PTO/SB/05 (09-04)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No.	CP391
First Inventor	BRITTAIN
Title	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
Express Mail Label No.	EV637773764US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ **Fee Transmittal Form (e.g., PTO/SB/17)**
   (Submit an original and a duplicate for fee processing)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* **63**]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** (*35 U.S.C.113*) [*Total Sheets* **6**]
5. **Oath or Declaration** [*Total Sheets* ___]
   a. ☐ Newly executed (original or copy)
   b. ☐ Copy from a prior application (37 CFR 1.63 (d))
   (for a continuation/divisional with Box 18 completed)
   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).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a.-c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATIONS PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee ___
10. ☐ **37 C.F.R. 3.73(b) Statement** ☐ **Power of Attorney**
    (when there is an assignee)
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☒ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. ☒ Other: Unsigned Declaration

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*
☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: ____ / ____
Prior application information: *Examiner* ____ *Art Unit:* ____

19. CORRESPONDENCE ADDRESS

☒ *Customer Number* 27573 *OR* ☐ *Correspondence address below*

Name					
Address					
City		*State*		*Zip Code*	
Country		*Telephone*		*Fax*	

Signature		Date	January 12, 2006
Name (Print/Type)	Scott K. Larsen	*Registration No. (Attorney/Agent)*	38,532

PTO/SB/17 (12-04v2)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 7,500.00

Complete if Known	
Application Number	UNKNOWN
Filing Date	January 12, 2006
First Named Inventor	BRITTAIN
Examiner Name	UNKNOWN
Art Unit	UNKNOWN
Attorney Docket No.	CP391

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 03-1195 Deposit Account Name: Cephalon, Inc.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	1,000.00
Design	200	100	100	50	130	65	____
Plant	200	100	300	150	160	80	____
Reissue	300	150	500	250	600	300	____
Provisional	200	100	0	0	0	0	____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 30 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee($)		Fee Paid ($)
78	-20 or HP=	58	x	$50	=	$2,900.00

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee($)		Fee Paid ($)
21	- 3 or HP=	18	x	$200	=	$3,600.00

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
____	____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), 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).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
62	- 100 =	0	/ 50 =	____	(round up to a whole number) x		=	____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	____
Other (e.g., late filing surcharge) : _____	____

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	38,532	Telephone	610-738-6463
Name (Print/Type)	Scott K. Larsen			Date	January 12, 2006

PTO/SB/05 (09-04)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No.	CP391
First Inventor	BRITTAIN
Title	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
Express Mail Label No.	EV637773764US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ **Fee Transmittal Form (e.g., PTO/SB/17)**
   (Submit an original and a duplicate for fee processing)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* **63**]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** (*35 U.S.C.113*) [*Total Sheets* **6**]
5. **Oath or Declaration** [*Total Sheets* ___]
   a. ☐ Newly executed (original or copy)
   b. ☐ Copy from a prior application (37 CFR 1.63 (d))
   (for a continuation/divisional with Box 18 completed)
   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).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a.-c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATIONS PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee ___
10. ☐ **37 C.F.R. 3.73(b) Statement** ☐ **Power of Attorney**
    (when there is an assignee)
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☒ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. ☒ Other: Unsigned Declaration

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*
☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: ____ / ____
Prior application information: *Examiner* ____ *Art Unit:* ____

19. CORRESPONDENCE ADDRESS

☒ *Customer Number* 27573 *OR* ☐ *Correspondence address below*

Name					
Address					
City		*State*		*Zip Code*	
Country		*Telephone*		*Fax*	

Signature		Date	January 12, 2006
Name (Print/Type)	Scott K. Larsen	*Registration No. (Attorney/Agent)*	38,532

PTO/SB/17 (12-04v2)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 7,500.00

Complete if Known	
Application Number	UNKNOWN
Filing Date	January 12, 2006
First Named Inventor	BRITTAIN
Examiner Name	UNKNOWN
Art Unit	UNKNOWN
Attorney Docket No.	CP391

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 03-1195 Deposit Account Name: Cephalon, Inc.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	1,000.00
Design	200	100	100	50	130	65	____
Plant	200	100	300	150	160	80	____
Reissue	300	150	500	250	600	300	____
Provisional	200	100	0	0	0	0	____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 30 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee($)		Fee Paid ($)
78	-20 or HP=	58	x	$50	=	$2,900.00

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee($)		Fee Paid ($)
21	- 3 or HP=	18	x	$200	=	$3,600.00

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
____	____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), 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).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
62	- 100 =	0	/ 50 =	____	(round up to a whole number) x		=	____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	____
Other (e.g., late filing surcharge) : _____	____

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	38,532	Telephone	610-738-6463
Name (Print/Type)	Scott K. Larsen			Date	January 12, 2006

BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

FIELD OF THE INVENTION

The present invention pertains to the field of pharmaceutical compositions for the treatment of various disease states, especially neoplastic diseases and autoimmune diseases. Particularly, it relates to pharmaceutical formulations comprising nitrogen mustards, particularly the nitrogen mustard bendamustine, e.g., bendamustine HCl.

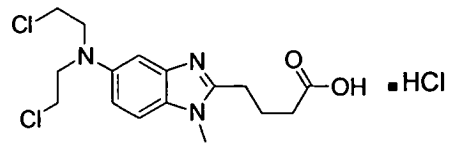
BACKGROUND OF THE INVENTION

The present invention claims the benefit of and priority to US Serial No. 60/644,354, filed January 14, 2005, entitled, "Bendamustine Pharmaceutical Compositions," which is incorporated herein by reference in its entirety, including figures and claims.

The following description includes information that may be useful in understanding the present invention. It is not an admission that any such information is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

Because of their high reactivity in aqueous solutions, nitrogen mustards are difficult to formulate as pharmaceuticals and are often supplied for administration in a lyophilized form that requires reconstitution, usually in water, by skilled hospital personal prior to administration. Once in aqueous solution, nitrogen mustards are subject to degradation by hydrolysis, thus, the reconstituted product should be administered to a patient as soon as possible after its reconstitution.

Bendamustine, (4-{5-[Bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl} butyric acid, is an atypical structure with a benzimidazole ring, whose structure includes an active nitrogen mustard (see Formula I, which shows bendamustine hydrochloride).



Formula I

Bendamustine was initially synthesized in 1963 in the German Democratic Republic (GDR) and was available from 1971 to 1992 in that location under the name Cytostasan®. Since that time, it has been marketed in Germany under the tradename Ribomustin®. It has been widely used in Germany to treat chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, and breast cancer.

Due to its degradation in aqueous solutions (like other nitrogen mustards), bendamustine is supplied as a lyophilized product. The current lyophilized formulation of bendamustine (Ribomustin®) contains bendamustine hydrochloride and mannitol in a sterile lyophilized form as a white powder for intravenous use following reconstitution. The finished lyophilisate is unstable when exposed to light. Therefore, the product is stored in brown or amber-colored glass bottles. The current lyophilized formulation of bendamustine contains degradation products that may occur during manufacturing of the drug substance and/or during the lyophilization process to make the finished drug product.

Currently bendamustine is formulated as a lyophilized powder for injection with 100 mg of drug per 50 mL vial or 25 mg of drug per 20 mL vial. The vials are opened and reconstituted as close to the time of patient administration as possible. The product is reconstituted with 40 mL (for the 100 mg presentation) or 10 mL (for the 25 mg presentation) of Sterile Water for Injection. The reconstituted product is further diluted into 500 mL, q.s., 0.9% Sodium Chloride for Injection. The route of administration is by intravenous infusion over 30 to 60 minutes.

Following reconstitution with 40 mL Sterile Water for Injection, vials of bendamustine are stable for a period of 7 hours under room temperature storage or for 6 days upon storage at 2-8°C. The 500 mL admixture solution must be administered to the patient within 7 hours of vial reconstitution (assuming room temperature storage of the admixture).

The reconstitution of the present bendamustine lyophilized powder is difficult. Reports from the clinic indicate that reconstitution can require at least fifteen minutes and may require as long as thirty minutes. Besides being burdensome and time-consuming for the healthcare professional responsible for reconstituting the product, the lengthy exposure of bendamustine to water during the reconstitution process increases the potential for loss of potency and impurity formation due to the hydrolysis of the product by water.

Thus, a need exists for lyophilized formulations of bendamustine that are easier to reconstitute and which have a better impurity profile than the current lyophilate (lyophilized powder) formulations of bendamustine.

German (GDR) Patent No. 34727 discloses a method of preparing ω-[5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-alkane carboxylic acids substituted in the 1-position.

German (GDR) Patent No. 80967 discloses an injectable preparation of γ-[1-methyl-5-bis-(β-chloroethyl)-amino-benzimaidazolyl-(2)]-butric acid hydrochloride.

German (GDR) Patent No. 159877 discloses a method for preparing 4-[1-methyl-5-bis (2-chloroethyl) amino-benzimidazolyl-2)-butyric acid.

German (GDR) Patent No. 159289 discloses an injectable solution of bendamustine.

Ribomustin® bendamustine Product monograph (updated 1/2002) http://www.ribosepharm.de/pdf/ribomustin_bendamustin/productmonograph.pdf provides information about Ribomustin® including product description.

Ni et al. report that the nitrosourea SarCNU was more stable in pure tertiary butanol than in pure acetic acid, dimethyl sulfoxide, methylhydroxy, water or in TBA/water mixtures (Ni et al. (2001) *Intl. J. Phamaceutics* 226:39-46).

Lyophilized cyclophoshamide is known in the art see e.g., US Patent Nos. 5,418,223; 5,413,995; 5,268,368; 5,227,374; 5,130,305; 4,659,699; 4,537,883; and 5,066,647.

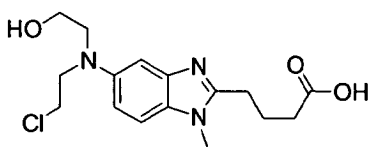
The lyophilized nitrogen mustard Ifosfamide is disclosed in International Publication No. WO 2003/066027; US Pat. Nos. 6,613,927; 5,750,131; 5,972,912; 5,227,373; and 5,204,335.

Teagarden et al. disclose lyophilized formulations of prostaglandin E-1 made by dissolving PGE-1 in a solution of lactose and tertiary butyl alcohol (US Pat. No. 5,770,230).

SUMMARY OF THE INVENTION

The present invention is directed to stable pharmaceutical compositions of nitrogen mustards, in particular lyophilized bendamustine and its use in treatment of various disease states, especially neoplastic diseases and autoimmune diseases.

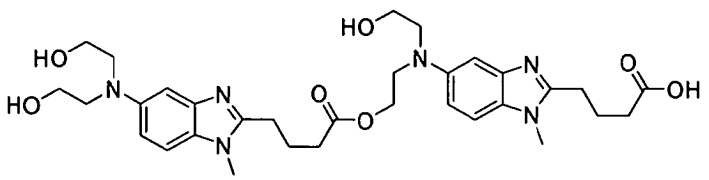
An embodiment of the invention is a pharmaceutical composition of bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine) HP1, as shown in Formula II,



Formula II

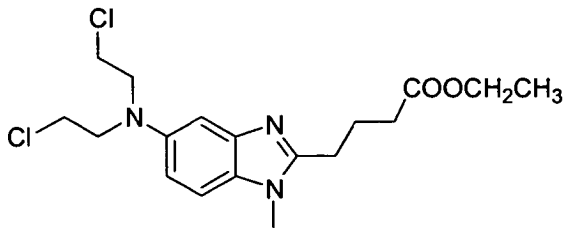
at the time of release or where the HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized pharmaceutical composition of bendamustine as described herein. In a preferred embodiment is a pharmaceutical composition of bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%.

Another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.1 % to about 0.3 % bendamustine dimer as shown in Formula III at release or at time zero after reconstitution



Formula III.

Yet another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.5%, preferably 0.15% to about 0.5%, bendamustine ethylester, as shown in Formula IV at release or at time zero after reconstitution



Formula IV.

Yet another embodiment of the invention is a lyophilized preparation of bendamustine wherein the concentration of bendamustine ethylester (Formula IV) is no more than 0.2%, preferably 0.1%, greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

In another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine) HP1 at the time of drug product release. In a preferred embodiment is a lyophilized preparation of bendamustine containing not more than about 0.50% (area percent of bendamustine) HP1, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%. An aspect of this embodiment is lyophilized preparations of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, (area percent of bendamustine) HP1 at the time of release of drug product where the lyophilized preparation is packaged in a vial or other pharmaceutically acceptable container.

In yet another aspect of the invention, the lyophilized preparations of bendamustine are stable with respect to the amount of HP1 for at least about 6 months, preferably 12 months, preferably 24 months, to about 36 months or greater when stored at about 2° to about 30°. Preferred temperatures for storage are about 5° C and about room temperature.

Another embodiment of the invention is a pharmaceutical dosage form that includes a pharmaceutical composition of bendamustine containing not more than about 0.5% to about 0.9% HP1, preferably not more than about 0.50%, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%, where the HP1 is the amount of HP1 present at release or at time zero after reconstitution of a lyophilized preparation of bendamustine of the present invention. In preferred aspects of the invention, the dosage form can be about 5 to about 500 mg of bendamustine, about 10 to about 300 mg of bendamustine, about 25 mg of bendamustine, about 100 mg of bendamustine, and about 200 mg of bendamustine.

Yet another embodiment of the invention is a pharmaceutical dosage form that includes a lyophilized preparation of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, HP1. Preferred dosage forms can be about 5 to about 500 mg of bendamustine, about 10 to about 300 mg of bendamustine, about 25 mg of bendamustine, about 100 mg of bendamustine, and about 200 mg of bendamustine.

In still another embodiment, the invention includes a pharmaceutical composition of bendamustine including bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine), preferably not more than about 0.50%, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%, and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release or time zero after reconstitution of a lyophilized pharmaceutical composition of bendamustine as disclosed herein. In different aspects of this embodiment, the organic solvent is selected from one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane. Preferred organic solvents include one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol. A more preferred organic solvent is tertiary butanol, also known as TBA, t-butanol, tert-butyl alcohol or tertiary butyl alcohol.

The present invention involves a method for obtaining agency approval for a bendamustine product, the improvement which includes setting a release specification for bendamustine degradants at less than about 4.0%, preferably about 2.0 % to about 4.0 %, (area percent bendamustine) or otherwise to achieve the pharmaceutical compositions described herein. An aspect of this embodiment is a method for obtaining agency approval for a bendamustine product which includes setting a release specification for HP1 to be less than or equal to 1.5% (area percent Bendamustine). The bendamustine product herein contains not more than about 0.5% (area percent of bendamustine) HP1 at release.

Another embodiment is a method for obtaining agency approval for a bendamustine product, the improvement which includes setting a shelf-life specification for bendamustine degradants at less than about 7.0%, preferably about 5.0% to about 7.0%, (area percent bendamustine) where the product is stored at about 2°C to about 30°C. Preferred temperatures for storage are about 5°C and about room temperature. The bendamustine product herein contains not more than about 0.5% (area percent of bendamustine) HP1 at release.

Another embodiment of the invention is a process for manufacturing a lyophilized preparation of bendamustine which includes controlling for the concentration of bendamustine degradants in the final product, such that the concentration of bendamustine degradants is less than about 4.0%, preferably no more than about 2.0 % to about 4.0 %, (area percent of bendamustine) at release or otherwise to achieve the pharmaceutical compositions described herein. The bendamustine product herein contains not more than about 0.5% to about 0.9%, preferably about 0.5%, (area percent of bendamustine) HP1 at release.

The present invention discloses a process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of HP1 is less than 0.9%, preferably 0.5%, (area percent of bendamustine) and, at the time of product expiration, the concentration of bendamustine degradants is less than about 7.0%, preferably no more than about 5.0% to about 7.0%; wherein said product is stored at about 2°C to about 30°C.

Another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization from about 0 to 24 hours does not exceed about 0.5% to about 0.9% (area percent of bendamustine) preferably 0.50%, preferably 0.45%, more preferably 0.40%, more preferably 0.35%, even more preferably 0.30%. An aspect of this embodiment is the lyophilized powder produced from the pre-lyophilization solution or dispersion.

Still another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of bendamustine ethylester produced during lyophilization from about 0 to 24 hours does not exceed about 0.5% (area percent bendamustine). An aspect of this embodiment is the lyophilized powder produced from the pre-lyophilization solution or dispersion.

Still another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of bendamustine ethylester (as shown in Formula IV) produced during lyophilization from about 0 to 24 hours is no more than 0.2%, preferably 0.1%, greater than the concentration of bendamustine ethylester as found in the drug substance used to make the pre-lyophilization solution. A preferred organic solvent is tertiary butanol.

The invention also discloses methods for preparing a bendamustine lyophilized preparation that includes dissolving bendamustine in a stabilizing concentration of an alcohol solvent of between about 5% to about 100% (v/v alcohol to form a pre-lyophilization solution; and lyophilizing the pre-lyophilization solution; wherein the bendamustine lyophilized preparation made from such methods contains not more than about 0.5% to about 0.9%, preferably 0.5%, (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release or at time zero

after reconstitution of the lyophilized pharmaceutical composition of bendamustine. Other alcohol concentrations include about 5% to about 99.9%, about 5% to about 70%, about 5% to about 60%, about 5% to about 50%, about 5% to about 40%, about 20% to about 35%. Preferred concentrations of alcohol are from about 20% to about 30%. Preferred alcohols include one or more of methanol, ethanol, propanol, iso-propanol, butanol, and tertiary-butanol. A more preferred alcohol is tertiary-butanol. A preferred concentration of tertiary-butanol is about 20% to about 30%, preferably about 30%. An aspect of this embodiment is the addition of an excipient before lyophilization. A preferred excipient is mannitol. Preferred pre-lyophilized concentrations of bendamustine are from about 2 mg/mL to about 50 mg/mL.

In a preferred method for preparing a bendamustine lyophilized preparation, lyophilizing the pre-lyophilization solution comprises i) freezing the pre-lyophilization solution to a temperature below about -40°C, preferably -50°C, to form a frozen solution; ii) holding the frozen solution at or below -40°C, preferably -50°C, for at least 2 hours; iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution; iv) holding for about 10 to about 70 hours; v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and vii) holding for about 5 to about 40 hours to form a bendamustine lyophilized preparation. In a more preferred method lyophilizing the pre-lyophilization solution comprises i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution; ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours; iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution; iv) holding at a primary drying temperature for about 10 to about 48 hours; v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours. A preferred alcohol is tertiary-butanol. A preferred concentration of tertiary-butanol is about 20% to about 30%, preferably about 30%. An aspect of this embodiment is the addition of an excipient before lyophilization. A preferred excipient is mannitol. Preferred pre-lyophilized concentrations of bendamustine are from about 2 mg/mL to about 50 mg/mL.

Another embodiment of the invention is the lyophilized powder or preparation obtained from the methods of preparing a bendamustine lyophilized preparation disclosed herein.

The invention also involves bendamustine formulations for lyophilization that include an excipient and a stabilizing concentration of an organic solvent. A preferred formulation includes bendamustine at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water. Included in this embodiment of the invention are the lyophilized preparations made from such bendamustine formulations.

Included in the inventions are methods of treating a medical condition in a patient that involve administering a therapeutically effective amount of a pharmaceutical composition of the invention where the condition is amenable to treatment with said pharmaceutical composition. Some conditions amenable to treatment with the compositions of the invention include chronic lymphocytic leukemia (CLL), Hodgkin's disease, non-Hodgkin's lymphoma (NHL), multiple myeloma (MM), breast cancer, small cell lung cancer, hyperproliferative disorders, and an autoimmune disease. Preferred conditions include NHL, CLL, breast cancer, and MM. Preferred autoimmune diseases include rheumatoid arthritis, multiple sclerosis or lupus.

Included in the inventions are the use of the pharmaceutical compositions or pharmaceutical preparations of the invention in the manufacture of a medicament for the treatment of a medical condition, as defined herein, in a patient that involve administering a therapeutically effective amount of a pharmaceutical composition of the invention where the condition is amenable to treatment with said pharmaceutical composition.

Also included in the invention are methods of treating in which the pharmaceutical compositions of the invention are in combination with one or more anti-neoplastic agents where the antineoplastic agent is given prior, concurrently, or subsequent to the administration of the pharmaceutical composition of the invention. Preferred antineoplastic agents are antibodies specific for CD20.

Another embodiment of the invention is a lyophilization cycle for producing lyophilized bendamustine preparations of the invention. A preferred lyophilization cycle includes a) freezing to about -50°C over about 8 hours; b) holding at -50°C for about 4 hours; c)

ramping to -25°C over about 3 hours; d) holding at -10°C for 30 hours; e) ramping to between about 25°C and about 40°C or higher for about 3 hours; f) holding between about 25°C and about 40°C for about 25 hours; g) ramping to about 20°C in 1 hour; h) unloading at about 20°C, at a pressure of 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying. An aspect of this cycle involves step (e) which is ramped to about 30-35°C for 3 hours and then ramped to 40°C for 5 hours. Another aspect of this embodiment is the lyophilized powered prepared from such lyophilization cycles. A more preferred lyophilization cycle includes i) starting with a shelf temperature of about 5°C for loading; ii) freezing to about -50°C over about 8 hours; iii) holding at -50°C for about 4 hours; iv) ramping to about -20°C over about 3 hours; v) holding at about -20°C for 6 hours; ramping to about -15°C over about 1 hour; vi) holding at -15°C for about 20 hours; vii) ramping to about -15°C over about 1 hour; viii) holding at about -15°C for about 20 hours; ix) ramping to about -12°C over about 0.5 hours; x) holding at about -12°C for about 15.5 hours; xi) ramping to between about 25°C and about 40°C or higher for about 15 hours; xii) holding between about 25°C and about 40°C for about 10 hours; xiii) ramping to about 40°C over about 1 hour; and xiv) holding at about 40°C for about 5 hours; unloading at about 5°C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying. In a preferred embodiment step (xi) is ramped to about 30-35°C for about 15 hours.

The invention also encompasses a pharmaceutical dosage form of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form. Preferred concentrations of bendamustine include about 10 to about 500 mg/container, about 100 mg/container, about 5 mg to about 2 g/container and about 170 mg/container.

The present invention also includes pre-lyophilized pharmaceutical compositions of bendamustine. A preferred pre-lyophilized composition includes bendamustine HCl

about 15 mg/mL, mannitol about 25.5 mg/mL, about 30% (v/v) tertiary-butyl alcohol, and water.

These and other embodiments of the invention are described hereinbelow or are evident to persons of ordinary skill in the art based on the following disclosures.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the solubility of bendamustine at various temperatures for two different solutions of bendamustine in tertiary butanol.

Fig. 2 shows the purity results of an HPLC analysis after incubating bendamustine in various alcohols for 24 hours at 5°C. Results are presented as the area percent of the bendamustine peak.

Fig. 3 shows HP1 (Formula II) formation after 24 hours in various alcohol/water co-solvents at 5°C

Fig 4 shows dimer (Formula III) formation after 24 hours in various alcohol/water co-solvents at 5°C

Fig. 5- shows a lyophilization cycle for bendamustine using a TBA/water co-solvent.

Fig. 6 shows a chromatogram for Ribomustin® using HPLC method No. 1.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms "formulate" refers to the preparation of a drug, e.g., bendamustine, in a form suitable for administration to a mammalian patient, preferably a human. Thus, "formulation" can include the addition of pharmaceutically acceptable excipients, diluents, or carriers.

As used herein, the term "lyophilized powder" or "lyophilized preparation" refers to any solid material obtained by lyophilization, i.e., freeze-drying of an aqueous solution. The aqueous solution may contain a non-aqueous solvent, i.e. a solution composed of aqueous and one or more non-aqueous solvent(s). Preferably, a lyophilized preparation is one in which the solid material is obtained by freeze-drying a solution composed of aqueous and one or more non-aqueous solvents, more preferably the non-aqueous solvent is an alcohol.

By "stable pharmaceutical composition" is meant any pharmaceutical composition having sufficient stability to have utility as a pharmaceutical product. Preferably, a stable pharmaceutical composition has sufficient stability to allow storage at a convenient temperature, preferably between -20°C and 40°C, more preferably about 2°C to about 30°C, for a reasonable period of time, e.g., the shelf-life of the product which can be as short as one month but is typically six months or longer, more preferably one year or longer even more preferably twenty-four months or longer, and even more preferably thirty-six months or longer. The shelf-life or expiration can be that amount of time where the active ingredient degrades to a point below 90% purity. For purposes of the present invention stable pharmaceutical composition includes reference to pharmaceutical compositions with specific ranges of impurities as described herein. Preferably, a stable pharmaceutical composition is one which has minimal degradation of the active ingredient, e.g., it retains at least about 85 % of un-degraded active, preferably at least about 90 %, and more preferably at least about 95%, after storage at 2-30°C for a 2-3 year period of time.

By "stable lyophilized preparation" is meant any lyophilized preparation having sufficient stability, such characteristics as similarly defined herein for a stable pharmaceutical composition, to have utility as a pharmaceutical product

By "degraded" is meant that the active has undergone a change in chemical structure.

