabolic flux from glycine to inosinic acid.

In the ribonucleotide pool studies, it was discovered that both MTX and LY309887 caused rapid depletion of both purines, ATP and GTP, and had moderate effects on the pyrimidines, UTP and CTP. However, MTA produced no significant effects on any of the ribonucleotide triphosphates at concentrations 10 times their IC_{50} in CEM cells. In contrast, all three compounds demonstrated more dramatic effects on deoxyribonucleotide pools. In response to LY309887, dATP declined rapidly, followed closely by dCTP, and then later by dGTP and dTTP at a slower rate. MTX rapidly depleted all four deoxyribonucleotide levels. The effect of MTA on deoxyribonucleotide levels is consistent with reports of other TS inhibitors *(33)*. It was found that MTA was able to induce rapid losses in dTTP, dCTP, and dGTP. However, an increase of dATP level was observed for cells treated with MTA. It will be interesting to examine the difference of the rate of accumulation of dATP induced by MTA or by other specific TS inhibitors, since Chong and Tattersall*(33)* reported that the combination of a GARFT inhibitor and TS inhibitor prevented the rise in the dATP pool seen with the TS inhibitor alone. The mechanism for the changes in dATP levels induced by TS inhibitors has not been well understood. However, it is noteworthy that for MTX, a drug with both antipyrimidine and antipurine effects, depletion of dTTP occurred without a concomitant increase in dATP. In summary, these studies showed that MTA exhibited unique metabolic effects that were quite distinct from those of MTX and LY309887. The folate pools (accumulation of 10-formylTHF) data suggest that in addition to the primary effect on the thymidylate synthesis, MTA may produce an antipurine effect by interfering with the enzymes along the *de novo* purine biosynthetic pathway.

2.5. Effects of MTA on Cell-Cycle Alterations and Cell Proliferation(34)

MTA affects the growth rate of CCRF-CEM leukemia cells in a concentration-dependent manner. When CEM cells were treated with concentrations of MTA greater than 100 n*M*, cell growth was completely inhibited and apoptosis occurred within 36 h of drug treatment. Multiple cell-cycle alterations occurred in CCRF-CEM populations when cells were treated with either a sublethal (30 n*M*) or a lethal (300 n*M*) dose of MTA. Within 8 h of drug addition, both treatments synchronized cells into G1 or G1/S population. At the sublethal dose, the cell population was distributed throughout S phase after 24 h of drug addition, and was able to complete DNA synthesis. In contrast, the population treated with 300 n*M* of MTA was effectively synchronized into early S phase after 24 h and was unable to complete DNA synthesis. Further studies also showed that levels of cyclin E, a G1/S-specific protein in cell-cycle control, were dramatically increased relative to control in cells treated with either sublethal or lethal dose of MTA. Dual parameter flow cytometry confirmed that cyclin E levels were 2–5-fold higher in treated vs. untreated cells. In contrast, cyclin A levels did not begin to increase until af-

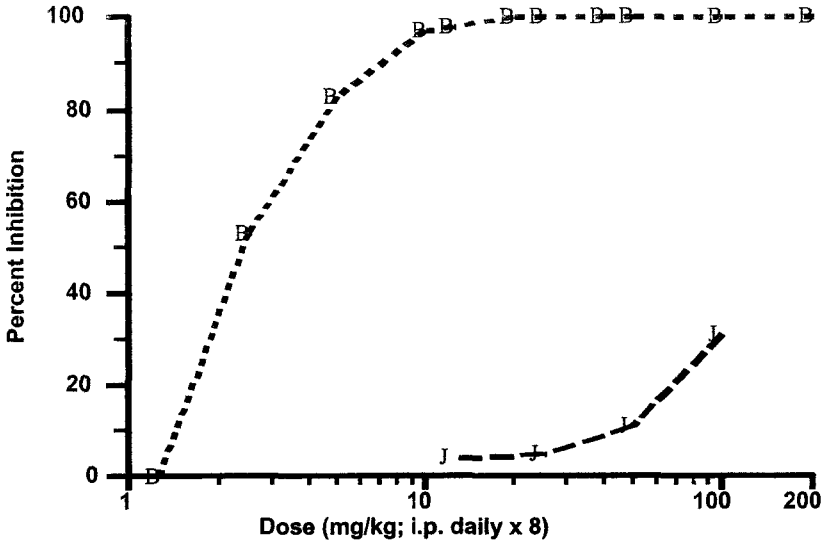

Fig. 3. Antitumor activity of MTA (LY231514, **B**) or CB3717 (**J**) against L5178Y/TK$^-$/HX$^-$, in DBA/2 mice. Both MTA and CB3717 were administered ip, daily × 10.

ter 24 h of treatment. These observations indicate that both lethal and sublethal concentrations of MTA are able to cause an initial blockade in the cell cycle prior to the G1/S checkpoint. This is followed by synchronization of the cells into the S phase under lethal concentrations. Continued exposure of the drug triggers major apoptotic events that eventually lead to cell death.

2.6. The in Vivo Antitumor Effects and the Role of Folic Acid in Modulating the Efficacy and Toxicity of MTA

MTA was found to be highly active against the L5178Y/TK-/HX-lymphoma in mice *(35)* (Fig. 3). An excellent therapeutic index was seen, along with antitumor activity in this thymidine kinase-deficient murine model, a result that is consistent with TS inhibition being the primary mode of action of MTA. Good antitumor activity was also observed for MTA in other human tumor xenografts that expressed normal level of thymidine kinase, including VRC5 (colon, 80% growth inhibition) and GC3 (colon, 94% growth inhibition), BXPC3 (pancreas), LX-1 (lung), and MX-1 (breast) xenografts (Table 5).

To evaluate the importance of dietary folate in modulating the toxicity of MTA, LD_{50} values were determined in mice maintained on standard diet (SD) or on a special low-folate diet (LFD) *(35)*. MTA was administered ip daily for 10 d. It is estimated that mice on LFD consumed an average of approx 0.003 mg/kg/d of folic acid vs 0.75–1.5 mg/kg/d for mice on SD. Thus mice on SD had a daily intake of approx 250–500 times more folic acid than mice on LFD. MTA was more toxic to several different strains of mice maintained on LFD (Table 6), with the LD_{50} values being 30- to 250-fold lower than mice maintained on SD. A similar effect had been observed for antipurine antifolates such as lometrexol. The MTD of lometrexol on LFD was 1000- to 5000-fold lower than in mice

Xenograft Mode
GC3 colon carci
VRC5 colon car
BXPC3 pancreat
LX-1 lung carcir
MX-1 mammary

+++: 95–100%
++: 80–94% in
+: 60–79% inhi
−: <60% inhibi
[a]MTA was give
[b]DDATHF was
[c]NCI data.

Strain of Mouse	
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CD1 nu/nu	S
	I
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Table 5
Comparison of Antitumor Activity of MTA with DDATHF and Methotrexate Against Human Tumor Xenografts

Xenograft Model	*MTA*[a]	*DDATHF*[b]	*Methotrexate*
GC3 colon carcinoma	+++	+++	−
VRC5 colon carcinoma	++	+++	−
BXPC3 pancreatic carcinoma	+	+	−
LX-1 lung carcinoma	+	++	[c]
MX-1 mammary carcinoma	+	+	[c]

+++: 95–100% inhibition.
++: 80–94% inhibition.
+: 60–79% inhibition.
−: <60% inhibition inactive.
[a]MTA was given ip, qd × 10 at doses up to 300 mg/kg/dose.
[b]DDATHF was given ip, q2d × 5 at doses up to 100 mg/kg/dose.
[c]NCI data.

Table 6
Effect of Dietary Folate on the Lethality of MTA

Strain of Mouse	*Diet*	*Route, Schedule*	*LD_{50} (mg/kg)*	*Ratio of LD_{50} (SD/LFD)*
DBA/2	Standard diet	ip qd × 10	approx 600	approx 60
	Low folate diet		approx 10	
CD1 nu/nu	Standard diet	ip qd × 10	approx 400	approx 250
	Low folate diet		1.56	
C3H	Standard diet	ip qd × 10	>1600	approx 30
	Low folate diet		50–100	

maintained on SD *(36)*. DHFR inhibitors such as methotrexate had a similar effect but to a lesser extent (50- to 100-fold, J.F. Worzalla, unpublished observation). The therapeutic index of MTA against the L5178Y/TK-/HX-tumor was greatly diminished when the mice were put on a LFD (2 wk) with no folate supplementation. Good antitumor activity was observed at 0.3 mg/kg and 1.0 mg/kg (ip daily × 10) doses only, and significant toxicity was observed for MTA at higher doses (Fig. 4). However, if daily folic acid supplementation (15 mg/d/mouse, po) was given in conjunction with MTA, excellent antitumor dose-response (10 mg/kg to 1000 mg/kg, with antitumor activity ranging from 80 to 100%) and no lethality were observed. This antitumor dose response (with folate supplementation) is identical to the dose response that was observed for MTA on mice fed with SD (Fig. 3). These data suggest that folate supplementation not only modulates the toxicity but also slightly enhances the antitumor response of MTA.

2.7. Drug Disposition and Metabolism of MTA

The metabolism and disposition of MTA was studied in mice and dogs *(37)*. Some selected pharmacokinetic parameters are summarized in Table 7. Intravenous injections of MTA gave high plasma levels of the drug, resulting in an AUC value of 30–33 μg-h/mL for mice and dogs after 20 and 7.5 mg/kg doses, respectively. In vitro protein binding of

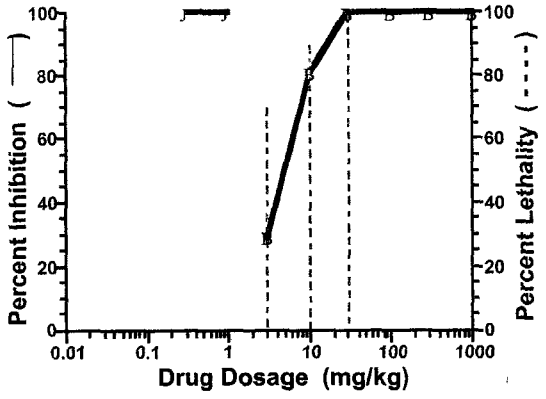


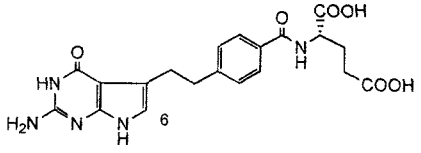
Fig. 4. Antitumor activity of MTA against L5178Y/TK$^-$/HX$^-$ lymphoma for mice on low folate diet (LFD) with no folate supplementation (**J**) and for mice on low folate diet that received 15 mg/kg/d daily folate supplementation (**B**); vertical dashed lines represent percent lethality in mice on low folate diet with no folate supplementation.

Table 7
Pharmacokinetic Parameters for Mouse and Dog with MTA

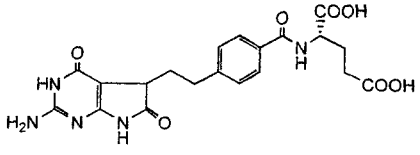
	Mouse		*Dog*	
Sex	M	F	M	F
Dose (mg/kg)	20	20	7.5	7.5
Route	iv	ip	iv	iv
t_{max} (h)	0.083	0.25	0.083	0.083
C_{max} (μg/mL)	41	33	38 ± 2	49 ± 9
$t_{1/2}$ (h) (interval)	7 (1–48)	7.8 (2–48)	2.8 (1–24)	1.8 (1–12)
AUC (μg/h/mL)	31	44	33 ± 1.9	30 ± 2
Cl (mL/h/kg)	645	—	230 ± 24	246 ± 29

[^{14}C]MTA in plasma was estimated at concentrations of 0.5 and 5 μg/mL using an ultracentrifugation procedure. In mouse plasma, the binding of [^{14}C]MTA was 54–58% and in dog plasma it was 46–47%. The binding was notably higher (81%) in human plasma. MTA was primarily eliminated unchanged in feces (57%) of mice after a single iv dose of [^{14}C]MTA. Urine was the major route of excretion (69%) in dogs. Half-life values were approx 7 and 2.8 h for mice and dogs, respectively.

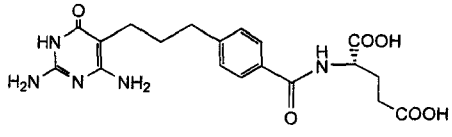
Unchanged MTA accounted for the majority of urinary radiocarbon in mice (90%) and dogs (68%), although two minor metabolites were found in these species. The first metabolite, LY338979, was formed by oxidation of the pyrrole ring of MTA (position 6) giving the corresponding lactam (Fig. 5). This type of oxidative transformation is also seen with tryptophans and other indole-containing compounds. Further oxidation of the lactam ring of LY338979, followed by one-carbon extrusion, gave a second metabolite, LY368962, with a complete loss of the pyrrole ring structure. The structures of both metabolites were confirmed by total synthesis and NMR (long-range heteronuclear coupling) experiments. Testing of the major metabolite LY338979 showed that this agent is



MTA (LY231514)



LY338979



LY368962

Fig. 5. Structures of the two minor metabolites of MTA isolated from the urine of mice and dogs, LY338979 and LY368962.

biologically inactive as a TS inhibitor (using a rhTS assay) and has no antiproliferative effects on CEM cells in culture.

2.8. Preclinical Toxicological Findings of MTA (38)

Genetic toxicology studies indicated that MTA is negative in the Ames test, in vitro chromosome aberration test, and mammalian HGPRT+ locus forward mutation assay. In acute toxicology studies, it was found that the iv median lethal dose (MLD) of MTA for female and male Fisher 344 rats was >1574 and 1322 mg/kg, respectively. In comparison, the MLD of MTX in rats is 6 to 25 mg/kg. The iv MLD for female and male CD-1 mice was >1574 mg/kg. The LD_{10} for mice could not be calculated, but was estimated to be >1574 mg/kg. The MLD of MTX for mice was 94 mg/kg.

Single and repeated dose studies of MTA were conducted in CD-1 mice and dogs. In pilot mice studies, groups of five mice of each sex per dose group were given daily ip doses of 0, 50, 100, and 150 mg/kg MTA for 2 wk. Daily doses were well tolerated by mice, all mice survived to study termination and had no clinical signs of toxicity. In longer duration studies, 10 mice/sex/dose group were dosed ip with 0, 10, and 25 mg/kg MTA daily, 105 mg/kg twice weekly, or 315 mg/kg once weekly for 6 wk. Again, all mice tolerated all doses and schedules with no compound-related mortality or clinical signs of toxicity. Intestinal necrosis was slight and was limited only to male and female mice treated daily with 10 and 25 mg/kg MTA. The higher levels of thymidine (1–5 μM) in mice is thought to reduce the impact of MTA on the proliferation and function of normal cells.

Dogs are more sensitive to MTA. Single iv slow-bolus doses of 10, 25, 50, or 100 mg/kg of MTA can be tolerated by dogs. Up to five daily doses of 5 or 10 mg/kg were given to one dog/dose and the dose of 10 mg/kg proved to be toxic. Dogs given single doses of 50 mg/kg or more had modest lymphopenia and leukopenia 6 d after dosing. The leukopenia was neither consistent nor dose related, and values returned to the reference range within 10 d of dosing. There were no important effects on erythrocyte or platelet parameters. For longer duration studies, one dog/sex/dose group was given either daily iv slow-bolus doses of 0.1, 0.5, 0.75, or 1.0 mg/kg MTA or 10 mg/kg twice weekly for up to 2 wk. Dogs treated with 0.75 or 1 mg/kg died after 7–10 daily doses. Mortality was preceded by clinical signs of decreased food consumption, hypoactivity, dehydration, emesis, abnormal stools, and increased salivation.

In summary, the toxicological profile of MTA is consistent with the known antiproliferative activities of folate antimetabolites. The major pathological effects associated with MTA occurred in the intestinal tract and lymphoid tissues; bone marrow was only minimally affected in dogs and mice given repeated doses. No hepatic or renal toxicity was observed in these preclinical toxicology studies by histopathalogic evaluation.

3. CLINICAL STUDIES OF MTA

3.1. MTA Phase I Experience

Three dosing schedules have been investigated in the phase I setting. In study JMAA, patients received MTA once every 21 d. Study JMAB looked at administering the drug once weekly for 4 wk out of every 6, and study BP-001 investigated a schedule of daily times five every 21 d.

The daily times five every 3 wk schedule resulted in a maximum tolerated dose (MTD) of 4 mg/m^2/day *(21)*. Dose-limiting toxicities on this schedule were reversible neutropenia and elevated liver enzymes. Nonhematologic toxicities were mild and included mucositis, diarrhea, rash, fatigue, and elevated transaminases. Minor responses were observed using this schedule in one patient with colorectal cancer and one patient with nonsmall-cell lung cancer (NSCLC). Phase II studies are ongoing to assess the efficacy of this schedule.

Studies JMAA and JMAB used the Modified Continual Reassessment Method for dose escalation. This involves treating a single patient at each minimally toxic dose level and adding more patients once significant toxicities are observed at a dose level. A minimum of three patients are treated at a dose level once moderate reversible toxicity is demonstrated (grade III hematologic toxicity or grade II nonhematologic toxicity excluding nausea, vomiting, and alopecia). Dose-limiting toxicity (DLT) was defined as grade IV hematologic or grade III nonhematologic toxicity, excluding nausea, vomiting, and alopecia. A minimum of six patients are treated once dose-limiting toxicities are demonstrated. In this manner, the number of patients exposed to lower, potentially less effective doses of drug is limited and more patients are treated at doses approaching the MTD *(39)*.

The DLT on the weekly times four, every 6 weeks schedule (study JMAB) was myelosuppression, particularly leukopenia and granulocytopenia *(19)*. Inability to maintain the weekly treatment schedule because of neutropenia limited dose escalation on this schedule. This schedule is not currently being pursued in phase II trials.

Schedule[a]
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Dose range
Recommended
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DLT

Responses

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Table 8
Initial Phase I Experience

	BP-001	*JMAA*	*JMAB*
Schedule[a]	Daily × 5, every 21 d	Once every 21 d	Weekly × 4, every 6 wk
Number of patients treated	38	37	24
Dose range	0.2–5.2 mg/m^2	50–700 mg/m^2	10–40 mg/m^2
Recommended phase II dose	4 mg/m^2	600 mg/m^2	30 mg/m^2
DLT	neutropenia	neutropenia, mucositis, fatigue	myelosuppression, particularly granulocytopenia
Responses	Minor responses in colorectal (1) and NSCLC (1)	Partial responses in pancreas (2), and colorectal (2)	Minor responses in colorectal (2)

[a] All doses administered as a 10-min infusion.

The once every 21 d schedule has been evaluated in phase II trials *(20)*. In the phase I trial investigating this dose (study JMAA), 37 patients were administered drug at doses ranging from 50–700 mg/m^2. Dose-limiting toxicities on this schedule were neutropenia, thrombocytopenia, and fatigue. The MTD on this schedule was determined to be 600 mg/m^2, and of the 20 patients treated at this dose, Common Toxicity Criteria (CTC) grade IV neutropenia and CTC grade IV thrombocytopenia occurred in four and one patients, respectively, in the first cycle. CTC grade II toxicities included rash, mucositis, nausea, vomiting, fatigue, anorexia, and elevations of liver transaminases. Patients experiencing rash were treated in subsequent cycles with 4 mg dexamethasone twice daily for 3 d, starting the day before MTA therapy. The severity of the rash was reduced or the rash prevented in these patients *(19)*. The phase I experience is summarized in Table 8.

Pharmacokinetic determinations were made in 20 patients who were treated at the MTD (600 mg/m^2) in study JMAA. A mean maximum plasma concentration of 137 μg/mL was attained, with a mean half-life of 3.1 h (range 2.2–7.2 h). Mean clearance and steady-state volume of distribution values of 40 mL/min/m^2 and 7.01/m^2 were also measured. This mean clearance value is similar to that of creatinine clearance in the age range of the patients enrolled (approx 45–55 mL/min/m^2) and the volume of distribution reflects limited distribution outside the bloodstream *(40)*.

Samples collected from the first dose in each course of therapy showed the disposition of MTA to be linear over the entire dose range (0.2–700 mg/m^2). The clearance of the drug is primarily renal, with 80% or more of the dose recovered unchanged in the urine during the first 24 h after dosing. No accumulation appears to occur with multiple courses, and the disposition of MTA does not change after multiple doses. Gender does not appear to affect MTA disposition. MTA clearance appears to decrease with age, although this decrease is most likely to be related to decreasing renal function *(40)*.

Partial responses were seen in one of the phase I studies (JMAA), with two in pancreas cancer patients of duration 2 and 6 mo, and two in colorectal cancer patients of duration 7 and 11 mo. Both patients with colorectal cancer had received prior

chemotherapy, one with intrahepatic FUDR and one with raltitrexed. One of the patients with pancreatic cancer had received prior therapy with 5FU.

3.2. Phase II Experience

Two phase II studies in colorectal cancer, one in pancreas cancer, two in NSCLC, and one in breast cancer began in late 1995. These studies were designed to include patients with advanced disease who were either chemonaive or had received prior chemotherapy in the metastatic setting, with a starting dose of 600 mg/m^2 once every 21 d. Results from these studies are preliminary.

Clinical activity of MTA in metastatic colorectal carcinoma has been demonstrated in two multicenter trials performed in Canada *(23)* and the U.S. *(24)*. Prior adjuvant chemotherapy was allowed if completed at least 1 yr prior to study entry. In the Canadian study, the starting dose of 600 mg/m^2 was reduced to 500 mg/m^2 after dose reductions were required in five of the first eight patients. Toxicities leading to these reductions included rash, mucositis, neutropenia, and febrile neutropenia. Responses were seen at this reduced dose in six patients for an overall response rate of 21% (8–39.7%) *(23)*. In the U.S. colorectal study, objective tumor responses were seen in 6 of 39 patients for an overall response rate of 16% *(24)*.

Two responses, one complete and one partial, were observed in 35 evaluable patients in the pancreatic cancer phase II study for an overall response rate of 6% *(25)*. Importantly, there were 13 additional patients with stable disease lasting for over 6 mo of treatment, suggesting a clinical benefit not immediately apparent from objective tumor measurements.