The term "therapeutically effective amount" as used herein refers to that amount of the compound being administered that will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to the treatment of neoplasms, a therapeutically effective amount refers to that amount which has the effect of (1) reducing the size of the tumor, (2) inhibiting (that is, slowing to some extent, preferably stopping) tumor metastasis, (3) inhibiting to some extent (that is, slowing to some extent, preferably stopping) tumor growth, and/or, (4) relieving to some extent (or, preferably, eliminating) one or more symptoms associated with the cancer. Therapeutically effective amount can also mean preventing the disease from occurring in an animal that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment). Further, therapeutically effective amount can be that amount that increases the

life expectancy of a patient afflicted with a terminal disorder. Typical therapeutically effective doses for bendamustine for the treatment of non-Hodgkin's lymphoma can be from about 60-120 mg/m^2 given as a single dose on two consecutive days. The cycle can be repeated about every three to four weeks. For the treatment of chronic lymphocytic leukemia (CLL) bendamustine can be given at about 80-100 mg/m^2 on days 1 and 2. The cycle can be repeated after about 4 weeks. For the treatment of Hodgkin's disease (stages II-IV), bendamustine can be given in the "DBVBe regimen" with daunorubicin 25 mg/m^2 on days 1 and 15, bleomycin 10 mg/m^2 on days 1 and 15, vincristine 1.4 mg/m^2 on days 1 and 15, and bendamustine 50 mg/m^2 on days 1-5 with repetition of the cycle about every 4 weeks. For breast cancer, bendamustine (120 mg/m^2) on days 1 and 8 can be given in combination with methotrexate 40 mg/m^2 on days 1 and 8, and 5-fluorouracil 600 mg/m^2 on days 1 and 8 with repetition of the cycle about every 4 weeks. As a second-line of therapy for breast cancer, bendamustine can be given at about 100-150 mg/m^2 on days 1 and 2 with repetition of the cycle about every 4 weeks.

As used herein "neoplastic" refers to a neoplasm, which is an abnormal growth, such growth occurring because of a proliferation of cells not subject to the usual limitations of growth. As used herein, "anti-neoplastic agent" is any compound, composition, admixture, co-mixture, or blend which inhibits, eliminates, retards, or reverses the neoplastic phenotype of a cell.

As used herein "hyperproliferation" is the overproduction of cells in response to a particular growth factor. "Hyperproliferative disorders" are diseases in which the cells overproduce in response to a particular growth factor. Examples of such "hyperproliferative disorders" include diabetic retinopathy, psoriasis, endometriosis, cancer, macular degenerative disorders and benign growth disorders such as prostate enlargement.

As used herein, the term "vial" refers to any walled container, whether rigid or flexible.

"Controlling" as used herein means putting process controls in place to facilitate achievement of the thing being controlled. For example, in a given case, "controlling" can mean testing samples of each lot or a number of lots regularly or randomly; setting the concentration of degradants as a release specification; selecting process conditions, e.g.,

use of alcohols and/or other organic solvents in the pre-lyophilization solution or dispersion, so as to assure that the concentration of degradants of the active ingredient is not unacceptably high; etc. Controlling for degradants by setting release specifications for the amount of degradants can be used to facilitate regulatory approval of a pharmaceutical product by a regulatory agency, such as the U.S. Food and Drug Administration and similar agencies in other countries or regions ("agency").

The term "pharmaceutically acceptable" as used herein means that the thing that is pharmaceutically acceptable, e.g., components, including containers, of a pharmaceutical composition, does not cause unacceptable loss of pharmacological activity or unacceptable adverse side effects. Examples of pharmaceutically acceptable components are provided in The United States Pharmacopeia (USP), The National Formulary (NF), adopted at the United States Pharmacopeial Convention, held in Rockville, Md. in 1990 and FDA Inactive Ingredient Guide 1990, 1996 issued by the U.S. Food and Drug Administration (both are hereby incorporated by reference herein, including any drawings). Other grades of solutions or components that meet necessary limits and/or specifications that are outside of the USP/NF may also be used.

The term "pharmaceutical composition" as used herein shall mean a composition that is made under conditions such that it is suitable for administration to humans, e.g., it is made under GMP conditions and contains pharmaceutically acceptable excipients, e.g., without limitation, stabilizers, bulking agents, buffers, carriers, diluents, vehicles, solubilizers, and binders. As used herein pharmaceutical composition includes but is not limited to a pre-lyophilization solution or dispersion as well as a liquid form ready for injection or infusion after reconstitution of a lyophilized preparation.

A "pharmaceutical dosage form" as used herein means the pharmaceutical compositions disclosed herein being in a container and in an amount suitable for reconstitution and administration of one or more doses, typically about 1-2, 1-3, 1-4, 1-5, 1-6, 1-10, or about 1-20 doses. Preferably, a "pharmaceutical dosage form" as used herein means a lyophilized pharmaceutical composition disclosed herein in a container and in an amount suitable for reconstitution and delivery of one or more doses, typically about 1-2, 1-3, 1-4, 1-5, 1-6, 1-10, or about 1-20 doses. The pharmaceutical dosage form can comprise a vial or syringe or other suitable pharmaceutically acceptable container. The

pharmaceutical dosage form suitable for injection or infusion use can include sterile aqueous solutions or dispersions or sterile powders comprising an active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The prevention of the growth of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

As used herein, the term "excipient" means the substances used to formulate active pharmaceutical ingredients (API) into pharmaceutical formulations; in a preferred embodiment, an excipient does not lower or interfere with the primary therapeutic effect of the API. Preferably, an excipient is therapeutically inert. The term "excipient" encompasses carriers, diluents, vehicles, solubilizers, stabilizers, bulking agents, and binders. Excipients can also be those substances present in a pharmaceutical formulation as an indirect or unintended result of the manufacturing process. Preferably, excipients are approved for or considered to be safe for human and animal administration, i.e., GRAS substances (generally regarded as safe). GRAS substances are listed by the Food and Drug administration in the Code of Federal Regulations (CFR) at 21 CFR § 182 and 21 CFR § 184, incorporated herein by reference. Preferred excipients include, but are not limited to, hexitols, including mannitol and the like.

As used herein "a stabilizing concentration of an organic solvent" or "a stabilizing concentration of an alcohol" means that amount of an organic solvent or alcohol that reduces the level of degradation of bendamustine to achieve a specified level of degradants in the final drug product. For example, with respect to the degradant HP1, a stabilizing concentration of an organic solvent is that amount which results in an HP1 concentration (area percent of bendamustine) of less than about 0.5%, preferably less than 0.45 %, preferably less than 0.40 %, more preferably less than 0.35%, more preferably less than 0.30%, and even more preferably less than 0.25%. With respect to the overall or total degradant concentration of the final drug product, a stabilizing concentration of an

organic solvent is that amount that results in a total degradant concentration (at the time of drug product release) of less than about 7% (area percent bendamustine), preferably less than about 6%, more preferably less than about 5%, and even more preferably less than about 4.0%. By "area percent of bendamustine" is meant the amount of a specified degradant, e.g., HP1, relative to the amount of bendamustine as determined, e.g., by HPLC.

The term "organic solvent" means an organic material, usually a liquid, capable of dissolving other substances.

As used herein, "trace amount of an organic solvent" means an amount of solvent that is equal to or below recommended levels for pharmaceutical products, for example, as recommended by ICH guidelines (International Conferences on Harmonization, Impurities-- Guidelines for Residual Solvents. Q3C. Federal Register. 1997;62(247):67377). The lower limit is the lowest amount that can be detected.

The term "release" or "at release" means the drug product has met the release specifications and can be used for its intended pharmaceutical purpose.

A. General

The invention provides stable, pharmaceutically acceptable compositions prepared from bendamustine. In particular, the invention provides formulations for the lyophilization of bendamustine HCl. The lyophilized powder obtained from such formulations is more easily reconstituted than the presently available lyophilized powder of bendamustine. Further, the lyophilized products of the present invention have a better impurity profile than Ribomustin® with respect to certain impurities, in particular HP1, bendamustine dimer, and bendamustine ethylester, prior to reconstitution, upon storage of the lyophilate, or following reconstitution and admixture.

The present invention further provides formulations of bendamustine useful for treating neoplastic diseases. The formulations described herein can be administered alone or in combination with at least one additional anti-neoplastic agent and/or radioactive therapy.

An aspect of the invention is conditions and means for enhancing the stability of bendamustine prior to and during the lyophilization process, upon shelf storage or upon reconstitution.

Anti-neoplastic agents which may be utilized in combination with the formulations of the invention include those provided in the Merck Index 11, pp 16-17, Merck & Co., Inc. (1989) and The Chemotherapy Source Book (1997). Both books are widely recognized and readily available to the skilled artisan.

There are large numbers of antineoplastic agents available in commercial use, in clinical evaluation and in pre-clinical development, which could be selected for treatment of neoplasia by combination drug chemotherapy. Such antineoplastic agents fall into several major categories, namely, antibiotic-type agents, covalent DNA-binding drugs, antimetabolite agents, hormonal agents, including glucocorticoids such as prednisone and dexamethasone, immunological agents, interferon-type agents, differentiating agents such as the retinoids, pro-apoptotic agents, and a category of miscellaneous agents, including compounds such as antisense, small interfering RNA, and the like. Alternatively, other anti-neoplastic agents, such as metallomatrix proteases (MMP) inhibitors, SOD mimics or $alpha_v$ $beta_3$ inhibitors may be used.

One family of antineoplastic agents which may be used in combination with the compounds of the inventions consists of antimetabolite-type antineoplastic agents. Suitable antimetabolite antineoplastic agents may be selected from the group consisting of alanosine, AG2037 (Pfizer), 5-FU-fibrinogen, acanthifolic acid, aminothiadiazole, brequinar sodium, carmofur, Ciba-Geigy CGP-30694, cyclopentyl cytosine, cytarabine phosphate stearate, cytarabine conjugates, Lilly DATHF, Merrel Dow DDFC, dezaguanine, dideoxycytidine, dideoxyguanosine, didox, Yoshitomi DMDC, doxifluridine, Wellcome EHNA, Merck & Co. EX-015, fazarabine, floxuridine, fludarabine phosphate, 5-fluorouracil, N-(2'-furanidyl)-5-fluorouracil, Daiichi Seiyaku FO-152, isopropyl pyrrolizine, Lilly LY-188011, Lilly LY-264618, methobenzaprim, methotrexate, Wellcome MZPES, norspermidine, NCI NSC-127716, NCI NSC-264880, NCI NSC-39661, NCI NSC-612567, Warner-Lambert PALA, pentostatin, piritrexim, plicamycin, Asahi Chemical PL-AC, Takeda TAC-788, thioguanine, tiazofurin, Erbamont TIF, trimetrexate, tyrosine kinase inhibitors, tyrosine protein kinase inhibitors, Taiho UFT and uricytin.

A second family of antineoplastic agents which may be used in combination with the compounds of the invention consists of covalent DNA-binding agents . Suitable

alkylating-type antineoplastic agents may be selected from the group consisting of Shionogi 254-S, aldo-phosphamide analogues, altretamine, anaxirone, Boehringer Mannheim BBR-2207, bestrabucil, budotitane, Wakunaga CA-102, carboplatin, carmustine, Chinoin-139, Chinoin-153, chlorambucil, cisplatin, cyclophosphamide, American Cyanamid CL-286558, Sanofi CY-233, cyplatate, Degussa D-19-384, Sumimoto DACHP(Myr)2, diphenylspiromustine, diplatinum cytostatic, Erba distamycin derivatives, Chugai DWA-2114R, ITI E09, elmustine, Erbamont FCE-24517, estramustine phosphate sodium, fotemustine, Unimed G-6-M, Chinoin GYKI-17230, hepsul-fam, ifosfamide, iproplatin, lomustine, mafosfamide, melphalan, mitolactol, Nippon Kayaku NK-121, NCI NSC-264395, NCI NSC-342215, oxaliplatin, Upjohn PCNU, prednimustine, Proter PTT-119, ranimustine, semustine, SmithKline SK&F-101772, Yakult Honsha SN-22, spiromustine, Tanabe Seiyaku TA-077, tauromustine, temozolomide, teroxirone, tetraplatin and trimelamol.

Another family of antineoplastic agents which may be used in combination with the compounds disclosed herein consists of antibiotic-type antineoplastic agents. Suitable antibiotic-type antineoplastic agents may be selected from the group consisting of Taiho 4181-A, aclarubicin, actinomycin D, actinoplanone, alanosine, Erbamont ADR-456, aeroplysinin derivative, Ajinomoto AN-201-II, Ajinomoto AN-3, Nippon Soda anisomycins, anthracycline, azino-mycin-A, bisucaberin, Bristol-Myers BL-6859, Bristol-Myers BMY-25067, Bristol-Myers BMY-25551, Bristol-Myers BMY-26605, Bristol-Myers BMY-27557, Bristol-Myers BMY-28438, bleomycin sulfate, bryostatin-1, Taiho C-1027, calichemycin, chromoximycin, dactinomycin, daunorubicin, Kyowa Hakko DC-102, Kyowa Hakko DC-79, Kyowa Hakko DC-88A, Kyowa Hakko DC89-Al, Kyowa Hakko DC92-B, ditrisarubicin B, Shionogi DOB-41, doxorubicin, doxorubicin-fibrinogen, elsamicin-A, epirubicin, erbstatin, esorubicin, esperamicin-Al, esperamicin-Alb, Erbamont FCE-21954, Fujisawa FK-973, fostriecin, Fujisawa FR-900482, glidobactin, gregatin-A, grincamycin, herbimycin, idarubicin, illudins, kazusamycin, kesarirhodins, Kyowa Hakko KM-5539, Kirin Brewery KRN-8602, Kyowa Hakko KT-5432, Kyowa Hakko KT-5594, Kyowa Hakko KT-6149, American Cyanamid LL-D49194, Meiji Seika ME 2303, menogaril, mitomycin, mitoxantrone, SmithKline M-TAG, neoenactin, Nippon Kayaku NK-313, Nippon Kayaku NKT-01, SRI International NSC-357704, oxalysine,

oxaunomycin, peplomycin, pilatin, pirarubicin, porothramycin, pyrindamycin A, Tobishi RA-I, rapamycin, rhizoxin, rodorubicin, sibanomicin, siwenmycin, Sumitomo SM-5887, Snow Brand SN-706, Snow Brand SN-07, sorangicin-A, sparsomycin, SS Pharmaceutical SS-21020, SS Pharmaceutical SS-7313B, SS Pharmaceutical SS-9816B, steffimycin B, Taiho 4181-2, talisomycin, Takeda TAN-868A, terpentecin, thrazine, tricrozarin A, Upjohn U-73975, Kyowa Hakko UCN-10028A, Fujisawa WF-3405, Yoshitomi Y-25024 and zorubicin.

A fourth family of antineoplastic agents which may be used in combination with the compounds of the invention include a miscellaneous family of antineoplastic agents selected from the group consisting of alpha-carotene, alpha-difluoromethyl-arginine, acitretin, arsenic trioxide, Avastin® (bevacizumab), Biotec AD-5, Kyorin AHC-52, alstonine, amonafide, amphethinile, amsacrine, Angiostat, ankinomycin, anti-neoplaston A10, antineoplaston A2, antineoplaston A3, antineoplaston A5, antineoplaston AS2-1, Henkel APD, aphidicolin glycinate, asparaginase, Avarol, baccharin, batracylin, benfluron, benzotript, Ipsen-Beaufour BIM-23015, bisantrene, Bristo-Myers BMY-40481, Vestar boron-10, bromofosfamide, Wellcome BW-502, Wellcome BW-773, caracemide, carmethizole hydrochloride, Ajinomoto CDAF, chlorsulfaquinoxalone, Chemes CHX-2053, Chemex CHX-100, Warner-Lambert CI-921, Warner-Lambert CI-937, Warner-Lambert CI-941, Warner-Lambert CI-958, clanfenur, claviridenone, ICN compound 1259, ICN compound 4711, Contracan, Yakult Honsha CPT-ll, crisnatol, curaderm, cytochalasin B, cytarabine, cytocytin, Merz D-609, DABIS maleate, dacarbazine, datelliptinium, didemnin- B, dihaematoporphyrin ether, dihydrolenperone, dinaline, distamycin, Toyo Pharmar DM-341, Toyo Pharmar DM-75, Daiichi Seiyaku DN-9693, elliprabin, elliptinium acetate, epothionesTsumura EPMTC, erbitux, ergotamine, erlotnib, etoposide, etretinate, fenretinide, Fujisawa FR-57704, gallium nitrate, genkwadaphnin, Gleevec® (imatnib), Chugai GLA-43, Glaxo GR-63178, gefitinib, grifolan NMF-5N, hexadecylphosphocholine, Green Cross HO-221, homoharringtonine, hydroxyurea, BTG ICRF-187, indanocine, ilmofosine, isoglutamine, isotretinoin, Otsuka JI-36, Ramot K-477, Otsuak K-76COONa, Kureha Chemical K-AM, MECT Corp KI-8110, American Cyanamid L-623, leukoregulin, lonidamine, Lundbeck LU-23-112, Lilly LY-186641, NCI (US) MAP, marycin, mefloquine, Merrel Dow MDL-27048, Medco MEDR-340,

merbarone, merocyanine derivatives, methylanilinoacridine, Molecular Genetics MGI-136, minactivin, mitonafide, mitoquidone, mopidamol, motretinide, Zenyaku Kogyo MST-16, N-(retinoyl)amino acids, Nisshin Flour Milling N-021, N- acylated-dehydroalanines, nafazatrom, Taisho NCU-190, nocodazole derivative, Normosang, NCI NSC-145813, NCI NSC-361456, NCI NSC-604782, NCI NSC-95580, octreotide, Ono ONO-112, oquizanocine, Akzo Org-10172, paclitaxel, pancratistatin, pazelliptine, Warner-Lambert PD-111707, Warner-Lambert PD-115934, Warner-Lambert PD-131141, Pierre Fabre PE-1001, ICRT peptide D, piroxantrone, polyhaematoporphyrin, polypreic acid, Efamol porphyrin, probimane, procarbazine, proglumide, Invitron protease nexin I, Tobishi RA-700, razoxane, Sapporo Breweries RBS, restrictin-P, retelliptine, retinoic acid, Rhone-Poulenc RP-49532, Rhone-Poulenc RP-56976, Rituxan® (and other anti CD20 antibodies, e.g. Bexxar®, Zevalin®), SmithKline SK&F-104864, statins (Lipitor® etc.), Sumitomo SM-108, Kuraray SMANCS, SeaPharm SP-10094, spatol, spirocyclopropane derivatives, spirogermanium, Unimed, SS Pharmaceutical SS-554, strypoldinone, Stypoldione, Suntory SUN 0237, Suntory SUN 2071, superoxide dismutase, Thalidomide, Thalidomide analogs, Toyama T-506, Toyama T-680, taxol, Teijin TEI-0303, teniposide, thaliblastine, Eastman Kodak TJB-29, tocotrienol, Topostin, Teijin TT-82, Kyowa Hakko UCN-01, Kyowa Hakko UCN-1028, ukrain, Eastman Kodak USB-006, vinblastine sulfate, vincristine, vindesine, vinestramide, vinorelbine, vintriptol, vinzolidine, withanolides and Yamanouchi YM-534, Zometa®.

Examples of radioprotective agents which may be used in the combination chemotherapy of this invention are AD-5, adchnon, amifostine analogues, detox, dimesna, 1-102, MM-159, N-acylated-dehydroalanines, TGF-Genentech, tiprotimod, amifostine, WR-151327, FUT-187, ketoprofen transdermal, nabumetone, superoxide dismutase (Chiron and Enzon).

Methods for preparation of the antineoplastic agents described above may be found in the literature. Methods for preparation of doxorubicin, for example, are described in U.S. Pat. Nos. 3,590,028 and 4,012,448. Methods for preparing metallomatrix protease inhibitors are described in EP 780386. Methods for preparing .alpha$_v$ .beta$_3$ inhibitors are described in WO 97/08174.

Preferred anti-neoplastic agents include, without limitation, one or more of daunorubicin, bleomycin, vincristine, doxorubicin, dacarbazine, prednisolone, mitoxantrone, prednisone, methotrexate, 5-flurouracil, dexamethasone, thalidomide, thalidomide derivatives, 2ME2, Neovastat, R 11 5777, arsenic trioxide, bortezomib, tamoxifen, G3139 (antisense), and SU5416, mitomycin, anti-CD20 antibodies, such as Rituxan® and R-etodolac.

Preferred drug regimens for which the present formulation may be used in conjunction with or as a replacement for one or more of the components includes, without limitation, ABVD (doxorubicin, bleomycin, vincristine, dacarbazine), DBV (daunorubicin, belomycin, vincristine), CVPP (cyclophosphamide, vinblastine, procarbazine, prednisolone), COP (cyclophosphamide, vincristine, prednisolone), CHOP (cyclophosphamide, doxorubicin,

vincristine and prednisone) and CMF (cyclophosphamide, methotrexate, 5-fluorouracil). Additional regimens are given in Table A below.

Table A- Cancer Therapeutic Regimens

Abbreviation	Drugs Used	Disease
AC	Doxorubicin & Cyclophosphamide	Breast cancer
CFM (CF, FNC)	Cyclophosphamide, Fluorouracil, Mitaxantrone	Breast cancer
CMF	Cyclophosphamide, Methotrexate, Fluorouracil	Breast cancer
NFL	Mitoxantrone, Fluorouracil, Leucovorin	Breast cancer
Sequential Dox-CMF	Doxorubicin	Breast cancer
VATH	Vinblastine, Doxorubicin, Thiotepa, Fluoxymesterone	Breast cancer
EMA-86	Etoposide, Mitoxantrone, Ctyarabine	AML (induction)

7 + 3	Cytarabine WITH Daunorubicin OR Idarobicin OR Mitoxantrone	AML (induction)
5 + 2	Cytarabine WITH Daunorubicin OR Mitoxantrone	AML (induction)
HiDAC	Cytarabine	AML (post-remission)
ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine	Hodgkin's
ChlVPP	Chlorambucil, Vinblastine, Procarbazine, Prednisone	Hodgkin's
EVA	Etoposide, Vinblastine, Doxorubicin	Hodgkin's
MOPP	Mechlorethamine, Vincristine, Procarbazine, Prednisone	Hodgkin's
MOPP/ABV Hybrid	Mechlorethamine, Vincristine, Procarbazine, Prednisone, Doxorubicin, Bleomycin, Vinblastine	Hodgkin's
MOPP/ABVD	Mechlorethamine, Doxorubicin, Vinblastine, Bleomycin, Etoposide, Prednisone	Hodgkin's
CNOP	Cyclophosphamide, Mitoxantrone, Vincristine, Prednisone	Non-Hodgkin's
COMLA	Cyclophosphamide, Vincristine, Methotrexate, Leucovorin, Cytarabine	Non-Hodgkin's

DHAP	Dexamethasone, Cisplatin, Cytarabine	Non-Hodgkin's
ESHAP	Etoposide, Methylprednisilone, Cisplatin, Cytarabine	Non-Hodgkin's
MACOP-B	Methotrexate, Leucovorin, Doxorubicin, Cyclophosphamide, Vincristine, Prednisone, Bleomycin, Septra, Ketoconazole	Non-Hodgkin's
m-BACOD	Methotrexate, Leucovorin, Bleomycin, Doxorubicin, Cyclophosphamide, Vincristine, Dexamethasone	Non-Hodgkin's
MINE-ESHAP	Mesna, Ifosfamide, Mitoxantrone, Etoposide	Non-Hodgkin's
NOVP	Mitoxantrone, Vinblastine, Prednisone, Vincristine	Non-Hodgkin's
ProMACE/cytaBOM	Prednisone, Doxorubicin, Cyclophosphamide, Etoposide, Cytarabine, Bleomycin, Vincristine, Methotrexate, Leucovorin, Septra	Non-Hodgkin's
M2	Vincristine, Carmustine, Cyclophosphamide,	Multiple Myeloma

	Melphalan, Prednisone	
MP	Melphalan, Prednisone	Multiple Myeloma
VAD	Vincristine, Doxorubicin, Dexamethasone	Multiple Myeloma
VBMCP	Vincristine, Carmustine, Melphalan, Cyclophosphamide, Prednisone	Multiple Myeloma

As described herein, a lyophilized formulation of bendamustine is achieved following removal of an organic solvent in water. The most typical example of the solvent used to prepare this formulation is tertiary butanol (TBA). Other organic solvents can be used including ethanol, n-propanol, n-butanol, isoproponal, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, acetone, 1-pentanol, methyl acetate, methanol, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, cyclohexane. These preceding solvents may be used individually or in combination. Useful solvents must form stable solutions with bendamustine and must not appreciably degrade or deactivate the API. The solubility of bendamustine in the selected solvent must be high enough to form commercially useful concentrations of the drug in solvent. Additionally, the solvent should be capable of being removed easily from an aqueous dispersion or solution of the drug product, e.g., through lyophilization or vacuum drying. Preferably, a solution having a concentration of about 2-80 mg/mL, preferably about 5 to 40 mg/mL, more preferably 5-20 mg/mL and even more preferably 12 to 17 mg/mL bendamustine is used.

A pharmaceutically acceptable lyophilization excipient can be dissolved in the aqueous phase. Examples of excipients useful for the present invention include, without limitation, sodium or potassium phosphate, citric acid, tartaric acid, gelatin, glycine, and

carbohydrates such as lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose and hetastarch. Mannitol is a preferred excipient. Other excipients that may be used if desired include antioxidants, such as, without limitation, ascorbic acid, acetylcysteine, cysteine, sodium hydrogen sulfite, butyl-hydroxyanisole, butyl-hydroxytoluene or alpha-tocopherol acetate, or chelators.

A typical formulation and lyophilization cycle useful in accordance with the present invention is provided below. Lyophilization can be carried out using standard equipment as used for lyophilization or vacuum drying. The cycle may be varied depending upon the equipment and facilities used for the fill/finish.

In accordance with a typical embodiment of the present invention, an aqueous pre-lyophilization solution or dispersion is first formulated in a pharmaceutically acceptable compounding vessel. The solution is aseptically filtered into a sterile container, filled into an appropriate sized vial, partially stoppered and loaded into the lyophilizer. Using lyophilization techniques described herein the solution is lyophilized until a moisture content in the range of about 0.1 to about 8.0 percent is achieved. The resulting lyophilization powder is stable as a lyophilized powder for about six months to greater than about 2 years, preferably greater than about 3 years at about 5°C to about 25° C and can be readily reconstituted with Sterile Water for Injection, or other suitable carrier, to provide liquid formulations of bendamustine, suitable for internal administration e.g., by parenteral injection. For intravenous administration, the reconstituted liquid formulation, i.e., the pharmaceutical composition, is preferably a solution.

The pre-lyophilization solution or dispersion normally is first formulated in a pharmaceutically acceptable container by: 1) adding an excipient, such as mannitol (about 0 to about 50 mg/mL) with mixing to water (about 65% of the total volume) at ambient temperature, 2) adding an organic solvent (0.5- 99.9% v/v), such as TBA to the aqueous solution with mixing at about 20°-35°C, 4) adding bendamustine HCl to the desired concentration with mixing, 5) adding water to achieve the final volume, and 6) cooling the solution to about 1°C to about 30°C, preferably about 5°C. Although the preceding steps are shown in a certain order, it is understood that one skilled in the art can change the order of the steps and quantities as needed. Quantities can be prepared on a weight basis also.

The pre-lyophilization solution or dispersion can be sterilized prior to lyophilization, sterilization is generally performed by aseptic filtration, e.g., through a 0.22 micron or less filter. Multiple sterilization filters can be used. Sterilization of the solution or dispersion can be achieved by other methods known in the art, e.g., radiation.

In this case, after sterilization, the solution or dispersion is ready for lyophilization. Generally, the filtered solution will be introduced into a sterile receiving vessel, and then transferred to any suitable container or containers in which the formulation may be effectively lyophilized. Usually the formulation is effectively and efficiently lyophilized in the containers in which the product is to be marketed, such as, without limitation, a vial, as described herein and as known in the art.

A typical procedure for use in lyophilizing the pre-lyophilization solutions or dispersions is set forth below. However, a person skilled in the art would understand that modifications to the procedure or process may be made depending on such things as, but not limited to, the pre-lyophilization solution or dispersion and lyophilization equipment.

Initially, the product is placed in a lyophilization chamber under a range of temperatures and then subjected to temperatures well below the product's freezing point, generally for several hours. Preferably, the temperature will be at or below about -40°C for at least 2 hours. After freezing is complete, the chamber and the condenser are evacuated through vacuum pumps, the condenser surface having been previously chilled by circulating refrigerant. Preferably, the condenser will have been chilled below the freezing point of the solution preferably to about -40°, more preferably to about -50°C or lower, even more preferably to about -60°C or lower. Additionally, evacuation of the chamber should continue until a pressure of about 10 to about 600 microns, preferably about 50 to about 150 microns is obtained.

The product composition is then warmed under vacuum in the chamber and condenser. This usually will be carried out by warming the shelves within the lyophilizer on which the product rests during the lyophilization process at a pressure ranging from about 10 to about 600 microns. The warming process will optimally take place very gradually, over the course of several hours. For example, the product temperature should initially be increased from about -30°C to about -10°C and maintained for about 10-70 hours. Additionally, the product temperature can be increased from the freezing

temperature to about 25°C-40°C over a period of 30-192 hours. To prevent powder ejection of the lyophilate from vials, complete removal of the organic solvent and water should be done during the initial drying phase. Complete drying can be confirmed by stabilization of vacuum, condenser temperature and product shelf temperature. After the initial drying, the product temperature should be increased to about 25°C-40°C and maintained for about 5-40 hours.

Once the drying cycle is completed, the pressure in the chamber can be slowly released to atmospheric pressure (or slightly below) with sterile, dry-nitrogen gas (or equivalent gas). If the product composition has been lyophilized in containers such as vials, the vials can be stoppered, removed and sealed. Several representative samples can be removed for purposes of performing various physical, chemical, and microbiological tests to analyze the quality of the product.

The lyophilized bendamustine formulation is typically marketed in pharmaceutical dosage form. The pharmaceutical dosage form of the present invention, although typically in the form of a vial, may be any suitable container, such as ampoules, syringes, co-vials, which are capable of maintaining a sterile environment. Such containers can be glass or plastic, provided that the material does not interact with the bendamustine formulation. The closure is typically a stopper, most typically a sterile rubber stopper, preferably a bromobutyl rubber stopper, which affords a hermetic seal.

After lyophilization, the bendamustine lyophilization powder may be filled into containers, such as vials, or alternatively the pre-lyophilization solution can be filled into such vials and lyophilized therein, resulting in vials which directly contain the lyophilized bendamustine formulation. Such vials are, after filling or lyophilization of the solution therein, sealed, as with a stopper, to provide a sealed, sterile, pharmaceutical dosage form. Typically, a vial will contain a lyophilized powder including about 10-500 mg/vial, preferably about 100 mg/vial, bendamustine and about 5mg-2g/vial, preferably about 170 mg/vial, mannitol.

The lyophilized formulations of the present invention may be reconstituted with water, preferably Sterile Water for Injection, or other sterile fluid such as co-solvents, to provide an appropriate solution of bendamustine for administration, as through parenteral

injection following further dilution into an appropriate intravenous admixture container, for example, normal saline.

B. Solubility

The solubility of bendamustine HCl (bendamustine) in water (alone) and with varying amounts of alcohols commonly used in lyophilization, e.g., methanol, ethanol, propanol, isopropanol, butanol and tertiary-butyl alcohol (TBA) was determined by visual inspection. Amounts of bendamustine at 15 mg/mL, combined with mannitol at 25.5 mg/mL were prepared in 10 mL of the indicated alcohol solutions at room temperature (see Table 1). Samples were then refrigerated at 5°C and inspected after 0, 3, 6 and 24 hours for particulates and/or precipitates.

The results shown in Table 1 indicate that bendamustine solubility is dependant on temperature and the amount of alcohol in aqueous solutions. For the alcohols tested, the solubility of bendamustine increased as the concentration of alcohol increased. The formation of a precipitant was also dependent on the temperature and time. Bendamustine did not precipitate immediately with any alcohol, but crystallized after storage at 5°C. Alcohols varied in their effect on solubility. Without wishing to be bound to any particular theory, smaller alcohols such as methanol and ethanol have less of an effect on solubility as compared with larger alcohols (tertiary-butanol and n-butanol). However, the shape of the alcohol is also important. For example n-propanol was found to be better than iso-propanol in preventing precipitation in this system. The two alcohols with the greatest effect on solubility were n-propanol and tertiary-butanol.

Table 1. Bendamustine solubility over a 24 hour period in various alcohols when stored at 5°C.

	Zero Time	3 Hours	6 Hours	24 Hours
Methanol (v/v)				
0% (Water Only)	CCS	CCS	Precipitate	Precipitate
5%	CCS	CCS	Precipitate	Precipitate
10%	CCS	CCS	CCS	Precipitate
20%	CCS	CCS	CCS	Precipitate
30%	CCS	CCS	CCS	CCS
Ethanol (v/v)				
1.9%	CCS	CCS	Precipitate	Precipitate
5%	CCS	CCS	Precipitate	Precipitate
10%	CCS	CCS	CCS	Precipitate
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
n-Propanol (v/v/)				
5%	CCS	CCS	CCS	Precipitate

10%	CCS	CCS	CCS	CCS
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
Iso-propanol (v/v)				
5%	CCS	Precipitate	Precipitate	Precipitate
10%	CCS	CCS	CCS	CCS
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
n-Butanol (v/v)				
5%	CCS	CCS	CCS	CCS
10%	CCS	CCS	CCS	CCS
20%	2 layers	2 layers	2 layers	2 layers
30%	2 layers	2 layers	2 layers	2 layers
Tert-Butanol (v/v)				
5%	CCS	CCS	CCS	Precipitate
10%	CCS	CCS	CCS	Precipitate
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS

CCS stands for clear colorless solution

Experiments to quantitatively determine the solubility of bendamustine at various temperatures for three different solutions are summarized in Figure 1 and Table 2. The amount of TBA, 20% (v/v) and 30% (v/v), used in the experiment was based on stability studies (results described below). For both solutions tested, the solubility of bendamustine decreased linearly with temperatures from 25°C to 0°C. This experiment confirmed the data shown in Table 1 and highlights the difference in bendamustine solubility for 20% and 30% TBA solutions.

Table 2. Solubility of bendamustine in TBA

	-8°C	0°C	5°C	25°C
20% (v/v) TBA 25.5 mg/mL mannitol Water, q.s. to desired volume	14 mg/mL	11 mg/mL	17 mg/mL	47 mg/mL
30% (v/v) TBA 25.5 mg/mL mannitol Water, q.s. to desired volume	20 mg/mL	18 mg/mL	27 mg/mL	65 mg/mL

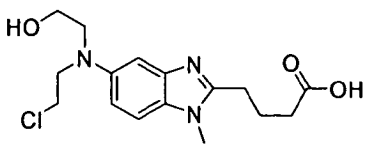
C. Stability

Because of its instability in aqueous solutions due to hydrolysis with water, bendamustine requires lyophilization in order to make a product suitable for

pharmaceutical use. However, during the manufacturing of lyophilized drug products, aqueous solutions are commonly needed for filling, prior to lyophilization. Thus, the use of aqueous solutions during the compounding and fill processes for bendamustine and other nitrogen mustards can result in degradation of the drug product. Consequently, the effect of various alcohols on the degradation of bendamustine was evaluated to determine if formulations could be found that would allow longer fill-finish times, provide lyophilate powders that could be reconstituted more quickly than the current Ribomustin® formulation, and/or provide lyophilized preparations of bendamustine with a better impurity profile with respect to certain impurities, e.g., HP1, and BM1 dimer than Ribomustin®.

Preferably, a lyophilized preparation of the invention is stable with respect to HP1, i.e., the amount of HP1 does not increase appreciably (does not exceed the shelf-life specifications), for 6 months, more preferably 12 months, and even more preferably greater than 24 months, e.g., 36 months, when stored at about 2°C to about 30°C, preferably 5°C.

Table 3 shows the stability results of bendamustine in water with no addition of alcohol over a 24 hour period at 5°C. Bendamustine degrades rapidly in water alone and forms predominantly the hydrolysis product, HP1 (monohydroxy bendamustine).



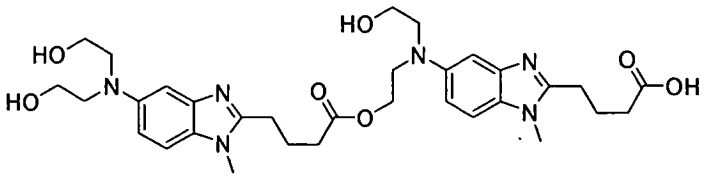
Monohydroxy bendamustine (HP1)

Formula II

Table 3. Stability of bendamustine in water

	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
0% Alcohol, i.e., Water Alone	0 hours	99.11	0.60	0.11
	3 hours	98.83	0.86	0.13
	6 hours	98.44	1.22	0.17
	24 hours	95.67	3.81	0.29

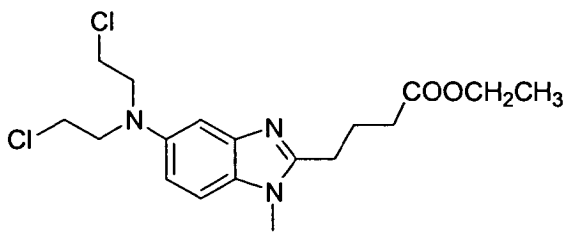
The other major degradant observed during this study and other long term stability studies was the dimer of bendamustine.



Bendamustine Dimer (BM1 Dimer)

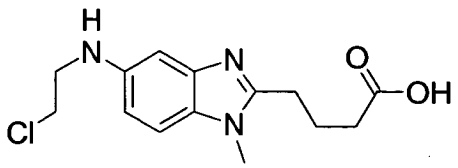
Formula III

Other degradants contained in the Ribomustin lyophilized product are bendamustine ethylester (BM1EE) (Formula IV) and BM1DCE (Formula V). BM1EE is formed when bendamustine reacts with ethyl alcohol.



Bendamustine ethylester (BM1EE)

Formula IV



BM1DCE

Formula V

Figure 2 summarizes the purity results of an HPLC analysis after incubating bendamustine in various alcohols for 24 hours at 5°C. Results are presented as the area percent of the total peak area. The numerical values for Figure 2 are provided in Tables 3-

9. The purity was highest in solutions containing higher concentration of alcohols, regardless of the alcohol. Of the alcohols evaluated, bendamustine degraded the least in a solution containing about 30% (v/v) TBA. In about 10% and about 20 % alcohol solutions, n-butanol was superior in preventing degradation of bendamustine. At 20% and 30% (v/v), n-butanol in water resulted in a biphasic system due to the insolubility of n-butanol in water at these concentrations.

Figures 3 and 4 show the amount of degradation of bendamustine as measured by HP1 and dimer formation quantified by HPLC (as described herein). HP1 and dimer formation increased as the amount of alcohol concentration decreased regardless of the alcohol. This increase in impurities occurred with an anticipated time dependence (see Tables 3-9). Tert-butanol and n-butanol appeared superior to other alcohols in preventing degradation of the product. As seen in Table 10, mannitol had no effect on the stabilization of bendamustine with TBA.

Table 4. HPLC stability results for the stability of bendamustine in various ethyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

V/V alcohol	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
1.9% Ethanol	0 hours	99.11	0.64	0.12
	3 hours	98.83	0.90	0.14
	6 hours	98.60	1.12	0.15
	24 hours	96.16	3.41	0.27
5% Ethanol	0 hours	99.31	0.44	0.12
	3 hours	99.10	0.64	0.13
	6 hours	98.87	0.86	0.14
	24 hours	96.89	2.68	0.25
10% Ethanol	0 hours	99.44	0.33	0.11
	3 hours	99.28	0.48	0.12
	6 hours	99.10	0.65	0.12
	24 hours	98.03	1.57	0.18
20% Ethanol	0 hours	99.54	0.22	0.10
	3 hours	99.45	0.30	0.11
	6 hours	99.36	0.39	0.11
	24 hours	98.61	0.96	0.15
30% Ethanol	0 hours	99.62	0.15	0.10
	3 hours	99.56	0.21	0.11
	6 hours	99.52	0.24	0.12
	24 hours	99.21	0.45	0.12

Table 5. HPLC stability results for bendamustine in various Tert-butanol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Tert-butanol	0 hours	99.34	0.41	0.12

	3 hours	99.10	0.64	0.14
	6 hours	98.85	0.88	0.13
	24 hours	97.58	2.09	0.20
10% Tert-butanol	0 hours	99.46	0.30	0.11
	3 hours	99.26	0.48	0.12
	6 hours	99.05	0.69	0.13
	24 hours	98.04	1.64	0.19
20% Tert-butanol	0 hours	99.59	0.17	0.11
	3 hours	99.48	0.29	0.11
	6 hours	99.35	0.40	0.12
	24 hours	98.35	1.27	0.20
30% Tert-butanol	0 hours	99.63	0.13	0.10
	3 hours	99.60	0.16	0.10
	6 hours	99.58	0.18	0.11
	24 hours	99.42	0.34	0.12

Table 6. HPLC stability results for various n-propyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% n-Propanol	0 hours	99.25	0.43	0.13
	3 hours	99.00	0.66	0.15
	6 hours	98.72	0.94	0.16
	24 hours	97.24	2.33	0.26
10% n-Propanol	0 hours	99.34	0.33	0.15
	3 hours	99.17	0.48	0.14
	6 hours	98.92	0.70	0.16
	24 hours	97.67	1.83	0.28
20% n-Propanol	0 hours	99.45	0.33	0.13
	3 hours	99.42	0.26	0.13
	6 hours	99.29	0.39	0.14
	24 hours	98.60	0.97	0.24
30% n-Propanol	0 hours	99.53	0.15	0.13
	3 hours	99.51	0.15	0.15
	6 hours	99.44	0.20	0.11
	24 hours	99.27	0.36	0.17

Table 7. HPLC stability results for bendamustine in various iso-propyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Iso-propanol	0 hours	99.21	0.48	0.13
	3 hours	98.65	0.72	0.14
	6 hours	98.56	1.02	0.14
	24 hours	96.14	3.35	0.26
10% Iso-propanol	0 hours	99.32	0.37	0.12
	3 hours	99.11	0.55	0.14
	6 hours	98.85	0.75	0.16
	24 hours	97.68	1.92	0.21
20% Iso-propanol	0 hours	99.49	0.21	0.11
	3 hours	99.39	0.31	0.12
	6 hours	99.22	0.42	0.13
	24 hours	98.61	1.04	0.17

30% Iso-propanol	0 hours	99.56	0.15	0.10
	3 hours	99.47	0.20	0.12
	6 hours	99.40	0.24	0.11
	24 hours	99.15	0.52	0.14

Table 8. HPLC stability results for bendamustine in various methyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Methanol	0 hours	99.35	0.40	0.12
	3 hours	98.97	0.70	0.14
	6 hours	98.66	0.95	0.14
	24 hours	96.65	2.83	0.23
10% Methanol	0 hours	99.42	0.34	0.11
	3 hours	99.01	0.59	0.12
	6 hours	98.86	0.80	0.12
	24 hours	97.65	1.85	0.18
20% Methanol	0 hours	99.56	0.22	0.11
	3 hours	99.31	0.38	0.11
	6 hours	98.99	0.50	0.12
	24 hours	98.31	1.15	0.16
30% Methanol	0 hours	99.59	0.18	0.10
	3 hours	99.43	0.27	0.11
	6 hours	99.25	0.34	0.11
	24 hours	98.65	0.76	0.13

Table 9. HPLC stability results for bendamustine in various n-butyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Butanol	0 hours	99.25	0.49	0.13
	3 hours	98.94	0.73	0.14
	6 hours	98.76	0.91	0.14
	24 hours	97.46	2.20	0.21
10% Butanol	0 hours	99.44	0.30	0.11
	3 hours	99.18	0.49	0.12
	6 hours	99.03	0.64	0.12
	24 hours	98.13	1.55	0.17
20% Butanol[a]	0 hours	99.54	0.23	0.10
	3 hours	99.45	0.31	0.11
	6 hours	99.30	0.40	0.11
	24 hours	98.81	0.91	0.14
30% Butanol[a]	0 hours	99.55	0.24	0.10
	3 hours	99.40	0.29	0.10
	6 hours	99.40	0.37	0.11
	24 hours	99.00	0.74	0.12

a – Both solutions had 2 layers/phases of liquids in the vial. Solutions were vortexed prior to sample preparation.

The results in Tables 1-9 indicate that the stability of bendamustine HCl with respect to HP1 and dimer improves with increasing alcohol concentration.

Table 10. HPLC stability results for bendamustine in TBA with and without mannitol over a 24 hour period.

Sample	Purity (%Area)	HP1 (%)
TBA 20% (v/v) with Mannitol		
0 hours	99.59	0.17
24 hours @ 5°C	99.35	1.27
TBA 20% (v/v) without Mannitol		
0 hours	100.0	0.00
24 hours @ 5°C	98.80	1.21

NOTE: The samples analyzed without mannitol were analyzed by HPLC using a normal phase method while the samples analyzed with mannitol used a reverse phase HPLC method. Slight variability may be seen in other samples analyzed between the two methods.

D. Lyophilization Cycle Development

Different pre-lyophilization formulations were prepared at various concentrations of bendamustine, mannitol, and alcohols in water. The cycle development was changed and optimized at each step for freezing (fast vs. slow), primary drying (both temperature and pressure), and secondary drying as described herein.

Based upon all of the information detailed above on solubility, stability, and ease of lyophilization, preferred formulations include the following:

Ingredients	Concentration
Bendamustine	about 2-40 mg/mL
Mannitol	about 0-50 mg/mL
Alcohol	about 0.5%-40% (v/v)
Water, q.s. to	desired volume

wherein the alcohol is selected from methanol, n-propanol, or isopropanol

Ingredients	Concentration
Bendamustine	about 5-20 mg/mL
Mannitol	10-30 mg/mL
Alcohol	1-20% (v/v)
Water, q.s. to	desired volume

wherein the alcohol is selected from methanol, n-propanol, or isopropanol

Ingredients	Concentration
Bendamustine	about 5-20 mg/mL

Mannitol	10-30 mg/mL
Alcohol	5-40% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Alcohol	about 5-15% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Alcohol	about 10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-40 mg/mL
Mannitol	about 0-50 mg/mL
Butanol	about 0.5-20% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 5-20 mg/mL
Mannitol	about 10-30 mg/mL
Butanol	about 1-10 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Butanol	about 1-10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration

Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Butanol	about 10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-100 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-99.9 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-99 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 90-99 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 5-20 mg/mL
Mannitol	about 10-30 mg/mL
Tertiary butanol	about 5-80 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Tertiary butanol	about 10-50 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12.5-15 mg/mL
Mannitol	about 0-30 mg/mL
Ethanol	about 20-30 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Tertiary butanol	about 30 % (v/v)
Water, q.s. to	desired volume

EXAMPLES

The following Examples are provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. These Examples are in no way to be considered to limit the scope of the invention in any manner.

Materials:

Bendamustine HCl, (Degussa, Lot #s 0206005 and 0206007)
Mannitol, NF or equivalent (Mallinckrodt)
Ethyl Alcohol Dehydrated (200 proof), USP or equivalent (Spectrum)
Tertiary-butyl alcohol, ACS (EM Science)
Methanol (Spectrum and EMD)
Propanol (Spectrum)
Iso-propanol (Spectrum)
Butanol (Spectrum)
Water, HPLC grade or equivalent (EMD)
Acetonitrile, HPLC grade or equivalent (EMD)
Trifluoroacetic Acid, J.T. Baker

Methanol, HPLC grade or equivalent (EM Science, Cat # MX0488P-1)
Trifluoroacetic Acid, HPLC grade or equivalent (JT Baker, Cat# JT9470-01)

Equipment:
Waters 2695 Alliance HPLC system with photodiode array detector
Waters 2795 Alliance HPLC system with dual wavelength detector
Analytical Balance (Mettler AG285, ID #1028) and (Mettler XS205)
VirTis Lyophilizer AdVantage
Agilent Zorbax SB-C18 5 μm 80Å 4.6 × 250 mm column, Cat# 880975-902

Example 1- HPLC Procedures

Method 1

Mobile Phase A: 0.1% TFA; H_2O
Mobile Phase B: 0.1% TFA; 50% ACN:50% H_2O
UV: 230 nm
Flow rate: 1.0 mL/min
Column temp.: 30 °C
Column: Zorbax SB-C18 5 μm 80 Å 4.6 × 250 mm
Sample temp.: 5 °C
Injection Volume: 10 μL
Sample Concentration: 0.25 mg/mL in MeOH
Gradient: 20%B for 1 min
20 – 90%B in 23 min
90%B for 6 min
back to 20%B in 1 min
hold at 20%B for 4 min
Run time: 30 min
Post run time: 5 min

Method 2

Mobile Phase A: 0.1% TFA; H_2O:ACN (9:1)
Mobile Phase B: 0.1% TFA; H_2O:ACN (5:5)
UV: 230 nm
Flow rate: 1.0 mL/min
Column: Zorbax SB-C18 5 μm 80 Å 4.6 × 250 mm

Column temp.: 30 °C
Sample temp.: 5 °C
Injection Volume: 10 μL
Sample Concentration:0.25 mg/mL in MeOH
Gradient: 0%B for 3 min
0 – 50%B in 13 min
50 – 70%B in 17 min
70 – 90%B in 2 min
90%B for 5 min
back to 0%B in 1 min
hold at 0%B for 4 min
Run time: 40 min
Post run time: 5 min

Method 3
Phase A: HPLC grade water with 0.1 % TFA(v/v)
Phase B: HPLC grade ACN / water(1:1v/v) with 0.1%TFA(v/v)
UV: 254 nm
Flow rate: 1.0 mL/min
Column: Zorbax SB-C18 5 μm 80 Å 4.6 × 250 mm
Column temp.: 30 °C
Sample temp.: 5 °C
Injection Volume: 5 μL
Acquisition time: 30 min
Post time: 9 min
Diluent: methanol

Gradient:

Time (min.)	% Phase A	% Phase B
0.0	82	18
7.0	60	40
11.0	60	40
15.0	20	80
30.0	20	80
31.0	82	18

Sample preparation- dissolve the drug product with 200 mL MeOH. Sonicate 6 minutes. The solution can be injected directly into the HPLC (ca. 0.5 mg/mL)

Method 4

Phase A: HPLC grade water with 0.1 % TFA(v/v)
Phase B: HPLC grade ACN with 0.1%TFA(v/v)
UV: 254 nm
Flow rate: 1.0 mL/min
Column: Zorbax Bonus RP-C14 5 μm 4.6 × 150 mm
Column temp.: 30°C
Sample temp.: 5°C
Injection Volume: 2 μL
Acquisition time:31 min
Post time: 5 min
Diluent: NMP/0.1% TFA in water (50:50 v/v)

Gradient:

Time (min.)	% Phase A	% Phase B
0.0	93	7
5	93	7
13	73	27
16	73	27
25	10	90
31	10	90

Sample preparation for method 4- dissolve the drug product with a known amount of diluent to prepare a concentration of 4.2 mg/mL for injection directly into the HPLC. It may be necessary to perform a second dilution (the 100 mg/vial dosage form) to obtain a 4.2 mg/mL sample concentration.