A phase II study in patients with locally advanced and/or metastatic breast cancer is ongoing and includes patients who have received prior adjuvant chemotherapy as well as one prior therapy for metastatic disease. Fourteen of 22 patients had received prior chemotherapy, 10 as adjuvant treatment, 7 for metastatic disease, and 3 patients who received both. Of the 18 patients evaluable for response, 1 complete and 5 partial responses have been documented for an overall response rate of 30%. Responses have been seen in pulmonary and hepatic metastases. Three of the six responding patients had received recent prior therapy with paclitaxel, docetaxel, or an anthracycline for metastatic disease *(27)*.

One multi-institutional study in NSCLC has been completed in Canada *(26)* and an additional study is ongoing in Australia and South Africa *(22)*. All patients were chemonaive. The majority of patients on the Canadian study used the lower starting dose of 500 mg/m^2, which was reduced from 600 mg/m^2 during the course of the study after one of the first three patients experienced CTC grade III mucositis and grade IV vomiting and myalgia. Seven partial responses have been observed in 30 evaluable patients for an overall response rate of 23.3% (95% CI 9.9–42.3%) *(26)*. All responding patients were treated at the 500 mg/m^2 dose level.

The second NSCLC study, which is being carried out jointly between Australia and South Africa, has enrolled 21 patients to date, with 20 evaluable for response. All patients are receiving 600 mg/m^2 every 3 wk in this study. Five partial responses have been noted for an overall response rate of 25% *(22)*. The phase II experience to date is summarized in Table 9.

Table 9
Phase II Experience

Study	*JMAC*	*JMAD*	*JMAN*	*JMAO*	*JMAG*	*JMAL*
Site	U.S.	U.S.	Canada	Canada	U.K.	Aus/S Africa
Tumor site	colorectal	pancreas	NSCLC	colorectal	breast	NSCLC
Number of evaluable patients	39	35	30	29	18	20
Median cycles	4	2	3	3	4	4
(Range)	(1–12)	(1–12)	(1–8)	(1–8)	(1–9)	(1–9)
CR	1	1	0	1	1	0
PR	5	1	7	5	5	5
Overall RR (%)	16	6	23	21	30	25
(95% CI, %)			(9.9–42.3)	(8–39.7)		

Table 10
Laboratory Toxicity (n = 209)

	Grade I (%)	*Grade II (%)*	*Grade III (%)*	*Grade IV (%)*
Alk Phos	49	13	4	
ALT	33	26	22	0
AST	42	30	10	0
Bilirubin		18	7.3	2
Creatinine	13	5	0	0
ANC	9	21	27	27
Hb	34	43	12	2.4
Platelets	31	6	7	8

A total of 209 patients have been treated on the once every 3 wk schedule in the phase II setting at 600 mg/m^2 and are evaluable for safety analysis. The most frequent, serious toxicity has been hematologic in nature. CTC grade III and IV hematologic toxicity included neutropenia (25 and 26%, respectively) and thrombocytopenia (7 and 10%, respectively). Although severe neutropenia is common, the frequency of serious infection has been low (grade IV infection 2%). Likewise, thrombocytopenia has been apparent, and yet serious episodes of bleeding have been rare (<1%). Whereas 8% of patients experienced grade III (4% with grade IV) skin rash, prophylactic dexamethasone is reported to ameliorate or prevent the rash in subsequent cycles. Other grade III and IV nonhematologic toxicities included stomatitis, diarrhea, vomiting, and infection. As seen in clinical studies of other antifolates, transient grade III and IV elevation of liver transaminases are common but not dose limiting. There have been no cases of persistent transaminase elevation. Tables 10 and 11 summarize the laboratory and nonlaboratory toxicity data from the phase II studies conducted at a starting dose of 600 mg/m^2.

4. CONCLUSION AND PERSPECTIVE

Extensive biochemical and pharmacological evidence has demonstrated that MTA is a novel antifolate that differs in its mode of action from other antifolates currently un-

Table 11
Nonlaboratory Toxicity (n = 209)

	Grade I (%)	*Grade II (%)*	*Grade III (%)*	*Grade IV (%)*
Cutaneous	19	39	11	5
Diarrhea	17	11	4	3
Infection	13	8	2	2
Nausea	33	30	9	0.5
Fatigue	13	11	6	0
Pulmonary	0.5	7	2	2
Stomatitis	23	16	6	1
Vomiting	13	30	2	3

dergoing investigation. MTA is transported into the cell mainly through the reduced folate carrier system and extensively metabolized to polyglutamated forms. The polyglutamates of MTA inhibit at least three key folate enzymes: TS, DHFR, and GARFT, and to a lesser extent AICARFT and C1-tetrahydrofolate synthase. The combined effects of the inhibition exerted by MTA at each target give rise to an unusual end-product reversal pattern at the cellular level that is distinct from those of other inhibitors such as methotrexate and the quinazoline antifolates. MTA is broadly active against murine solid tumors and human tumor xenografts in vivo. Many lines of evidence indicate that MTA does not behave like a pure TS inhibitor nor does it act like a conventional DHFR inhibitor, such as methotrexate, which also inhibits multiple folate enzymes. The important biochemical, pharmacological and clinical characteristics of MTA include:

- Potent inhibition of TS, DHFR, and enzymes in the *de novo* purine biosynthetic pathway including GARFT, with TS as the primary target.
- Unique end-product reversal pattern which is distinct from all other antifolates.
- Different cross-resistance pattern to cells resistant to TS inhibitors.
- Distinct metabolic effects on folate and nucleotide pools compared to other antifolates.
- Early evidence of clinical activity in NSCLC, a tumor type considered resistant to TS-based antifolate antimetabolites.
- Single-agent activity in phase II studies in patients with a broad spectrum of solid tumors including colorectal, breast, nonsmall-cell lung, and pancreatic cancers.

MTA is, therefore, a novel antifolate with unique biochemical and pharmacological properties. The efficient polyglutamation, longer cellular retention and multiple folate enzyme inhibition mechanism may contribute directly to the exciting antitumor responses now being observed in patients. Specifically, the multitargeted inhibition mechanism of MTA is intriguing. This new level of mechanistic insight evolving and around MTA prompts us to challenge the traditional approach to antifolate drug discovery and development, which has focused on developing potent and selective inhibitors of a single folate enzyme target. Given the complex nature of folate metabolism and the role of folates in maintaining the physiological functions of living systems, it is reasonable to expect that agents that can interfere with multiple enzymes in the folate pathway may

trigger more biochemical imbalance of the cellular DNA and RNA synthesis of malignant cells than agents acting on a single control point.

In conclusion, MTA is a new generation antifolate with inhibitory activity against multiple folate enzymes including TS, DHFR, and GARFT. In current phase II studies, MTA is broadly active as a single agent and has shown encouraging antitumor activity in multiple solid tumors. More advanced and extensive clinical trials of MTA are currently in progress, including trials in which the effects of MTA in combination with other agents such as cisplatin and gemcitabine are under investigation. The combination of a novel mode of action, preclinical and clinical activity, manageable and tolerable side effects and a dosing schedule that is easy to administer, indicates that MTA will play an important role in the treatment of patients with solid tumors.

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REFERENCES

1. Taylor EC, Harrington PJ, Fletcher SR, Beardsley GP, Moran RG. Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. *J Med Chem* 1985; 28:914–921.
2. Shih C, Grindey GB, Houghton PT, Houghton JA. The in vivo activity of 5,10-dideazatetrahydrofolic acid (DDATHF) and its diastereomers. *Proc Am Assoc Cancer Res* 1988; 29:283.
3. Beardsley GP, Moroson BA, Taylor EC, Moran RG. A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydofolate is a potent inhibitor of *de novo* purine synthesis. *J Biol Chem* 1989; 264:328–333.
4. Habeck LL, Leitner TA, Shackleford KA, Gossett LS, Schultz RM, Andis SL, Shih C, Grindey GB, Mendelsohn LG. A novel class of monoglutamated antifolates exhibits tight-binding inhibition of human glycinamide ribonucleotide formyltransferase and potent activity against solid tumors. *Cancer Res* 1994; 54:1021–1026.
5. Boritzki TJ, Bartlett CA, Howland EF, Margosiak SA, Palmer CL, Romines WH, Varney MD, Webber S, Jackson RC. Biological properties of AG2034: a new inhibitor of glycinamide ribonucleotide formyltransferase. *Proc Am Assoc Cancer Res* 1996; 37:385.
6. Eisenhauser EA, Wierzbicki R, Knowling M. Phase II trials of trimetrexate in advanced adult soft tissue sarcoma. *Ann Oncol* 1991; 2:689–690.
7. Sirotnak FM, DeGraw JI, Schmid FA, Goutas LJ, Moccio DM. New folate analogs of 10-deazaaminopterin series, further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models. *Can Chemother Pharmacol* 1984; 12:26–30.
8. Kris MG, Kinghan JJ, Gralla RJ, Fanucchi MP, Wertheim MS, O'Connel JP, Marks LD, Williams L, Farag F, Young CW, Sirotnak FM. Phase I trial and clinical pharmacological evaluation of 10-ethyl-10-deazaaminopterin in adult patients with advanced cancer. *Cancer Res* 1988; 48:5573–5579.
9. Rosowsky A, Bader H, Chen G, Mota CE, Vaidys C, Wright JE. Dihydrofolate reductase inhibition and in vivo antitumor activity of non-polyglutamatable analogs of aminopterin against human non-small cell lung carcinoma. *Proc Am Assoc Cancer Res* 1995; 36:376.
10. Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson I, Hughes LR. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo:* a new agent for clinical study. *Cancer Res* 1991; 51:5576–5586.
11. Marsham PR, Hughes LR, Jackman AL, Hayter AJ, Oldfield J, Wardleworth JM, Bishop JA, O'Connor BM, Calvert AH. Quinazoline antifolate thymidylate synthase inhibitors: heterocyclic benzoyl ring modifications. *J Med Chem* 1991; 34:1594–1605.

12. Calvete JA, Balmanno K, Taylor GA, Rafi I, Newell DR, Lind MJ, Calvert AH, Webber S, Clendennin NJ. Preclinical and clinical studies of prolonged administration of the novel thymidylate synthase inhibitor, AG337. *Proc Am Assoc Cancer Res* 1994; 35:306.
13. Duch DS, Banks S, Dec IK, Dickerson SH, Ferone R, Heath LS, Humphreys J, Knick V, Pendergast W, Singer S, Smith G, Waters K, Wilson R. Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. *Cancer Res* 1993; 53:810–818.
14. Jackman AL, Aherne GW, Kimbell R, Brunton L, Hardcastle A, Wardleworth JW, Stephens TC, Boyle FT. ZD9331, a novel non-polyglutamatable quinazoline thymidylate synthase inhibitor. *Proc Am Assoc Cancer Res* 1994; 35:301.
15. Baldwin SW, Tse A, Taylor EC, Rosowsky A, Gossett LS, Shih C, Moran RG. Structure features of 5,10-dideazatetrahydrofolate that determine inhibition of mammalian glycinamide ribonucleotide formyltransferase. *Biochemistry* 1991; 30:1997–2006.
16. Taylor EC, Kuhnt D, Shih C, Rinzel SM, Grindey GB, Barredo J, Jannatipour M, Moran RG. A dideazatetrahydrofolate analogue lacking a chiral center at C-6; N-{4-[2-(2-Amino-1,7-dihydro-4-oxopyrrolo[2,3-d]pyrimidin-6-yl)ethy l]benzoyl}glutamic acid, a new and potent inhibitor of thymidylate synthase. *J Med Chem* 1992; 35:4450–4454.
17. Habeck LL, Shih C, Gossett LS, Leitner TA, Schultz RM, Andis SL, Moran RG, Mendelsohn LG. Substrate specificity of mammalian folylpolyglutamate synthetase for 5,10-dideazatetrahydrofolate analogs. *Mol Pharmacol* 1995; 48:326–333.
18. Shih C, Chen VJ, Gossett LS, Gates SB, MacKellar WC, Habeck LL, Shackelford KA, Mendelsohn LG, Soose DJ, Patel VF, Andis SL, Bewley JR, Rayl EA, Morrison BA, Beardsley GP, Kohler W, Ratnam M, Schultz RM. LY231514, a pyrrolo[2,3-d]pyrimidine based antifolate that inhibits multiple folate requiring enzymes. *Cancer Res* 1997; 57:1116–1123.
19. Rinaldi DA, Burris HA, Dorr FA, Wordworth JR, Kuhn JG, Eckardt JR, et al. Initial phase I evaluation of the novel thymidylate synthase inhibitor, LY231514, using the modified continual reassessment method for dose escalation. *J Clin Oncol* 1995; 13:2842–50.
20. Rinaldi DA, Burris HA, Dorr FA, Rodriguez G, Eckhardt SG, Fields SM, et al. A phase I evaluation of LY231514, a novel multitargeted antifolate, administered every 21 days. *Proc Am Soc Clin Oncol* 1996; 16:A489.
21. McDonald AC, Vasey PA, Walling J, Woodworth JR, Abrahams T, Bailey NP, Siddiqui N, Lind MJ, Cassidy J, Twelves CJ. Phase I and pharmacokinetic study of LY2331514, the multi-targeted antifolate, administered by daily ×5, q21 schedule. *Ann Oncol* 1996; 7(suppl 5):20.
22. Clarke S, Boyer M, Millward M, Findlay M, Ackland S, Childs A, Brew S, Walcher V, Watt D, Thornton D. Phase II study of LY231514 in patients with advanced non-small cell lung cancer (NSCLC). *Lung Cancer* 1997; 18(suppl 1), 12, A34.
23. Cripps MC, Burnell M, Jolivet J, Lofters W, Fisher B, Panasci L, Iglesias J, Eisenhauer E. Phase II study of a multi-targeted antifolate (LY231514) (MTA) as first line therapy in patients with locally advanced or metastatic colorectal cancer (MCC). *Eur J Cancer* 1997; 33(suppl 8), S172, A768.
24. John W, Clark J, Burris H, Picus J, Schulamn L, Thornton D, Lochrer P. A phase II trial of LY231514 in patients with metastatic colorectal cancer. *Proc Am Soc Clin Oncol* 1997; 16:A1038.
25. Miller KD, Loehrer PJ, Picus J, Blanke C, John J, Schulman L, Burris H, Thornton D. A phase II trial of LY231514 in patients with unresectable pancreatic cancer. *Proc Am Soc Clin Oncol* 1997; 16:A1060.
26. Rusthoven J, Eisenhauer E, Bitts C, Gregg R, Dancey J, Fisher B, Iglesias J. A phase II study of the multi-targeted antifolate in patients with advanced non-small cell lung cancer. *Proc Am Soc Clin Oncol* 1997; 16:A1728.
27. Smith IE, Miles DW, Coleman RE, Lind MJ, McCarthy S Chick J. Phase II study of LY231514 (MTA) in patients with locally recurrent or metastatic breast cancer (LR/MBC)—an interim report. *Proc Am Soc Clin Oncol* 1997; 16:A671.
28. Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, Drake JC, Jolivet J. Polyglutamation of methotrexate, Is methotrexate a prodrug? *J Clin Invest* 1985; 76:907–912.
29. $H630_{R10}$ cells were obtained from Dr. Patrick G. Johnston, Queen's University, Belfast, UK. The characterization of the $H630_{R10}$ cell line can be found in reference: *Cancer Res* 1991; 51:66–68.
30. Pizzorno G, Moroson BA, Cashmore AR, Russello O, Mayer JR, Galivan J, Bunni MA, Priest DG, Beardsley GP. Multifactorial resistance to 5,10-dideazatetrahydrofolic acid in cell lines derived from human lymphoblastic leukemia CCRF-CEM. *Cancer Res* 1995; 55:566–573.

31. Dixon
lowing
cancer
32. Chen
of MT
Pools i
33. Chong
triphos
Biochε
34. Tonkin
fects c
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35. Worza
icity a
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36. Alati
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56:23
37. Wood
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38. *Clinic*
39. Zervo
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Soc C
40. Wooc
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col 19

31. Dixon KH, Mulligan T, Chung KN, Elwood PC, Cowan KH. Effects of folate receptor expression following stable transfection into wild type and methotrexate transport-deficient ZR-75-1 human breast cancer cells. *J Biol Chem* 1992; 26:24,140–24,147.
32. Chen VJ, Bewley JR, Andis SL, Schultz RM, Shih C, Mendelsohn LG, Seitz DE, Tonkinson JL. Effects of MTA (Multitargeted Antifolate, LY231514) on Intracellular Folate and Nucleoside Triphosphate Pools in CCRF-CEM cells, *Assoc Cancer Res Annual Meeting,* 1997.
33. Chong L and Tattersall MHN, 5,10-dideazatetrahydrofolic acid reduces toxicity and deoxyadenosine triphosphate pool expansion in cultured L1210 cells treated with inhibitors of thymidylate synthase. *Biochem Pharmacol* 1995; 49:819–827.
34. Tonkinson JL, Marder P, Andis SL, Schultz RM, Gossett LS, Shih C, Mendelsohn LG. Cell cycle effects of antifolate antimetabolites: implications for cytotoxicity and cytostasis. *Cancer Chemother Pharmacol* 1997; 39:521–540.
35. Worzalla JF, Self TD, Theobald KS, Schultz RM, Mendelsohn LG, Shih C. Effects of folic acid on toxicity and antitumor activity of LY231514 multitargeted antifolate (MTA). *Proc Am Assoc Cancer Res* 1997; 38:478.
36. Alati T, Worzalla JF, Shih C, Bewley JR, Lewis S, Moran RG, Grindey GB. Augmentation of the therapeutic activity of lometrexol [(6R)5,10-dideazatetrahydrofolate] by oral folic acid. *Cancer Res* 1996; 56:2331–2335.
37. Woodland JM, Barnett CJ, Dorman DE, Gruber JM, Shih C, Spangle LA, Wilson T, Ehlhardt WJ. The metabolism and disposition of the antifolate LY231514 (MTA) in mice and dogs. *Drug Metab Dispos* 1997; 25(6):693–700.
38. *Clinical Investigators' Brochure for LY231514.* Eli Lilly and Company, Indianapolis, IN.
39. Zervos PH, Faries D, Dorr FA, Rinaldi D, Storniolo AM, VonHoff DD. Practical use of the modified continual reassessment method (mCRM) for dose escalation in a phase I trial with LY231514. *Proc Am Soc Clin Oncol* 1995; 14:A473.
40. Woodworth J, Rinaldi D, Burris H, Thornton D, Reddy S, Kuhn J, VonHoff D. Assessments of hemotoxicity and relationships to pharmacokinetics from a LY231514 phase I study. *Proc Am Soc Clin Oncol* 1997; 16:A734.

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The master key effect of vitamin B12 in treatment of malignancy — A potential therapy?

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Summary Vitamin B_{12} plays a functional role in a variety of organs and body systems and the list of these organs and body systems is growing. According to our working hypothesis (''Master Key Effect'') vitamin B_{12} has some unique functions, which are still not accepted; vitamin B12 functions to keep body systems in balance, even under the stress of severe pathology. What is the explanation for elevation of cobalamin level in oncological patients?

1. It is well known that there is a high level of vitamin B12 in different kinds of malignancy.
2. There is a positive correlation between level of vitamin B12 and the severity of the disease, the more severe the disease the higher the level of B12.
3. A number of the experimental laboratory studies indicate an inhibition in the growth of malignant cells upon use of vitamin B12.
4. There are no experimental results indicating the opposite, that vitamin B12 stimulates growth of malignant cells.
5. There is no data about toxic effect of vitamin B12 in the treatment of various diseases.

As yet I have not been able to find another explanation for high level of vitamin B12 in oncology patients other than that it is a compensatory mechanism.

Perhaps following this body's ''warning sign'', we should start treatment with high doses of vitamin B12 to try to help the stabilization of normal function of the organs and systems. Laboratory researches should be continued to substantiate introduction of cobalamin as preliminary treatment of particular diseases.

Researchers have attempted to correlate vitamin B12 with malignancy ever since the multifunctional role of cobalamin has begun to be understood. Much research has been performed in laboratories *in vitro* and *in vivo*. There are many hypotheses about role of vitamin B12 in growth of malignancy, but we still have many more questions than we have answers. What is the explanation for elevation of cobalamine level in oncological patients? Is it a risk factor or an indication of developing neoplasms? Is it a result of liver cell damage, resulting in the release of free vitamin B12 in the serum? What about patients without hepatic damage? Is it marker of malignancy? Maybe human body is

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cleverer than we have thought and elevated vitamin B12 is a sign that the body is fighting disease, and that as result of this ''battle'', there is a mobilization of resources, and an attempt to compensate by activating biologically active substances for ''repair'' of defects!?

Multifunctional systems in the human body need to maintain homeostasis. Man is an ideal example of a system that constantly aspires to attain optimal regulation, even under the stress of severe pathology. There seems to be universal, interchangeable, biologically active substances that regulate the system and keep it in balance. I propose that one of these substances is vitamin B12.

Why vitamin B_{12}? Vitamin B_{12} plays a functional role in a variety of organs and body systems and the list of these organs and body systems is growing. It affects the peripheral and central nervous systems, bone marrow, skin and mucous membranes, bones, and vessels, as well as normal development during childhood. Vitamin B12 (cobalamin) is unique among all the vitamins in that it contains not only a complex organic molecule, but also an essential trace element, cobalt. Vitamin B12 plays an important role in DNA synthesis and has important immunomodulatory and neurotrophic effects. According to our ''working hypothesis'' vitamin B_{12} has some unique, but still unrecognized functions. It is possible that even when the serum cobalamin level is normal, treatment with vitamin B_{12} can correct defects caused by other biologically active substances. In our studies treatment with vitamin B12 has been successful in the treatment of recurrent aphthous stomatitis of any etiology, irrespective of B12 level in the blood before treatment! We call this phenomenon the ''Master Key Effect'' [1]. In this article I would like to review known data concerning the connection between vitamin B12 and malignancy and I will attempt to explain this according to the ''Master Key Effect'' hypothesis.

Elevated levels of serum cobalamin may be a sign of a serious, even life-threatening, disease. Hematologic disorders, like chronic myelogeneous leukemia, promyelocytic leukemia, polycythemia vera and also the hypereosinophilic syndrome, can result in elevated levels of cobalamin. Not surprisingly, a rise of the cobalamin concentration in serum is one of the diagnostic criteria for the latter two diseases. Several liver diseases, like acute hepatitis, cirrhosis, hepatocellular carcinoma and metastatic liver disease, can also be accompanied by an increase in circulating cobalamin. This phenomenon is caused predominantly by cobalamin release during hepatic cytolysis and/or decreased cobalamin clearance by the affected liver. Altogether it can be concluded that an observed elevation of cobalamin in blood merits a full diagnostic examination to assess the presence of disease [2].

Carmel et al. studied 139 patients with nonhematologic malignancy in order to define the incidence of vitamin B12-related abnormalities and correlate them with clinical findings. High serum vitamin B12 level usually implied a poor prognosis in a patient with cancer. However, while most such patients had hepatic and other metastases, hepatic involvement was not universal nor did most of the patients with hepatic disease have high vitamin B12 levels [3].

The relationship between vitamin B12 levels and survival was studied in a group of 161 terminally ill cancer patients. Their average age was 74.7 years. The length of survival decreased with the increase in serum vitamin B12 levels. In multivariate analyses, C-reactive protein was the most important prognostic factor in this population, and vitamin B12 provided information independent of CRP in predicting survival. These data indicate that an elevated serum vitamin B12 level is a predictive factor for mortality in patients with cancer, independent of CRP or other factors [4].

In one prospective study [5] researchers found high vitamin B12 levels in patients suffering from prostate cancer, and their conclusion was that cobalamin stimulates prostate cancer development...

The effect of cobalamin on the proliferation of malignant cells has been examined *in vivo* and *in vitro* in numerous studies [6—8]. Methylcobalamin inhibited the proliferation of androgen-sensitive SC-3 cells (a cloned cell line from Shionogi mouse mammary tumor, SC115) in culture at the concentration of 100—300 μg/ml. An inhibitory activity of methylcobalamin on the proliferation was also observed in other cell lines (estrogen-sensitive B-1F cells from mouse Leydig cell tumor and MCF-7 cells from human mammary tumor) at the concentration of 500 μg/ml. Moreover, large doses of methylcobalamin injected intraperitoneally (100 mg/kg body weight/day) were non-toxic and suppressed the tumor growth of SC115 and B-1F cells in mice fed a vitamin B12 deficient diet. These results indicate that methylcobalamin inhibits the proliferation of malignant cells in culture and *in vivo* and propose the possibility of methylcobalamin as a candidate of potentially useful agents for the treatment for some malignant tumors [6].

Malignancies are common in the digestive tube, although with unequal distribution among segments. The aim of a study conducted by Kurbel et al. was to compare available interpretations of the low cancer incidence in the small bowel and

high incidence in the large bowel. Small bowel mucosa is the main absorptive part of the digestive tube with absorption rates for various nutrients so high that they can even be considered as clearances from the intestinal content. Consequently, these nutrients are not present in the large bowel. An alternative explanation is that an absorbable protective substance from the intraluminal content might protect the mucosa from malignant transformations. It can be speculated that if there are any cytoprotective substances in the digested food their effect would be expressed mostly in the absorptive small intestine, leaving the large bowel mucosa unprotected. Vitamin B12 might be a possible candidate for this role. The results indicated that cobalamin availability showed similar distribution, available in low incidence segments and unavailable in high incidence segments [9].

Cobalamin carrier proteins, the transcobalamins (TC), were found to be elevated during trauma, infections and chronic inflammatory conditions. This remains un-explained. It is proposed that such TC elevations signal a need for cobalamin central to the resolution of inflammation [10]. Animal and human clinical data suggest that high dose cobalamin may prove a promising approach to systemic inflammatory response syndrome (SIRS), sepsis, septic and traumatic shock. Septic shock has an extremely high mortality rate, with approximately 200,000 people dying from sepsis annually in the US. The high mortality results in part from severe hypotension secondary to high serum nitric oxide (NO) concentrations. Initially, vitamin B12 was proposed for use as a scavenger and cytoprotective agent to bind and inactivate NO [11]. The use of vitamin B12 as a carrier to deliver nitric oxide into tumor cells is novel [12,13]. A number of studies have demonstrated that cobalamin is important in maintaining differentiation, proliferation, and metabolic status of cells. NO inactivates vitamin B12 and methionine synthase, thereby impairing DNA formation and, consequently, new cell formation. The vitamins: folic acid, B12 and B6 and B2 are the source of coenzymes which participate in one carbon metabolism [14]. The overall functions of vitamin B12, as a source of coenzymes in intracellular recycling of methionine, in methionine synthase reaction, in the prevention of chromosome breakage, in methylation, and in maintaining a one-carbon metabolic balance, have been reviewed. NO can cause both apoptosis and necrosis, making it a good candidate for antitumor therapy. In one investigational study it was shown that complex NO-cobalamin inhibited tumor growth *in vivo* and *in vitro* by activating the extrinsic apoptotic pathway [12]. Inhibition of methionine synthase also creates a ''methylfolate trap'', analogous to what occurs in vitamin B12 deficiency [15,16]. Haematological sequel of vitamin B12 deficiency are attributed to disturbed DNA synthesis, but vitamin B12 itself plays no role in DNA biosynthesis. A proposed explanation for this is the methylfolate trap hypothesis. This hypothesis states that B12 deficiency impairs overall folate metabolism, because 5-methyltetrahydrofolate (5MTHF) becomes metabolically trapped. This trap results from the fact that 5MTHF can neither be metabolised via the methionine synthase pathway, nor can it be reconverted to its precursor, methylenetetrahydrofolate [17]. Methionine metabolism and transmethylation are frequently altered in cancer cells. The alteration is often expressed as an inability of the cancer cells to grow when methionine is replaced by homocysteine in the culture medium, a condition that allows the growth of normal cells. This metabolic defect is termed methionine dependence [18]. Methionine dependence is unique to cancer cells and defined as the inability to grow in a methionine-deprived environment, even if supplemented with the metabolic precursor homocysteine. Cobalamin-dependent methionine synthase catalyses the formation of methionine and tetrahydrofolate from homocysteine and methyltetrahydrofolate, thus linking the methionine and folate pathways [19].

Interactions between adaptative and selective processes are illustrated in the model of recursive causality as defined in Rupert Riedl's systems theory of evolution [20]. One of the main features of this theory, also termed the theory of evolving complexity, is central to the notion of recursive or feedback causality – the idea that every biological effect in living systems in some way feeds back to its own cause. There is a hypothesis that ''recursive'' or ''feedback'' causality provides a model for explaining the consequences of interacting genetic and epigenetic mechanisms, which are known to play a key role in development of cancer [21]. Epigenetics includes any process that alters gene activity without changes of the DNA sequence. The most important epigenetic mechanisms are DNA-methylation and chromatin remodeling. Hypomethylation of so-called oncogenes and hypermethylation of tumor suppressor genes appear to be critical determinants of cancer. Folic acid, vitamin B12 and other nutrients influence the function of enzymes that participate in various methylation processes by affecting the supply of methyl groups into a variety of molecules which may be directly or indirectly associated with cancerogenesis. The enzymes also play a role in development and differentiation of cells and organisms and thus illustrate the close association between evolutionary and developmental

mechanisms. This enabled new ways to understand the interaction between the genome and environment and may improve biomedical concepts, including environmental health aspects where epigenetic and genetic modifications are closely associated. For example, recent observations showed that methylated nucleotides in the gene promoter may serve as a target for solar UV-induced mutations of the p53 tumor suppressor gene [22]. This illustrates the close interaction of genetic and epigenetic mechanisms in cancerogenesis resulting from changes in transcriptional regulation and its contribution to a phenotype at the micro- or macro-evolutionary level. The above-mentioned interactions of genetic and epigenetic mechanisms in oncogenesis defy explanation by simple linear causality, like the continuing adaptability of complex systems. They can be explained by the concept of recursive causality and has introduced molecular biology into the realm of cognition science and systems theory; based on the notion of feedback or recursive causality, a model for epigenetic mechanisms with relevance for oncology and biomedicine is provided.

As was mentioned above, every system in mechanics and nature seeks some sort of balance. The human body, as an example of a multifunctional system, is no exception to this rule. Upon an imbalance or disease, the organism tries to compensate by mobilization of its inner resources. There is an on-going process of accumulation of biologically active substances to fight disease. Parenthetically, one of conventional, as well as non-conventional, ways of treating diseases is using a medicine (substance) or different physical methods which are able to activate these substances. The human organism has been evolved over millions of years and thus, it is reasonable to expect it to react appropriately. Unfortunately, this battle for balance is not always successful. Being stressed the organism ''shows'' us signs of distress and ''calls for help''. The only thing that we must do is to know how to read them.

I propose that a high level of vitamin B12 in oncological diseases is such a sign. What is the basis for such hypothesis? The basis for such a proposal can be summarized as follows:

1. The well known fact that a high level of vitamin B12 is present in different kinds of malignancy.
2. There is a positive correlation between level of vitamin B12 and the severity of the disease, the more severe the disease the higher the level of B12.
3. A number of the experimental laboratory studies indicate an inhibition in the growth of malignant cells upon use of vitamin B12.
4. There are no experimental results indicating the opposite, that vitamin B12 stimulates growth of malignant cells.
5. There is no data about toxic effect of vitamin B12 in the treatment of various diseases. Sometimes there is necessary to use very high doses to achieve therapeutic effect [23]. Vitamin B12 is the only known vitamin that does not have any toxic effect even in such cases.

As yet I have not been able to find another explanation for high level of vitamin B12 in oncology patients other than that it is a compensatory mechanism.

Possibly following this body's ''warning sign'', we should start preliminary treatment with high doses of vitamin B12 to try to stabilize normal body functioning of the various organs and organ systems.

Analyzing the literature of research in cancer which investigated the association with vitamin B12, I do not understand why these studies indicating a positive results did not investigate this association further. Perhaps this can be explained as follows:

1. Preference for treatment with vitamin B12 to modern perspective medicines does not seem appropriate for oncology patients who do not have time for such kind of experiments (an ethical question).
2. The unconvincing or unequivocal results of the research, which could be a consequence of using not high enough doses of vitamin B12, do not encourage oncologists to try vitamin B12 treatment.
3. The paradoxical dilemma, in which the solution is so close, well-known, accessible, and cheap, makes it hard to believe that vitamin B12 may be effective in the treatment of oncology diseases.

Laboratory research should be continued even if it might disprove the above-stated hypothesis. And who knows? It may in fact provide us with more evidence of the effectiveness of vitamin B12 in the treatment of malignant diseases.

References

[1] Volkov I, Press Y, Rudoy I. Vitamin B_{12} could be a ''MASTER KEY'' in the regulation of multiple pathological processes. J Nippon Med School 2006;73(2):65—9.

[2] Ermens AA, Vlasveld LT, van Marion-Kievit JA, Lensen CJ, Lindemans J. The significance of an elevated cobalamin concentration in the blood. Ned Tijdschr Geneeskd 2002;146(10):459—64.

[3] Carmel R, Eisenberg L. Serum vitamin B12 and transcobalamin abnormalities in patients with cancer. Cancer 1977;40(3):1348—53.
[4] Geissbuhler P, Mermillod B, Rapin CH. Elevated serum vitamin B12 levels associated with CRP as a predictive factor of mortality in palliative care cancer patients: a prospective study over five years. J Pain Symptom Manage 2000;20(2):93—103.
[5] Hultdin J, Van Guelpen B, Bergh A, Hallmans G, Stattin P. Plasma folate, vitamin B12, and homocysteine and prostate cancer risk: a prospective study. Int J Cancer 2005;113(5): 819—24.
[6] Nishizawa Y, Yamamoto T, Terada N, Fushiki S, Matsumoto K, Nishizawa Y. Effects of methylcobalamin on the proliferation of androgen-sensitive or estrogen-sensitive malignant cells in culture and in vivo. Int J Vitam Nutr Res 1997;67(3):164—70.
[7] Liteplo RG, Hipwell SE, Rosenblatt DS, Sillaots S, Lue-Shing H. Changes in cobalamin metabolism are associated with the altered methionine auxotrophy of highly growth autonomous human melanoma cells. J Cell Physiol 1991;149(2): 332—8.
[8] Fiskerstrand T, Christensen B, Tysnes OB, Ueland PM, Refsum H. Development and reversion of methionine dependence in a human glioma cell line: relation to homocysteine remethylation and cobalamin status. Cancer Res 1994;54(18):4899—906.
[9] Kurbel S, Kovacic D, Radic R, Drenjancevic I, Glavina K, Ivandic A. Cancer incidences in the digestive tube: is cobalamin a small intestine cytoprotector? Med Hypotheses 2000;54(3):412—6.
[10] Wheatley C. A scarlet pimpernel for the resolution of inflammation? The role of supra-therapeutic doses of cobalamin, in the treatment of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic or traumatic shock. Med Hypotheses 2006;67(1):4—42. Epub 2006 Mar 20.
[11] Broderick KE, Feala J, McCulloch A, Paternostro G, Sharma VS, Pilz RB, et al. The nitric oxide scavenger cobinamide profoundly improves survival in *Drosophila melanogaster* model of bacterial sepsis. FASEB J 2006;20(11):1865—73.
[12] Bauer JA, Morrison BH, Grane RW, Jacobs BS, Dabney S, Gamero AM, et al. Effects of interferon beta on transcobalamin II-receptor expression and antitumor activity of nytrosylcobalamin. J Natr Cancer Inst 2002;94(13): 1010—9.
[13] Tang Z, Bauer JA, Morrison B, Lindner DJ. Nitrosylcobalamin promotes cell death via S nitrosylation of Apo2L/TRAIL receptor DR4. Mol Cell Biol 2006;26(15): 5588—94.
[14] Selhub J. Folate, vitamin B12 and vitamin B6 and one carbon metabolism. J Nutr Health Aging 2002;6(1):39—42.
[15] Friso S, Choi SW. The potential cocarcinogenic effect of vitamin B(12) deficiency. Clin Chem Lab Med 2005;43(10): 1158—63.
[16] Mason JB, Choi SW. Effects of alcohol on folate metabolism: implications for carcinogenesis. Alcohol 2005;35(3): 235—41. Review.
[17] Smulders YM, Smith DE, Kok RM, Teerlink T, Swinkels DW, Stehouwer CD, et al. Cellular folate vitamer distribution during and after correction of vitamin B12 deficiency: a case for the methylfolate trap. Br J Haematol 2006;132(5): 623—9.
[18] Hoffman RM. Altered methionine metabolism and transmethylation in cancer. Anticancer Res 1985;5(1):1—30.
[19] Blackburn A, Bibby MC, Lucock MD, Nicolaou A. Temporal evaluation of methionine synthase and related metabolites in the MAC15A mouse adenocarcinoma animal model. Int J Cancer 2004;112(4):577—84.
[20] Wagner GP, Laubichler MD. Rupert Riedl and the resynthesis of evolutionary and developmental biology: body plans and evolvability. J Exp Zool B Mol Dev Evol 2004;302(1):92—102.
[21] Haslberger A, Varga F, Karlic H. Recursive causality in evolution: A model for epigenetic mechanisms in cancer development. Med Hypotheses, 2006, July 14.
[22] Benjamin CL, Ananthaswamy HN. p53 and the pathogenesis of skin cancer. Toxicol Appl Pharmacol., 2006, December 15 [Epub ahead of print].
[23] Kira J, Tobimatsu S, Goto I. Vitamin B_{12} metabolism and massive-dose methyl vitamin B_{12} therapy in Japanese patients with multiple sclerosis. Intern Med., 1994.

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use ALIMTA safely and effectively. See full prescribing information for ALIMTA.

ALIMTA (pemetrexed disodium) Injection, Powder, Lyophilized, For Solution for Intravenous Use

Initial U.S. Approval: 2004

RECENT MAJOR CHANGES

Indications and Usage, Locally Advanced or Metastatic Nonsquamous Non-Small Cell Lung Cancer - Combination with Cisplatin (1.1) 09/2008

Indications and Usage, Locally Advanced or Metastatic Nonsquamous Non-Small Cell Lung Cancer - Maintenance (1.2) MM/YYYY

Indications and Usage, Locally Advanced or Metastatic Nonsquamous Non-Small Cell Lung Cancer - After Prior Chemotherapy (1.3) 09/2008

Dosage and Administration, Combination Use with Cisplatin (2.1) 09/2008

INDICATIONS AND USAGE

ALIMTA® is a folate analog metabolic inhibitor indicated for:

- Locally Advanced or Metastatic Nonsquamous Non-Small Cell Lung Cancer:
  - Initial treatment in combination with cisplatin. (1.1)
  - Maintenance treatment of patients whose disease has not progressed after four cycles of platinum-based first-line chemotherapy. (1.2)
  - After prior chemotherapy as a single agent. (1.3)
- Mesothelioma: in combination with cisplatin. (1.4)

Limitations of Use:

- ALIMTA is not indicated for the treatment of patients with squamous cell non-small cell lung cancer. (1.5)

DOSAGE AND ADMINISTRATION

- Combination use in Non-Small Cell Lung Cancer and Mesothelioma: Recommended dose of ALIMTA is 500 mg/m^2 i.v. on Day 1 of each 21-day cycle in combination with cisplatin 75 mg/m^2 i.v. beginning 30 minutes after ALIMTA administration. (2.1)
- Single-Agent use in Non-Small Cell Lung Cancer: Recommended dose of ALIMTA is 500 mg/m^2 i.v. on Day 1 of each 21-day cycle. (2.2)
- Dose Reductions: Dose reductions or discontinuation may be needed based on toxicities from the preceding cycle of therapy. (2.4)

DOSAGE FORMS AND STRENGTHS

- 100 mg vial for injection (3)
- 500 mg vial for injection (3)

CONTRAINDICATIONS

History of severe hypersensitivity reaction to pemetrexed. (4)

WARNINGS AND PRECAUTIONS

- Premedication regimen: Instruct patients to take folic acid and vitamin B_{12}. Pretreatment with dexamethasone or equivalent reduces cutaneous reaction. (5.1)
- Bone marrow suppression: Reduce doses for subsequent cycles based on hematologic and nonhematologic toxicities. (5.2)
- Renal function: Do not administer when CrCl <45 mL/min. (2.4, 5.3)
- NSAIDs with renal insufficiency: Use caution in patients with mild to moderate renal insufficiency (CrCl 45-79 mL/min). (5.4)
- Lab monitoring: Do not begin next cycle unless ANC ≥1500 cells/mm^3, platelets ≥100,000 cells/mm^3, and CrCl ≥45 mL/min. (5.5)
- Pregnancy: Fetal harm can occur when administered to a pregnant woman. Women should be advised to use effective contraception measures to prevent pregnancy during treatment with ALIMTA. (5.6)

ADVERSE REACTIONS

The most common adverse reactions (incidence ≥20%) with single-agent use are fatigue, nausea, and anorexia. Additional common adverse reactions when used in combination with cisplatin include vomiting, neutropenia, leukopenia, anemia, stomatitis/pharyngitis, thrombocytopenia, and constipation. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Eli Lilly and Company at 1-800-LillyRx (1-800-545-5979) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- NSAIDs: Use caution with ibuprofen or other NSAIDs. (7.1)
- Nephrotoxic drugs: Concomitant use of these drugs and/or substances which are tubularly secreted may result in delayed clearance. (7.2)

See 17 for PATIENT COUNSELING INFORMATION and FDA-approved patient labeling

Revised: 00/0000

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Nonsquamous Non-Small Cell Lung Cancer - Combination with Cisplatin

ALIMTA is indicated in combination with cisplatin therapy for the initial treatment of patients with locally advanced or metastatic nonsquamous non-small cell lung cancer.

1.2 Nonsquamous Non-Small Cell Lung Cancer - Maintenance

ALIMTA is indicated for the maintenance treatment of patients with locally advanced or metastatic nonsquamous non-small cell lung cancer whose disease has not progressed after four cycles of platinum-based first-line chemotherapy.

1.3 Nonsquamous Non-Small Cell Lung Cancer - After Prior Chemotherapy

ALIMTA is indicated as a single-agent for the treatment of patients with locally advanced or metastatic nonsquamous non-small cell lung cancer after prior chemotherapy.

1.4 Mesothelioma

ALIMTA in combination with cisplatin is indicated for the treatment of patients with malignant pleural mesothelioma whose disease is unresectable or who are otherwise not candidates for curative surgery.

1.5 Limitations of Use

ALIMTA is not indicated for the treatment of patients with squamous cell non-small cell lung cancer. *[see Clinical Studies (14.1, 14.2, 14.3)]*

2 DOSAGE AND ADMINISTRATION

2.1 Combination Use with Cisplatin

Nonsquamous Non-Small Cell Lung Cancer and Malignant Pleural Mesothelioma

The recommended dose of ALIMTA is 500 mg/m^2 administered as an intravenous infusion over 10 minutes on Day 1 of each 21-day cycle. The recommended dose of cisplatin is 75 mg/m^2 infused over 2 hours beginning approximately 30 minutes after the end of ALIMTA administration. Patients should receive appropriate hydration prior to and/or after receiving cisplatin. See cisplatin package insert for more information.

2.2 Single-Agent Use

Nonsquamous Non-Small Cell Lung Cancer

The recommended dose of ALIMTA is 500 mg/m^2 administered as an intravenous infusion over 10 minutes on Day 1 of each 21-day cycle.

2.3 Premedication Regimen

Vitamin Supplementation

To reduce toxicity, patients treated with ALIMTA must be instructed to take a low-dose oral folic acid preparation or multivitamin with folic acid on a daily basis. At least 5 daily doses of folic acid must be taken during the 7-day period preceding the first dose of ALIMTA; and dosing should continue during the full course of therapy and for 21 days after the last dose of ALIMTA. Patients must also receive one (1) intramuscular injection of vitamin B_{12} during the week preceding the first dose of ALIMTA and every 3 cycles thereafter. Subsequent vitamin B_{12} injections may be given the same day as ALIMTA. In clinical trials, the dose of folic acid studied ranged from 350 to 1000 mcg, and the dose of vitamin B_{12} was 1000 mcg. The most commonly used dose of oral folic acid in clinical trials was 400 mcg *[see Warnings and Precautions (5.1)]*.