Results

The retention times for some Bendamustine impurities using HPLC Method 1 described above are shown in Table 11. An HPLC chromatograph for Ribomustin® using the HPLC procedure described herein is shown in Fig. 6.

Table 11: Retention Time for Bendamustine and some of its Impurities using HPLC Method 1

Sample Name	Retention Time (min)
HP1	14.110
Bendamustine	22.182
BM1 Dimer	24.824
BM1EE	26.968

Although HPLC Method 1 was capable of resolving impurities found in bendamustine it was not capable of separating a potential impurity formed during analysis, the methyl ester of bendamustine (BM1ME). The retention time difference between BM1ME and BM1 Dimer was only 0.3 minutes. In order to resolve BM1 Dimer, another HPLC method (# 2) was developed. HPLC method #2 was capable of separating all the impurities but required a longer run time of 45 minutes (Table 12).

Table 12: Retention Time for bendamustine and impurities using HPLC Method 2.

Sample Name	Retention Time (min)
HP1	15.694
BM1	25.420
BM1ME	31.065
BM1 Dimer	32.467
BM1EE	36.038

The impurity profile of various lots of Ribomustin using HPLC Method 3 are shown in Table 13.

Table13- Ribomustine Impuirty Profile using HPLC Method 3

% Area					
Batch	**Bendamustine(HCl)**	**HP1**	**BM1EE**	**BM1 Dimer**	**BM1DCE**
03H08	98.14	1.07	0.21	0.34	0.03
03H07	97.67	1.5	0.2	0.33	0.04
02K27	96.93	0.93	0.29	1.18	0.08
03C08	97.61	1.24	0.19	0.46	0.02

Example 2- Solubility

The solubility of bendamustine HCl (bendamustine) in water (alone) and with varying amounts of methanol, ethanol, propanol, isopropanol, butanol and tertiary-butyl alcohol (TBA) was determined by visual inspection. Amounts of bendamustine at 15 mg/mL, mannitol at 25.5 mg/mL were prepared in 10 mL of the indicated alcohol solutions (Table 1) at room temperature. Samples were then refrigerated at 5°C and inspected after 0, 3, 6 and 24 hours for particulates and/or precipitates.

Results summarized in Table 1 indicate that bendamustine solubility is dependant on temperature and the amount of alcohol in aqueous solutions. For all alcohols the solubility of bendamustine increased as the concentration of alcohol increased. The formation of a precipitant was also dependent on the temperature and time.

The solubility of bendamustine was also determined in 20% (v/v) TBA containing 25.5 mg/mL mannitol in water, and 30% (v/v) TBA containing 25.5 mg/mL mannitol in water (Fig 1). Bendamustine was added to 4 mL of each solution while mixing until it would no longer dissolve. The saturated solutions were allowed to mix for 1 hour at -8°C, 0°C, 5°C, or 25°C. The samples were centrifuged and placed back at the original temperature for a minimum of 30 minutes. The -8°C sample was placed into an ice bath containing sodium chloride, which lowers the temperature of the ice bath, and the temperature was measured when the sample was pulled for analysis. An aliquot of each sample was taken and prepared for HPLC analysis.

The results of these experiments are shown in Figure 1 and Table 2. The amount of TBA, 20% (v/v) and 30% (v/v), used in the experiment (Fig. 1) was based on stability studies described herein.

As indicated in Fig. 1, the solubility of bendamustine decreased linearly with temperature (25°C to 0°C). The solubility of bendamustine was temperature dependant whether it was dissolved in water alone or with an alcohol. The 20% (v/v) TBA may likely be the lower limit required for efficient and robust pharmaceutical manufacturing due to the stability and solubility of bendamustine. A filling solution of 15 mg/mL bendamustine is close to the saturation limit of 17.2 mg/mL bendamustine at 5°C but higher than the limit at 0°C. The 30% (v/v) TBA is the recommended concentration of TBA for the final formulation and is well within the solubility limit regardless of temperature.

Example 3-Stability

A. Stability in Water

Solutions of bendamustine (15 mg/mL), and mannitol (25.5 mg/mL) were prepared in water at room temperature and immediately placed in an ice bath (to lower the temperature quickly to about 5°C) for 10 minutes and then refrigerated at 5°C. A sample of each formulation was analyzed by HPLC using the methods described herein after 0, 3, 6 and 24 hours when stored at 5°C.

B. Stability in Alcohols

Solutions containing 15 mg/mL bendamustine, 25.5 mg/mL mannitol, and 1.9%, 5%, 10%, 20% or 30% (v/v) ethyl alcohol in water or 5%, 10%, 20% or 30% (v/v) TBA, methanol, propanol, iso-propanol, or butanol in water were prepared at room temperature, placed into an ice bath for 10 minutes and then refrigerated at 5°C. A sample of each formulation was analyzed by HPLC after 0, 3, 6 and 24 hours when stored at 5°C.

C. Stability Results

Table 3 shows the stability results of bendamustine in water with no addition of alcohol over a 24 hour period at 5°C. Bendamustine degrades quickly in water but the stability of bendamustine increases with increasing alcohol concentrations (Figs. 2, 3 and 4). Although alcohols are frequently used in lyophilization to aid in solubility problems, the effect of alcohols on bendamustine stability is unique, unexpected and useful in manufacturing bendamustine with fewer impurities since an aqueous solution can be used while maintaining the stability of bendamustine. TBA was found to be the best stabilizer of the six alcohols tested (Figs. 2, 3, and 4). All alcohols at 30% (v/v) reduced the formation of impurities HP1 and Dimer at 5°C for up to 24 hours. With respect to TBA, HP1 reaches only about 0.4% when stored at 5°C for up to 24 hours. Lower concentrations of alcohol may not be efficient, when formulated at 15 mg/mL bendamustine and stored at 5°C due to bendamustine precipitation and impurity formation.

Example 4- Formulation Optimization

After the solubility and stability of bendamustine were determined, the formulation was optimized for lyophilization. Since the concentration of bendamustine is higher in a 30% TBA/water saturated solution as compared with other alcohol solutions, it is

anticipated that the vial size required to fill 100 mg of bendamustine can be decreased from the current Ribomustin® presentation. Although a saturated solution of bendamustine contains 18 mg/mL at 0°C, a concentration of 15 mg/mL was selected for the formulation to compensate for slight differences in API solubility due to differences in bulk API purity as a result of batch differences. A concentration of 15 mg/mL bendamustine requires 6.67 mL to fill 100 mg of bendamustine HCl per vial.

The surface (sublimation) area to volume ratio is critical to producing a lyophilized product with good appearance that freeze dries quickly. Generally, lyophilized products occupy between 30% to 50% of the vial volume. A 20 mL vial with 6.67 mL contains about 30% of its capacity and has a surface area ratio of 0.796 cm^2/mL.

Mannitol was selected as the bulking agent in order to maintain a formulation similar to Ribomustin®. Studies were performed to evaluate the effect of mannitol on bendamustine solubility and appearance of the product. Mannitol decreases the solubility of bendamustine (at 15 mg/mL) in both ethanol and TBA aqueous solutions. For example, solutions containing 5% and 10% ethanol and TBA without mannitol did not precipitate over 24 hours. However, for samples with mannitol (Table 1) precipitate was observed within 24 hours. There was no precipitate with aqueous solutions containing 30% (v/v) TBA, 15 mg/mL bendamustine, and 25.5 mg/mL mannitol. In order to maintain a well formed cake resistant to breakage during handling, a minimum of 134 mg/vial of mannitol was required with no difference observed in vials up to 200 mg/vial of mannitol.

All alcohols tested increased the stability and solubility of bendamustine. However, a significant mole fraction was required to affect the stability of the filling solution and the ease of manufacturing. Smaller alcohols have the undesirable effect of lowering the freezing point of the bulk solution and thus requiring long lyophilization cycles at lower temperatures. Higher concentrations of methanol and ethanol produced unattractive cakes that were difficult to reconstitute. 10% ethanol, 20% ethanol, 10% iso-propanol, 20% iso-propanol, or 30% TBA aqueous solutions containing bendamustine (15 mg/mL), mannitol (25.5 mg/mL) were prepared and lyophilized. The lyophilized vials filled from solutions of 10% ethanol, 20% ethanol, 10% iso-propanol, 20% iso-propanol produced either a collapsed cake or a film residue. The only solvent system producing an

acceptable cake was 30% TBA. Additionally, reconstitution of 10% ethanol, 20% ethanol, 10% iso-propanol, 20% iso-propanol lyophilized vials were difficult and did not fully dissolve until >45 minutes.

The ability to utilize a smaller vial is constrained by the concentration or solubility of bendamustine in the aqueous/organic solution. At lower concentrations of ethanol, methanol, isopropanol and n-propanol, which produced acceptable cake appearance, a more dilute solution of bendamustine is required due to solubility limitations. To maintain a presentation with 100 mg of bendamustine per vial, a vial larger than 50 mL would be required. Also, stability studies herein indicated that at the lower alcohol concentration, the chemical stability was not sufficient to allow for acceptable filling times.

One of the factors affecting the ease of reconstitution is the porosity of the lyophilate. In general, amorphously precipitated solids with little surface area are more difficult to solubilize. Most lyophilates containing mannitol will reconstitute within 3-5 minutes as long as there is no precipitate formed during lyophilization, frequently caused by evaporation of a liquid (melt back). Based on our experience with several lyophilization solvent systems and not wishing to be bound to any particular theory, the problems associated with Ribomustin® reconstitution may be associated with precipitation caused by melt back during lyophilization. Most organic solvents do not lyophilize efficiently and cause melt back because of their low melting point. TBA (tertiary butyl alcohol) has a high melting point and a similar vapor pressure as compared to water. TBA is removed by sublimation, not evaporation, at about the same rate as water. Lyophilates produced with 30% (v/v) TBA according to the invention reconstitute within 3-10 minutes as compare to commercially available Ribomustin which may take 30-45 minutes.

Based upon the solubility, stability, ease of reconstitution and manufacturing considerations, the following is a preferred pre-lyophilization formulation of the present invention: bendamustine HCl about 15 mg/mL, mannitol about 25.5 mg/mL, about 30% (v/v) tertiary-butyl alcohol, and q.s. using water for Injection. The formulation is then filled at 5°C using 6.67 mL in an amber 20 mL, 20 mm vial and partially stoppered with a bromobutyl stopper and loaded into a pre-chilled lyophilizer.

Example 5- Impurity assessment

Major impurities introduced during Ribomustin® manufacturing, compounding, fill, and lyophilization procedure, as determined by HPLC analysis (Fig. 6), are the hydrolysis product HP1, the Dimer, and the ethyl ester of bendamustine, BM1EE. BM1EE can be formed during drug substance manufacturing, e.g., during recrystalization and/or purification processes. BM1EE is known to be a more potent cytotoxic drug than bendamustine. Experiments were undertaken to determine if the use of a 30% TBA aqueous filling solution would lead to the formation of bendamustine t-butyl ester.

Experiments were performed using traditional Fisher esterfication reaction conditions required for the formation of t-butyl ester of bendamustine. Bendamustine was heated in 60°C TBA with HCl for 20 hours. No reaction was observed. This result indicated that it would be very difficult to form the tert-butyl ester of bendamustine during the fill/finish process. No new impurities in drug product manufactured from TBA have been observed in stability studies to date.

To aid in the testing of the drug product, synthetic routes using more reactive sources of the t-butyl moiety were developed. Another attempt to make tert-butyl ester was carried out by formation of the acyl chloride of bendamustine. A suspension of bendamustine in methylene chloride was treated with oxalyl chloride and N,N-dimethylformamide. After acyl chloride was formed, the solvent was concentrated. The residue was added to methylene chloride, tert-butanol, triethylamine, and 4-dimethylaminopyridine and the mixture was stirred at room temperature overnight. After adding all solvents and purification, an unknown compound was given. The LC-MS did not match the molecular weight of bendamustine tert-butyl ester and the proton NMR did not showed the peak for tert-butyl. Therefore, this attempt also failed to produce the bendamustine tert-butyl ester. Thus, using TBA as the co-solvent has an additional benefit of not forming the ester from the alcohol.

Example 6- Lyophilization Cycle Development

Numerous lyophilization cycles were performed to evaluate the critical stages of lyophilization and achieve the most efficient drying cycle. Experiments were performed to evaluate the effect of the freezing rate, primary drying temperature, time, and pressure on the product.

A. Freezing Rate

The literature reports that TBA adopts different crystal forms depending on the freeze rate. In some TBA solutions, the slower the product froze, the quicker it dried. Larger crystals formed during slow freezing producing bigger pores allowing more efficient sublimation. However, during studies with bendamustine, the freezing rate was not found to be a critical processing parameter when evaluated at 2 and 8 hours.

B. Primary and Secondary Drying

During the first attempts to lyophilize from 30% TBA solutions, the lyophilized cake fractured and powder was ejected from the vial. These cakes appeared to contain amorphous particles within the lyophilate, an indication of melt back. This phenomenon was reproducible and occurred when the product reached about -10°C (refer to Fig. 5) independent of the warming rate. Several variables were tested to determine the cause and solution to the problem of the powder ejection. The pressure was raised from 50 μm to 150 μm during primary drying, but powder ejection was still observed but to a lesser extent. This experiment was then repeated except the freezing rate was extended to 8 hours from 2 hours. This change had no effect.

The length of primary drying was next evaluated. For example, the following very slow drying cycle was evaluated: freezing from +25°C to -50°C in eight hours; holding at -50°C for 5 hours, warming and drying from-50°C to -25°C in seven hours; holding for twenty hours at -25°C, warming and drying from -25°C to -15°C in two hours and holding for twenty hours at -15°C, warming and drying from -15°C to 40°C in six hours and holding for twenty hours at 40°C while maintaining a chamber pressure of 150 μm throughout drying. No powder ejection (Fig 5) was observed. This cycle resulted in a well-formed cake without fracture that reconstituted readily. Without wishing to be bound to a particular theory, the problems with powder ejection and difficulty with reconstitution may be the result of drying the lyophilate too quickly, thus resulting in strong vapor flow out of the cake as well as melt back. With the use of a less aggressive drying cycle an aesthetic, stable, and easy to reconstitute cake was reproducibly formed. Thus, removing all unbound water and tertiary-butyl alcohol prior to secondary drying may prevent melt back as well as powder ejection. The lyophilization cycle was further optimized under

these gentle conditions (Fig. 5). There were no immediate degradation products as a result of drying at 40°C for up to 20 hours.

Example 7- Lyophilization cycle

Step	Description	Time (Hour)	Temperature (°C)	Pressure (Microns)
1	Hold	0.25	5°C	-
2	Ramp	8	-50°C	-
3	Hold	4	-50°C	-
4	Ramp	3	-20°C	150
5	Hold	6	-20°C	150
6	Ramp	1	-15°C	150
7	Hold	20	-15°C	150
8	Ramp	0.5	-12°C	150
9	Hold	15.5	-12C	150
10	Ramp	15	35C	50
11	Hold	10	35°C	50
12	Ramp	1	40C	50
	Hold	5	40C	50
Total		89.25	-	-

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the spirit and scope of the invention. More specifically, it will be apparent that certain solvents which are both chemically and physiologically related to the solvents disclosed herein may be substituted for the solvents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit and scope of the invention as defined by the appended claims.

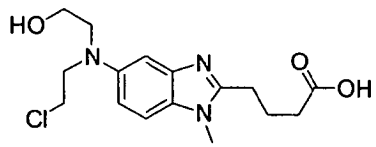
All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications are herein incorporated by reference to

the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

What is claimed is:

1. A pharmaceutical composition of bendamustine containing not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II,



Formula II

wherein said HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized preparation of bendamustine.

2. The composition according to claim 1, wherein the amount of HP1 is not more than 0.5% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

3. The composition according to claim 1, wherein the amount of HP1 is not more than 0.4% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

4. The composition according to claim 1, wherein the amount of HP1 is not more than 0.3% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

5. A lyophilized preparation of bendamustine containing not more than about 0.9% (area percent of bendamustine) HP1 at release.

6. A lyophilized preparation of bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

7. The lyophilized preparation according to claim 5, wherein the preparation is packaged in a vial or other pharmaceutically acceptable container.

8. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about six months when stored at 5° C.

9. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 12 months when stored at 5° C.

10. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 24 months when stored at 5° C.

11. A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine containing not more than about 0.9% HP1, wherein said HP1 is the amount of HP1 present at release.

12. A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine containing not more than about 0.5% HP1, wherein said HP1 is the amount of HP1 present at release.

13. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 5 mg to about 500 mg of bendamustine.

14. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 10 mg to about 300 mg of bendamustine.

15. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 25 mg of bendamustine.

16. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 100 mg of bendamustine.

17. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 200 mg of bendamustine.

18. A pharmaceutical dosage form comprising the lyophilized preparation of claim 5.

19. A pharmaceutical composition of bendamustine comprising bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1 and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release.

20. A pharmaceutical composition of bendamustine according to claim 19 wherein the organic solvent is selected from the group consisting of one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane.

21. A pharmaceutical composition according to claim 20, wherein the organic solvent is selected from the group consisting of one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol.

22. A pharmaceutical composition according to claim 19, wherein the organic solvent is tertiary butanol.

23. A lyophilized preparation according to claim 5 further comprising a trace amount of an organic solvent.

24. A lyophilized preparation according to claim 23 wherein said organic solvent is tertiary butanol.

25. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a release specification for bendamustine degradants at less than 4.0 % (area percent bendamustine) for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

26. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a release specification for bendamustine of HP1 at less than or equal to 1.5% for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

27. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a shelf-life specification for bendamustine degradants at less than 7.0% (area percent bendamustine) for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

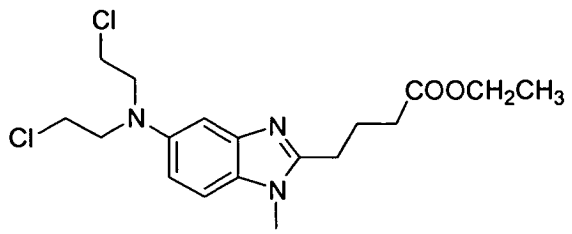
28. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of bendamustine degradants is less than 4.0 % (area percent of bendamustine) and the concentration of HP1 is less than 0.5% (area percent of bendamustine).

29. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.9% (area percent of bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

30. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.5% (area percent of

bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

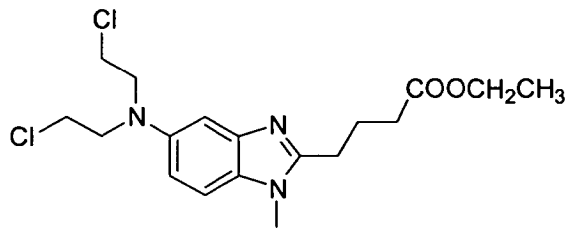
31. A lyophilized preparation of bendamustine wherein the concentration of bendamustine ethylester (as shown in Formula IV)



Formula IV

is no more than 0.2% greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

32. A lyophilized preparation of bendamustine according to claim 5 containing not more than about 0.5% bendamustine ethylester as shown in Formula IV



Formula IV.

33. A bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.9% (area percent bendamustine).

34. A bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.5% (area percent bendamustine).

35. The lyophilized powder produced from the pre-lyophilization solution or dispersion according to claim 33.

36. A method of preparing a bendamustine lyophilized preparation comprising,

a) dissolving bendamustine in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine lyophilized preparation contains not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

37. A method of preparing a bendamustine lyophilized preparation comprising,

a) dissolving bendamustine in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine lyophilized preparation contains not more than about 0.5% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

38. A method according to claim 36, wherein the alcohol concentration is between about 5% to about 99.9%.

39. A method according to claim 36, wherein said alcohol is selected from one or more of methanol, ethanol, propanol, iso-propanol, butanol, and tertiary-butanol.

40. A method according to claim 39, wherein said alcohol is tertiary-butanol.

41. A method according to claim 40, wherein said tertiary butanol is at a concentration of about 20% to 30%.

42. A method according to claim 40, wherein said tertiary butanol is at a concentration of about 30%.

43. A method according to claim 36, wherein an excipient is added before lyophilization.

44. A method according to claim 43, wherein the excipient is mannitol.

45. A method according to claim 36, wherein the bendamustine concentration is about 2 to 50 mg/mL.

46. The lyophilized powder obtained from the method according to claim 36.

47. A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to a temperature below about -40°C to form a frozen solution;

ii) holding the frozen solution at or below -40°C for at least 2 hours;

iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution;

iv) holding for about 10 to about 70 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vii) holding for about 5 to about 40 hours to form a bendamustine lyophilized preparation.

48. A method according to claim 47, wherein said alcohol is tertiary-butanol.

49. A method according to claim 48, wherein said tertiary butanol is at a concentration of about 20% to 30%.

50. A method according to 49, wherein said tertiary butanol is at a concentration of about 30%.

51. The lyophilized powder obtained from the method according to claim 47.

52. A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution;

ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours;

iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution;

iv) holding at a primary drying temperature for about 10 to about 48 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours.

53. A method according to claim 52, wherein said alcohol is tertiary-butanol.

54. A method according to claim 53, wherein said tertiary butanol is at a concentration of about 20% to 30%.

55. A method according to 54, wherein said tertiary butanol is at a concentration of about 30%.

56. The lyophilized powder obtained from the method according to claim 53.

57. A method according to claim 36 wherein step b) comprises: i) starting with a shelf temperature of about 5°C for loading; ii) freezing to about -50°C over about 8 hours; iii) holding at -50°C for about 4 hours; iv) ramping to about -20°C over about 3 hours; v) holding at about -20°C for 6 hours; ramping to about -15°C over about 1 hour; vi) holding at -15°C for about 20 hours; vii) ramping to about -15°C over about 1 hour; viii) holding at about -15°C for about 20 hours; ix) ramping to about -12°C over about 0.5 hours; x) holding at about -12°C for about 15.5 hours; xi) ramping to between about 25°C and about 40°C or higher for about 15 hours; xii) holding between about 25°C and about 40°C for about 10 hours; xiii) ramping to about 40°C over about 1 hour; andxiv) holding at about 40°C for about 5 hours; unloading at about 5°C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying.

58. A lyophilization cycle according to claim 57, wherein step (xi) is ramped to about 30-35°C for about 15 hours.

59. The lyophilized powder prepared from the lyophilization cycle of claim 57.

60. A formulation for lyophilization comprising bendamustine at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water.

61. A lyophilized preparation made from the formulation according to claim 60.

62. A method of treating a medical condition in a patient comprising dissolving the preparation of claim 5 in a pharmaceutically acceptable solvent to produce a pharmaceutically acceptable solution and administering to said patient a therapeutically effective amount of said solution, wherein said condition is amenable to treatment with said preparation.

63. A method of treating according to claim 62, wherein said condition is selected from chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, breast cancer, small cell lung cancer, and an autoimmune disease.

64. A method of treating according to claim 63, wherein said condition is non-Hodgkin's lymphoma.

65. A method of treating according to claim 63, wherein said condition is chronic lymphocytic leukemia.

66. A method of treating according to claim 63, wherein said condition is multiple myeloma.

67. A method of treating according to claim 62 further comprising administering the dissolved preparation of claim 5 in combination with one or more anti-neoplastic agents wherein said antineoplastic agent is given prior, concurrently, or subsequent to the administration of the dissolved preparation of claim 5.