Corticosteroid

Skin rash has been reported more frequently in patients not pretreated with a corticosteroid. Pretreatment with dexamethasone (or equivalent) reduces the incidence and severity of cutaneous reaction. In clinical trials, dexamethasone 4 mg was given by mouth twice daily the day before, the day of, and the day after ALIMTA administration *[see Warnings and Precautions (5.1)]*.

2.4 Laboratory Monitoring and Dose Reduction/Discontinuation Recommendations

Monitoring

Complete blood cell counts, including platelet counts, should be performed on all patients receiving ALIMTA. Patients should be monitored for nadir and recovery, which were tested in the clinical study before each dose and on days 8 and 15 of each cycle. Patients should not begin a new cycle of treatment unless the ANC is $\geq$1500 cells/mm^3, the platelet count is $\geq$100,000 cells/mm^3, and creatinine clearance is $\geq$45 mL/min. Periodic chemistry tests should be performed to evaluate renal and hepatic function *[see Warnings and Precautions (5.5)]*.

Dose Reduction Recommendations

Dose adjustments at the start of a subsequent cycle should be based on nadir hematologic counts or maximum nonhematologic toxicity from the preceding cycle of therapy. Treatment may be delayed to allow sufficient time for recovery. Upon recovery, patients should be retreated using the guidelines in Tables 1-3, which are suitable for using ALIMTA as a single-agent or in combination with cisplatin.

Table 1: Dose Reduction for ALIMTA (single-agent or in combination) and Cisplatin - Hematologic Toxicities

Nadir ANC <500/mm^3 and nadir platelets ≥50,000/mm^3.	75% of previous dose (pemetrexed and cisplatin).
Nadir platelets <50,000/mm^3 without bleeding regardless of nadir ANC.	75% of previous dose (pemetrexed and cisplatin).
Nadir platelets <50,000/mm^3 with bleeding[a], regardless of nadir ANC.	50% of previous dose (pemetrexed and cisplatin).

[a] These criteria meet the CTC version 2.0 (NCI 1998) definition of ≥CTC Grade 2 bleeding.

If patients develop nonhematologic toxicities (excluding neurotoxicity) ≥Grade 3, treatment should be withheld until resolution to less than or equal to the patient's pre-therapy value. Treatment should be resumed according to guidelines in Table 2.

Table 2: Dose Reduction for ALIMTA (single-agent or in combination) and Cisplatin - Nonhematologic Toxicities[a,b]

	Dose of ALIMTA (mg/m^2)	Dose of Cisplatin (mg/m^2)
Any Grade 3 or 4 toxicities except mucositis	75% of previous dose	75% of previous dose
Any diarrhea requiring hospitalization (irrespective of Grade) or Grade 3 or 4 diarrhea	75% of previous dose	75% of previous dose
Grade 3 or 4 mucositis	50% of previous dose	100% of previous dose

[a] NCI Common Toxicity Criteria (CTC).

[b] Excluding neurotoxicity (see Table 3).

In the event of neurotoxicity, the recommended dose adjustments for ALIMTA and cisplatin are described in Table 3. Patients should discontinue therapy if Grade 3 or 4 neurotoxicity is experienced.

Table 3: Dose Reduction for ALIMTA (single-agent or in combination) and Cisplatin - Neurotoxicity

CTC Grade	Dose of ALIMTA (mg/m^2)	Dose of Cisplatin (mg/m^2)
0-1	100% of previous dose	100% of previous dose
2	100% of previous dose	50% of previous dose

Discontinuation Recommendation

ALIMTA therapy should be discontinued if a patient experiences any hematologic or nonhematologic Grade 3 or 4 toxicity after 2 dose reductions or immediately if Grade 3 or 4 neurotoxicity is observed.

Renally Impaired Patients

In clinical studies, patients with creatinine clearance ≥45 mL/min required no dose adjustments other than those recommended for all patients. Insufficient numbers of patients with creatinine clearance below 45 mL/min have been treated to make dosage recommendations for this group of patients *[see Clinical Pharmacology (12.3)]*. Therefore, ALIMTA should not be administered to patients whose creatinine clearance is <45 mL/min using the standard Cockcroft and Gault formula (below) or GFR measured by Tc99m-DPTA serum clearance method:

$$\text{Males: } \frac{[140 - \text{Age in years}] \times \text{Actual Body Weight (kg)}}{72 \times \text{Serum Creatinine (mg/dL)}} = \text{mL/min}$$

Females: Estimated creatinine clearance for males × 0.85

Caution should be exercised when administering ALIMTA concurrently with NSAIDs to patients whose creatinine clearance is <80 mL/min *[see Drug Interactions (7.1)]*.

2.5 Preparation and Administration Precautions

As with other potentially toxic anticancer agents, care should be exercised in the handling and preparation of infusion solutions of ALIMTA. The use of gloves is recommended. If a solution of ALIMTA contacts the skin, wash the skin immediately and thoroughly with soap and water. If ALIMTA contacts the mucous membranes, flush thoroughly with water. Several published guidelines for handling and disposal of anticancer agents are available *[see References (15)]*.

ALIMTA is not a vesicant. There is no specific antidote for extravasation of ALIMTA. To date, there have been few reported cases of ALIMTA extravasation, which were not assessed as serious by the investigator. ALIMTA extravasation should be managed with local standard practice for extravasation as with other non-vesicants.

2.6 Preparation for Intravenous Infusion Administration

1. Use aseptic technique during the reconstitution and further dilution of ALIMTA for intravenous infusion administration.
2. Calculate the dose of ALIMTA and determine the number of vials needed. Vials contain either 100 mg or 500 mg of ALIMTA. The vials contain an excess of ALIMTA to facilitate delivery of label amount.

3. Reconstitute each 100-mg vial with 4.2 ml of 0.9% Sodium Chloride Injection (preservative free). Reconstitute each 500-mg vial with 20 mL of 0.9% Sodium Chloride Injection (preservative free). Reconstitution of either size vial gives a solution containing 25 mg/mL ALIMTA. Gently swirl each vial until the powder is completely dissolved. The resulting solution is clear and ranges in color from colorless to yellow or green-yellow without adversely affecting product quality. The pH of the reconstituted ALIMTA solution is between 6.6 and 7.8. FURTHER DILUTION IS REQUIRED.
4. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. If particulate matter is observed, do not administer.
5. An appropriate quantity of the reconstituted ALIMTA solution must be further diluted into a solution of 0.9% Sodium Chloride Injection (preservative free), so that the total volume of solution is 100 ml. ALIMTA is administered as an intravenous infusion over 10 minutes.
6. Chemical and physical stability of reconstituted and infusion solutions of ALIMTA were demonstrated for up to 24 hours following initial reconstitution, when stored at refrigerated or ambient room temperature [see USP Controlled Room Temperature] and lighting. When prepared as directed, reconstitution and infusion solutions of ALIMTA contain no antimicrobial preservatives. Discard any unused portion.

Reconstitution and further dilution prior to intravenous infusion is only recommended with 0.9% Sodium Chloride Injection (preservative free). ALIMTA is physically incompatible with diluents containing calcium, including Lactated Ringer's Injection, USP and Ringer's Injection, USP and therefore these should not be used. Coadministration of ALIMTA with other drugs and diluents has not been studied, and therefore is not recommended. ALIMTA is compatible with standard polyvinyl chloride (PVC) administration sets and intravenous solution bags.

3 DOSAGE FORMS AND STRENGTHS

ALIMTA, pemetrexed for injection, is a white to either light-yellow or green-yellow lyophilized powder available in sterile single-use vials containing 100 mg or 500 mg pemetrexed.

4 CONTRAINDICATIONS

ALIMTA is contraindicated in patients who have a history of severe hypersensitivity reaction to pemetrexed or to any other ingredient used in the formulation.

5 WARNINGS AND PRECAUTIONS

5.1 Premedication Regimen

Need for Folate and Vitamin B_{12} Supplementation

Patients treated with ALIMTA must be instructed to take folic acid and vitamin B_{12} as a prophylactic measure to reduce treatment-related hematologic and GI toxicity *[see Dosage and Administration (2.3)]*. In clinical studies, less overall toxicity and reductions in Grade 3/4 hematologic and nonhematologic toxicities such as neutropenia, febrile neutropenia, and infection with Grade 3/4 neutropenia were reported when pretreatment with folic acid and vitamin B_{12} was administered.

Corticosteroid Supplementation

Skin rash has been reported more frequently in patients not pretreated with a corticosteroid in clinical trials. Pretreatment with dexamethasone (or equivalent) reduces the incidence and severity of cutaneous reaction *[see Dosage and Administration (2.3)]*.

5.2 Bone Marrow Suppression

ALIMTA can suppress bone marrow function, as manifested by neutropenia, thrombocytopenia, and anemia (or pancytopenia) *[see Adverse Reactions (6.1)]*; myelosuppression is usually the dose-limiting toxicity. Dose reductions for subsequent cycles are based on nadir ANC, platelet count, and maximum nonhematologic toxicity seen in the previous cycle *[see Dosage and Administration (2.4)]*.

5.3 Decreased Renal Function

ALIMTA is primarily eliminated unchanged by renal excretion. No dosage adjustment is needed in patients with creatinine clearance ≥45 mL/min. Insufficient numbers of patients have been studied with creatinine clearance <45 mL/min to give a dose recommendation. Therefore, ALIMTA should not be administered to patients whose creatinine clearance is <45 mL/min *[see Dosage and Administration (2.4)]*.

One patient with severe renal impairment (creatinine clearance 19 mL/min) who did not receive folic acid and vitamin B_{12} died of drug-related toxicity following administration of ALIMTA alone.

5.4 Use with Non-Steroidal Anti-Inflammatory Drugs with Mild to Moderate Renal Insufficiency

Caution should be used when administering ibuprofen concurrently with ALIMTA to patients with mild to moderate renal insufficiency (creatinine clearance from 45 to 79 mL/min). Other NSAIDs should also be used with caution *[see Drug Interactions (7.1)]*.

5.5 Required Laboratory Monitoring

Patients should not begin a new cycle of treatment unless the ANC is ≥1500 cells/mm^3, the platelet count is ≥100,000 cells/mm^3, and creatinine clearance is ≥45 mL/min *[see Dosage and Administration (2.4)]*.

5.6 Pregnancy Category D

Based on its mechanism of action, ALIMTA can cause fetal harm when administered to a pregnant woman. Pemetrexed administered intraperitoneally to mice during organogenesis was embryotoxic, fetotoxic and teratogenic in mice at greater than

1/833rd the recommended human dose. If ALIMTA is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus. Women of childbearing potential should be advised to avoid becoming pregnant. Women should be advised to use effective contraceptive measures to prevent pregnancy during treatment with ALIMTA *[see Use in Specific Populations (8.1)]*.

5.7 Third Space Fluid

The effect of third space fluid, such as pleural effusion and ascites, on ALIMTA is unknown. In patients with clinically significant third space fluid, consideration should be given to draining the effusion prior to ALIMTA administration.

6 ADVERSE REACTIONS

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reactions rates cannot be directly compared to rates in other clinical trials and may not reflect the rates observed in clinical practice.

In clinical trials, the most common adverse reactions (incidence ≥20%) during therapy with ALIMTA as a single-agent were fatigue, nausea, and anorexia. Additional common adverse reactions (incidence ≥20%) during therapy with ALIMTA when used in combination with cisplatin included vomiting, neutropenia, leukopenia, anemia, stomatitis/pharyngitis, thrombocytopenia, and constipation.

Non-Small Cell Lung Cancer (NSCLC) - Combination with Cisplatin

Table 4 provides the frequency and severity of adverse reactions that have been reported in >5% of 839 patients with NSCLC who were randomized to study and received ALIMTA plus cisplatin and 830 patients with NSCLC who were randomized to study and received gemcitabine plus cisplatin. All patients received study therapy as initial treatment for locally advanced or metastatic NSCLC and patients in both treatment groups were fully supplemented with folic acid and vitamin B_{12}.

Table 4: Adverse Reactions in Fully Supplemented Patients Receiving ALIMTA plus Cisplatin in NSCLC[a]

Reaction[b]	ALIMTA/cisplatin (N=839)		Gemcitabine/cisplatin (N=830)	
	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)
All Adverse Reactions	90	37	91	53
Laboratory				
Hematologic				
Anemia	33	6	46	10
Neutropenia	29	15	38	27
Leukopenia	18	5	21	8
Thrombocytopenia	10	4	27	13
Renal				
Creatinine elevation	10	1	7	1
Clinical				
Constitutional Symptoms				
Fatigue	43	7	45	5
Gastrointestinal				
Nausea	56	7	53	4
Vomiting	40	6	36	6
Anorexia	27	2	24	1
Constipation	21	1	20	0
Stomatitis/Pharyngitis	14	1	12	0
Diarrhea	12	1	13	2
Dyspepsia/Heartburn	5	0	6	0
Neurology				
Neuropathy-sensory	9	0	12	1
Taste disturbance	8	0[c]	9	0[c]
Dermatology/Skin				
Alopecia	12	0[c]	21	1[c]
Rash/Desquamation	7	0	8	1

[a] For the purpose of this table a cut off of 5% was used for inclusion of all events where the reporter considered a possible relationship to ALIMTA.

[b] Refer to NCI CTC Criteria version 2.0 for each Grade of toxicity.

[c] According to NCI CTC Criteria version 2.0, this adverse event term should only be reported as Grade 1 or 2.

No clinically relevant differences in adverse reactions were seen in patients based on histology.

In addition to the lower incidence of hematologic toxicity on the ALIMTA and cisplatin arm, use of transfusions (RBC and platelet) and hematopoietic growth factors was lower in the ALIMTA and cisplatin arm compared to the gemcitabine and cisplatin arm.

The following additional adverse reactions were observed in patients with non-small cell lung cancer randomly assigned to receive ALIMTA plus cisplatin.

Incidence 1% to 5%

Body as a Whole — febrile neutropenia, infection, pyrexia
General Disorders — dehydration
Metabolism and Nutrition — increased AST, increased ALT
Renal — creatinine clearance decrease, renal failure
Special Senses — conjunctivitis

Incidence Less than 1%

Cardiovascular — arrhythmia
General Disorders — chest pain
Metabolism and Nutrition — increased GGT
Neurology — motor neuropathy

Non-Small Cell Lung Cancer (NSCLC) - Maintenance

Table 5 provides the frequency and severity of adverse reactions that have been reported in >5% of 438 patients with NSCLC who received ALIMTA and 218 patients with NSCLC who received placebo. All patients received study therapy immediately following 4 cycles of platinum-based treatment for locally advanced or metastatic NSCLC. Patients in both study arms were fully supplemented with folic acid and vitamin B_{12}.

Table 5: Adverse Reactions in Patients Receiving ALIMTA versus Placebo in NSCLC[a]

Reaction[b]	ALIMTA (N=438)		Placebo (N=218)	
	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)
All Adverse Reactions	66	16	37	4
Laboratory				
Hematologic				
Anemia	15	3	6	1
Neutropenia	6	3	0	0
Leukopenia	6	2	1	1
Hepatic				
Increased ALT	10	0	4	0
Increased AST	8	0	4	0
Clinical				
Constitutional Symptoms				
Fatigue	25	5	11	1
Gastrointestinal				
Nausea	19	1	6	1
Anorexia	19	2	5	0
Vomiting	9	0	1	0
Mucositis/stomatitis	7	1	2	0
Diarrhea	5	1	3	0
Infection	5	2	2	0
Neurology				
Neuropathy-sensory	9	1	4	0
Dermatology/Skin				
Rash/Desquamation	10	0	3	0

[a] For the purpose of this table a cut off of 5% was used for inclusion of all events where the reporter considered a possible relationship to ALIMTA.

[b] Refer to NCI CTCAE Criteria version 3.0 for each Grade of toxicity.

No clinically relevant differences in Grade 3/4 adverse reactions were seen in patients based on age, gender, ethnic origin, or histology except a higher incidence of Grade 3/4 fatigue for Caucasian patients compared to non-Caucasian patients (6.5% versus 0.6%).

Safety was assessed by exposure for patients who received at least one dose of ALIMTA (N=438). The incidence of adverse reactions was evaluated for patients who received ≤6 cycles of ALIMTA, and compared to patients who received >6 cycles of

ALIMTA. Increases in adverse reactions (all grades) were observed with longer exposure; however no clinically relevant differences in Grade 3/4 adverse reactions were seen.

Consistent with the higher incidence of anemia (all grades) on the ALIMTA arm, use of transfusions (mainly RBC) and erythropoiesis stimulating agents (ESAs; erythropoietin and darbepoetin) were higher in the ALIMTA arm compared to the placebo arm (transfusions 9.5% versus 3.2%, ESAs 5.9% versus 1.8%).

The following additional adverse reactions were observed in patients with non-small cell lung cancer who received ALIMTA.

Incidence 1% to 5%

Dermatology/Skin — alopecia, pruritis/itching
Gastrointestinal — constipation
General Disorders — edema, fever (in the absence of neutropenia)
Hematologic — thrombocytopenia
Renal — decreased creatinine clearance, increased creatinine, decreased glomerular filtration rate
Special Senses — ocular surface disease (including conjunctivitis), increased lacrimation

Incidence Less than 1%

Cardiovascular — supraventricular arrhythmia
Dermatology/Skin — erythema multiforme
General Disorders — febrile neutropenia, allergic reaction/hypersensitivity
Neurology — motor neuropathy
Renal — renal failure

Non-Small Cell Lung Cancer (NSCLC) – After Prior Chemotherapy

Table 6 provides the frequency and severity of adverse reactions that have been reported in >5% of 265 patients randomly assigned to receive single-agent ALIMTA with folic acid and vitamin B_{12} supplementation and 276 patients randomly assigned to receive single-agent docetaxel. All patients were diagnosed with locally advanced or metastatic NSCLC and received prior chemotherapy.

Table 6: Adverse Reactions in Fully Supplemented Patients Receiving ALIMTA versus Docetaxel in NSCLC[a]

Reaction[b]	ALIMTA (N=265)		Docetaxel (N=276)	
	All Grades Toxicity (%)	Grades 3-4 Toxicity (%)	All Grades Toxicity (%)	Grades 3-4 Toxicity (%)
Laboratory				
Hematologic				
Anemia	19	4	22	4
Leukopenia	12	4	34	27
Neutropenia	11	5	45	40
Thrombocytopenia	8	2	1	0
Hepatic				
Increased ALT	8	2	1	0
Increased AST	7	1	1	0
Clinical				
Gastrointestinal				
Nausea	31	3	17	2
Anorexia	22	2	24	3
Vomiting	16	2	12	1
Stomatitis/Pharyngitis	15	1	17	1
Diarrhea	13	0	24	3
Constipation	6	0	4	0
Constitutional Symptoms				
Fatigue	34	5	36	5
Fever	8	0	8	0
Dermatology/Skin				
Rash/Desquamation	14	0	6	0
Pruritis	7	0	2	0
Alopecia	6	1[c]	38	2[c]

[a] For the purpose of this table a cut off of 5% was used for inclusion of all events where the reporter considered a possible relationship to ALIMTA.
[b] Refer to NCI CTC Criteria for lab values for each Grade of toxicity (version 2.0).
[c] According to NCI CTC Criteria version 2.0, this adverse event term should only be reported as Grade 1 or 2.

No clinically relevant differences in adverse reactions were seen in patients based on histology.

Clinically relevant adverse reactions occurring in <5% of patients that received ALIMTA treatment but >5% of patients that received docetaxel include CTC Grade 3/4 febrile neutropenia (1.9% ALIMTA, 12.7% docetaxel).

The following additional adverse reactions were observed in patients with non-small cell lung cancer randomly assigned to receive ALIMTA.

Incidence 1% to 5%

Body as a Whole — abdominal pain, allergic reaction/hypersensitivity, febrile neutropenia, infection

Dermatology/Skin — erythema multiforme

Neurology — motor neuropathy, sensory neuropathy

Renal — increased creatinine

Incidence Less than 1%

Cardiovascular — supraventricular arrhythmias

Malignant Pleural Mesothelioma (MPM)

Table 7 provides the frequency and severity of adverse reactions that have been reported in >5% of 168 patients with mesothelioma who were randomly assigned to receive cisplatin and ALIMTA and 163 patients with mesothelioma randomly assigned to receive single-agent cisplatin. In both treatment arms, these chemonaive patients were fully supplemented with folic acid and vitamin B_{12}.

Table 7: Adverse Reactions in Fully Supplemented Patients Receiving ALIMTA plus Cisplatin in MPM[a]

Reaction[b]	ALIMTA/cisplatin (N=168)		Cisplatin (N=163)	
	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)	All Grades Toxicity (%)	Grade 3-4 Toxicity (%)
Laboratory				
Hematologic				
Neutropenia	56	23	13	3
Leukopenia	53	15	17	1
Anemia	26	4	10	0
Thrombocytopenia	23	5	9	0
Renal				
Creatinine elevation	11	1	10	1
Creatinine clearance decreased	16	1	18	2
Clinical				
Eye Disorder				
Conjunctivitis	5	0	1	0
Gastrointestinal				
Nausea	82	12	77	6
Vomiting	57	11	50	4
Stomatitis/Pharyngitis	23	3	6	0
Anorexia	20	1	14	1
Diarrhea	17	4	8	0
Constipation	12	1	7	1
Dyspepsia	5	1	1	0
Constitutional Symptoms				
Fatigue	48	10	42	9
Metabolism and Nutrition				
Dehydration	7	4	1	1
Neurology				
Neuropathy-sensory	10	0	10	1
Taste Disturbance	8	0[c]	6	0[c]
Dermatology/Skin				
Rash	16	1	5	0
Alopecia	11	0[c]	6	0[c]

[a] For the purpose of this table a cut off of 5% was used for inclusion of all events where the reporter considered a possible relationship to ALIMTA.

[b] Refer to NCI CTC Criteria version 2.0 for each Grade of toxicity except the term "creatinine clearance decreased" which is derived from the CTC term "renal/genitourinary-other".

[c] According to NCI CTC Criteria version 2.0, this adverse event term should only be reported as Grade 1 or 2.

The following additional adverse reactions were observed in patients with malignant pleural mesothelioma randomly assigned to receive ALIMTA plus cisplatin.