68. A method of treating according to claim 67 wherein the antineoplastic agent is an antibody specific for CD20, wherein said antibody is given prior, concurrently or subsequent to the administration of the dissolved preparation of claim 5.

69. A method of treating according to claim 62 wherein the autoimmune disease is rheumatoid arthritis, multiple sclerosis or lupus.

70. A method of treating according to claim 62, wherein the medical condition is a hyperproliferative disorder.

71. A pharmaceutical dosage form of bendamustine containing not more than about 0.9% HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other

pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

72. A pharmaceutical dosage form of bendamustine containing not more than about 0.5% HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

73. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine at a concentration of about 10 to about 500 mg/container.

74. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine at a concentration of about 100 mg/container.

75. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 5 mg to about 2 g/container.

76. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 170 mg/container.

77. A pre-lyophilized pharmaceutical composition of bendamustine comprising about 15 mg/mL bendamustine HCl, about 25.5 mg/mL mannitol, about 30% (v/v) tertiary-butyl alcohol, and water.

78. The preparation of claim 5 which is a pharmaceutical composition.

Abstract

The present invention provides pharmaceutical formulations of lyophilized bendamustine suitable for pharmaceutical use. The present invention further provides methods of producing lyophilized bendamustine. The pharmaceutical formulations can be used for any disease that is sensitive to treatment with bendamustine, such as neoplastic diseases.

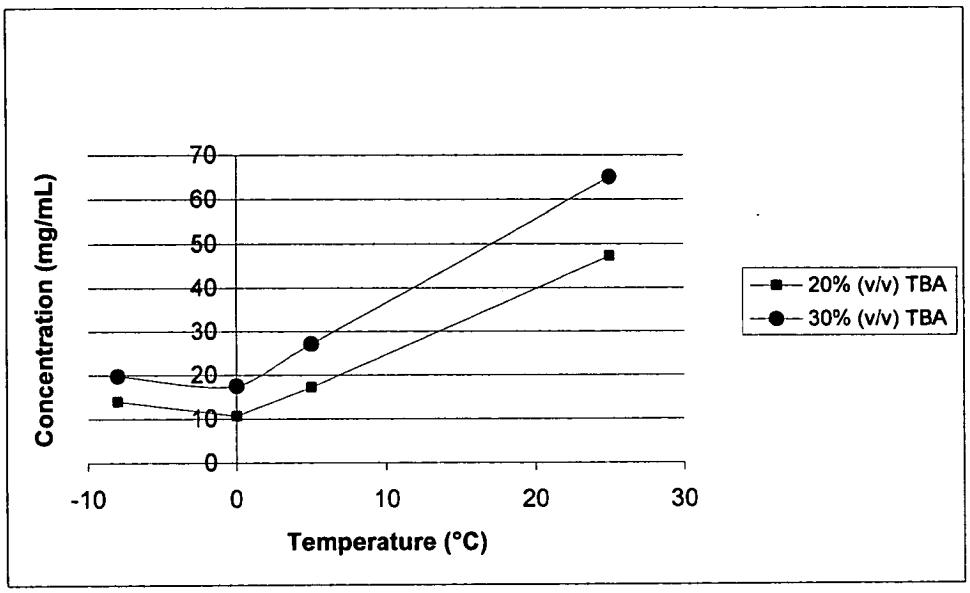


Fig. 1

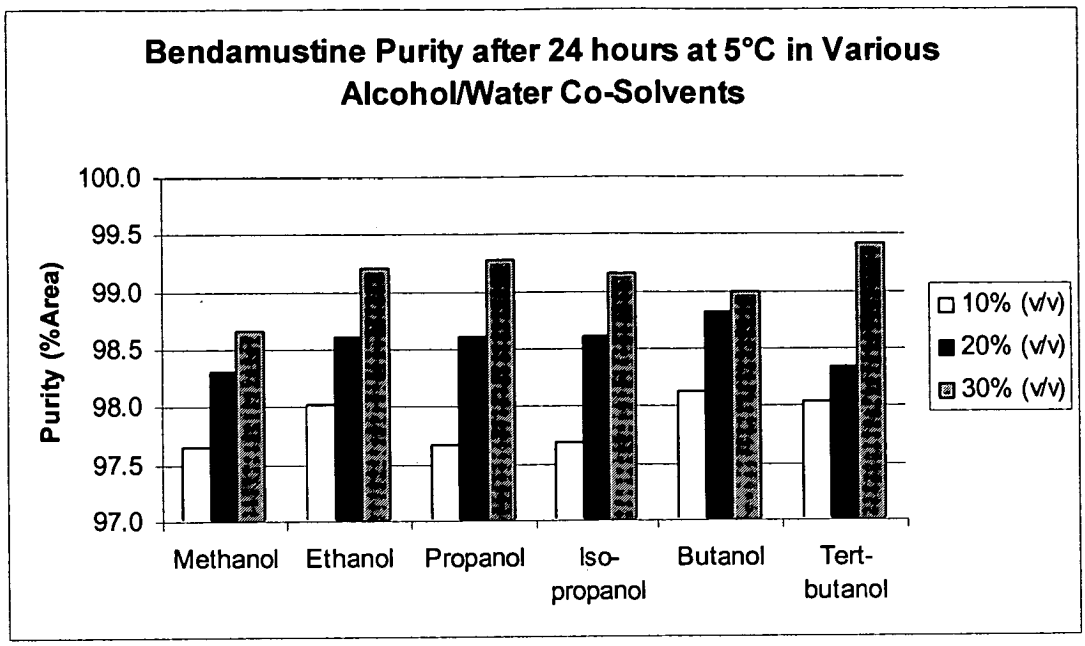


Fig 2

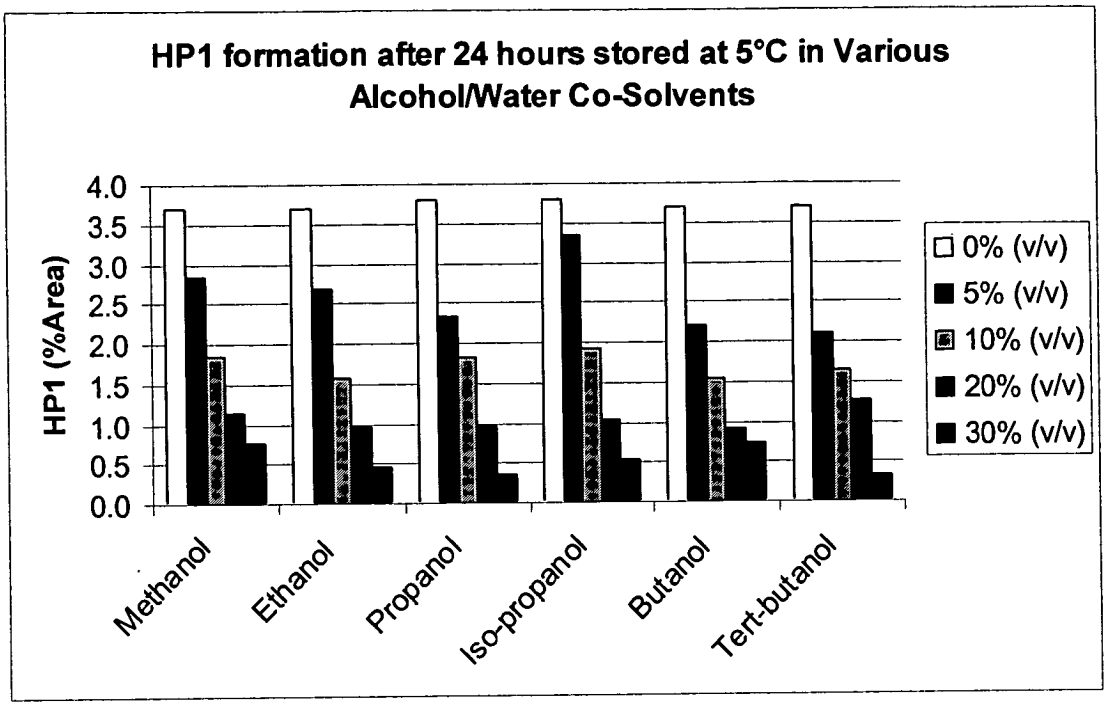
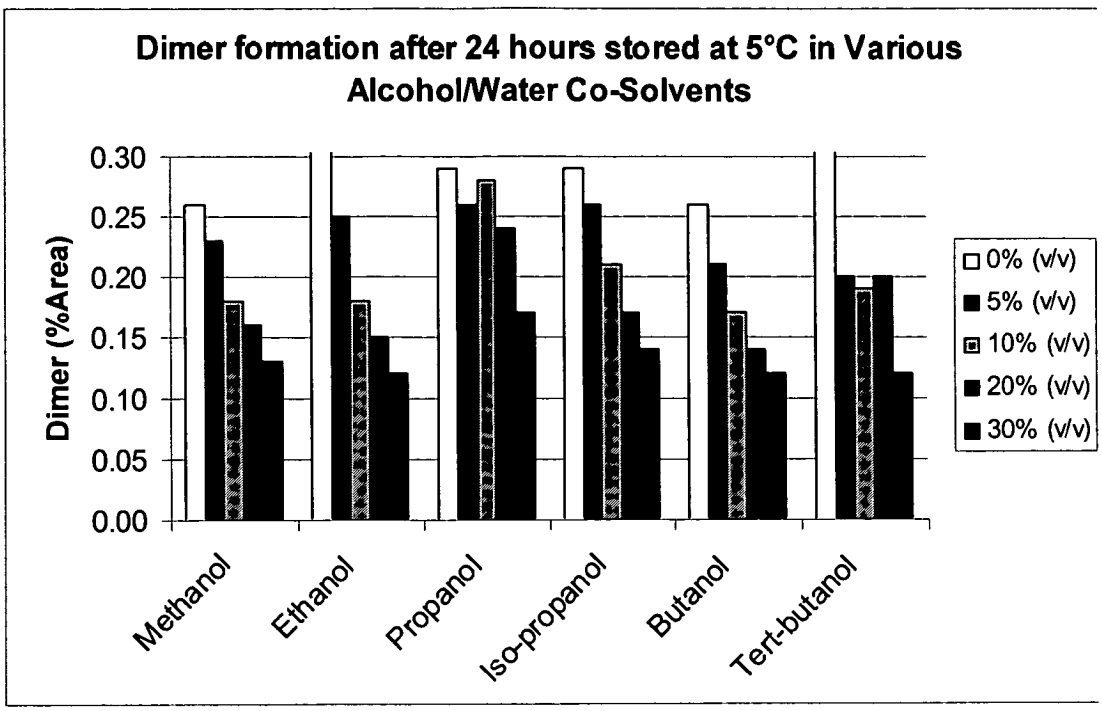


Fig 3

Figure 4.



The numerical values for Figure 4 are provided in Tables 3-9 in Appendix 1.

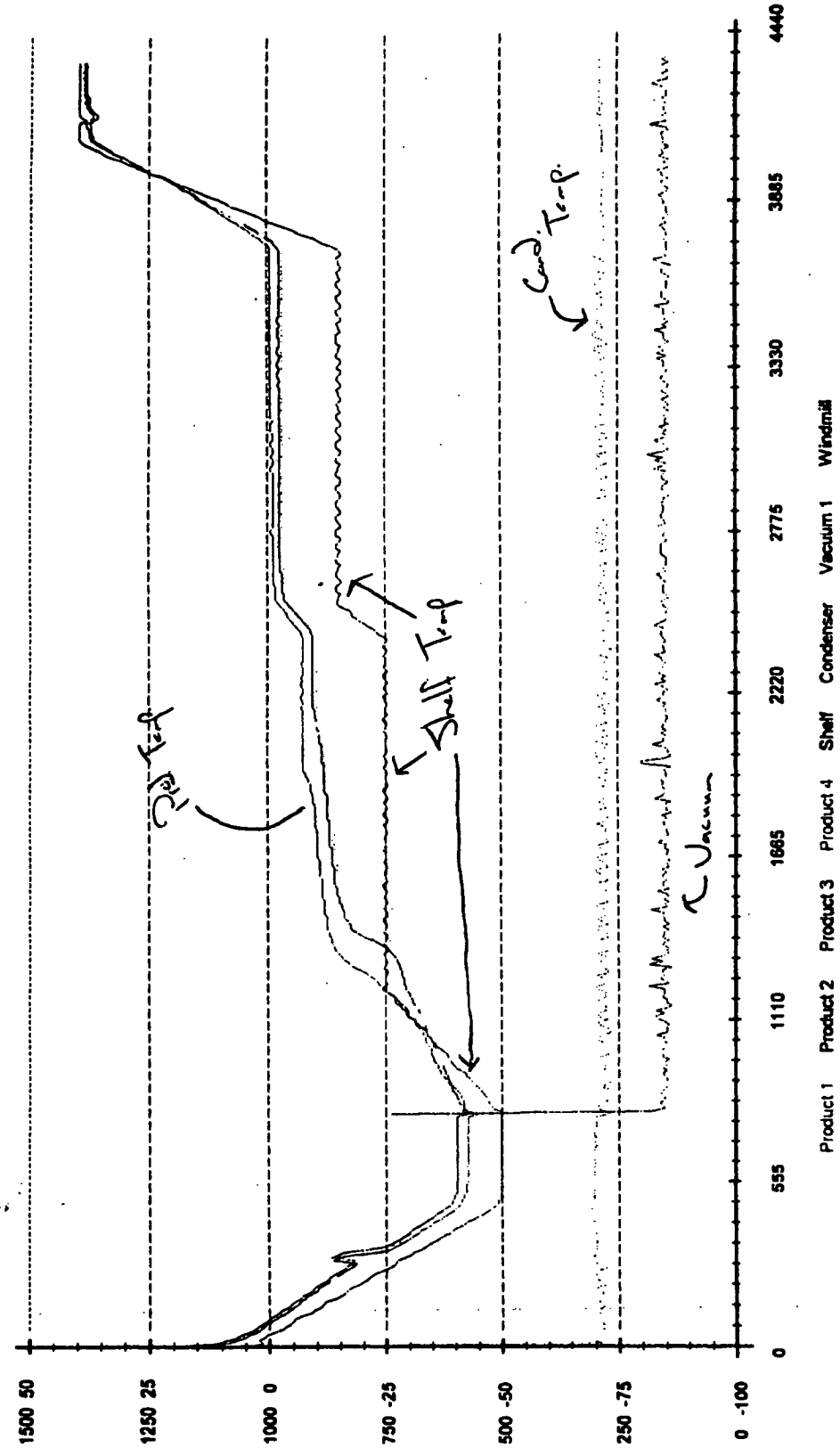


Figure 5

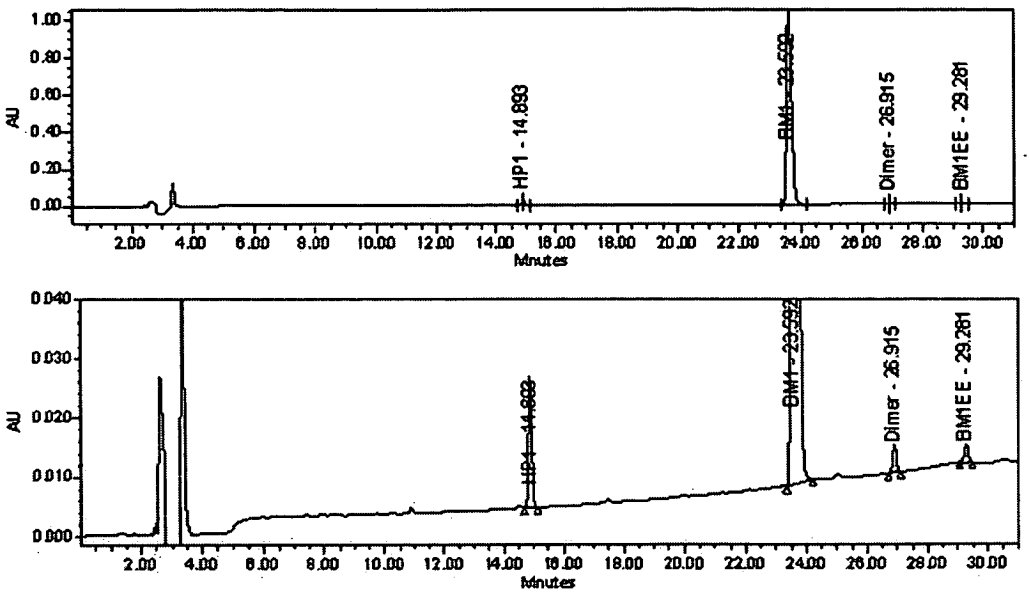


Fig. 6

CP391

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Brittain et al. **Group Art Unit:** Not Assigned

For: BENDAMUSTINE PHARMACEUTICAL COMPOSITION **Examiner:** Not Assigned

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

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

I believe that 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

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☒ is attached hereto.
☐ was filed on _____ as Serial No. _____.
☐ said application having been amended on _____ .

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

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned

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

Serial Number	Date Filed
60/644354	January 14, 2005

I hereby appoint all the practitioners associated with Customer Number 27573 (which is the Customer Number assigned to Cephalon, Inc.) to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith. Each practitioner associated with Customer Number 27573 is an attorney registered before the United States Patent and Trademark Office.

Address all telephone calls and correspondence to:

Robert T. Hrubiec
CEPHALON, INC.
145 Brandywine Parkway
West Chester, PA 19380
Telephone No.: **(610) 738-6356**
Facsimile No.: **(610) 738-6590**

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.

<table>
<tr><td>Name: Jason Edward Brittain</td><td rowspan="3">

_____
Signature

Date of Signature:_____

Citizenship: United States of America</td></tr>
<tr><td>Mailing Address:
1580 Chiswick Ct.
El Cajon, CA 92020</td></tr>
<tr><td>City/State of Actual Residence:
El Cajon, California</td></tr>
</table>

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: Joe Craig Franklin	
Mailing Address: 11519 Kirby Place San Diego, CA 92126	_____ **Signature** **Date of Signature:**_____
City/State of Actual Residence: San Diego, California	**Citizenship:** United States of America

PATENT APPLICATION SERIAL NO _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

01/17/2006 YPOLITE1 00000054 031195 11330868

01 FC:1011	300.00	DA
02 FC:1111	500.00	DA
03 FC:1311	200.00	DA
04 FC:1202	2900.00	DA
05 FC:1201	3600.00	DA

PTO-1556
(5/87)

U.S. Government Printing Office: 2002 — 466-267/68033

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875 Effective December 8, 2004

Application or Docket Number: 11330868

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	150.00		N/A	300.00
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	$250		N/A	$500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	$100		N/A	$200
TOTAL CLAIMS (37 CFR 1.16(i))	78 minus 20 =	58	X$ 25 =		OR	X$50 =	2900
INDEPENDENT CLAIMS (37 CFR 1.16(h))	29 minus 3 =	15	X100 =			X200 =	3600
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))			+180=			+360=	
			TOTAL			TOTAL	7500

If the difference in column 1 is less than zero, enter "0" in column 2.

1 5 6 11 12 19 25 26 27 28 29 30 31 33 34 36 37 60 71 72 77

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$ 25 =		OR	X$50 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(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 B	Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
						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.

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested Classification	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	
Computer Readable Form (CRF)?::	No
Number of copies of CRF::	
Title::	Bendamustine Pharmaceutical Compositions
Attorney Docket Number::	CP391
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	3
Total Drawing Sheets::	6
Small Entity::	No
Petition Included::	No
Secrecy Order in Parent Appl.::	No

Applicant Information

Applicant Authority type::	Inventor
Primary Citzenship Country::	US
Status::	Full Capacity
Given Name::	Jason Edward
Family Name::	Brittain

Name Suffix::	
City of Residence::	El Cajon
State or Province of Residence::	CA
Country of Residence::	US
Street of Mailing Address::	1580 Chiswick Ct.
City of Mailing Address::	El Cajon
State or Province of Mailing Address::	CA
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	92020
Applicant Authority type::	Inventor
Primary Citzenship Country::	US
Status::	Full Capacity
Given Name::	Joe Craig
Family Name::	Franklin
Name Suffix::	
City of Residence::	San Diego
State or Province of Residence::	CA
Country of Residence::	US
Street of Mailing Address::	11519 Kirby Place
City of Mailing Address::	San Diego
State or Province of Mailing Address::	CA
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	92126

Correspondence Information

Correspondence Customer Number::	27573
Phone Number::	610-738-6463

Fax Number:: 610-738-6590

E-Mail address:: intprop@cephalon.com

Representative Information

Representative Customer Number:: 27573

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Application claiming benefit under 35 USC 119(e)	60644354	01/14/05

Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::
			Yes
			Yes
			Yes

Assignee Information::

Assignee Name:: Salmedix, Inc.

Street of Mailing Address:: 41 Moores Road

City of Mailing Address:: Frazer

State or Province of Mailing Address:: PA

Country of Mailing Address:: United States of America

Postal or Zip Code of Mailing Address:: 19355

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

FORMALITIES LETTER

27573
CEPHALON, INC.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

Date Mailed: 02/28/2006

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. 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 oath or declaration is unsigned.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

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

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
    - The drawings must be reasonably free from erasures and must be free from alterations, overwriting, interlineations, folds, and copy marks. See Figure(s) 5.

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.

The applicant needs to satisfy supplemental fees problems indicated below.

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

- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is **$130** for a Large Entity

- **$130** Surcharge.

Replies should be mailed to: Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

A copy of this notice MUST be returned with the reply.

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199, or 1-800-972-6382
PART 3 - OFFICE COPY

PTO/SB/21 (04-04)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

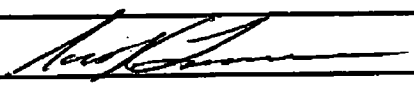
Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Brittain
Art Unit	1616
Examiner Name	Not Assigned
Total Number of Pages in This Submission	15
Attorney Docket Number	CP391

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment / Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☒ Response to Missing Parts under 37 CFR1.52 or 1.53
  - ☒ Copy of Missing Parts
  - ☒ Declaration/POA
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Technology Center (TC)
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:
  Supplemental Application Data Sheet

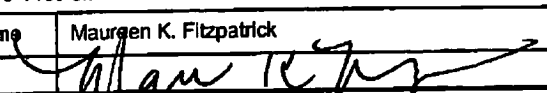
Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Scott K. Larsen, Registration No. 38,532
Signature	
Date	March 14, 2006

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below.

Typed or printed name	Maureen K. Fitzpatrick		
Signature		Date	March 14, 2006

Attorney Docket: CP391 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Brittain **Confirmation No. 9998**

Serial No.: 11/330,868 **Group Art Unit: 1616**

Filing Date: January 12, 2006 **Examiner: Not Assigned**

For: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

MAIL STOP MISSING PARTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

In response to the "Notice to File Missing Parts of Non-Provisional Application -- Filing Date Granted" Dated February 28, 2006, a response to which is due April 28, 2006, enclosed herewith for filing is the Original Combined Declaration and Power of Attorney, executed by the inventor(s) and a copy of the Notice to File Missing Parts of Non-Provisional Application.

Please charge the surcharge in the amount of **$130.00**, any deficiency or credit any overpayment to my Deposit Account No. 03-1195.

Date: March 14, 2006

Scott K. Larsen
Registration No. 38,532

CEPHALON, Inc.
145 Brandywine Parkway
West Chester, PA 19380
Phone: (610) 738-6463
Fax: (610) 727-7651

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

FORMALITIES LETTER

27573
CEPHALON, INC.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

Date Mailed: 02/28/2006

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

03/15/2006 HTECKLU1 00000123 031195 11330868
01 FC:1051 130.00 DA

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. 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 oath or declaration is unsigned.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

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

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - The drawings must be reasonably free from erasures and must be free from alterations, overwriting, interlineations, folds, and copy marks. See Figure(s) 5.

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.

The applicant needs to satisfy supplemental fees problems indicated below.

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

- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is **$130** for a Large Entity

- **$130** Surcharge.

Replies should be mailed to: Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

A copy of this notice MUST be returned with the reply.

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199, or 1-800-972-6382
PART 2 - COPY TO BE RETURNED WITH RESPONSE

RECEIVED
CENTRAL FAX CENTER
MAR 14 2006

CP391

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Brittain et al. **Group Art Unit:** Not Assigned

For: **BENDAMUSTINE PHARMACEUTICAL COMPOSITION** **Examiner:** Not Assigned

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

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

I believe that 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

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☐ is attached hereto.
☒ was filed on January 12, 2006 as Serial No. 11/330,868.
☐ said application having been amended on _____.

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

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned

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

Serial Number	Date Filed
60/644354	January 14, 2005

I hereby appoint all the practitioners associated with Customer Number 27573 (which is the Customer Number assigned to Cephalon, Inc.) to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith. Each practitioner associated with Customer Number 27573 is an attorney registered before the United States Patent and Trademark Office.