Incidence 1% to 5%

Body as a Whole — febrile neutropenia, infection, pyrexia
Dermatology/Skin — urticaria
General Disorders — chest pain
Metabolism and Nutrition — increased AST, increased ALT, increased GGT
Renal — renal failure

Incidence Less than 1%

Cardiovascular — arrhythmia
Neurology — motor neuropathy

Effects of Vitamin Supplementations

Table 8 compares the incidence (percentage of patients) of CTC Grade 3/4 toxicities in patients who received vitamin supplementation with daily folic acid and vitamin B_{12} from the time of enrollment in the study (fully supplemented) with the incidence in patients who never received vitamin supplementation (never supplemented) during the study in the ALIMTA plus cisplatin arm.

Table 8: Selected Grade 3/4 Adverse Events Comparing Fully Supplemented versus Never Supplemented Patients in the ALIMTA plus Cisplatin arm (% incidence)

Adverse Event[a] (%)	**Fully Supplemented Patients** (N=168)	**Never Supplemented Patients** (N=32)
Neutropenia/granulocytopenia	23	38
Thrombocytopenia	5	9
Vomiting	11	31
Febrile neutropenia	1	9
Infection with Grade 3/4 neutropenia	0	6
Diarrhea	4	9

[a] Refer to NCI CTC criteria for lab and non-laboratory values for each grade of toxicity (Version 2.0).

The following adverse events were greater in the fully supplemented group compared to the never supplemented group: hypertension (11%, 3%), chest pain (8%, 6%), and thrombosis/embolism (6%, 3%).

Subpopulations

No relevant effect for ALIMTA safety due to gender or race was identified, except an increased incidence of rash in men (24%) compared to women (16%).

6.2 Post-Marketing Experience

The following adverse reactions have been identified during post-approval use of ALIMTA. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure.

These reactions have occurred with ALIMTA when used as a single-agent and in combination therapies.

Gastrointestinal — colitis
General Disorders and Administration Site Conditions — edema
Injury, poisoning, and procedural complications — Radiation recall has been reported in patients who have previously received radiotherapy.
Respiratory — interstitial pneumonitis

7 DRUG INTERACTIONS

7.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Ibuprofen

Although ibuprofen (400 mg four times a day) can decrease the clearance of pemetrexed, it can be administered with ALIMTA in patients with normal renal function (creatinine clearance ≥80 mL/min). Caution should be used when administering ibuprofen concurrently with ALIMTA to patients with mild to moderate renal insufficiency (creatinine clearance from 45 to 79 mL/min) *[see Clinical Pharmacology (12.3)]*.

Other NSAIDs

Patients with mild to moderate renal insufficiency should avoid taking NSAIDs with short elimination half-lives for a period of 2 days before, the day of, and 2 days following administration of ALIMTA.

In the absence of data regarding potential interaction between ALIMTA and NSAIDs with longer half-lives, all patients taking these NSAIDs should interrupt dosing for at least 5 days before, the day of, and 2 days following ALIMTA administration. If concomitant administration of an NSAID is necessary, patients should be monitored closely for toxicity, especially myelosuppression, renal, and gastrointestinal toxicity.

7.2 Nephrotoxic Drugs

ALIMTA is primarily eliminated unchanged renally as a result of glomerular filtration and tubular secretion. Concomitant administration of nephrotoxic drugs could result in delayed clearance of ALIMTA. Concomitant administration of substances that are also tubularly secreted (e.g., probenecid) could potentially result in delayed clearance of ALIMTA.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Teratogenic Effects - Pregnancy Category D *[see Warnings and Precautions (5.6)]*

Based on its mechanism of action, ALIMTA can cause fetal harm when administered to a pregnant woman. There are no adequate and well controlled studies of ALIMTA in pregnant women. Pemetrexed was embryotoxic, fetotoxic, and teratogenic in mice. In mice, repeated intraperitoneal doses of pemetrexed when given during organogenesis caused fetal malformations (incomplete ossification of talus and skull bone; about 1/833rd the recommended intravenous human dose on a mg/m^2 basis), and cleft palate (1/33rd the recommended intravenous human dose on a mg/m^2 basis). Embryotoxicity was characterized by increased embryo-fetal deaths and reduced litter sizes. If ALIMTA is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus. Women of childbearing potential should be advised to use effective contraceptive measures to prevent pregnancy during the treatment with ALIMTA.

8.3 Nursing Mothers

It is not known whether ALIMTA or its metabolites are excreted in human milk. Because many drugs are excreted in human milk, and because of the potential for serious adverse reactions in nursing infants from ALIMTA, a decision should be made to discontinue nursing or discontinue the drug, taking into account the importance of the drug for the mother.

8.4 Pediatric Use

The safety and effectiveness of ALIMTA in pediatric patients have not been established.

8.5 Geriatric Use

ALIMTA is known to be substantially excreted by the kidney, and the risk of adverse reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection. Renal function monitoring is recommended with administration of ALIMTA. No dose reductions other than those recommended for all patients are necessary for patients 65 years of age or older *[see Dosage and Administration (2.4)]*.

In the initial treatment non-small cell lung cancer clinical trial, 37.7% of patients treated with ALIMTA plus cisplatin were ≥65 years and Grade 3/4 neutropenia was greater as compared to patients <65 years (19.9% versus 12.2%). For patients <65 years, the HR for overall survival was 0.96 (95% CI: 0.83, 1.10) and for patients ≥65 years the HR was 0.88 (95% CI: 0.74, 1.06) in the intent to treat population.

In the maintenance non-small cell lung cancer trial 33.3% of patients treated with ALIMTA were ≥65 years and no differences were seen in Grade 3/4 adverse reactions as compared to patients <65 years. For patients <65 years, the HR for overall survival was 0.74 (95% CI: 0.58, 0.93) and for patients ≥65 years the HR was 0.88 (95% CI: 0.65, 1.21) in the intent to treat population.

In the non-small cell lung cancer trial after prior chemotherapy, 29.7% patients treated with ALIMTA were ≥65 years and Grade 3/4 hypertension was greater as compared to patients <65 years. For patients <65 years, the HR for overall survival was 0.95 (95% CI: 0.76, 1.19), and for patients ≥65 years the HR was 1.15 (95% CI: 0.79, 1.68) in the intent to treat population.

The mesothelioma trial included 36.7% patients treated with ALIMTA plus cisplatin that were ≥65 years, and Grade 3/4 fatigue, leukopenia, neutropenia, and thrombocytopenia were greater as compared to patients <65 years. For patients <65 years, the HR for overall survival was 0.71(95% CI: 0.53, 0.96) and for patients ≥65 years, the HR was 0.85 (95% CI: 0.59, 1.22) in the intent to treat population.

8.6 Patients with Hepatic Impairment

There was no effect of elevated AST, ALT, or total bilirubin on the pharmacokinetics of pemetrexed *[see Clinical Pharmacology (12.3)]*.

Dose adjustments based on hepatic impairment experienced during treatment with ALIMTA are provided in Table 2 *[see Dosage and Administration (2.4)]*.

8.7 Patients with Renal Impairment

ALIMTA is known to be primarily excreted by the kidneys. Decreased renal function will result in reduced clearance and greater exposure (AUC) to ALIMTA compared with patients with normal renal function *[see Dosage and Administration (2.4) and Clinical Pharmacology (12.3)]*. Cisplatin coadministration with ALIMTA has not been studied in patients with moderate renal impairment.

8.8 Gender

In the initial treatment non-small cell lung cancer trial, 70% of patients were males and 30% females. For males the HR for overall survival was 0.97 (95% CI: 0.85, 1.10) and for females the HR was 0.86 (95% CI: 0.70, 1.06) in the intent to treat population.

In the maintenance non-small cell lung cancer trial, 73% of patients were males and 27% females. For males the HR for overall survival was 0.78 (95% CI: 0.63, 0.96) and for females the HR was 0.83 (95% CI: 0.56, 1.21) in the intent to treat population.

In the non-small cell lung cancer trial after prior chemotherapy, 72% of patients were males and 28% females. For males the HR for overall survival was 0.95 (95% CI: 0.76, 1.19) and for females the HR was 1.28 (95% CI: 0.86, 1.91) in the intent to treat population.

In the mesothelioma trial, 82% of patients were males and 18% females. For males the HR for overall survival was 0.85 (95% CI: 0.66, 1.09) and for females the HR was 0.48 (95% CI: 0.27, 0.85) in the intent to treat population.

8.9 Race

In the initial treatment non-small cell lung cancer trial, 78% of patients were Caucasians, 13% East/Southeast Asians, and 9% others. For Caucasians, the HR for overall survival was 0.92 (95% CI: 0.82, 1.04), for East/Southeast Asians the HR was 0.86 (95% CI: 0.61, 1.21), and for others the HR was 1.24 (95% CI: 0.84, 1.84) in the intent to treat population.

In the maintenance non-small cell lung cancer trial, 65% of patients were Caucasians, 23% East Asian, and 12% others. For Caucasians the HR for overall survival was 0.77 (95% CI: 0.62, 0.97), for East Asians was 1.05 (95% CI: 0.70, 1.59) and for others the HR was 0.46 (95% CI: 0.26, 0.79) in the intent to treat population.

In the non-small cell lung cancer trial after prior chemotherapy, 71% of patients were Caucasians and 29% others. For Caucasians the HR for overall survival was 0.91 (95% CI: 0.73, 1.15) and for others the HR was 1.27 (95% CI: 0.87, 1.87) in the intent to treat population.

In the mesothelioma trial, 92% of patients were Caucasians and 8% others. For Caucasians, the HR for overall survival was 0.77 (95% CI: 0.61, 0.97) and for others the HR was 0.86 (95% CI: 0.39, 1.90) in the intent to treat population.

10 OVERDOSAGE

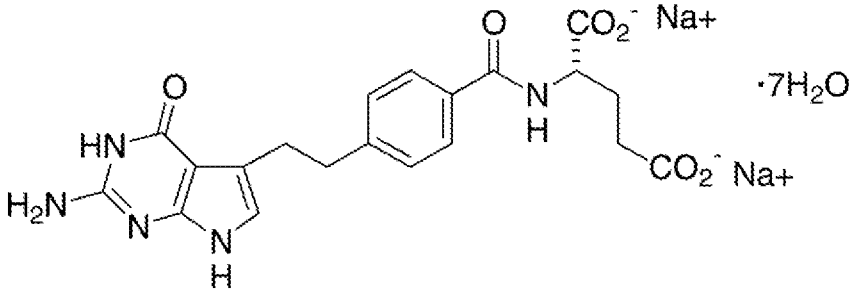
There have been few cases of ALIMTA overdose. Reported toxicities included neutropenia, anemia, thrombocytopenia, mucositis, and rash. Anticipated complications of overdose include bone marrow suppression as manifested by neutropenia, thrombocytopenia, and anemia. In addition, infection with or without fever, diarrhea, and mucositis may be seen. If an overdose occurs, general supportive measures should be instituted as deemed necessary by the treating physician.

In clinical trials, leucovorin was permitted for CTC Grade 4 leukopenia lasting ≥3 days, CTC Grade 4 neutropenia lasting ≥3 days, and immediately for CTC Grade 4 thrombocytopenia, bleeding associated with Grade 3 thrombocytopenia, or Grade 3 or 4 mucositis. The following intravenous doses and schedules of leucovorin were recommended for intravenous use: 100 mg/m^2, intravenously once, followed by leucovorin, 50 mg/m^2, intravenously every 6 hours for 8 days.

The ability of ALIMTA to be dialyzed is unknown.

11 DESCRIPTION

Pemetrexed disodium heptahydrate has the chemical name L-Glutamic acid, *N*-[4-[2-(2-amino-4,7-dihydro-4-oxo-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-, disodium salt, heptahydrate. It is a white to almost-white solid with a molecular formula of $C_{20}H_{19}N_5Na_2O_6 \cdot 7H_2O$ and a molecular weight of 597.49. The structural formula is as follows:



ALIMTA is supplied as a sterile lyophilized powder for intravenous infusion available in single-dose vials. The product is a white to either light yellow or green-yellow lyophilized solid. Each 100-mg or 500-mg vial of ALIMTA contains pemetrexed disodium equivalent to 100 mg pemetrexed and 106 mg mannitol or 500 mg pemetrexed and 500 mg mannitol, respectively. Hydrochloric acid and/or sodium hydroxide may have been added to adjust pH.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

ALIMTA, pemetrexed for injection, is a folate analog metabolic inhibitor that exerts its action by disrupting folate-dependent metabolic processes essential for cell replication. In vitro studies have shown that pemetrexed inhibits thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), which are folate-dependent enzymes involved in the de novo biosynthesis of thymidine and purine nucleotides. Pemetrexed is taken into cells by membrane carriers such as the reduced folate carrier and membrane folate binding protein transport systems. Once in the cell, pemetrexed is converted to polyglutamate forms by the enzyme folylpolyglutamate synthetase. The polyglutamate forms are retained in cells and are inhibitors of TS and GARFT. Polyglutamation is a time- and concentration-dependent process that occurs in tumor cells and, is thought to occur to a lesser extent, in normal tissues. Polyglutamated metabolites are thought to have an increased intracellular half-life resulting in prolonged drug action in malignant cells.

12.2 Pharmacodynamics

Preclinical studies have shown that pemetrexed inhibits the in vitro growth of mesothelioma cell lines (MSTO-211H, NCI-H2052). Studies with the MSTO-211H mesothelioma cell line showed synergistic effects when pemetrexed was combined concurrently with cisplatin.

Absolute neutrophil counts (ANC) following single-agent administration of ALIMTA to patients not receiving folic acid and vitamin B_{12} supplementation were characterized using population pharmacodynamic analyses. Severity of hematologic toxicity, as measured by the depth of the ANC nadir, correlates with the systemic exposure, or area under the curve (AUC) of pemetrexed. It was also observed that lower ANC nadirs occurred in patients with elevated baseline cystathionine or homocysteine concentrations. The levels of these substances can be reduced by folic acid and vitamin B_{12} supplementation. There is no cumulative effect of pemetrexed exposure on ANC nadir over multiple treatment cycles.

Time to ANC nadir with pemetrexed systemic exposure (AUC), varied between 8 to 9.6 days over a range of exposures from 38.3 to 316.8 mcg•hr/mL. Return to baseline ANC occurred 4.2 to 7.5 days after the nadir over the same range of exposures.

12.3 Pharmacokinetics

Absorption

The pharmacokinetics of ALIMTA administered as a single-agent in doses ranging from 0.2 to 838 mg/m^2 infused over a 10-minute period have been evaluated in 426 cancer patients with a variety of solid tumors. Pemetrexed total systemic exposure (AUC) and maximum plasma concentration (C_{max}) increase proportionally with dose. The pharmacokinetics of pemetrexed do not change over multiple treatment cycles.

Distribution

Pemetrexed has a steady-state volume of distribution of 16.1 liters. In vitro studies indicate that pemetrexed is approximately 81% bound to plasma proteins. Binding is not affected by degree of renal impairment.

Metabolism and Excretion

Pemetrexed is not metabolized to an appreciable extent and is primarily eliminated in the urine, with 70% to 90% of the dose recovered unchanged within the first 24 hours following administration. The clearance decreases, and exposure (AUC) increases, as renal function decreases. The total systemic clearance of pemetrexed is 91.8 mL/min and the elimination half-life of pemetrexed is 3.5 hours in patients with normal renal function (creatinine clearance of 90 mL/min).

The pharmacokinetics of pemetrexed in special populations were examined in about 400 patients in controlled and single arm studies.

Effect of Age

No effect of age on the pharmacokinetics of pemetrexed was observed over a range of 26 to 80 years.

Effect of Gender

The pharmacokinetics of pemetrexed were not different in male and female patients.

Effect of Race

The pharmacokinetics of pemetrexed were similar in Caucasians and patients of African descent. Insufficient data are available to compare pharmacokinetics for other ethnic groups.

Effect of Hepatic Insufficiency

There was no effect of elevated AST, ALT, or total bilirubin on the pharmacokinetics of pemetrexed. However, studies of hepatically impaired patients have not been conducted *[see Dosage and Administration (2.4) and Use in Specific Populations (8.6)]*.

Effect of Renal Insufficiency

Pharmacokinetic analyses of pemetrexed included 127 patients with reduced renal function. Plasma clearance of pemetrexed decreases as renal function decreases, with a resultant increase in systemic exposure. Patients with creatinine clearances of 45, 50, and 80 mL/min had 65%, 54%, and 13% increases, respectively in pemetrexed total systemic exposure (AUC) compared to patients with creatinine clearance of 100 mL/min *[see Warnings and Precautions (5.4) and Dosage and Administration (2.4)]*.

Pediatric

Pediatric patients were not included in clinical trials.

Effect of Ibuprofen

Ibuprofen doses of 400 mg four times a day reduce pemetrexed's clearance by about 20% (and increase AUC by 20%) in patients with normal renal function. The effect of greater doses of ibuprofen on pemetrexed pharmacokinetics is unknown *[see Drug Interactions (7.1)]*.

Effect of Aspirin

Aspirin, administered in low to moderate doses (325 mg every 6 hours), does not affect the pharmacokinetics of pemetrexed. The effect of greater doses of aspirin on pemetrexed pharmacokinetics is unknown.

Effect of Cisplatin

Cisplatin does not affect the pharmacokinetics of pemetrexed and the pharmacokinetics of total platinum are unaltered by pemetrexed.

Effect of Vitamins

Coadministration of oral folic acid or intramuscular vitamin B_{12} does not affect the pharmacokinetics of pemetrexed.

Drugs Metabolized by Cytochrome P450 Enzymes

Results from in vitro studies with human liver microsomes predict that pemetrexed would not cause clinically significant inhibition of metabolic clearance of drugs metabolized by CYP3A, CYP2D6, CYP2C9, and CYP1A2.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No carcinogenicity studies have been conducted with pemetrexed. Pemetrexed was clastogenic in the in vivo micronucleus assay in mouse bone marrow but was not mutagenic in multiple in vitro tests (Ames assay, CHO cell assay). Pemetrexed administered

at i.v. doses of 0.1 mg/kg/day or greater to male mice (about 1/1666 the recommended human dose on a mg/m^2 basis) resulted in reduced fertility, hypospermia, and testicular atrophy.

14 CLINICAL STUDIES

14.1 Non-Small Cell Lung Cancer (NSCLC) - Combination with Cisplatin

A multi-center, randomized, open-label study in 1725 chemonaive patients with Stage IIIb/IV NSCLC was conducted to compare the overall survival following treatment with ALIMTA in combination with cisplatin (AC) versus gemcitabine in combination with cisplatin (GC). ALIMTA was administered intravenously over 10 minutes at a dose of 500 mg/m^2 with cisplatin administered intravenously at a dose of 75 mg/m^2 after ALIMTA administration, on Day 1 of each 21-day cycle. Gemcitabine was administered at a dose of 1250 mg/m^2 on Day 1 and Day 8, and cisplatin was administered intravenously at a dose of 75 mg/m^2 after administration of gemcitabine, on Day 1 of each 21-day cycle. Treatment was administered up to a total of 6 cycles, and patients in both treatment arms received folic acid, vitamin B_{12}, and dexamethasone *[see Dosage and Administration (2.3)]*.

Patient demographics of the intent to treat (ITT) population are shown in Table 9. The demographics and disease characteristics were well balanced.

Table 9: First-Line Therapy: Summary of Patient Characteristics in Study of NSCLC

Patient characteristic	ALIMTA plus Cisplatin (AC) (N=862)	Gemcitabine plus Cisplatin (GC) (N=863)
Age (yrs)		
Median (range)	61.1 (28.8-83.2)	61.0 (26.4-79.4)
Gender		
Male/Female	70.2%/29.8%	70.1%/29.9%
Origin		
Caucasian	669 (77.6%)	680 (78.8%)
Hispanic	27 (3.1%)	23 (2.7%)
Asian	146 (16.9%)	141 (16.3%)
African descent	18 (2.1%)	18 (2.1%)
Stage at Entry		
IIIb/IV	23.8%/76.2%	24.3%/75.7%
Histology		
Nonsquamous NSCLC[a]	618 (71.7%)	634 (73.5%)
Adenocarcinoma	436 (50.6%)	411 (47.6%)
Large cell	76 (8.8%)	77 (8.9%)
Other[b]	106 (12.3%)	146 (16.9%)
Squamous	244 (28.3%)	229 (26.5%)
ECOG PS[c,d]		
0/1	35.4%/64.6%	35.6%/64.3%
Smoking History[e]		
Ever/never smoker	83.1%/16.9%	83.9%/16.1%

[a] Includes adenocarcinoma, large cell, and other histologies except those with squamous cell type.

[b] The subgroup of "other" represents patients with a primary diagnosis of NSCLC whose disease did not clearly qualify as adenocarcinoma, squamous cell carcinoma, or large cell carcinoma.

[c] Eastern Cooperative Oncology Group Performance Status.

[d] ECOG PS was not reported for all randomized patients. Percentages are representative of N=861 for the ALIMTA plus cisplatin arm, and N=861 for the gemcitabine plus cisplatin arm.

[e] Smoking history was collected for 88% of randomized patients (N=757 for the ALIMTA plus cisplatin arm and N=759 for the gemcitabine plus cisplatin arm).

Patients received a median of 5 cycles of treatment in both study arms. Patients treated with ALIMTA plus cisplatin received a relative dose intensity of 94.8% of the protocol-specified ALIMTA dose intensity and 95.0% of the protocol-specified cisplatin dose intensity. Patients treated with gemcitabine plus cisplatin received a relative dose intensity of 85.8% of the protocol-specified gemcitabine dose intensity and 93.5% of the protocol-specified cisplatin dose intensity.

The primary endpoint in this study was overall survival. The median survival time was 10.3 months in the ALIMTA plus cisplatin treatment arm and 10.3 months in the gemcitabine plus cisplatin arm, with an adjusted hazard ratio of 0.94.

Table 10: First-Line Therapy: Efficacy in NSCLC - ITT Population

	ALIMTA plus Cisplatin (N=862)	Gemcitabine plus Cisplatin (N=863)
Median overall survival (95% CI)	10.3 mos (9.8-11.2)	10.3 mos (9.6-10.9)
Adjusted hazard ratio (HR)[a,b] (95% CI)	0.94 (0.84-1.05)	
Median progression-free survival (95% CI)	4.8 mos (4.6-5.3)	5.1 mos (4.6-5.5)

Adjusted hazard ratio (HR)[a,b] (95% CI)	1.04 (0.94-1.15)	
Overall response rate (95% CI)	27.1% (24.2-30.1)	24.7% (21.8-27.6)

[a] Adjusted for gender, stage, basis of diagnosis, and performance status.

[b] A HR that is less than 1.0 indicates that survival is better in the AC arm than in the GC arm. Alternatively, a HR that is greater than 1.0 indicates survival is better in the GC arm than in the AC arm.

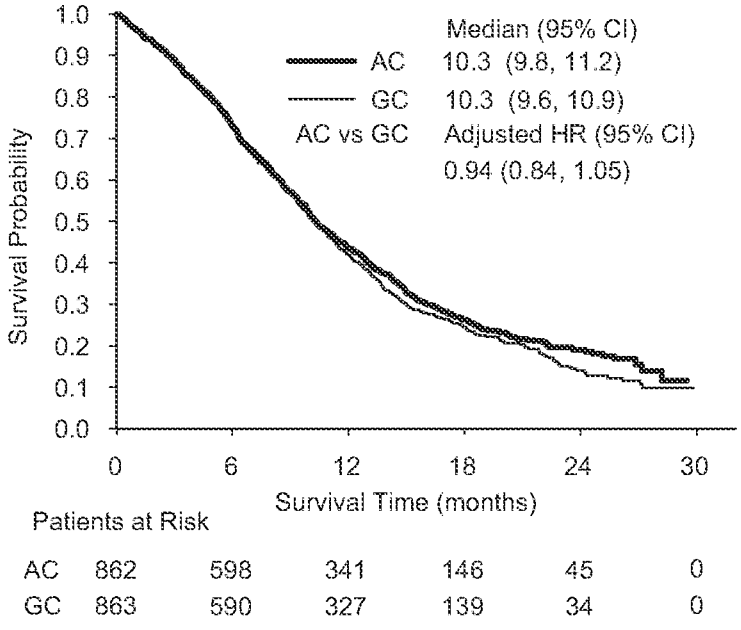


Figure 1: Kaplan-Meier Curves for Overall Survival ALIMTA plus Cisplatin (AC) versus Gemcitabine plus Cisplatin (GC) in NSCLC - ITT Population.

A pre-specified analysis of the impact of NSCLC histology on overall survival was examined. Clinically relevant differences in survival according to histology were observed and are shown in Table 11. This difference in treatment effect for ALIMTA based on histology demonstrating a lack of efficacy in squamous cell histology was also observed in the single-agent, second-line study and the maintenance study *[see Clinical Studies (14.2, 14.3)]*.

Table 11: First-Line Therapy: Overall Survival in NSCLC Histologic Subgroups

Histology Subgroup	Median Overall Survival in Months (95% CI)				Unadjusted Hazard Ratio (HR)[a,b] (95% CI)	Adjusted Hazard Ratio (HR)[a,b,c] (95% CI)
	ALIMTA plus Cisplatin		Gemcitabine plus Cisplatin			
Nonsquamous NSCLC[d] (N=1252)	11.0 (10.1-12.5)	N=618	10.1 (9.3-10.9)	N=634	0.84 (0.74-0.96)	0.84 (0.74-0.96)
Adenocarcinoma (N=847)	12.6 (10.7-13.6)	N=436	10.9 (10.2-11.9)	N=411	0.84 (0.71-0.98)	0.84 (0.71-0.99)
Large Cell (N=153)	10.4 (8.6-14.1)	N=76	6.7 (5.5-9.0)	N=77	0.68 (0.48-0.97)	0.67 (0.48-0.96)
Other[e] (N=252)	8.6 (6.8-10.2)	N=106	9.2 (8.1-10.6)	N=146	1.12 (0.84-1.49)	1.08 (0.81-1.45)
Squamous Cell (N=473)	9.4 (8.4-10.2)	N=244	10.8 (9.5-12.1)	N=229	1.22 (0.99-1.50)	1.23 (1.00-1.51)

[a] A HR that is less than 1.0 indicates that survival is better in the AC arm than in the GC arm. Alternatively, a HR that is greater than 1.0 indicates survival is better in the GC arm than in the AC arm.

[b] Unadjusted for multiple comparisons.

[c] HRs adjusted for ECOG PS, gender, disease stage, and basis for pathological diagnosis (histopathological/cytopathological).

[d] Includes adenocarcinoma, large cell, and other histologies except those with squamous cell type.

[e] The subgroup of "other" represents patients with a primary diagnosis of NSCLC whose disease did not clearly qualify as adenocarcinoma, squamous cell carcinoma, or large cell carcinoma.

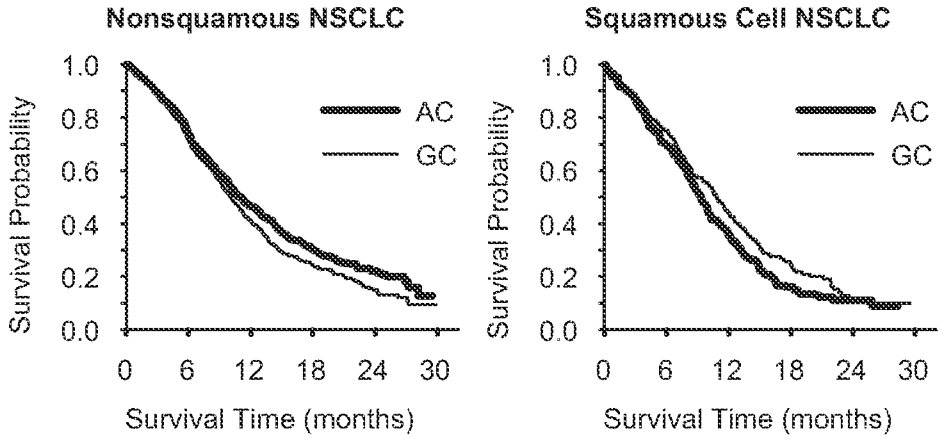


Figure 2: Kaplan-Meier Curves for Overall Survival ALIMTA plus Cisplatin (AC) versus Gemcitabine plus Cisplatin (GC) in NSCLC - Nonsquamous NSCLC and Squamous Cell NSCLC.

14.2 Non-Small Cell Lung Cancer - Maintenance

A multi-center, randomized, double-blind, placebo-controlled study was conducted in 663 patients with Stage IIIb/IV NSCLC who did not progress after four cycles of platinum-based chemotherapy. Patients who did not progress were randomized 2:1 to receive ALIMTA or placebo immediately following platinum-based chemotherapy. ALIMTA was administered intravenously over 10 minutes at a dose of 500 mg/m^2 on Day 1 of each 21-day cycle, until disease progression. Patients in both study arms received folic acid, vitamin B_{12}, and dexamethasone *[see Dosage and Administration (2.3)]*.

The study was designed to demonstrate superior progression-free survival and overall survival of ALIMTA over placebo. Progression-free survival (PFS) was assessed by independent review. Patient characteristics of the intent to treat (ITT) population are shown in Table 12. The demographics and baseline disease characteristics were well balanced between study arms.

Table 12: Maintenance Therapy: Summary of Patient Characteristics in Study of NSCLC

Patient characteristic	ALIMTA (N=441)	Placebo (N=222)
Age (yrs)		
Median (range)	60.6 (25.6-82.6)	60.4 (35.4-78.5)
Gender		
Male/Female	73.0%/27.0%	72.5%/27.5%
Ethnic Origin		
Caucasian	279 (63.3%)	149 (67.1%)
East Asian	104 (23.6%)	50 (22.5%)
Other	58 (13.2%)	23 (10.4%)
Stage at Entry[a]		
IIIb/IV	18.0%/82.0%	21.2%/78.8%
Histology (%)		
Nonsquamous NSCLC[b]	325 (73.7%)	156 (70.3%)
Adenocarcinoma	222 (50.3%)	106 (47.7%)
Large cell	10 (2.3%)	10 (4.5%)
Other[c]	93 (21.1%)	40 (18.0%)
Squamous	116 (26.3%)	66 (29.7%)
ECOG PS[d]		
0/1	40.1%/59.9%	38.3%/61.7%
Smoking History[e]		
Ever/never smoker	74.1%/25.9%	71.5%/28.5%
Time from start of induction therapy to study randomization (months)		
Median (range)	3.25 (1.6-4.8)	3.29 (2.7-5.1)

[a] Stage at Entry was not reported for all randomized patients. Percentages are representative of N=440 for the ALIMTA arm and N=222 for the placebo arm.

[b] Includes patients with adenocarcinoma, large cell, and other histologic diagnoses.

[c] The subgroup of "Other" represents patients with a primary diagnosis of NSCLC whose disease did not clearly qualify as adenocarcinoma, large cell carcinoma, or squamous cell carcinoma.

[d] Eastern Cooperative Oncology Group Performance Status (ECOG PS) was not reported for all randomized patients. Percentages are representative of N=439 for the ALIMTA arm, and N=222 for the placebo arm.

[e] Smoking history was not reported for all randomized patients. Percentages are representative of N=437 for the ALIMTA arm and N=221 for the placebo arm.

Patients received a median of 5 cycles of ALIMTA and 3.5 cycles of placebo. Patients randomized to ALIMTA received a relative dose intensity of 95.7%. A total of 213 patients (48.3%) completed ≥6 cycles and a total of 98 patients (22.6%) completed ≥10 cycles of treatment with ALIMTA.

In the overall study population, ALIMTA was statistically superior to placebo in terms of overall survival (OS) (median 13.4 months versus 10.6 months, HR=0.79 (95% CI: 0.65-0.95), p-value=0.012) and PFS (median 4.0 months versus 2.0 months, HR=0.60 (95% CI: 0.49-0.73), p-value<0.00001). A difference in treatment outcomes was observed according to histologic classification. For the population of patients with nonsquamous NSCLC, ALIMTA was superior to placebo for OS (median 15.5 months versus 10.3 months, HR=0.70 (95% CI: 0.56-0.88)) and PFS (median 4.4 months versus 1.8 months, HR=0.47 (95% CI: 0.37-0.60)). For the population of patients with squamous NSCLC, ALIMTA did not improve OS compared to placebo (median 9.9 months versus 10.8 months, HR=1.07 (95% CI: 0.77-1.50)) or PFS (median 2.4 months versus 2.5 months, HR=1.03 (95% CI: 0.71-1.49)). This difference in treatment effect for ALIMTA based on histology demonstrating lack of benefit in squamous cell histology was also observed in the first-line and second line studies. *[see Clinical Studies (14.1, 14.3)]*

Efficacy results for the overall patient population are presented in Table 13 and Figure 3, and efficacy results by pre-specified histologic subgroups are presented in Table 14 and Figure 4, below.

Table 13: Maintenance Therapy: Efficacy of ALIMTA versus Placebo in NSCLC - ITT Population

Efficacy Parameter[a,b]	**ALIMTA (N=441)**	**Placebo (N=222)**
Median overall survival[c] (95% CI)	13.4 mos (11.9-15.9)	10.6 mos (8.7-12.0)
Hazard ratio (HR)[c] (95% CI)	0.79 (0.65-0.95)	
p-value	p=0.012	
Median progression-free survival (95% CI)	4.0 mos (3.1-4.4)	2.0 mos (1.5-2.8)
Hazard ratio (HR)[c] (95% CI)	0.60 (0.49-0.73)	
p-value	p<0.00001	

[a] PFS and OS were calculated from time of randomization, after completion of 4 cycles of induction platinum-based chemotherapy.

[b] Values for PFS given based on independent review (ALIMTA N=387, Placebo N=194).

[c] Unadjusted hazard ratios are provided. A HR <1.0 indicates that the result is better in the ALIMTA arm than in the placebo arm.

Table 14: Maintenance Therapy: Efficacy in NSCLC by Histologic Subgroups[a]

	Overall Survival		**Progression-Free Survival[b]**	
	ALIMTA	**Placebo**	**ALIMTA**	**Placebo**
	Median (months)	**Median (months)**	**Median (months)**	**Median (months)**
	HR[c] (95% CI)		**HR[c] (95% CI)**	
Nonsquamous NSCLC[d] N=481	**15.5**	**10.3**	**4.4**	**1.8**
	0.70 (0.56-0.88)		**0.47 (0.37-0.60)**	
Adenocarcinoma N=328	16.8	11.5	4.6	2.7
	0.73 (0.56-0.96)		0.51 (0.38-0.68)	
Large cell carcinoma N=20	8.4	7.9	4.5	1.5
	0.98 (0.36-2.65)		0.40 (0.12-1.29)	
Other[e] N=133	11.3	7.7	4.1	1.6
	0.61 (0.40-0.94)		0.44 (0.28-0.68)	
Squamous cell N=182	**9.9**	**10.8**	**2.4**	**2.5**
	1.07 (0.77-1.50)		**1.03 (0.71-1.49)**	

[a] PFS and OS were calculated from time of randomization, after completion of 4 cycles of induction platinum-based chemotherapy. All results unadjusted for multiple comparisons.

[b] Values for PFS are given based on independent review (ALIMTA N=387, Placebo N=194).

[c] Unadjusted hazard ratios are provided. A HR <1.0 indicates that the result is better in the ALIMTA arm than in the placebo arm. A HR >1.0 indicates that the result is better in the placebo arm than in the ALIMTA arm.

[d] Includes patients with adenocarcinoma, large cell carcinoma, and other histology.

[e] The subgroup of "Other" represents patients with a primary diagnosis of NSCLC whose disease did not clearly qualify as adenocarcinoma, large cell carcinoma, or squamous cell carcinoma.

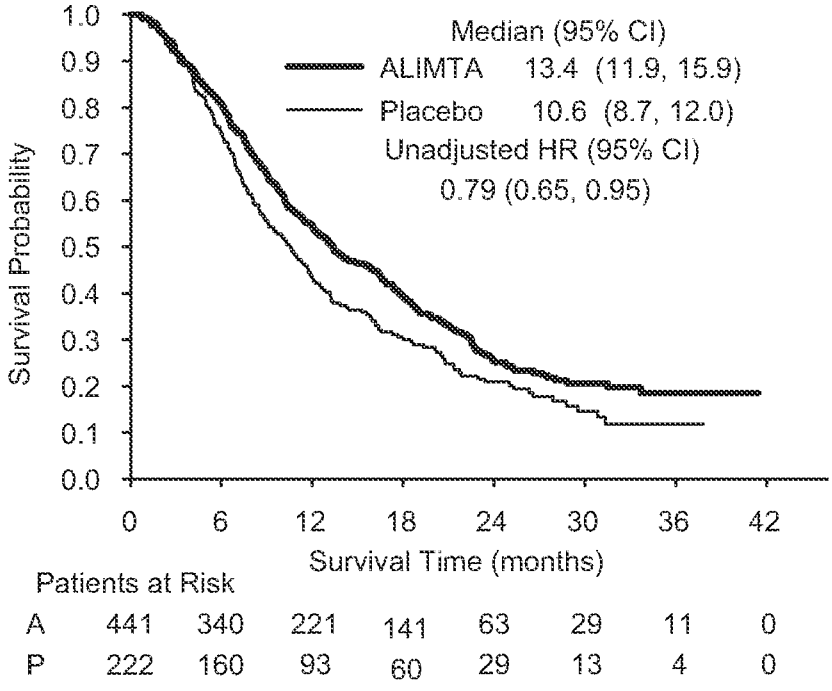


Figure 3: Kaplan-Meier Curve for Overall Survival ALIMTA (A) versus Placebo (P) in NSCLC - ITT Population.

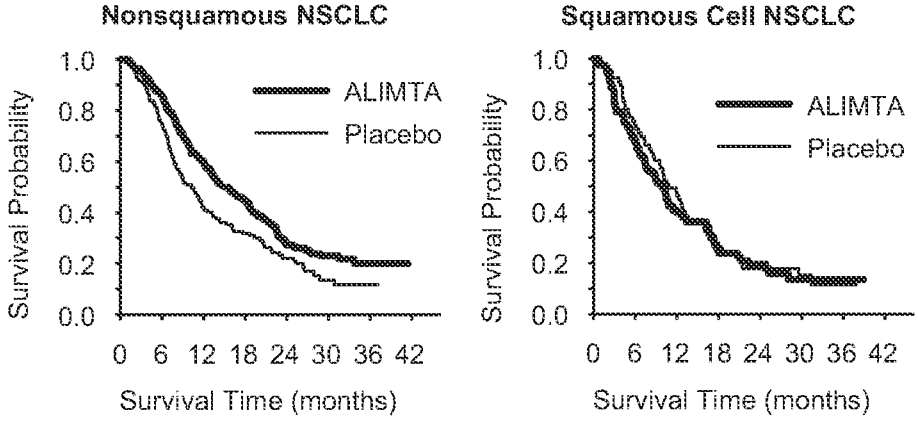


Figure 4: Kaplan-Meier Curves for Overall Survival ALIMTA versus Placebo in NSCLC - Nonsquamous NSCLC and Squamous Cell NSCLC.

14.3 Non-Small Cell Lung Cancer - After Prior Chemotherapy

A multi-center, randomized, open label study was conducted in patients with Stage III or IV NSCLC after prior chemotherapy to compare the overall survival following treatment with ALIMTA versus docetaxel. ALIMTA was administered intravenously over 10 minutes at a dose of 500 mg/m^2 and docetaxel was administered at 75 mg/m^2 as a 1-hour intravenous infusion. Both drugs were given on Day 1 of each 21-day cycle. All patients treated with ALIMTA received vitamin supplementation with folic acid and vitamin B_{12}. The study was intended to show either an overall survival superiority or non-inferiority of ALIMTA to docetaxel. Patient demographics of the intent to treat (ITT) population are shown in Table 15.

Table 15: Second-Line Therapy: Summary of Patient Characteristics in NSCLC Study

Patient characteristic	**ALIMTA** (N=283)	**Docetaxel** (N=288)
Age (yrs)		
Median (range)	59 (22-81)	57 (28-87)
Gender (%)		
Male/Female	68.6/31.4	75.3/24.7
Stage at Entry (%)		

III/IV	25.1/74.9	25.3/74.7
Diagnosis/Histology (%)		
Adenocarcinoma	154 (54.4)	142 (49.3)
Squamous	78 (27.6)	94 (32.6)
Bronchoalveolar	4 (1.4)	1 (0.3)
Other	47 (16.6)	51 (17.7)
Performance Status (%)[a]		
0-1	234 (88.6)	240 (87.6)
2	30 (11.4)	34 (12.4)

[a] Performance status was not reported for all randomized patients. Percentages are representative of N=264 for the ALIMTA arm and N=274 for the docetaxel arm.

The primary endpoint in this study was overall survival. The median survival time was 8.3 months in the ALIMTA treatment arm and 7.9 months in the docetaxel arm, with a hazard ratio of 0.99 (see Table 16). The study did not show an overall survival superiority of ALIMTA.

Table 16: Efficacy of ALIMTA versus Docetaxel in Non-Small Cell Lung Cancer - ITT Population

	ALIMTA (N=283)	Docetaxel (N=288)
Median overall survival (95% CI)	8.3 mos (7.0-9.4)	7.9 mos (6.3-9.2)
Hazard ratio (HR) (95% CI)	0.99 (0.82-1.20)	
Median progression-free survival (95% CI)	2.9 mos (2.4-3.1)	2.9 mos (2.7-3.4)
Hazard ratio (HR) (95% CI)	0.97 (0.82-1.16)	
Overall response rate (95% CI)	8.5% (5.2-11.7)	8.3% (5.1-11.5)

A retrospective analysis of the impact of NSCLC histology on overall survival was examined. Clinically relevant differences in survival according to histology were observed and are shown in Table 17. This difference in treatment effect for ALIMTA based on histology demonstrating a lack of efficacy in squamous cell histology was also observed in the first-line combination study and in the maintenance study *[see Clinical Studies (14.1, 14.2)]*.

Table 17: Second Line Therapy: Overall Survival of ALIMTA versus Docetaxel in NSCLC by Histologic Subgroups

Histology Subgroup	Median Overall Survival in Months (95% CI)				Unadjusted Hazard Ratio (HR)[a,b] (95% CI)	Adjusted Hazard Ratio (HR)[a,b,c] (95% CI)
	ALIMTA		Docetaxel			
Nonsquamous NSCLC[d] (N=399)	9.3 (7.8-9.7)	N=205	8.0 (6.3-9.3)	N=194	0.89 (0.71-1.13)	0.78 (0.61-1.00)
Adenocarcinoma (N=301)	9.0 (7.6-9.6)	N=158	9.2 (7.5-11.3)	N=143	1.09 (0.83-1.44)	0.92 (0.69-1.22)
Large Cell (N=47)	12.8 (5.8-14.0)	N=18	4.5 (2.3-9.1)	N=29	0.38 (0.18-0.78)	0.27 (0.11-0.63)
Other[e] (N=51)	9.4 (6.0-10.1)	N=29	7.9 (4.0-8.9)	N=22	0.62 (0.32-1.23)	0.57 (0.27-1.20)
Squamous Cell (N=172)	6.2 (4.9-8.0)	N=78	7.4 (5.6-9.5)	N=94	1.32 (0.93-1.86)	1.56 (1.08-2.26)

[a] A HR that is less than 1.0 indicates that survival is better in the ALIMTA arm than in the docetaxel arm. Alternatively, a HR that is greater than 1.0 indicates survival is better in the docetaxel arm than in the ALIMTA arm.

[b] Unadjusted for multiple comparisons.

[c] HRs adjusted for ECOG PS, time since prior chemotherapy, disease stage, and gender.

[d] Includes adenocarcinoma, large cell, and other histologies except those with squamous cell type.

[e] The subgroup of "other" represents patients with a primary diagnosis of NSCLC whose disease did not clearly qualify as adenocarcinoma, squamous cell carcinoma, or large cell carcinoma.

14.4 Malignant Pleural Mesothelioma

A multi-center, randomized, single-blind study in 448 chemonaive patients with malignant pleural mesothelioma (MPM) compared survival in patients treated with ALIMTA in combination with cisplatin to survival in patients receiving cisplatin alone. ALIMTA was administered intravenously over 10 minutes at a dose of 500 mg/m^2 and cisplatin was administered intravenously over 2 hours at a dose of 75 mg/m^2 beginning approximately 30 minutes after the end of administration of ALIMTA. Both drugs were

given on Day 1 of each 21-day cycle. After 117 patients were treated, white cell and GI toxicity led to a change in protocol whereby all patients were given folic acid and vitamin B_{12} supplementation.