Address all telephone calls and correspondence to:

Robert T. Hrubiec
CEPHALON, INC.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Telephone No.: **(610) 738-6356**
Facsimile No.: **(610) 738-6590**

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: **Jason Edward Brittain**	
Mailing Address: 1580 Chiswick Ct. El Cajon, CA 92020	_____ **Signature** **Date of Signature:**_____
City/State of Actual Residence: El Cajon, California	**Citizenship:** United States of America

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: **Joe Craig Franklin**	 **Signature**
Mailing Address: 3708 East 45th Street Tulsa, OK 74135	
City/State of Actual Residence: Tulsa, Oklahoma	**Date of Signature:**_____ **Citizenship:** United States of America

CP391

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Brittain et al. **Group Art Unit:** Not Assigned

For: **BENDAMUSTINE PHARMACEUTICAL COMPOSITION** **Examiner:** Not Assigned

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

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

I believe that 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

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☐ is attached hereto.
☒ was filed on January 12, 2006 as Serial No. 11/330,868.
☐ said application having been amended on _____.

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

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

CP391

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned

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

Serial Number	Date Filed
60/644354	January 14, 2005

I hereby appoint all the practitioners associated with Customer Number 27573 (which is the Customer Number assigned to Cephalon, Inc.) to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith. Each practitioner associated with Customer Number 27573 is an attorney registered before the United States Patent and Trademark Office.

Address all telephone calls and correspondence to:

Robert T. Hrubiec
CEPHALON, INC.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Telephone No.: **(610) 738-6356**
Facsimile No.: **(610) 738-6590**

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: **Jason Edward Brittain**	 **Signature**
Mailing Address: 1580 Chiswick Ct. El Cajon, CA 92020	**Date of Signature:** 04 February 2006
City/State of Actual Residence: El Cajon, California	**Citizenship:** United States of America

 CP391

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: Joe Craig Franklin	
Mailing Address: 11519 Kirby Place San Diego, CA 92126	_____ **Signature** **Date of Signature:**_____
City/State of Actual Residence: San Diego, California	**Citizenship:** United States of America

Supplemental Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested Classification	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	
Computer Readable Form (CRF)?::	No
Number of copies of CRF::	
Title::	Bendamustine Pharmaceutical Compositions
Attorney Docket Number::	CP391
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	3
Total Drawing Sheets::	6
Small Entity::	No
Petition Included::	No
Secrecy Order in Parent Appl.::	No

Applicant Information

Applicant Authority type::	Inventor
Primary Citzenship Country::	US
Status::	Full Capacity
Given Name::	Jason Edward
Family Name::	Brittain

Name Suffix::	
City of Residence::	El Cajon
State or Province of Residence::	CA
Country of Residence::	US
Street of Mailing Address::	1580 Chiswick Ct.
City of Mailing Address::	El Cajon
State or Province of Mailing Address::	CA
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	92020
Applicant Authority type::	Inventor
Primary Citzenship Country::	US
Status::	Full Capacity
Given Name::	Joe Craig
Family Name::	Franklin
Name Suffix::	
City of Residence::	Tulsa
State or Province of Residence::	OK
Country of Residence::	US
Street of Mailing Address::	3708 East 45th Street
City of Mailing Address::	Tulsa
State or Province of Mailing Address::	OK
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	74135

Correspondence Information

Correspondence Customer Number::	27573
Phone Number::	610-738-6463

Fax Number::	610-738-6590
E-Mail address::	intprop@cephalon.com

Representative Information

Representative Customer Number::	27573

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Application claiming benefit under 35 USC 119(e)	60644354	01/14/05

Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::
			Yes
			Yes
			Yes

Assignee Information::

Assignee Name::	Cephalon, Inc.
Street of Mailing Address::	41 Moores Road
City of Mailing Address::	Frazer
State or Province of Mailing Address::	PA
Country of Mailing Address::	United States of America
Postal or Zip Code of Mailing Address::	19355

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

27573
CEPHALON, INC.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

FORMALITIES LETTER

Date Mailed: 03/27/2006

NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

Filing Date Granted

The U.S. Patent and Trademark Office has received your reply on 03/14/2006 to the Notice to File Missing Parts (Notice) mailed 02/28/2006 and it has been entered into the nonprovisional application.The reply, however, does not include the following items required in the Notice.

The period of reply remains as set forth in the Notice. You may, however, obtain EXTENSIONS OF TIME under the provisions of 37 CFR 1.136 (a) accompanied by the appropriate fee (37 CFR 1.17(a)).

A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application. Replies should be mailed to: Mail Stop Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria VA 22313-1450.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

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

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
    - The drawings must be reasonably free from erasures and must be free from alterations, overwriting, interlineations, folds, and copy marks. See Figure(s) 5.

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
P.O. Box 1450
Alexandria VA 22313-1450

A copy of this notice MUST be returned with the reply.

YL

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199, or 1-800-972-6382

PART 3 - OFFICE COPY

PTO/SB/21 (04-04)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Brittain
Art Unit	1616
Examiner Name	Not Assigned
Total Number of Pages in This Submission: 5	Attorney Docket Number: CP391

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment / Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☐ Response to Missing Parts under 37 CFR1.52 or 1.53
  - ☐ Copy of Missing Parts
  - ☐ Declaration/POA
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Technology Center (TC)
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:
  - **Response to Notice of Incomplete Reply**
  - **Figure 5**
  - **Copy of the Notice of Incomplete Reply**

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Scott K. Larsen, Registration No. 38,632
Signature	
Date	April 4, 2006

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below.

Typed or printed name	Maureen K. Fitzpatrick		
Signature		Date	April 4, 2006

Attorney Docket: CP391 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Brittain et al. **Confirmation No. 9998**

Serial No.: 11/330,868 **Group Art Unit: 1616**

Filing Date: January 12, 2006 **Examiner: Unknown**

For: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

MAIL STOP MISSING PARTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

RESPONSE TO NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

In response to the "Notice of Incomplete Reply -- Filing Date Granted" Dated March 27, 2006, a response to which is due April 28, 2006, enclosed herewith for filing is Figure 5 and a copy of the Notice of Incomplete Reply.

Please charge any deficiency or credit any overpayment to Deposit Account No. 03-1195.

Date: April 4, 2006

Scott K. Larsen
Registration No. 38,532

CEPHALON, Inc.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Phone: (610) 738-6463
Fax: (610) 727-7651

UNITED STATES PATENT AND TRADEMARK OFFICE
UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

FORMALITIES LETTER

27573
CEPHALON, INC.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

Date Mailed: 03/27/2006

NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

Filing Date Granted

The U.S. Patent and Trademark Office has received your reply on 03/14/2006 to the Notice to File Missing Parts (Notice) mailed 02/28/2006 and it has been entered into the nonprovisional application.The reply, however, does not include the following items required in the Notice.

The period of reply remains as set forth in the Notice. You may, however, obtain EXTENSIONS OF TIME under the provisions of 37 CFR 1.136 (a) accompanied by the appropriate fee (37 CFR 1.17(a)).

A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application. Replies should be mailed to: Mail Stop Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria VA 22313-1450.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

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

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
    - The drawings must be reasonably free from erasures and must be free from alterations, overwriting, interlineations, folds, and copy marks. See Figure(s) 5.

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
P.O. Box 1450
Alexandria VA 22313-1450

A copy of this notice MUST be returned with the reply.

Office of Initial Patent Examination (571) 272-4000, or 1-800-PTO-9199, or 1-800-972-6382
PART 2 - COPY TO BE RETURNED WITH RESPONSE

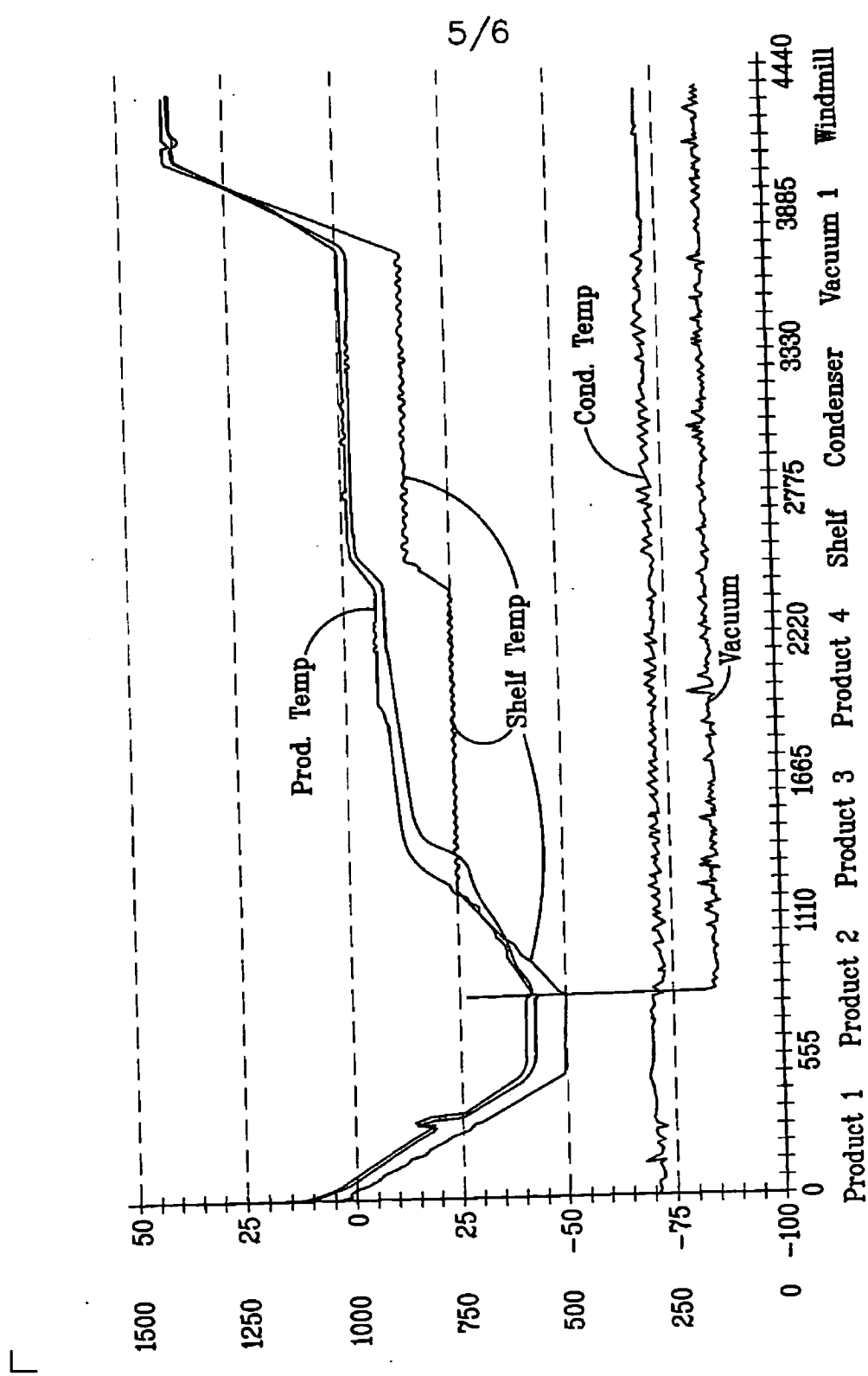


FIG. 5

01-25-08

IFW

PTO/SB/21 (09-04)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	470

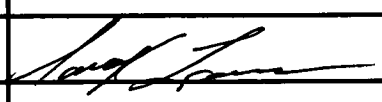
Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Brittain
Art Unit	1616
Examiner Name	A. Soroush
Attorney Docket Number	CP391

ENCLOSURES ***(check all that apply)***

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment / Reply
  - ☐ After Final
- ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☒ **Information Disclosure Statement**
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
- ☐ Reply to Missing Parts under 37 CFR1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ **Other Enclosure(s)** ***(please identify below)*:**
  45 References
  Return Postcard

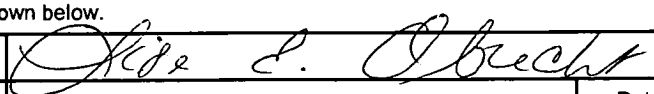
Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm	CEPHALON, INC.		
Signature			
Printed Name	Scott K. Larsen		
Date	January 23, 2008	Reg. No.	38,532

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as Express Mail Label Number EV 637825095 US in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below.

Signature			
Typed or printed name	Lisa E. Obrecht	Date	January 23, 2008

Attorney Docket No.: CP391 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	Brittain et al.
Serial No.:	11/330,868
Filing Date:	January 12, 2006
For.:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
Confirmation No.:	9998
Group Art Unit.:	1616
Examiner:	A. Soroush

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

Dear Sir:

INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 C.F. R. §1.56 and in accordance with 37 C.F.R. §§1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as a representation that a search has been made or an admission that this information is material to the patentability as defined in 37 C.F.R. § 1.56(b).

In accordance with §1.97(b), since this Information Disclosure Statement is being filed either within three months of the filing date of the above-identified application, within three months of the date of entry into the national stage of the above-identified application as set forth in § 1.491, or before the mailing date of a first Office Action on the merits of the above-identified application. No additional fee is required.

A copy of each cited non-US patent reference is enclosed.

Applicant would like to make of record the following co-pending and commonly owned application: USSN 11/267,010 filed on Nov. 4, 2005.

Please charge any deficiency or credit any overpayment to Deposit Account No. 03-1195.

Date: January 23, 2008

Scott K. Larsen
Attorney for Applicant
Registration No. 38,532

CEPHALON, Inc.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Telephone: (610) 738-6463
Facsimile: (610) 727-7651

FORM PTO-1449	Attorney Docket: CP391
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Application Number: 11/330,868
	Filing Date: January 12, 2006
List of Patent and Publications Cited by Applicant	First Named Inventor: Brittain
	Group Art Unit: 1616
	Examiner Name: A. Soroush
	Sheet: 1 of 5

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number	Name	Date of Publication	Class	Subclass
	A1	US-5204335	Sauerbier et al.	Apr. 20, 1993		
	A2	US-5227373	Alexander et al.	Jul. 13, 1993		
	A3	US-5750131	Wichert et al.	May 12, 1998		
	A4	US-5770230	Teagarden et al.	Jun. 23, 1998		
	A5	US-5776456	Anderson et al.	Jul. 7, 1998		
	A6	US-5955504	Wechter et al.	Sep. 21, 1999		
	A7	US-5972912	Marek et al.	Oct. 26, 1999		
	A8	US-6034256	Masferrer	Mar. 7, 2000		
	A9	US-6077850	Masferrer	Jun. 20, 2000		
	A10	US-6090365	Kaminski et al.	Jul. 18, 2000		
	A11	US-6271253 B1	Masferrer	Aug. 7, 2001		
	A12	US-6380210	Desimone et al.	Apr. 30, 2002		
	A13	US-6492390 B2	Masferrer	Dec. 12, 2002		
	A14	US-6545034 B1	Carson et al.	Apr. 8, 2003		
	A15	US-6569402	Cheesman et al.	May 27, 2003		
	A16	US-6573292 B1	Nardella	Jun. 3, 2003		
	A17	US-6613927 B1	Kwok	Sep. 2, 2003		
	A18	US-2003/0232874	Nardella	Dec 18, 2003		
	A19	US-2004/0053972	Nara	Mar. 18, 2004		
	A20	US-2004/0058956	Akiyama et al.	Mar. 25, 2004		
	A21	US-2004072889	Masjerrer	Apr. 15, 2004		
	A22	US-2004/0096436 A1	Carson et al.	May 20, 2004		
	A23	US-2004152672	Carson et al.	Aug. 5, 2004		
	A24	US-2004/0247600	Leoni	Dec. 9, 2004		
	A25	US-2005/0060028 A1	Horres et al.	Mar. 17, 2005		
	A26	US-2005/0176678	Horres et al.	Aug 11, 2005		
	A27	US-2006/0051412	Petereit et al.	Mar 9, 2006		

Examiner's Signature		**Date:**	

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	2 of 5

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Document No.	Date	Country	Translation YES	Translation NO
	B1	DD 159289	Jun. 1, 1981	Germany	x	
	B2	DD 159877	Apr. 13, 1983	Germany	x	
	B3	DD 293808	Sep. 12, 1991	Germany		x
	B4	DE 80967	Jun. 1, 1970	Germany	x	
	B5	DE 10016077	Dec. 13, 2001	Germany		x
	B6	DE 10304403	Aug. 5, 2004	Germany	A27	
	B7	DE 10306724	Sep. 18, 2003	Germany		x
	B8	EP 1354952	Oct. 22, 2003	Germany		
	B9	EP 1444989	Aug. 11, 2004	Italy		
	B10	WO 96/28148	Mar. 13, 1998	Australia		
	B11	WO 03/066027 A1	Feb. 7, 2003	PCT		
	B12	WO 03/081238	Oct. 2, 2003	PCT		
	B13	WO 03/086470 A3	May 6, 2004	PCT		
	B14	WO 03/094990	Mar. 11, 2003	PCT	A26	

OTHER DOCUMENTS Non-Patent Literature Documents

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), Title of Article, Title of Journal (book, magazine, catalog, etc.) Date, Pertinent Pages, Volume-Issue Number, publisher, city and/or country where published.
	C1	AIVADO, MANUEL et al., *Bendamustine in the treatment of chronic lymphocytic leukemia: Results and future perspectives*, Seminars in Oncology, **2002**, pp. 19-22, Vol. 29 No. 4, Suppl. 13.
	C2	BARMAN BALFOUR, JULIA A. et al., *Bendamustine*, Drugs, **2001**, pp. 631-638, Vol. 61(5), Auckland, New Zealand
	C3	BREMER, KARL, *High rates of long-lasting remissions after 5-day bendamustine chemotherapy cycles in pre-treated low-grade non-hodgkin's-lymphomas*, Journal of Cancer Research and Clinical Oncology, **2002**, pp.603-609, Vol. 128(11).

Examiner's Signature		**Date:**	

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	3 of 5

	C4	CHOW, KAI et al., *Anti-CD20 antibody (IDEC-C2B8, rituximab) enchances efficacy of cytotoxic drugs on neoplastic lymphocytes in vitro: role of cytokines complement, and caspases*, Haematologica, **Jan. 2002**, pp. 33-43, Vol. 87(1)
	C5	CHOW, KAI U. et al., *In AML Cell Lines Ara-C Combined with Purine Analogues is Able to Exert Synergistic as Well as Antagonistic Effects on Proliferation, Apoptosis and Disruption of Mitochondrial Membrane Potential*, Leukemia & Lymphoma, **2003**, pp.165-173, Vol. 44(1).
	C6	CHOW, KAI U. et al., *In vitro induction of apoptosis of neoplastic cells in low-grade non-Hodkin's lymphomas by combinations of established cytotoxic drugs with bendamustine*, Haematologica, **2001**, pp. 485-493, Vol. 86(5).
	C7	CHOW, KAI UWE et al., *Synergistic effects of chemotherapeutic drugs in lymphoma cells are associated with down-regulation of inhibitor of apoptosis proteins (IAPs), prostate-apoptosis-response-gene 4(Par-4), death-associated protein (Dazz) and with enforced caspase activation*, Biochemical Pharmacology, **2003**, pp.711-724, Vol. 66(5).
	C8	DIEHL, VOLKER et al., *Bendamustine in the Treatment of Hematologic Malignancies*, Semin. Oncol., **2002**, Vol. 29(4) Suppl. 13, Saundes, Philadelphia, PA.
	C9	FICHTNER, I. et al., *Antineoplastic activity and toxicity of some alkylating cytostatics (cyclophosphamide, CCNU, cytostasan) encapsulated in liposomes in different murine tumor models*, Journal of Microencapsulation, **1986**, pp. 77-87, Vol. 3(2).
	C10	GHANDI, VARSHA, *Metabolism and mechanisms of action of bendamustine: Rationales for combination therapies*, Seminars in Oncology, **2002**, pp. 4-11, Vol. 29, No. 4, Suppl. 13.
	C11	GUST, R. et al., *Investigations on the Stability of Bendamustin, a Cytostatic Agent of the Nitrogen Mustard Type, I. Synthesis, Isolation, and Characterization of Reference Substances*, Monatshefte fur Chemie, **1997**, pp. 291-299, Vol. 128(3)
	C12	HEIDER, ANDREA et al., *Efficacy and toxicity of bendamustine in patients with relapsed low-grade non-Hodgkin's lymphomas*, Anti-Cancer Drugs, **2001**, pp. 725-729, Vol. 12(9).
	C13	KATH, R. et al., *Bendamustine monotherapy in advanced and refractory chronic lymphocytic leukemia*, Journal of Cancer Research and Clinical Oncology, **2001**, pp. 48-54, Vol. 127(1).

Examiner's Signature		**Date:**	

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	4 of 5

	C14	KOENIGSMAN, MICHAEL et al., *Fludarabine and Bendamustine in Refractory and Relapsed Indolent Lymphoma – a Multicenter Phase I/II Trial of the East German Society of Hematology and Oncology (OSHO)*, Leukemia & Lymphoma. **2004**, pp.1821-1827, Vol.45(9)
	C15	KOESTER, WOLF et al., *Carboplatin in combination with bendamustine in previously untreated patients with extensive-stage small lung cancer (SCLC)*, Clinical Drug Investigation, **2004**, pp.611-618, Vol. 24(10).
	C16	KOLLMANNSBERGER, CHRISTIAN et al., *Phase II study of bendamustine in patients with relapsed or cisplatin-refractory germ cell cancer*, Anti-Cancer Drugs, **2000**, pp. 535-539, Vol. 11(7).
	C17	KONSTANTINOV, S. M. et al., *Cytotoxic efficacy of bendamustine in human leukemia and breast cancer cell lines*, Journal of Cancer Research and Clinical Oncology, **2002**, pp. 271-278, Vol. 128(5).
	C18	LEONI, LM et al., *Sdx-105 (Trenda), Active in Non-Hodgkins Lymphoma Cells, Induces the Mitotic Catastrophe Death Pathway*, Blood, **2004**, Vol. 104(11): Abs 4593.
	C19	MAAS, *Stabilitat von Benamustinhydrochlorid in Infusionslosungen* Pharmazie, vol. 49, no. 10, **1994**, pp. 775-777. (translation included)
	C20	NIEMEYER, CHRISTINA C. et al., *SDX-105 (bendamustine) is a clinically active chemotherapeutic agent with a distinct mechanism of action*, Proc Annu Meet Am Assoc Cancer Res, **2004**, Vol. 45:1st ed.
	C21	NOWAK, DANIEL et al., *Upon Drug-Induced Apoptosis in Lymphoma Cells X-linked Inhibitor of Apoptosis (XIAP) Translocates from the Cytosol to the Nucleus*, Leukemia & Lymphoma, **2004**, pp.1429-1436, Vol.45(7).
	C22	PONISCH, WOLFRAM et al., *Bendamustine in the treatment of Multiple Myeloma: Results and future perspectives*, Seminars in Oncology, **2002**, pp.23-26, Vol. 29 No. 4 Suppl. 13.
	C23	PREISS, R. et al., *Pharmacokinetics of bendamustin (Cytostasan) in patients*, Pharmazie, **1985**, pp. 782-784, Vol. 40(11). TRANSLATION
	C24	*Ribomustin: bendamustine Product Monograph*, **Jan. 2002**, pp. 3-54, Ribosepharm GMBH, Munchen, Germany
	C25	*Ribomustin: bendamustine Product Monograph*, **Mar. 2005**, pp. 3-73, Ribosepharm MBH, Munchen, Germany

Examiner's Signature		**Date:**	

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	5 of 5

	C26	RUMMEL, MATHIAS J. et al., *Bendamustine in the treatment of non-Hodgkin's lymphoma: Results and future perspectives*, Seminars in Oncology, **2002**, pp.27-32, Vol. 29 No. 4 Suppl. 13.
	C27	RUMMEL, MATHIAS J. et al., *In Vitro Studies With Bendaustine: Enhanced Activity in Combination With Rituximab*, Seminars in Oncology, **Aug. 2002**, pp. 12-14, Vol. 29 No. 4 Suppl 13
	C28	SCASNAR et al., *Radiochemical Assay of Stability of ^{14}C-Cytostasan Solutions During Preparation and Storage*, vol. 121, no. 2m 1988 pgs 489-497.
	C29	SCHMIDT-HIEBER, MARTIN et al., *A phase II study of bendamustine chemotherapy as second-line treatment in metastatic uveal melanoma*, Melanoma Research, **2004**, pp.439-442, Vol.14(6)
	C30	SCHOFFSKI, P., *Repeated administration of short infusions of Bendamustine: a phase I study in patients with advanced progressive solid tumors*, Journal of Cancer Research and Clinical Oncology, **2000**, pp. 41-47, Vol. 126(1).
	C31	SCHRIJVERS, DIRK et al., *Phase I studies with bendamustine: An update*, Seminars in Oncology, **2002**, pp. 15-18, Vol. 29 No. 4 Suppl. 13.
	C32	STRUMBERG, DIRK et al., *Bendamustine hydrochloride activity against doxorubicin-resistant human breast carcinoma cell lines*, Anti-Cancer Drugs, **1996**, pp. 415-421, Vol. 7(4)
	C33	WEIDE, RUDOLF et al., *Bendamustine mitoxantrone and rituximab (BMR): a new effective regimen for refractory or relapsed indolent lymphomas*, Leukemia & Lymphoma, **2002**, pp.327-331, Vol. 43(2)
	C34	WEIDE, RUDOLF et al., *Bendamustine/Mitoxantrone/Rituximab (BMR): A Very Effective, Well Tolerated Outpatient Chemoimmunotherapy for Relapsed and Refractory CD20-positive Indolent Malignancies. Final Results of a Pilot Study*, Leukemia & Lymphoma, **2004**, pp. 2445-2449, Vol. 45(12)
	C35	WERNER, W. et al., *Hydrolyseprodukte des Cancerostaticums Cytostasan (Bendamustin)*, Pharmazie, **1987**, pp. 272-273, Vol. 42 (Translation included)
	C36	ZULKOWSKI, K. et al., *Regression of brain metastases from breast carcinoma after chemotherapy with bendamustine*, Journal of Cancer Research and Clinical Oncology, **2002**, pp. 111-113, Vol. 128(2).