The primary analysis of this study was performed on the population of all patients randomly assigned to treatment who received study drug (randomized and treated). An analysis was also performed on patients who received folic acid and vitamin B_{12} supplementation during the entire course of study therapy (fully supplemented), as supplementation is recommended *[see Dosage and Administration (2.3)]*. Results in all patients and those fully supplemented were similar. Patient demographics are shown in Table 18.

Table 18: Summary of Patient Characteristics in MPM Study

Patient characteristic	Randomized and Treated Patients		Fully Supplemented Patients	
	ALIMTA/cis (N=226)	Cisplatin (N=222)	ALIMTA/cis (N=168)	Cisplatin (N=163)
Age (yrs)				
Median (range)	61 (29-85)	60 (19-84)	60 (29-85)	60 (19-82)
Gender (%)				
Male	184 (81.4)	181 (81.5)	136 (81.0)	134 (82.2)
Female	42 (18.6)	41 (18.5)	32 (19.0)	29 (17.8)
Origin (%)				
Caucasian	204 (90.3)	206 (92.8)	150 (89.3)	153 (93.9)
Hispanic	11 (4.9)	12 (5.4)	10 (6.0)	7 (4.3)
Asian	10 (4.4)	4 (1.9)	7 (4.2)	3 (1.8)
African descent	1 (0.4)	0	1 (0.6)	0
Stage at Entry (%)				
I	16 (7.1)	14 (6.3)	15 (8.9)	12 (7.4)
II	35 (15.6)	33 (15.0)	27 (16.2)	27 (16.8)
III	73 (32.4)	68 (30.6)	51 (30.5)	49 (30.4)
IV	101 (44.9)	105 (47.2)	74 (44.3)	73 (45.3)
Unspecified	1 (0.4)	2 (0.9)	1 (0.6)	2 (1.2)
Diagnosis/Histology[a] (%)				
Epithelial	154 (68.1)	152 (68.5)	117 (69.6)	113 (69.3)
Mixed	37 (16.4)	36 (16.2)	25 (14.9)	25 (15.3)
Sarcomatoid	18 (8.0)	25 (11.3)	14 (8.3)	17 (10.4)
Other	17 (7.5)	9 (4.1)	12 (7.1)	8 (4.9)
Baseline KPS[b] (%)				
70-80	109 (48.2)	97 (43.7)	83 (49.4)	69 (42.3)
90-100	117 (51.8)	125 (56.3)	85 (50.6)	94 (57.7)

[a] Only 67% of the patients had the histologic diagnosis of malignant mesothelioma confirmed by independent review.
[b] Karnofsky Performance Scale.

Table 19 and Figure 5 summarize the survival results for all randomized and treated patients regardless of vitamin supplementation status and those patients receiving vitamin supplementation from the time of enrollment in the trial.

Table 19: Efficacy of ALIMTA plus Cisplatin versus Cisplatin in Malignant Pleural Mesothelioma

Efficacy Parameter	Randomized and Treated Patients		Fully Supplemented Patients	
	ALIMTA/cis (N=226)	Cisplatin (N=222)	ALIMTA/cis (N=168)	Cisplatin (N=163)
Median overall survival (95% CI)	12.1 mos (10.0-14.4)	9.3 mos (7.8-10.7)	13.3 mos (11.4-14.9)	10.0 mos (8.4-11.9)
Hazard ratio	0.77		0.75	
Log rank p-value[a]	0.020		0.051	

[a] p-value refers to comparison between arms.

Similar results were seen in the analysis of patients (N=303) with confirmed histologic diagnosis of malignant pleural mesothelioma. There were too few non-white patients to assess possible ethnic differences. The effect in women (median survival 15.7 months with the combination versus 7.5 months on cisplatin alone), however, was larger than the effect in males (median survival 11 versus 9.4 respectively). As with any exploratory analysis, it is not clear whether this difference is real or is a chance finding.

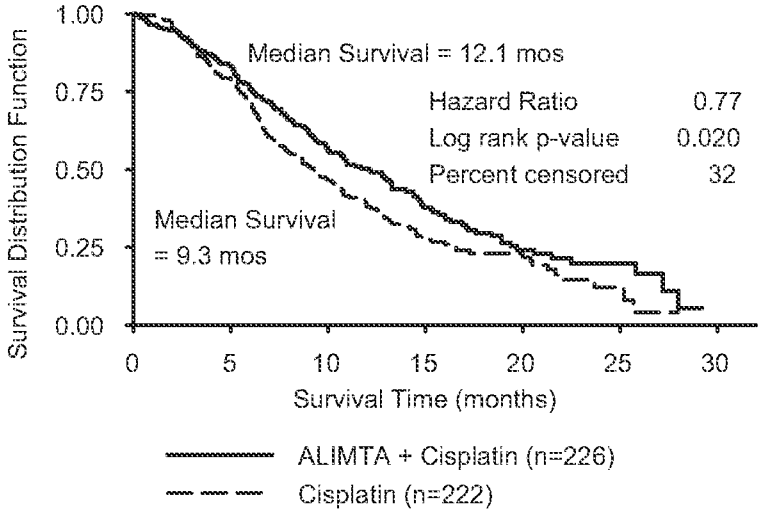


Figure 5: Kaplan-Meier Estimates of Survival Time for ALIMTA plus Cisplatin and Cisplatin Alone in all Randomized and Treated Patients.

Objective tumor response for malignant pleural mesothelioma is difficult to measure and response criteria are not universally agreed upon. However, based upon prospectively defined criteria, the objective tumor response rate for ALIMTA plus cisplatin was greater than the objective tumor response rate for cisplatin alone. There was also improvement in lung function (forced vital capacity) in the ALIMTA plus cisplatin arm compared to the control arm.

Patients who received full supplementation with folic acid and vitamin B_{12} during study therapy received a median of 6 and 4 cycles in the ALIMTA/cisplatin (N=168) and cisplatin (N=163) arms, respectively. Patients who never received folic acid and vitamin B_{12} during study therapy received a median of 2 cycles in both treatment arms (N=32 and N=38 for the ALIMTA/cisplatin and cisplatin arm, respectively). Patients receiving ALIMTA in the fully supplemented group received a relative dose intensity of 93% of the protocol specified ALIMTA dose intensity; patients treated with cisplatin in the same group received 94% of the projected dose intensity. Patients treated with cisplatin alone had a dose intensity of 96%.

15 REFERENCES

1. Preventing Occupational Exposures to Antineoplastic and Other Hazardous Drugs in Health Care Settings. NIOSH Alert 2004-165.
2. OSHA Technical Manual, TED 1-0.15A, Section VI: Chapter 2. Controlling Occupational Exposure to Hazardous Drugs. OSHA, 1999. http://www.osha.gov/dts/osta/otm/otm_vi/otm_vi_2.html
3. American Society of Health-System Pharmacists. ASHP guidelines on handling hazardous drugs. *Am J Health-Syst Pharm*. 2006; 63:1172-1193.
4. Polovich, M., White, J. M., & Kelleher, L. O. (eds.) 2005. Chemotherapy and biotherapy guidelines and recommendations for practice (2nd. ed.) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

ALIMTA, pemetrexed for injection, is available in sterile single-use vials containing 100 mg pemetrexed.
NDC 0002-7640-01 (VL7640): single-use vial with ivory flip-off cap individually packaged in a carton.
ALIMTA, pemetrexed for injection, is available in sterile single-use vials containing 500 mg pemetrexed.
NDC 0002-7623-01 (VL7623): single-use vial with ivory flip-off cap individually packaged in a carton.

16.2 Storage and Handling

ALIMTA, pemetrexed for injection, should be stored at 25°C (77°F); excursions permitted to 15-30°C (59-86°F) [see USP Controlled Room Temperature].

Chemical and physical stability of reconstituted and infusion solutions of ALIMTA were demonstrated for up to 24 hours following initial reconstitution, when stored refrigerated, 2-8°C (36-46°F), or at 25°C (77°F), excursions permitted to 15-30°C (59-86°F) [see USP Controlled Room Temperature]. When prepared as directed, reconstituted and infusion solutions of ALIMTA contain no antimicrobial preservatives. Discard unused portion *[see Dosage and Administration (2.5)]*.

ALIMTA is not light sensitive.

17 PATIENT COUNSELING INFORMATION

693 See FDA-Approved Patient Labeling
694 Patients should be instructed to read the patient package insert carefully.

695 **17.1 Need for Folic Acid and Vitamin B_{12}**
696 Patients treated with ALIMTA must be instructed to take folic acid and vitamin B_{12} as a prophylactic measure to reduce
697 treatment-related hematologic and gastrointestinal toxicity *[see Dosage and Administration (2.3)]*.

698 **17.2 Low Blood Cell Counts**
699 Patients should be adequately informed of the risk of low blood cell counts and instructed to immediately contact their
700 physician should any sign of infection develop including fever. Patients should also contact their physician if bleeding or symptoms of
701 anemia occur.

702 **17.3 Gastrointestinal Effects**
703 Patients should be instructed to contact their physician if persistent vomiting, diarrhea, or signs of dehydration appear.

704 **17.4 Concomitant Medications**
705 Patients should be instructed to inform the physician if they are taking any concomitant prescription or over-the-counter
706 medications including those for pain or inflammation such as non-steroidal anti-inflammatory drugs *[see Drug Interactions (7.1)]*.

707 Literature revised Month dd, yyyy

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INFORMATION FOR PATIENTS AND CAREGIVERS

ALIMTA® (uh-LIM-tuh)

(pemetrexed for injection)

Read the Patient Information that comes with ALIMTA before you start treatment and each time you get treated with ALIMTA. There may be new information. This leaflet does not take the place of talking to your doctor about your medical condition or treatment. Talk to your doctor if you have any questions about ALIMTA.

What is ALIMTA?

ALIMTA is a treatment for:

- **Malignant pleural mesothelioma.** This cancer affects the inside lining of the chest cavity. ALIMTA is given with cisplatin, another anti-cancer medicine (chemotherapy).
- Nonsquamous **non-small cell lung cancer.** This cancer is a disease in which malignant (cancer) cells form in the tissues of the lung. If you are having initial treatment for your lung cancer, ALIMTA may be given alone or in combination with another chemotherapy drug. If this is the first time you have been treated for your lung cancer, ALIMTA may be given with another anti-cancer drug called cisplatin. If you have completed initial treatment for your lung cancer, ALIMTA may be given alone immediately following your initial treatment. If you are being treated because your cancer has come back or you had trouble tolerating a prior treatment, ALIMTA may be given alone. Your doctor will speak to you about whether ALIMTA is appropriate for your specific type of non-small cell lung cancer.

To lower your chances of side effects of ALIMTA, you must also take folic acid and vitamin B_{12} prior to and during your treatment with ALIMTA. Your doctor will prescribe a medicine called a "corticosteroid" to take for 3 days during your treatment with ALIMTA. Corticosteroid medicines lower your chances of getting skin reactions with ALIMTA.

ALIMTA has not been studied in children.

What should I tell my doctor before taking ALIMTA?

Tell your doctor about all of your medical conditions, including if you:

- **are pregnant or planning to become pregnant.** ALIMTA may harm your unborn baby.
- **are breastfeeding.** It is not known if ALIMTA passes into breast milk. You should stop breastfeeding once you start treatment with ALIMTA.
- **are taking other medicines,** including prescription and nonprescription medicines, vitamins, and herbal supplements. ALIMTA and other medicines may affect each other causing serious side effects. Especially, tell your doctor if you are taking medicines called "nonsteroidal anti-inflammatory drugs" (NSAIDs) for pain or swelling. There are many NSAID medicines. If you are not sure, ask your doctor or pharmacist if any of your medicines are NSAIDs.

How is ALIMTA given?

- ALIMTA is slowly infused (injected) into a vein. The injection or infusion will last about 10 minutes. You will usually receive ALIMTA once every 21 days (3 weeks).
- If you are being treated with ALIMTA and cisplatin for the initial treatment of either mesothelioma or non-small cell lung cancer, ALIMTA will be given first as a 10 minute infusion into your vein and cisplatin (another anti-cancer drug) will also be given through your vein starting about 30 minutes after ALIMTA and ending about 2 hours later.
- If you have completed initial treatment for your non-small cell lung cancer, you may receive ALIMTA alone, given as a 10 minute infusion into your vein.
- If you are being treated because your non-small cell lung cancer has returned, you may receive ALIMTA alone, given as a 10 minute infusion into your vein.
- Your doctor will prescribe a medicine called a "corticosteroid" to take for 3 days during your treatment with ALIMTA. Corticosteroid medicines lower your chances for getting skin reactions with ALIMTA.
- **It is very important to take folic acid and vitamin B_{12} during your treatment with ALIMTA to lower your chances of harmful side effects.** You must start taking 350-1000 micrograms of folic acid every day for at least 5 days out of the 7 days before your first dose of ALIMTA. You must keep taking folic acid every day during the time you are getting treatment with ALIMTA, and for 21 days after your last treatment. You can get folic acid vitamins over-the-counter. Folic acid is also found in many multivitamin pills. Ask your doctor or pharmacist for help if you are not sure how to choose a folic acid product. Your doctor will give you vitamin B_{12} injections while you are getting treatment with ALIMTA. You will get your first vitamin B_{12} injection during the week before your first dose of ALIMTA, and then about every 9 weeks during treatment.
- You will have regular blood tests before and during your treatment with ALIMTA. Your doctor may adjust your dose of ALIMTA or delay treatment based on the results of your blood tests and on your general condition.

What should I avoid while taking ALIMTA?

- **Women who can become pregnant should not become pregnant during treatment with ALIMTA.** ALIMTA may harm the unborn baby.
- **Ask your doctor before taking medicines called NSAIDs.** There are many NSAID medicines. If you are not sure, ask your doctor or pharmacist if any of your medicines are NSAIDs.

What are the possible side effects of ALIMTA?

Most patients taking ALIMTA will have side effects. Sometimes it is not always possible to tell whether ALIMTA, another medicine, or the cancer itself is causing these side effects. **Call your doctor right away if you have a fever, chills, diarrhea, or mouth sores.** These symptoms could mean you have an infection.

The most common side effects of ALIMTA when given alone or in combination with cisplatin are:

- **Stomach upset, including nausea, vomiting, and diarrhea.** You can obtain medicines to help control some of these symptoms. Call your doctor if you get any of these symptoms.
- **Low blood cell counts:**
  - **Low red blood cells.** Low red blood cells may make you feel tired, get tired easily, appear pale, and become short of breath.
  - **Low white blood cells.** Low white blood cells may give you a greater chance for infection. If you have a fever (temperature above 100.4°F) or other signs of infection, call your doctor right away.
  - **Low platelets.** Low platelets give you a greater chance for bleeding. Your doctor will do blood tests to check your blood counts before and during treatment with ALIMTA.
- **Tiredness.** You may feel tired or weak for a few days after your ALIMTA treatments. If you have severe weakness or tiredness, call your doctor.
- **Mouth, throat, or lip sores** (stomatitis, pharyngitis). You may get redness or sores in your mouth, throat, or on your lips. These symptoms may happen a few days after ALIMTA treatment. Talk with your doctor about proper mouth and throat care.
- **Loss of appetite.** You may lose your appetite and lose weight during your treatment. Talk to your doctor if this is a problem for you.
- **Rash.** You may get a rash or itching during treatment. These usually appear between treatments with ALIMTA and usually go away before the next treatment. Call your doctor if you get a severe rash or itching.

Talk with your doctor, nurse or pharmacist about any side effect that bothers you or that doesn't go away.

These are not all the side effects of ALIMTA. For more information, ask your doctor, nurse or pharmacist.

General information about ALIMTA

Medicines are sometimes prescribed for conditions other than those listed in patient information leaflets. ALIMTA was prescribed for your medical condition.

This leaflet summarizes the most important information about ALIMTA. If you would like more information, talk with your doctor. You can ask your doctor or pharmacist for information about ALIMTA that is written for health professionals. You can also call 1-800-LILLY-RX (1-800-545-5979) or visit www.ALIMTA.com.

Patient information revised Month dd, yyyy

Eli Lilly and Company
Indianapolis, IN 46285, USA

www.ALIMTA.com

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2 Folate Biochemistry in Relation to Antifolate Selectivity

Roy L. Kisliuk

CONTENTS

1. INTRODUCTION

This review will deal with advances in folate biochemistry related to antifolate toxicity and selectivity. Because of the interrelatedness of reactions of folate metabolism, alterations in the activity of any folate enzyme, cellular transport system, as well as the concentration of any folate metabolite may be relevant to antifolate cytotoxicity and selectivity. Therefore, it is difficult to predict the results of inhibiting a given folate enzyme on antifolate selectivity. For example, in many experimental systems, the *cytotoxicity* of methotrexate is caused by its ability to inhibit dihydrofolate reductase, resulting

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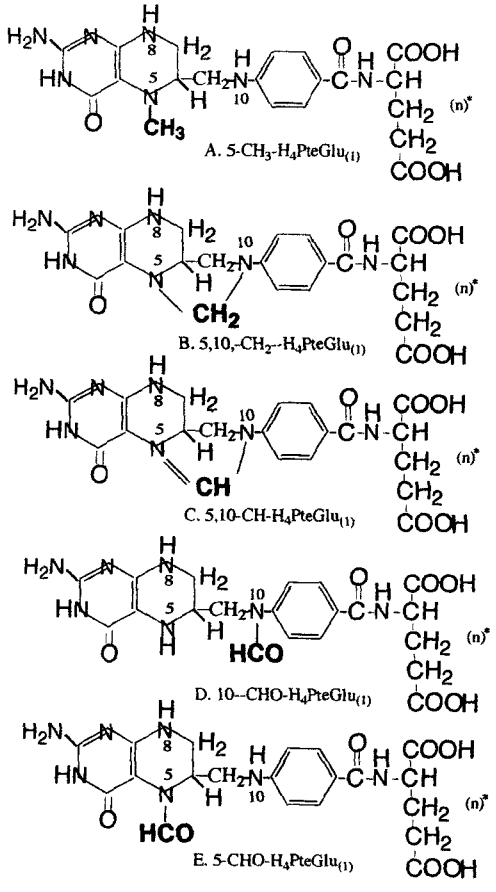

Fig. 1. Structures of tetrahydrofolic acid (THF) derivatives: **(A)** 5-methylTHF; **(B)** 5,10-methyleneTHF; **(C)** 5,10-methenylTHF; **(D)** 10-formylTHF; **(E)** 5-formylTHF (also called folinic acid, leucovorin or citrovorum factor). Pte stands for pteroic acid (p-[(2-amino-4-oxy-6-pteridyl-methyl)amino]benzoic acid).**n* refers to the total number of glutamate residues attached to pteroic acid. All additional Glu residues are joined by amide bonds to the γ-carboxyl group of Glu(1).

in lowered thymidylate formation, leading to lethal defects in DNA. However, its *selectivity* is often dependent on differential cellular uptake and polyglutamylation. Favorable clinical results with aminopterin, the forerunner of methotrexate, in acute leukemia in children were reported by Farber et al. *(1)* in 1948. This work depended on knowledge generated at the American Cyanamid Company, Pearl River, NY, on the structure of folic acid and the chemical synthesis of analogs in addition to the insightful clinical observations of the Farber group *(1)*. This work was done before the role of tetrahydrofolates in the metabolism of single carbon units was known. The present discussion of the current literature on folates is offered in the hope that, given the powerful analytical, structural, molecular genetic, and synthetic methods now available, new approaches to selective toxicity can be generated.

We focus on the metabolic interconversions and enzymology of three areas of folate metabolism, areas related to the essential metabolites methionine, thymidylate, and purine

1. methylen reductase
2. methioni
3. serine hy (Figs. 5,6
4. thymidyl
5. dihydrofc

[a]*C1THF synth*

6. *methylen dehydrog*
7. *methenyl cyclohydr*
8. *10-formyl synthetas*
9. 10-formy dehydrog
10. 5,10-meth synthetas
11. *glycinami formyltra*
12. *aminoimi ribonucle formyltra*
13. methionyl formyltra
14. *formiminc*
15. *formiminc*
16. *glycine cl*
17. dimethylg
18. sarcosine
19. folylpolyg synthetase
20. folylpolyg hydrolase

[a] Italics indic complex.

nucleotides. In encountered in (methanol leve level) (Fig. 1). ysis. The enzyr

Table 1
Folate Enzymes in Mammalian Cells

Enzyme	*Abbreviation*	*Cellular Location*	*Review Section*
1. methylenetetrahydrofolate reductase (Fig. 2)	MTHFR	cytoplasm	(2.2.)
2. methionine synthase (Fig. 2)	MS	cytoplasm	(2.2.)
3. serine hydroxymethyltransferase (Figs. 5,6)	SHMT	cytoplasm, mitochondria	(3.2.)
4. thymidylate synthase (Fig. 5)	TS	cytoplasm, nucleus	(3.3.)
5. dihydrofolate reductase (Fig. 5)	DHFR	cytoplasm	(3.4.)
[a]C_1THF synthase: [c]*(Fig. 6)*			(4.)
6. *methylenetetrahydrofolate dehydrogenase*	D	cytoplasm, mitochondria	
7. *methenyltetrahydrofolate cyclohydrolase*	C	cytoplasm, mitochondria	
8. *10-formyltetrahydrofolate synthetase*	S	cytoplasm	
9. 10-formylterahydrofolate dehydrogenase (Fig. 6)	FDH	cytoplasm	(5.)
10. 5,10-methenyltetrahydrofolate synthetase (Fig. 6)	MTHFS	cytoplasm, mitochondria	(6.)
11. *glycinamide ribonucleotide formyltransferase*[c] *(Fig. 7)*	GARFT	cytoplasm	(7.)
12. *aminoimidazolecarboxamide ribonucleotide formyltransferase*[b] *(Fig. 7)*	AICARFT	cytoplasm	(8.)
13. methionyl $tRNA_f^{met}$ formyltransferase	FMT	mitochondria	(9.)
14. *formiminotransferase*[b]	FTCD	cytoplasm	(10.)
15. *formiminocyclodeaminase*[b]	FTCD	cytoplasm	(10.)
16. *glycine cleavage system*[d]		mitochondria	(11.)
17. dimethylglycine dehydrogenase		mitochondria	(12.)
18. sarcosine dehydrogenase (Fig.3)		mitochondria	(12.)
19. folylpolyglutamate synthetase	FPGS	cytoplasm, mitochondria	(13.)
20. folylpolyglutamate hydrolase	GH	lysosomes, excreted	(14.)

[a] Italics indicate that the enzymes are part of a bifunctional[b], trifunctional[c], or tetrafunctional[d] protein complex.

nucleotides. In this order we proceed from the most reduced form of the single carbon unit encountered in mammalian metabolism to the more oxidized forms, that is, from methyl (methanol level) through methylene (formaldehyde level) to methenyl and formyl (formate level) (Fig. 1). We then consider the enzymes of folylpolyglutamate formation and hydrolysis. The enzymes discussed are listed in Table 1 with abbreviations, cellular location, and

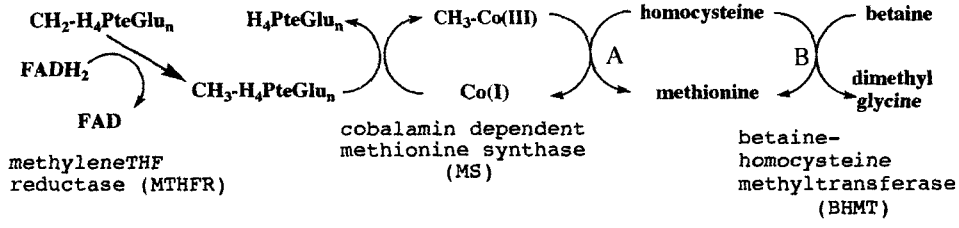

Fig. 2. Methylation of homocysteine to form methionine: **(A)** from 5-CH_3-H_4PteGlu$_n$ via methylcobalamin (CH_3-Co[III]), **(B)** from betaine.

location in the text. Human systems are emphasized. In order to keep the number of citations within bounds, key references serve as the source of additional current literature. Earlier folate studies are summarized in the treatise edited by Blakley and Benkovic *(2)* as well as in reviews by Kisliuk *(3)*, Shane *(4)*, and Wagner *(5)*.

2. METHIONINE

2.1 Introduction

In addition to its essential role as a constituent of proteins, methionine is a major metabolite, being at the confluence of the metabolism of folate, cobalamin, methyl groups, and polyamines. Our discussion will be divided into three sections: methionine methyl formation (Fig. 2), methionine methyl donation (Fig. 3), and propylamine donation (Fig. 4).

2.2 Methionine Methyl Formation

Methionine is required in the diet for normal growth but it is only the homocysteine portion that animals are unable to synthesize. Given adequate dietary folate and cobalamin, methyl groups are readily formed from CH_2-H_4PteGlu$_n$ by sequential reactions catalyzed by CH_2-H_4PteGlu reductase (MTHFR) and cobalamin-dependent methionine synthase (MS) (Fig. 2), which result in the methylation of homocysteine to form methionine. Interest in these enzymes is intense because low activity of either one leads to elevated blood levels of homocysteine, an important correlate in coronary disease *(6)*. Homocysteine has been implicated as a toxin of the endothelium of blood vessels *(7)* and has been shown to inhibit growth and p21ras methylation in cultured vascular endothelial cells *(8)*. The incidence of both coronary disease *(6)* and neural tube defects *(9)* is diminished by supplemental dietary folate.

Another enzyme catalyzing the methylation of homocysteine to methionine is betaine-homocysteine methyltransferase (Fig. 2). This enzyme has so far only been found in liver and kidney and its activity does not suffice to alleviate homocysteinemia *(7,10)*.

MTHFR catalyzes the reduction of CH_2-H_4PteGlu$_n$ to CH_3-H_4PteGlu$_n$ (Fig. 2). The hydrogen donor bound to the pig liver enzyme is FAD, which is itself reduced by NADPH. The K_m for CH_2-H_4PteGlu$_6$ is 0.1 μM as compared with 7 μM for CH_2-H_4PteGlu$_1$ *(11)*. MTHFR can catalyze the reverse reaction, the formation of CH_2-H_4PteGlu$_n$ from CH_3-H_4PteGlu$_n$, in the presence of the artificial electron acceptor menadione *(12)*, but this reaction is insignificant under physiological conditions in vitro *(7)* and in vivo *(13)*.

Sequencing of the cDNA for human MTHFR led to the discovery of an important variant (Ala222Val) which, in the homozygous state, leads to decreased MTHFR activ-

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Fig. 4. M
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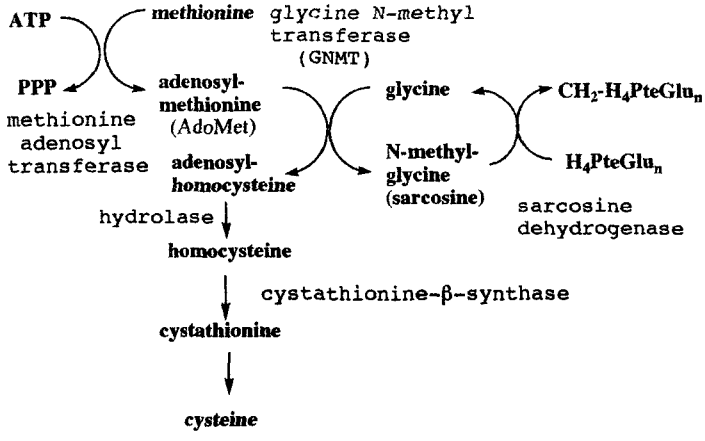

Fig. 3. Methionine as methyl donor; formation of homocysteine.

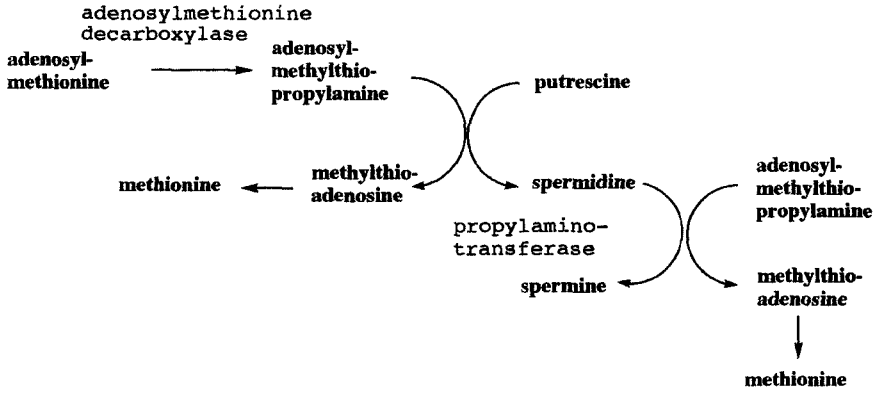

Fig. 4. Methionine as propylamine donor; formation of polyamines; putrescine, spermidine, and spermine.

ity which can lead to homocysteinemia *(14)*. Studies with an analogous mutation in *E. coli* MTHFR showed that FAD is more loosely bound to the mutant enzyme, leading to lower activity *(15)*. Addition of CH_3-$H_4PteGlu_1$ greatly slowed FAD dissociation. These results provide a reasonable explanation for the ability of high folate diets to lower blood levels of homocysteine in that MTHFR activity would be maintained even in the presence of the variant enzyme provided that, as is likely, CH_2-$H_4PteGlu_n$ in vivo also slows FAD dissociation and stabilizes MTHFR.

MTHFR is inhibited by adenosylmethionine (AdoMet) providing a system for decreasing methionine methyl group synthesis when dietary methionine is provided *(11)*.

Methionine synthase (Fig. 2) catalyzes the transfer of the methyl group of CH_3-$H_4PteGlu_n$ to homocysteine to form methionine. The human enzyme has been cloned in three laboratories *(16–18)* and consists of 1265 amino acids with a kDa of 140. Based on studies with the *E. coli* enzyme, which is highly homologous to the human enzyme, the cobalamin prosthetic group in the central portion of the protein interacts with homocysteine, CH_3-$H_4PteGlu_n$ and AdoMet, each of which is activated by a spe-

cific region of the protein *(19)*. The C-terminal AdoMet binding region is required because MS is occasionally inactivated by oxidation of the cob(I)alamin cofactor to an inactive cob(II)alamin form. Reactivation requires reductive methylation of cob(II)alamin to methylcob(I)alamin. The methyl group is provided by AdoMet. The reducing system is provided by a flavodoxin system in *E. coli*. In animals the auxiliary redox proteins have not been characterized, but defects in these proteins lead to functional MS deficiency *(20)*. The AdoMet requirement for the maintenance of MS activity is important in view of the essential role of MS in the incorporation of CH_3-$H_4PteGlu_1$ into cellular metabolism.

MS provides the only pathway by which the methyl group of CH_3-$H_4PteGlu_n$ can be removed in vivo, which makes MS activity essential for providing $H_4PteGlu_n$ from CH_3-$H_4PteGlu_n$. When MS activity is low because of either defective enzyme(s), cobalamin deficiency, or nitrous oxide inhibition, CH_3-$H_4PteGlu_n$ accumulates. The major source of cellular folates is usually blood CH_3-$H_4PteGlu_1$ which must be demethylated and then polyglutamylated before functioning as a coenzyme in thymidylate and purine nucleotide formation. Thus MS deficiency causes folate deficiency by trapping methyl groups in the form of CH_3-$H_4PteGlu_1$. CH_3-$H_4PteGlu_1$ is not retained in cells because it is a poor substrate for folylpolyglutamate synthetase and the monoglutamate form is not as tightly bound to folate enzymes or other folate binding proteins in cells as are the polyglutamate forms. Polyglutamate forms of CH_3-$H_4PteGlu$ are reported to have lower K_m values than the monoglutamate form for *E. coli* and bovine brain MS, but studies on the effect of polyglutamate chain length on substrate activity for human MS have not been reported. However, the fact that cultured cells can grow when supplied with CH_3-$H_4PteGlu_1$ indicates that it is demethylated to some extent by the action of MS.

Two interrelated aspects of methionine metabolism that have received attention in relation to chemotherapy are: the inhibition of MS by nitrous oxide *(21)* and the inability of many tumor cell lines to synthesize the methionine methyl group *(22)*. These cell lines are unable to grow when supplied with homocysteine and must be provided with methionine in the medium. In one such instance, a glioma cell line requires methionine because of lowered levels of cobalamin on MS *(23)*. MS-deficient cells should be especially sensitive to inhibition of methionine adenosyl transferase, which would deprive cells of AdoMet rendering them incapable of utilizing exogenous methionine as a source of methyl groups or polyamines.

Nitrous oxide specifically inactivates MS by reacting with cob(I)alamin and exposure to nitrous oxide leads to megaloblastic anemia *(24)*. Short-term remissions were observed in cases of chronic myeloid leukemia and childhood acute myeloid leukemia after treatment with nitrous oxide *(21)*. Methotrexate enhances nitrous oxide toxicity *(23)*. Methotrexate decreases MS activity by depleting cells of CH_3-$H_4PteGlu_n$ and of methylcobalamin. It is proposed that this leads to lower AdoMet levels and decreased methylation reactions that contribute to the cytotoxic action of methotrexate *(23)*. A reasonable explanation for methotrexate enhancement of nitrous oxide toxicity would be that both agents reduce MS activity, methotrexate by lowering cofactor levels, and nitrous oxide by inactivating cobalamin remaining on MS.

Recently cobalamin analogs were shown to inhibit the growth of BW5147 mouse lymphoma cells in culture *(25)*.

2.3 Methionine Methyl Donation

Methionine is converted to the active methyl donor, AdoMet, through the action of methionine adenosyl transferase (Fig. 3). Acceptors of the methyl group include glycine, DNA, RNA, norepinephrine, and at least 100 additional compounds *(5)* including arsenic compounds *(26)*. We limit our discussion to methylation of glycine and DNA. Glycine methylation serves as a route to recycle uneeded methionine methyl groups and is regulated by CH_3-$H_4PteGlu_n$ *(5)*. DNA methylation regulates gene expression *(27)*.

Glycine *N*-methyltransferase (GNMT) makes up 1–3% of the soluble protein in rat liver cytosol and is a major folate-binding protein even though it does not require a folate coenzyme for its activity *(5)*. It is a tetramer containing identical 292-amino acid residue 34-kDa subunits. When dietary methionine intake is high, glycine is methylated to sarcosine which is metabolized to CH_2-$H_4PteGlu_n$ and glycine in mitochondria. When dietary methionine is low, synthesis of CH_3-$H_4PteGlu_n$ is elevated because the inhibition of MTHFR by AdoMet is released. CH_3-$H_4PteGlu_n$ is an allosteric inhibitor of GNMT under conditions in which conserving methionine is advantageous. Thus AdoMet and CH_3-$H_4PteGlu_n$ regulate methionine methyl synthesis and disposal, respectively.

GNMT is also present at high levels in the exocrine pancreas in which methylation plays a role in exocrine secretion *(28)*.

Rat liver GNMT appears to be identical to the cytosolic receptor for benzo[a]pyrene which induces cytochrome P450 1A1 gene expression. Consistent with this proposed role for GNMT, its subunits are transported into the nucleus *(29)*.

The methylation of specific CpG sites in the DNA of the tumor suppressor gene p53 decreases when rats are maintained on a diet deficient in folate and methyl donors. The amount of p53 protein increases in response to many signals including DNA damage and nucleotide depletion *(30)*. p53 function is lost in about half of all human cancers. Alteration of the pattern of DNA methylation is postulated to be a factor in the increase in spontaneous liver cancer and in susceptibility to dimethylhydrazine-induced colon tumors *(31)*.

The adenosylhomocysteine arising from the action of AdoMet-dependent methyltransferases is hydrolyzed to homocysteine and adenosine (Fig. 3), an important reaction because adenosylhomocysteine is a potent inhibitor of AdoMet-dependent methylation reactions. Homocysteine may either be remethylated or metabolized to cysteine through the transsulfuration pathway that requires the action of cystathionine-β-synthase (Fig. 3) *(7)*. In mammalian liver, about half of the homocysteine is remethylated and half proceeds through the transsulfuration pathway. Many tissues however, lack cystathionine-β-synthase and are incapable of transsulfuration *(7)*. Genetic polymorphisms can result in decreased cystathionine-β-synthase activity which leads to homocysteinemia. One type of cystathionine-β-synthase deficiency responds to an increased supply of vitamin B_6 because the variant enzyme in this instance has a lowered affinity for its coenzyme, pyridoxal phosphate *(7)*.

2.4 Propylamine Donation

The polyamines putrescine, spermidine, and spermine are necessary for the growth of all cells *(32)*. Putrescine arises from the decarboxylation of ornithine. Spermidine and spermine are formed by sequential addition of propylamine residues to putrescine (Fig. 4). Adenosylmethylthiopropylamine arising from the decarboxylation of AdoMet is the

donor of propylamine. Thus ornithine decarboxylase and AdoMet decarboxylase are key enzymes involved in polyamine synthesis and the level of these enzymes is regulated by hormones, tumor promoters, and by polyamine levels *(32)*. A polyamine-responsive element has been identified in the 5'-leader sequence of the mRNA for AdoMet decarboxylase. This element codes for the peptide MAGDIS which plays a role in polyamine regulation of polyamine biosynthesis.

Spermine stimulates the activity of partially purified rat liver MS *(33)*. Since methionine provides propylamine for spermine synthesis (Fig. 4), stimulation of methionine synthesis by spermine could be part of a positive feedback loop related to the association of polyamine formation and growth. Polyamine analogs can cause cell-cycle arrest and apoptosis in human melanoma cells. One such analog is currently in Phase I clinical trial against solid tumors *(34)*.

3. THYMIDYLATE CYCLE

3.1 Introduction

Growing cells undergo apoptosis if *de novo* thymidylate synthesis is blocked. In colon carcinoma cells, thymineless apoptosis is mediated via Fas signaling *(35)*. Although cell death can be prevented if thymidine is supplied, under the usual in vivo conditions, plasma thymidine levels are too low for protection *(36)*. The three enzymes of the thymidylate synthesis cycle (Fig. 5) are serine hydroxymethytransferase (SHMT) *(37)*, thymidylate synthase (TS) *(38)*, and dihydrofolate reductase (DHFR) *(39)*. SHMT catalyzes the formation of CH_2-$H_4PteGlu_n$ from serine and $H_4PteGlu_n$. CH_2-$H_4PteGlu_n$ is then the substrate for the reductive methylation of dUMP to dTMP. Reducing equivalents are supplied by the conversion of $H_4PteGlu_n$ to $H_2PteGlu_n$, which makes it necessary to regenerate a molecule of $H_4PteGlu_n$ for every molecule of dTMP formed. DHFR enables completion of the cycle by catalyzing the reduction of $H_2PteGlu_n$ to $H_4PteGlu_n$, utilizing NADPH as the reductant.

3.2 Serine Hydroxymethyltransferase

Serine and glycine both provide CH_2-$H_4PteGlu_n$ which, in turn, is the major source of single carbon units for methionine, thymidylate and purine nucleotide synthesis. SHMT catalyzes the conversion of serine to glycine and CH_2-$H_4PteGlu_n$, whereas glycine is metabolized to CH_2-$H_4PteGlu_n$, CO_2, and NH_3^+ in mitochondria via the glycine cleavage system *(40)*. SHMT is found both in mitochondria (mSHMT) and in the cytosol (cSHMT). Chinese hamster ovary cells become auxotrophic for glycine when mSHMT is absent, which shows that the presence of cSHMT is insufficient to provide the cells need for glycine *(4)*. cSHMT may serve in gluconeogenesis by catalyzing the conversion of dietary glycine to serine, which then gives rise to pyruvate.

Human cDNAs that encode the two isozymes of SHMT have been cloned and sequenced *(41,42)*. Human mSHMT and cSHMT monomers (approx 55 kDa) contain 474 and 483 amino acid residues, respectively, and the deduced amino acid sequences show 63% identity. SHMT is a highly conserved enzyme, the human isozymes having approx 43% sequence identity with *E. coli* SHMT. All of the purified enzymes have four identical subunits that are yellow because of the presence of one molecule of pyridoxal phosphate on each subunit. Experiments with pig liver SHMT show that folylpolyglutamate

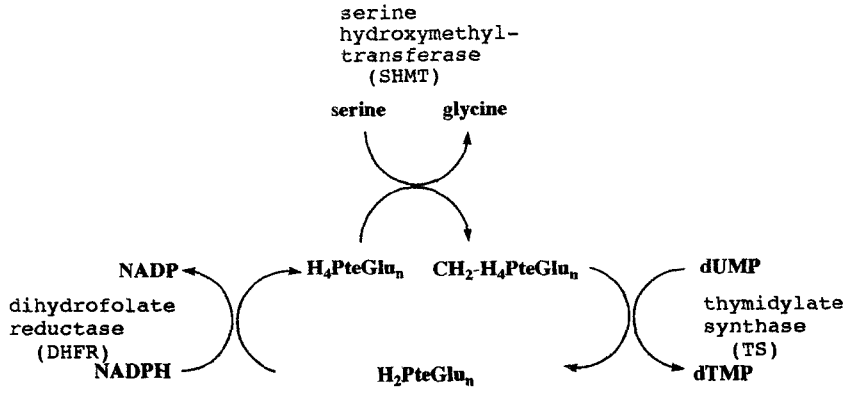

Fig. 5. The thymidylate cycle.

substrates and inhibitors bind more tightly to the enzyme than the monoglutamate forms *(43)*. Similar results were obtained with rabbit liver SHMT where it was shown, in addition, that cytosolic and mitochondrial SHMT have similar affinities for polyglutamate forms *(44)*.

An additional reaction catalyzed by both mSHMT and cSHMT is the hydrolysis of 5,10-CH-H_4PteGlu$_n$ to form 5-CHO-H_4PteGlu$_n$ (Fig. 6) *(45)*. A hydrated form of 5,10-CH-H_4PteGlu$_n$, (6R,11R)-5,10-hydroxymethylene-H_4PteGlu$_n$ is a likely intermediate in this reaction *(46)*. 5-CHO-H_4PteGlu$_{(n)}$ inhibits SHMT *(47)*, TS *(48)*, and AICARFT *(49)* and could therefore be an important compound in regulating one-carbon metabolism in that it could diminish both the formation and utilization of single-carbon units *(50)*. 5-CH_3-H_4PteGlu$_n$ also inhibits SHMT and thus could also act to diminish formation of single-carbon units along with 10-CHO-H_4PteGlu$_n$ which inhibits L1210 dihydrofolate reductase *(48)*. 5-CHO-H_4PteGlu$_n$, 10-CHO-H_4PteGlu$_n$ and 5-CH_3-H_4PteGlu$_n$ could all be feedback-signaling agents responding to increased levels of single-carbon folate metabolites at the formyl, hydroxymethyl, and methyl levels of oxidation. Consistent with this view, depletion of 5-CHO-H_4PteGlu$_n$ in neuroblastoma cells by overexpressing the cDNA for methenyltetrahydrofolate synthetase enhanced serine levels but lowered methionine levels, leading to the suggestion that serine synthesis and homocysteine remethylation compete for one-carbon units in the cytoplasm *(51)*.

Although SHMT as provider of the methylene group for the methyl group of thymidylate has been considered as a target for chemotherapeutic agents, specific antifolate inhibitors of SHMT have not been described. Perhaps an analog that bridges the folate site with the pyridoxal phosphate site would be effective. The serine analogs, D-fluoroalanine *(52)* and 4-chlorothreonine *(53)* are mechanism-based inhibitors of SHMT at millimolar levels.

3.3 Thymidylate Synthase

Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP. It is a dimeric enzyme (70 kD) with two catalytic sites per dimer, whose amino acid sequences are highly conserved *(54)*. Each monomer contributes amino acids to the catalytic site *(54)*. X-ray crystal structures of the *Lactobacillus casei* and *E. coli* enzymes show the folate cofactor bound above and trapping dUMP. Polyglutamate forms of the folate co-