Examiner's Signature		**Date:**	

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 444 989 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
11.08.2004 Bulletin 2004/33

(21) Application number: **03002603.3**

(22) Date of filing: **07.02.2003**

(51) Int Cl.[7]: **A61K 45/00**, A61K 39/395, A61K 45/06, A61P 35/00, A61P 37/06

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT SE SI SK TR
Designated Extension States:
AL LT LV MK RO

(71) Applicants:
- **Stassi, Giorgio, Dr.**
  90127 Palermo (IT)
- **Todaro, Matilde, Dr.**
  90127 Palermo (IT)

(72) Inventors:
- **Stassi, Giorgio, Dr.**
  90127 Palermo (IT)
- **Todaro, Matilde, Dr.**
  90127 Palermo (IT)

(74) Representative: **Isenbruck, Günter, Dr.**
Isenbruck, Bösl, Hörschler, Wichmann, Huhn, Patentanwälte
Theodor-Heuss-Anlage 12
68165 Mannheim (DE)

(54) Sensitizing cells for apoptosis by selectively blocking cytokines

(57) The invention refers to the use of a cytokine antagonist which modulates the expression and/or the function of a cytokine, particularly a Th2 helper cell cytokine, in a cell and causes the down-regulation of anti-apoptotic proteins in said cell through the cytokine modulation for sensitizing cells for apoptosis. In particular, the cells that can be treated with the cytokine antagonists are drug-resistant cancer cells which fail to undergo apoptosis.

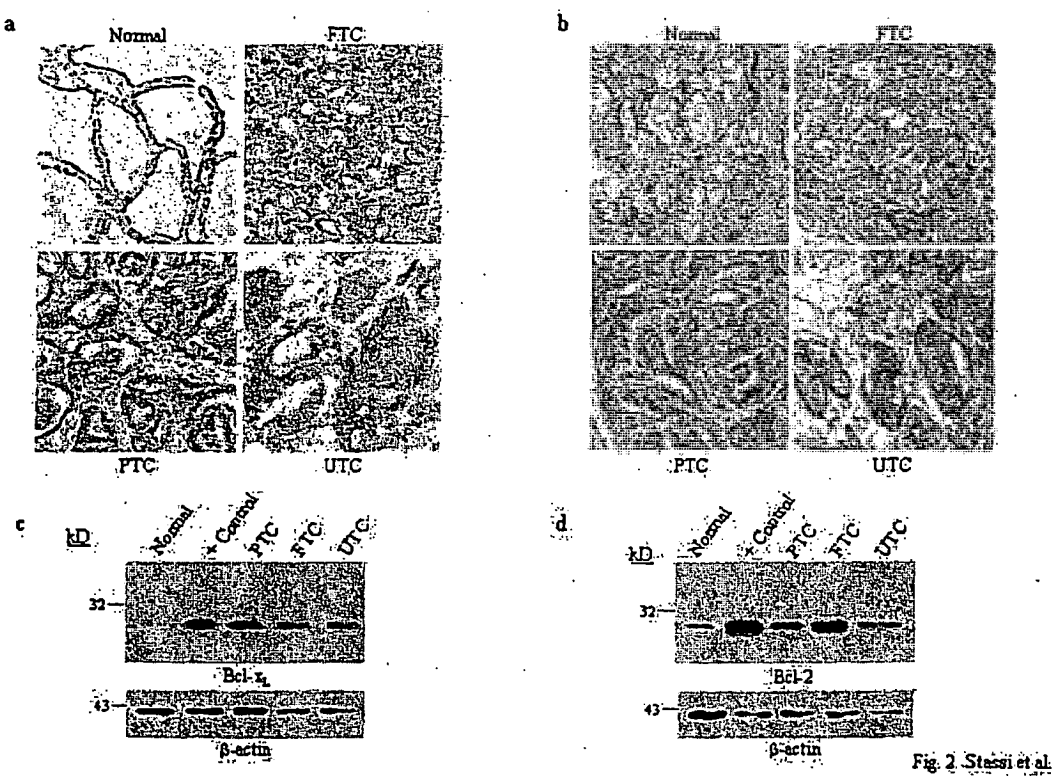


Fig. 2 Stassi et al.

EP 1 444 989 A1

Description

[0001] The present invention relates to a method of sensitizing cells for apoptosis by using compounds that selectively block cytokines, in particular interleukins, and the use of said compounds for the treatment of cancer and autoimmune diseases.

[0002] The molecular mechanisms controlling the balance between cell survival and cell death play a key role in a number of physiological and pathological processes. Crucial for the cellular ability to induce death of supernumerary, misplaced or damaged cells with high specificity and efficiency is the machinery of so-called "apoptosis" or "programmed cell death".

[0003] Diseases and conditions in which apoptosis has been implicated fall into two categories, those in which there is increased cell survival (i.e. apoptosis is reduced) and those in which there is excess cell death (i.e. apoptosis is increased). Diseases in which there is an excessive accumulation of cells due to increased cell survival include cancer, autoimmune disorders and viral infections. For these and other conditions in which insufficient apoptosis is believed to be involved, promotion of apoptosis is desired. This can be achieved, for example, by promoting cellular apoptosis or by increasing the sensitivity of cells to endogenous or exogenous apoptotic stimuli, for example, cell signaling molecules or other cytokines, cytotoxic drugs or radiation. Promotion of or sensitization to apoptosis is believed to have clinical relevance in sensitizing cancer cells to chemotherapeutic drugs or radiation.

[0004] In the second category, AIDS and neurodegenerative disorders like Alzheimer's or Parkinson's disease represent disorders for which an excess of cell death due to promotion of apoptosis (or unwanted apoptosis) is likely to be involved. Amyotrophic lateral sclerosis, retinitis pigmentosa, and epilepsy are other neurologic disorders in which apoptosis has been implicated. Apoptosis has been reported to occur in conditions characterized by ischemia, e.g. myocardial infarction and stroke. For these and other diseases and conditions in which unwanted apoptosis is believed to be involved, inhibitors of apoptosis are desired.

[0005] Currently, a major treatment for cancerous tumors is surgical removal of the affected areas of the tissue, organ, or gland. However, high recurrence rates are a major obstacle to the complete eradication of cancerous cells. It is believed that although the cancer cells in the malignant tumors can be removed surgically, cancerous cells that have invaded the surrounding tissue or lymph nodes frequently cause tumor recurrence. One reason for frequent tumor recurrence may be that during the development of the primary cancer, complete removal of all the cancer cells by surgical procedures is extremely difficult. Although irradiation, chemotherapy and appropriate hormone therapy all induce apoptosis to some extent in tumor cells, higher doses of the drugs or radiation may be required for suppressing the growth of cancer cells, which, in turn, can cause severe side effects on patients.

[0006] Thus, the problem underlying the present invention refers to the identification of compounds that specifically modulate distinct steps in the apoptosis pathway without causing the described deleterious side effects.

[0007] The effective cure of patients suffering from cancer is often difficult since many tumor cells have developed a resistance to anti-cancer drugs used for chemotherapy. The described phenotype involves a variety of strategies that tumor cells use to evade the cytostatic effects of anti-cancer drugs. Mechanisms for drug resistance include modifications in detoxification and DNA repair pathways, changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, and accelerated removal or secretion of drugs. In addition, cancer cells commonly fail to undergo apoptosis. Thus, apoptosis defects appear to be a major problem in cancer therapy as they confer resistance to many tumors against current treatment protocols, leading to tumor progression.

[0008] Apoptosis pathways involve diverse groups of molecules. One set of mediators implicated in apoptosis are so-called caspases, cysteine proteases that cleave their substrate specifically at aspartate residues. Caspases convey the apoptic signal in a proteolytic cascade, with caspases cleaving and activating other caspases which subsequently degrade other cellular targets eventually resulting in cellular breakdown. Caspase activation itself can be triggered by external stimuli affecting certain self-surface receptors, known to the person skilled in the art as so-called death receptors, or by intracellular stress response via the mitochondria leading to the release of mitochondrial proteins. Known death receptors mediating apoptosis include members of the tumor necrosis factor (TNF) receptor super family such as, e.g. CD95 (APO-1/Fas) or TRAIL (TNF-related apoptosis inducing ligand) receptors 1 and 2. Stimulation of death receptors with apoptosis-inducing substances leads, among others, to the activation of caspase 8, which in turn activates other downstream-acting caspases.

[0009] The induction or inhibition of apoptosis is controlled in part by the Bcl-2 family members. A number of such genes, including Bcl-2 and Bcl-x_L, counteract apoptosis by preserving mitochondrial membrane integrity and preventing cytochrome c release in the cytoplasm. In contrast, the pro-apoptotic members such as Bax and Bad antagonize the function of Bcl-2 and Bcl-x_L inducing heterodimer formation and mitochondrial membrane permeabilization with cytochrome c release.

[0010] In human cancers, a high expression of the anti-apoptotic members of the Bcl-2 family is commonly found and contributes to both neoplastic cell expansion and resistance to the therapeutic action of chemotherapeutic drugs. Overexpression of Bcl-2 can render cells

resistant to apoptosis, thereby favoring malignant growth. Moreover, since many chemotherapeutic agents kill tumor cells by inducing apoptosis, overexpression of Bcl-2 or Bcl-x_L can lead to a multi-drug resistant phenotype.

[0011] The expression of a variety of genes involved in the survival or death of different target cells, including members of the Bcl-2 family, is regulated by so-called cytokines. Cytokines belong to a diverse group of soluble, non-antibody proteins secreted by a variety of cell types of the immune system, which modulate the functional activities of individual cells by interaction with specific cell surface receptors, e.g. interferon, interleukin. The person skilled in the art knows two functionally distinct subsets of so-called T-helper cells that have been characterized on the basis of cytokine production. One subset, Th1 cells, secrete IFN-γ and other cytokines associated with inflammation and cell-mediated immune responses, whereas Th2 cells promote humoral response releasing IL-4, IL-5 and IL-10.

[0012] With respect to solve the problem underlying the present invention, namely the identification of compounds that specifically modulate distinct apoptosis steps, the inventors have surprisingly found that thyroid cancer cells autocrinely produce high levels of IL-4 and IL-10, as compared with normal tissues, while IFN-γ was barely detectable in those cancer cells. Thyroid cancer is the most common endocrine malignancy, responsible for about 60 % of the death secondary to endocrine cancer. Three major types of malignant tumors originate from the thyroid epithelium. The more differentiated papillary (PTC) and follicular (FTC) thyroid carcinomas account for the vast majority of malignant tumors, while the undifferentiated anaplastic carcinomas (UTC) are extremely rare. The high levels of IL-4 and IL-10 in thyroid cancer cells correlated with an overexpression of Bcl-x_L and Bcl-2 which in turn protects thyroid cancer cells against the cytotoxic effect of chemotherapeutic drugs suggesting a potential role of these anti-apoptotic proteins in thyroid cancer resistance from drug-induced cytotoxicity.

[0013] Thus, a first object of the present invention refers to the use of a cytokine antagonist which modulates the expression and/or the function of a cytokine in a cell for the down-regulation of a cell death preventing protein in a cell.

[0014] As a result of the down-regulation of a cell death preventing protein the cell is for sensitized for cell death. In the context of the present invention, the term "cell death" refers to any mechanism and process which can cause a cell to die. The skilled artisan distinguishes two processes named apoptosis and necrosis both of which are addressed within the scope of the present invention. However, the use of a cytokine antagonist according to the present invention is particularly effective if the death process the cell should be sensitized for is apoptosis. Thus, in a preferred embodiment of the present invention the "cell death preventing" proteins refer to "anti-apoptotic" proteins.

[0015] In a particular embodiment of the present invention the term "cell" refers to cells, that fail to undergo apoptosis as described in the introduction. In this respect, the cells encompass, for example, cancer cells and self-reacting cells of the immune system. Most preferably, the cell of the present invention is a cancer cell.

[0016] If the cell is a cancer cell, the defect in undergoing apoptosis may have rendered the cell resistant to various treatment strategies exploiting anti-neoplastic compounds and/or radiation therapies. The cancer cell to which the cytokine is preferably applied to can also be resistant to compounds which do not necessarily lead to cell death directly, but which sensitize these cells for apoptosis. The skilled artisan knows that such compounds include naturally occurring agonists for death receptors, i.e. receptor ligands or agonistic antibodies to said death receptors, as well as chemotherapeutic drugs.

[0017] The "cytokine" of the present invention belongs to the group of cytokines that are predominantly secreted by Th2 helper cells. More preferably, the cytokine is selected from the group consisting of IL-4, IL-5, IL-6, IL-10, and IL-13, as well as combinations thereof. For the efficient use of the cytokine antagonist of the present invention it is most preferred, if the cytokine is IL-4, IL-10 and/or IL-13, as well as combinations thereof.

[0018] Within the scope of the present invention, "anti-apoptotic proteins" include members of the Bcl family such as Bcl-2, Bcl-x_L, cFLIP, Mcl-1, Bcl-w, A1/BFL1, BOO/DIVA, NR-13, sentrin, TOSO, CPAN, PED, DFF45, and the like. The anti-apoptotic proteins of the present invention also include so-called "Inhibitors of Apoptosis Proteins" (IAPs). IAPs bind to early active caspases, thereby preventing the ongoing of the apoptosis process. They are expressed at high levels in many tumors and, by inhibition of caspases, contribute to the resistance of cancers against apoptosis induction. Examples of IAPs include NAIP, XIAP (hILP), cIAP-1, cIAP-2, ML-IAP (livin), KIAP, BIRC5 (survivin), TIAP, and Apollon. Finally, anti-apoptotic proteins can be others such as fortilin, and the like.

[0019] In a preferred embodiment of the present invention, the anti-apoptotic proteins include FED, cFLIP, Bcl-2 and Bcl-x_L, and combinations thereof. Most preferably, the anti-apoptotic proteins which are down-regulated by the cytokine antagonist are Bcl-2 and/or Bcl-x_L.

[0020] The term "cytokine antagonist" refers to any compound that is capable of directly modulating the expression and/or the function of the cytokine, thus leading to the down-regulation of anti-apoptotic proteins. It is further contemplated within the scope of the present invention that the cytokine antagonist refers to any compound that modulates the expression and/or the function of a cytokine indirectly, namely by affecting the expression and/or the function of the respective cytokine

receptor. It is obvious to the person skilled in the art that a down-regulation of the cytokine receptor directly interferes with the function of the cytokine itself. Therefore, the hereinafter described mechanisms and molecules, respectively, that modulate the expression and/or the function of a cytokine may also be extrapolated to cytokine receptors. In this respect, the term "cytokine" encompasses also cytokine receptors, unless otherwise indicated.

[0021] In the context of the present invention, the modulation of the expression and/or the function of the cytokine/cytokine receptor, hereinafter referred to as the "modulation", by the use of the cytokine antagonist according to the present invention can occur on the protein and/or on the nucleic acid level.

[0022] If the modulation occurs on the nucleic acid level, the cytokine antagonist according to the present invention can be a peptide or a nucleic acid that regulates the transcription of the cytokine gene by binding to up-stream and/or down-stream regulatory sequences of the coding region of the cytokine. Such regulatory sequences are known to the person skilled in the art and include so-called promoter, operator, enhancer or silencer regions. For example, the cytokine antagonist may interfere with the binding of the RNA polymerase to the promoter region of the cytokine gene, either by binding directly to the RNA polymerase binding region, by binding to the polymerase itself or by binding to other factors, e.g. transcription factors, which are required for efficient RNA polymerase binding and function. Furthermore, the cytokine antagonist may bind to the operator region and act as a so-called repressor of cytokine gene expression.

[0023] In a further embodiment of the present invention, the modulation on the nucleic acid level can occur by the use of nucleic acid molecules that hybridize to, and are therefore complementary to the coding sequence of the cytokine. These nucleic acid molecules may encode or act as cytokine gene antisense molecules useful, for example, in cytokine gene regulation. With respect to cytokine gene regulation, such techniques can be used to modulate, for example, the phenotype and metastatic potential of cancer cells. The use of antisense molecules as inhibitors is a specific, genetically based therapeutic approach. The present invention provides the therapeutic and prophylactic use of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding one of the aforementioned cytokines.

[0024] Similarly, a cytokine antagonist of the present invention that modulates the expression and/or the function of a cytokine on the nucleic acid level can be a dsRNA molecule which is complementary to the cytokine mRNA. Such molecules are also known in the art as small interfering RNA (siRNA). This technology to inhibit the expression of certain mRNAs is known to the person skilled in the art as RNA interference (RNAi). Preferably, the dsRNA molecules which are complementary to the mRNA of the cytokines of the present invention have a length between 10 and 30 base pairs, more preferably, they have a length between 19 and 25 base pairs. The cytokine antagonist being siRNA may be delivered to the target cell by any method known to the one of skilled art. Applicable is, for instance, the delivery by using cationic liposome reagents. It is also conceivable that the siRNA directed against the cytokine mRNA is obtained by using the DNA encoding it. In this case, a DNA construct comprising both a stretch of 19 to 25 nucleotides of the desired cytokine coding region, and the antisense stretch being separated from the sense stretch by a suitable linker which is able to form a hairpin loop, is inserted into a vector. The vector can be introduced into the target cell by methods well known the skilled artisan. The design of such a construct is further described e.g. in Brummelkamp et al. (Science 2002 Vol. 296, pages 550-553).

[0025] Furthermore, the present invention encompasses so-called ribozymes as cytokine antagonists. Ribozymes are naturally occurring RNA fragments that can be designed as human therapeutics to recognize, bind and digest any disease-causing mRNA sequence, in this case the cytokine mRNA. Ribozymes are designed to target the cytokine mRNA through complementary base pair hybridization. After binding to the target, the enzymatic activity of the ribozyme cleaves the cytokine mRNA thus preventing its translation into protein. The cytokine mRNA ribozymes can be chemically synthesized to selectively inhibit the cytokine production. In addition, the ribozymes may be chemically modified allowing the ribozymes to be more stable and active. Included are also ribozymes that do not only cleave cytokine-specific RNA molecules but also form carbon-carbon bonds in a covalent fashion, which raises the possibility of ribozymes that can catalyze other types of chemical reactions.

[0026] In a further embodiment of the present invention the translation of the cytokine gene can be reduced or eliminated by binding of an RNA-binding protein to one or more operator sequences in the 5'-UTR of the cytokine mRNA transcript. The bound RNA-binding protein interferes with translation, likely by preventing ribosome assembly or blocking the movement of the ribosome along the transcript from 5' to 3'. Such RNA-binding proteins may be multimeric, e.g. dimers of a particular RNA-binding protein. It is also possible within the scope of the present invention that the cytokine antagonist inhibits the cytokine expression by promoting or at least being involved in the degradation of cytokine mRNA.

[0027] If the modulation occurs on the protein level, the present invention encompasses antibodies or fragments thereof capable of specifically recognizing one or more epitopes of the cytokine gene products, epitopes of conserved variants of the cytokine gene products, epitopes of mutant cytokine gene products, or peptide fragments of cytokine gene products. Such antibodies may include, but are not limited to, polyclonal antibod-

ies, monoclonal antibodies (mAbs), human, humanized or chimeric antibodies, single-chain antibodies, Fab fragments, F(ab')2 fragments, Fv fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The cytokine antagonist being an antibody as described above can be used to capture and neutralize excessive amounts of cytokines that are overexpressed in drug-resistant cancer cells. It may be desirable for the present invention if the antibody recognizes more than one of the above mentioned cytokines. In order to capture and neutralize more than one overexpressed cytokine, the antibody used as a cytokine antagonist of the present invention can possess more than one specificities, i.e. being, for example, bispecific, trispecific or multispecific.

[0028] Epitopes and antigenic regions useful for generating antibodies can be found within the cytokine amino acid sequences (e.g. SWISS-PROT numbers P05112 for IL-4, P22301 for IL-10 or P35225 for IL-13) by procedures available to one of skill in the art. For example, short, unique peptide sequences can be identified in the amino acid sequences that have little or no homology to known amino acid sequences. Preferably the region of a protein selected to act as a peptide epitope or antigen is not entirely hydrophobic; hydrophilic regions are preferred because those regions likely constitute surface epitopes rather than internal regions of the present proteins and polypeptides. These surface epitopes are more readily detected in samples tested for the presence of the present proteins and polypeptides.

[0029] Peptides can be made by any procedure known to one of skill in the art, for example, by using in vitro translation or chemical synthesis procedures. Short peptides which provide an antigenic epitope but which by themselves are too small to induce an immune response may be conjugated to a suitable carrier. Suitable carriers and methods of linkage are well known in the art. Suitable carriers are typically large macromolecules such as proteins, polysaccharides and polymeric amino acids. Examples include serum albumins, keyhole limpet hemocyanin, ovalbumin, polylysine and the like. One of skill in the art can use available procedures and coupling reagents to link the desired peptide epitope to such a carrier. For example, coupling reagents can be used to form disulfide linkages or thioether linkages from the carrier to the peptide of interest. If the peptide lacks a disulfide group, one may be provided by the addition of a cysteine residue. Alternatively, coupling may be accomplished by activation of carboxyl groups.

[0030] The minimum size of peptides useful for obtaining antigen specific antibodies can vary widely. The minimum size must be sufficient to provide an antigenic epitope which is specific to the protein or polypeptide. The maximum size is not critical unless it is desired to obtain antibodies to one particular epitope. For example, a large polypeptide may comprise multiple epitopes, one epitope being particularly useful and a second epitope being immunodominant.

[0031] In a preferred embodiment of the present invention, the cytokine antagonist refers to an antibody against IL-4, IL-5, IL-6, IL-10, and/or IL-13, as well as combinations thereof. Even more preferred, the cytokine antagonist refers to an antibody against IL-4, IL-10, and/or IL-13, as well as combinations thereof. Most preferably, the cytokine antagonist refers to an antibody against IL-4 and/or IL-10, and combinations thereof. It is understood that the antibody being used as a cytokine antagonist can possess more than one specificities, as described supra, i.e. being directed to more than one of the mentioned IL, e.g. a bispecific antibody to IL4 and IL10.

[0032] In a further embodiment of the present invention, the cytokine antagonist that modulates the expression and/or the function of the cytokine can be a so-called aptamer, either a peptide-based aptamer or an oligonucleotide-based aptamer. Peptide aptamers are defined as protein-based recognition agents that consist of constrained combinatorial peptide libraries displayed on the surface of a scaffold protein. Peptide aptamers function *in trans*, interacting with and inactivating gene products without mutating the DNA that encodes them. In principle, combinatorial libraries of peptide aptamers should contain aptamers that interact with any given gene product, thus allowing peptide aptamers to be generated against an organism's entire proteome. Oligonucleotide-based aptamers being used as cytokine antagonist according to the present invention comprise DNA as well as RNA aptamers. In this respect, the present invention encompasses also mirror-image L-DNA or L-RNA aptamers, so-called spiegelmers.

[0033] The aptamers that are useful as cytokine antagonists for the present invention include those which interact with specific proteins and thus prevent or disrupt the specific protein interaction between the cytokine and its receptor. They can interact with the cytokine itself, preferably with that region of the cytokine that is involved in the receptor binding. The aptamers can also prevent/disrupt the interaction between the cytokine and its receptor by binding to the receptor, preferably with that region of the receptor that is involved in the cytokine binding. It is also possible that the aptamers bind to other factors/proteins that are required for successful cytokine/receptor interaction.

[0034] In the context of the described aptamers, it is also feasable the the cytokine antagonist comprises so-called small molecule inhibitors that may exhibit similar properties as aptamers, namely binding to either the cytokine or to the cytokine receptor, thereby inhibiting their proper interaction and, thus, function. The small molecule inhibitor can be a peptide or a small chemical compound, which has been identified by methods known to the skilled artisan, e.g. by computational combinatorial chemistry in combination with screening of compound libraries.

[0035] In a further embodiment of the present invention the cytokine antagonist that modulates the expression and/or the function of the cytokine, comprises at least one receptor, a derivative or fragment thereof, of any of the cytokines included in the present invention. Similarly to the proposed and described effect for using antibodies as cytokine antagonists, the cytokine receptor, a fragment or derivative thereof, can be used to capture and neutralize excessive amounts of cytokines which are overexpressed in drug-resistant cancer cells. Examples for suitable receptors and receptor subunits, respectively, include CD 124 which binds both IL-4 and IL-13 (data base accession number P24394), CD132 which represents the common gamma subunit shared by IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (data base accession number P31785), IL-13 receptor alpha-2 chain (data base accession number Q14627) and IL-10 receptor alpha chain (data base accession number Q13651).

[0036] In the context of the present invention the term "derivative or fragment" of a cytokine receptor refers to peptides the length of which and/or the amino acid composition of which can differ from the originally disclosed amino acid sequence, provided that the function of the receptor, namely the binding of the cytokine, is neither reduced nor eliminated. Therefore, the term "derivative or fragment" includes peptides which are extended or shortened on either the amino- or the carboxyterminal end or which possess deletions or insertions internally. In addition, the term "derivative or fragment" includes peptides with one or more amino acids being different from the originally disclosed sequence. Particularly advantageous for the present invention, especially if the receptor is used therapeutically, are soluble receptors lacking the transmembrane region. In this case, the receptor comprises the proposed extracellular binding domain, a fragment or derivative thereof, optionally being directly or via a spacer linked to the proposed intracellular domain or to the Fc part of an antibody.

[0037] With respect to receptors, derivatives or fragments thereof, the present invention also comprises so-called cytokine traps, which make use of the fact that the signalling cascade triggered by cytokines is initiated with the cytokine binding to a first subunit, said binding leading to the recruitment of the second subunit, whereby only the complex of the cytokine bound to both receptor subunit chains initiate the subsequent cascade. As described in Nature Medicine 2003, Vol. 9, pages 20-22 and pages 47-52, cytokine traps consist of the two relevant receptor subunits which are linked together by fusion with the Fc portion (complement binding domain) of the immunoglobulin IgG1. Therefore, the cytokine antagonist of the present invention can be a so-called "heterodimeric trap" consisting of two receptor subunits each of which is fused to the Fc portion of an antibody comprising the heavy chain c6nstant regions CH2 and CH3 and the hinge region of IgG1, whereby the constructs are paired via disulfide bridges between the hinge regions. The cytokine antagonist of the present invention can also be a so-called "inline trap", where the two receptor extracellular domains are fused in-line followed by the human IgG1 Fc. For example, a cytokine antagonist for the cytokine IL-4 would consist of the extracellular domains of CD124, as specified above, and CD132, as specified above, linked to IgG1 in the described manner.

[0038] Furthermore, the modulation of the cytokine can be achieved by using so-called muteins of the cytokines. Muteins are derivatives of biologically active proteins the amino acid composition of which has been artificially altered. The muteins of the present invention are still able to bind to their respective cell surface receptor, but are incapable of triggering an internal signal cascade which would lead to the up-regulation of anti-apoptotic proteins. In this respect, the muteins compete with the endogenously expressed cytokines for the binding sites on the respective receptor. The muteins can be made via bacterial expression of mutant genes that encode the muteins that have been synthesized from the genes for the parent proteins by oligonucleotide-directed mutagenesis.

[0039] In line with the above disclosures, the present invention furthermore refers to a method for the down-regulation of a cell death preventing protein in a cell, the method comprising

(a) providing a sample of tissue or cells from a subject
(b) contacting the cell or the sample with a cytokine antagonist

In a particularly preferred embodiment of the present invention the cell, to which the disclosed method should be applied to, is a cancer cell.

[0040] In order to act properly as a cytokine antagonist and in order to perform the described method it is desirable that the cytokine antagonist is delivered to the site of action namely to the proximity of a cell and/or into a cell. The person skilled in the art is aware of a variety of methods how to deliver the disclosed cytokine antagonists into or in the proximity of the target cell. In general, the appropriate method depends on whether the cytokine antagonist is a nucleic acid or a peptide. Furthermore, if the cytokine antagonist is a peptide it can be delivered into or in the proximity of the target cell by introducing the nucleic acid encoding it either to the target cell itself or to other cells being suitable to produce the peptide. For peptide production, both eukaryotic and prokaryotic host cells are contemplated.

[0041] There are several well-known methods of introducing nucleic acids into animal cells, any of which may be used in the present invention and which depend on the host. Typical hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like. At the simplest, the nucleic acid can be directly injected into the target cell /

target tissue, or by so-called microinjection into the nucleus. Other methods include fusion of the recipient cell with bacterial protoplasts containing the nucleic acid, the use of compositions like calcium chloride, rubidium chloride, lithium chloride, calcium phosphate, DEAE dextran, cationic lipids or liposomes or methods like receptor-mediated endocytosis, biolistic particle bombardment ("gene gun" method), infection with viral vectors, electroporation, and the like.

[0042] For the introduction of the cytokine antagonist, respectively the nucleic acid encoding it, into the cell and its expression it can be advantageous if the nucleic acid is integrated in an expression vector. The expression vector is preferably a eukaryotic expression vector, or a retroviral vector, a plasmid, bacteriophage, or any other vector typically used in the biotechnology field. If necessary or desired, the nucleic acid encoding the cytokine antagonist can be operatively linked to regulatory elements which direct the transcription and the synthesis of a translatable mRNA in pro- or eukaryotic cells. Such regulatory elements are promoters, enhancers or transcription termination signals, but can also comprise introns or similar elements, for example those, which promote or contribute to the stability and the amplification of the vector, the selection for successful delivery and/or the integration into the host's genome, like regions that promote homologous recombination at a desired site in the genome. For therapeutic purposes, the use of retroviral vectors has been proven to be most appropriate to deliver a desired nucleic acid into a target cell.

[0043] If the cytokine antagonist is a peptide that shall be directly introduced into the target cell it can be fused to a carrier peptide that mediates the cellular uptake of the peptide. Appropriate carriers are known to the person skilled in the art and include TAT, fibroblast growth factor, galparan (transportan), poly-arginine, and Pep-1, and functional fragments and derivatives of any of said carriers. Furthermore, the cytokine may be fused to a ligand for a cell surface receptor, or a functional portion thereof, and thus internalized by receptor-mediated endocytosis.

[0044] The cytokine antagonist as disclosed in the present invention can be used as a pharmaceutical, optionally in combination with at least one active compound, for the treatment of cancer. This is a further embodiment of the present invention. The term "active compound" refers to a compound other than the cytokine antagonist which is able to induce or sensitize for cell death, preferably apoptosis, or which inhibits cell proliferation. Active compounds which are able to induce or sensitize for cell death, preferably apoptosis are known to the person skilled in the art.

[0045] First, the phrase "active compound" refers to the use of electromagnetic or particulate radiation in the treatment of neoplasia. Radiation therapy is based on the principle that high-dose radiation delivered to a target area will result in the death of reproductive cells in both tumor and normal tissues. The radiation dosage regimen is generally defined in terms of radiation absorbed dose (rad), time and fractionation, and must be carefully defined by the oncologist. The amount of radiation a patient receives will depend on various consideration but the two most important considerations are the location of the tumor in relation to other critical structures or organs of the body, and the extent to which the tumor has spread. Examples of radiotherapeutic agents are provided in, but not limited to, radiation therapy and is known in the art (Hellman, Principles of Radiation Therapy, Cancer, in Principles and Practice of Oncology, 24875 (Devita et al., ed., 4th ed., vl, 1993). Recent advances in radiation therapy include three-dimensional conformal external beam radiation, intensity modulated radiation therapy (IMRT), stereotactic radiosurgery and brachytherapy (interstitial radiation therapy), the latter placing the source of radiation directly into the tumor as implanted "seeds." These newer treatment modalities deliver greater doses of radiation to the tumor, which accounts for their increased effectiveness when compared to standard external beam radiation therapy. Beta-emitting radionuclides are considered the most useful for radiotherapeutic applications because of the moderate linear energy transfer (LET) of the ionizing particle (electron) and its intermediate range (typically several millimeters in tissue). Gamma rays deliver dosage at lower levels over much greater distances. Alpha particles represent the other extreme; they deliver very high LET dosage, but have an extremely limited range and must, therefore, be in intimate contact with the cells of the tissue to be treated. In addition, alpha emitters are generally heavy metals, which limits the possible chemistry and presents undue hazards from leakage of radionuclide from the area to be treated. Depending on the tumor to be treated all kinds of emitters are conceivable within the scope of the present invention.

[0046] Generally, radiation therapy can been combined temporally with other active compounds listed below to improve the outcome of treatment. There are various terms to describe the temporal relationship of administering radiation therapy together with other active compounds, and the following examples are the preferred treatment regimens and are generally known by those skilled in the art and are provided for illustration only and are not intended to limit the use of other combinations. Administration of radiation therapy with other active compounds can be "sequential", i.e. separately in time in order to allow the separate administration, "concomitant" which refers to the administration on the same day, and, finally,"alternating" which refers to the administration of radiation therapy on the days in which other active compounds would not have been administered.

[0047] Another class of active compounds are chemical compounds having a cytostatic or anti-neoplastic effect ("cytostatic compound"). Cytostatic compounds included in the present invention comprise, but are not re-

stricted to (i) antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, such as bleomycin, (iii) DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; (iv) intercalating agents such as adriamycin (doxorubicin) or mitoxantrone; (v) protein synthesis inhibitors, such as L-asparaginase, cycloheximide, puromycin or diphteria toxin; (vi) topoisomerase I poisons, such as camptothecin or topotecan; (vii) topoisomerase II poisons, such as etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; (ix) kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents such as thioplatin, PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; (xi) hormones such as glucocorticoids or fenretinide; (xii) hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists.

[0048] Other cytostatic compounds include plant-derived cytostatics (from Viscum and derivatives); alcaloids such as vindesine; podophyllotoxins such as vinorelbin; alkylants such as nimustrine, carmustrine, lomustine, estramustrine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics such as aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs such as methotrexate, purine analogs such as cladribin, mercaptopurin, tioguanine and pyrimidine analogs such as cytarabine, fluorouracil, docetaxel; platinum compounds such as carboplatin, oxaliplatin; amsacrine, irinotecane, interferon-α, tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies such as trastuzumab, rituximab, or derived from enyzmes such as pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens such as polyestradiol, fosfestriol, ethinylestradiol, gestagens such as medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones such as triptoreline, leuproreline, busereline, gosereline, other hormones such as testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens such as toremifen; antiandrogens such as flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors such as anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives such as calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives such as retinolpalmitate, thymus D9, amilomer.

[0049] In a preferred embodiment of the present invention, the active compound having a cytostatic effect is selected from the group consisting of cisplatin, doxorubicin and paclitaxel (taxol).

[0050] Another class of active compounds which can be used in the present invention are those which are able to sensitize for or induce apoptosis by binding to death receptors ("death receptor agonists"). Agonists of death receptors include death receptor ligands such as tumor necrosis factor α (TNF-α), tumor necrosis factor β (TNF-β, lymphotoxin-α), LT-β (lymphotoxin-β), TRAIL (Apo2L, DR4 ligand), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR6 ligand as well as fragments and derivatives of any of said ligands. Furthermore, death receptors agonists comprise agonistic antibodies to death receptors such as anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R1 (p55 TNF-R) antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies. Preferably, the agonistic antibodies are selected from the group consisting of anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti TNF-R1 antibody and fragments and derivatives of any of said antibodies.

[0051] Another class of active compounds which can be used in combination with the cytokine antagonist are peptides, proteins or small molecule inhibitors which negatively regulate or inhibit the above described anti-apoptotic proteins. Examples of negatively regulating peptides include Smac/DIABLO, NRAGE and TAK1, fragments and derivatives thereof, which particularly inhibit the above described IAPs. These peptides may be modified in a way that they can be rapidly internalized into tumor cells by cellular uptake. The modification can occur by attaching a carrier peptide that mediates cellular uptake as disclosed above to the active compound.

[0052] The cytokine antagonist can be administered alone or in combination with one or more active compounds. The latter can be administered before, after or simultaneously with the administration of the cytokine antagonist. The dose of either the cytokine antagonist or the active compound as well as the duration and the temperature of incubation can be variable and depends on the target that is to be treated.

[0053] A further object of the present invention are pharmaceutical preparations which comprise an effective dose of at least one cytokine antagonist, optionally in combination with at least one active compound and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

[0054] The pharmaceutical according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspen-

sions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

[0055] The preparation of the pharmaceutical compositions can be carried out in a manner known per se. To this end, the cytokine antagonist and/or the active compound, together with one or more solid or liquid pharmaceutical carrier substances and/or additives (or auxiliary substances) and, if desired, in combination with other pharmaceutically active compounds having therapeutic or prophylactic action, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human or veterinary medicine.

[0056] For the production of pills, tablets, sugar-coated tablets and hard gelatin capsules it is possible to use, for example, lactose, starch, for example maize starch, or starch derivatives, talc, stearic acid or its salts, etc. Carriers for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, etc. Suitable carriers for the preparation of solutions, for example of solutions for injection, or of emulsions or syrups are, for example, water, physiological sodium chloride solution, alcohols such as ethanol, glycerol, polyols, sucrose, invert sugar, glucose, mannitol, vegetable oils, etc. It is also possible to lyophilize the cytokine antagonist and/or the active compound and to use the resulting lyophilisates, for example, for preparing preparations for injection or infusion. Suitable carriers for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid.

[0057] The pharmaceutical preparations can also contain additives, for example fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, dispersants, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, agents for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants.

[0058] The dosage of the cytokine antagonist, in combination with one or more active compounds to be administered, depends on the individual case and is, as is customary, to be adapted to the individual circumstances to achieve an optimum effect. Thus, it depends on the nature and the severity of the disorder to be treated, and also on the sex, age, weight and individual responsiveness of the human or animal to be treated, on the efficacy and duration of action of the compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to the cytokine antagonist.

[0059] The cytokine antagonists according to the present invention, respectively the medicaments containing the latter, can be used for the treatment of all cancer types which are resistant to apoptosis due to the expression of anti-apoptotic proteins. Examples of such cancer types comprise neuroblastoma, intestine carcinoma such as rectum carcinoma, colon carcinoma, familiary adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tong carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, follicular thyroid carcinoma, anaplastic thyroid carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeolid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma.

[0060] Examples of cancer types where the use of the cytokine antagonists according to the present invention, respectively the medicaments containing the latter, is particularly advantageous include all forms of thyroid carcinomas (medullary thyroid carcinoma, papillary thyroid carcinoma, follicular thyroid carcinoma, anaplastic thyroid carcinoma), breast carcinoma, lung carcinoma, prostate carcinoma and colon carcinoma. Most preferably, the cytokine antagonists are useful for the treatment of thyroid carcinomas.

[0061] The cytokine antagonists according to the present invention, respectively the medicaments containing the latter, can also be used for the treatment of all autoimmune diseases which are resistant to apoptosis due to the expression of anti-apoptotic proteins. Examples of such autoimmune diseases are collagen diseases such as rheumatoid arthritis, Lupus erythematodes disseminatus, Sharp syndrome, CREST syndrome (calcinosis, Raynaud syndrome, esophageal dysmotility, teleangiectasia), dermatomyositis, vasculitis (Morbus Wegener) and Sjögren syndrome, renal diseases such as Goodpasture syndrome, rapidly-pro-

gressing glomerulonephritis and membrane-proliferative glomerulonephritis type II, endocrine diseases such as type-I diabetes, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), autoimmune parathyreoidism, pernicious anemia, gonad insufficiency, idiopathic Morbus Addison, hyperthyreosis, Hashimoto thyroiditis and primary myxedemia, skin diseases such as Pemphigus vulgaris, bullous pemphigoid, Herpes gestationis, Epidermolysis bullosa and Erythema multiforme major, liver diseases such as primary biliary cirrhosis, autoimmune cholangitis, autoimmune hepatitis type-1, autoimmune hepatitis type-2, primary sclerosing cholangitis, neuronal diseases such as multiple sclerosis, Myastenia gravis, myasthenic Lambert-Eaton syndrome, acquired neuromyotony, Guillain-Barré syndrome (Müller-Fischer syndrome), Stiff-man syndrome, cerebellar degeneration, ataxia, opsoklonus, sensoric neuropathy and achalasia, blood diseases such as autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura (Morbus Werlhof), infectious diseases with associated autoimmune reactions such as AIDS, Malaria and Chagas disease.

[0062] A further object of the present invention is the use of the cytokine antagonist hybridizing with or binding to the cytokine, or the nucleic acid encoding it, as a diagnostic tool to detect and quantify the expression level of a cytokine present in the drug-resistant tumor cell. It is also possible to detect and quantify the expression level of a cytokine and thus, the susceptibility for cancer, by analyzing any of a potential patient's body fluid, such as serous effusions (blood), semen, vaginal secretions, saliva, cerebrospinal fluid, pleural and pericardial fluid, peritoneal fluid, synovial fluid and amniotic fluid.

[0063] The cytokine antagonist may therefore be useful to predict whether a patient suffering from a certain cancer type would be susceptible to a certain therapy and whether it would be required to change the treatment strategies. Binding and hybridization assays can be used to detect, prognose, diagnose, or monitor disease (including conditions and disorders) associated with the overexpression of the cytokines in tumor cells or body fluids. This requires the detection of nucleic acids that encode the cytokines, and the detection of the cytokine proteins.

[0064] Cytokine nucleic acids are detected and quantified herein by any of a number of means well known to those of skill in the art. Appropriate detection methods include biochemical methods such as spectrophotometry, radiography, gel electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitation reactions, immunodiffusion, immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays, tissue array, and the like.

[0065] Hybridization techniques are frequently used for detecting nucleic acids and the present invention contemplates all available hybridization techniques, including Southern, Northern and *in situ* hybridization techniques, dot blot analysis, cDNA arrays. Expression of cytokine mRNAs may be detected, for example, by Northern analysis, or by reverse transcription and amplification by PCR. Also contemplated are nucleic acid detection and quantification methods which employ signal moieties that are conjugated to nucleic acid probes, e.g. by incorporation of radioactively labeled nucleotides. Nucleic acids in a sample can be immobilized on a solid support and hybridized to such probes. The signal moiety can be detected directly, for example by fluorescence. Alternatively, the signal moiety may be detected indirectly by its enzymatic activity, for example in an ELISA or other colorimetric assay.

[0066] Hybridization techniques are usually performed by providing a sample of tissue or cells, contacting the sample with a labeled probe, that binds to said nucleic acid molecule, and determining the presence or amount of the probe bound to said nucleic acid molecule, thereby determining the presence or amount of the nucleic acid molecule in said sample.

[0067] Methods to quantify the presence and amount of a cytokine protein in a given sample are well known to the person skilled in the art. Briefly, a sample is provided, said sample is contacted with an antibody that immunospecifically binds to a given cytokine and the presence or amount of antibody bound to said cytokine is determined, whereby the presence or amount of cytokines in said sample is determined. Methods to determine the amount and presence of polypeptides comprise, among others, FACS, Western blotting, immunoprecipitation, ELISA, and RIA. It is advantageous if the antibody used for detection is conjugated to a molecule that enables and contributes to the detection. Suitable molecules comprise biotin, horseradish peroxidase, alkaline phosphatase, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), diamidinophenylindol (DAPI) and phycoerythrin.

[0068] Thus, the present invention finally embodies a diagnostic kit containing at least one cytokine antagonist being a nucleic acid or a peptide/protein, optionally in combination with suitable buffers, enzymes and other compounds facilitating the detection and quantification of the cytokine in a drug-resistant tumor cell or in a body fluid such as serous effusions (blood), semen, vaginal secretions, saliva, cerebrospinal fluid, pleural and pericardial fluid, peritoneal fluid, synovial fluid and amniotic fluid.

[0069] The invention is further illustrated in the following examples:

EXAMPLES

Example 1: Thyroid cancer cells are resistant to chemotherapy-induced cell death

[0070] Although clinical trials with single agents or with combinations of chemotherapeutic drugs have produced rare and limited positive response, without increase in median and mean survival time in comparison with the natural history of the disease, some compounds have shown a few beneficial effects in terms of partial response rates and reduction of metastatic tumor expansion.

[0071] To investigate the sensitivity of the different histological variants of thyroid epithelial carcinomas to the conventional chemotherapeutic drugs, we measured the viability of freshly purified normal and neoplastic thyrocytes exposed to cisplatin (300 ng/ml), doxorubicin (5 μM) and taxol (5 μM), using dosages compatible with *the in vivo* levels observed during cancer treatment. In line with the modest clinical efficacy reported in clinical trials, primary neoplastic cells derived from all the histological variants of thyroid epithelial carcinomas showed a considerable resistance to chemotherapeutic drugs as compared with normal thyrocytes (Fig. 1). Such resistance persisted for some days and was generally lost after eight to ten days of *in vitro* culture (results not shown).

Example 2: Thyroid cancer cells express Bcl-2 and Bcl-x_L

[0072] Refractoriness to chemotherapy of thyroid carcinoma cells may result from the inhibitory action of anti-apoptotic genes. Therefore, we evaluated the expression of relevant anti-apoptotic proteins, potentially able to protect thyroid cancer cells from the cytotoxic activity of chemotherapeutic drugs. Immunohistochemical analysis of PTC, FTC and UTC paraffin embedded sections showed that Bcl-2 and Bcl-x_L were considerably upregulated in thyroid carcinoma cells (Fig.2a and b). To determine more accurately the difference between normal and malignant thyrocytes, freshly purified control and neoplastic thyroid cells were lysed and analyzed by immunoblot. As shown in fig. 2c and d, Bcl-x_L was weakly expressed in normal cells and four to five fold upregulated in all the histological cancer variants, while Bcl-2 was found about threefold higher in FTC cells and twofold higher in PTC and UTC cells, as compared with normal thyrocytes. Hearts from Bel-x_L and Bcl-2 transgenic mice were used as positive control. The ability of Bcl-x_L and Bcl-2 overexpression to protect some cell types against the cytotoxic effect of chemotherapeutic drugs suggests a potential role of these anti-apoptotic proteins in thyroid cancer resistance from drug-induced cytotoxicity.

Example 3: Exogenous Bcl-2 and Bcl-x_L protect thyrocytes from cell death induced by chemotherapeutic agents

[0073] To prove that Bel-x_L and Bcl-2 up-regulation protect thyrocytes from apoptosis induced by chemotherapeutic drugs and may be responsible for thyroid cancer cell survival, normal thyrocytes were transduced with a retroviral vector (PINCO) that carried the green fluorescent protein (GFP) as a reporter gene. After infection, thyrocytes transduced with empty vector, Bcl-x_L and Bcl-2 were sorted by flow cytometry and exposed to cisplatinum, doxorubicin and taxol to evaluate the extent of chemotherapy-induced apoptosis. The infections were monitored by immunoblot analysis to confirm the efficiency of gene delivery (Fig. 3a). Thyrocytes transduced with either Bcl-x_L or Bcl-2 were almost completely protected from the cytotoxic effects of chemotherapeutic agents (Fig. 3b and c), indicating that overexpression of any of the two genes was sufficient to prevent thyroid cancer cell destruction. Thus, Bcl-x_L and Bcl-2 represent likely candidates for mediating refractoriness of thyroid cancer cells to chemotherapy.

Example 4: Autocrine production of IL-4 and IL-10 in thyroid cancer cells

[0074] To investigate whether the tumor microenvironment can influence thyroid cancer cell phenotype and function, we next evaluated the presence of those cytokines previously found to modulate thyrocyte susceptibility to apoptosis. The presence of Th1 and Th2 cytokines in the neoplastic thyroid gland was investigated by immunohistochemistry on paraffin embedded sections of thyroid carcinomas and by immunocytochemistry and immunoblot analysis on freshly isolated thyroid carcinoma cells. All the histological variants analyzed by immunohistochemistry, exhibited a high reactivity for IL-4 and IL-10, as compared with normal tissues, while IFN-γ was barely detectable (Fig. 4a). Interestingly, the reactivity against Th2 cytokines localized in thyroid follicles, suggesting that neoplastic thyroid cells were the source of production for both IL-4 and IL-10 (Fig. 4a). To rule out the possibility that these cytokines were released by infiltrating T cells, freshly purified thyroid cancer cells were analyzed by immunocytochemistry and immunoblot for expression of Th1 and Th2 cytokines. As observed in the immunohistochemistry experiments, purified thyroid cancer cells showed intense reactivity for both IL-4 and IL-10, while no expression of IFN-γ was detectable (Fig. 4b and c). Twenty nanograms of recombinant human IL-4, IL-10 and IFN-γ were used as positive controls for the immunoblot analysis. The comparison between positive controls and cancer samples indicated that malignant thyroid cells produce considerable amounts of those Th2 cytokines that have shown anti-apoptotic activity on thyroid follicular cells.

Example 5: IL-4 and IL-10 protect thyrocytes from cell death induced by chemotherapeutic agents

[0075] We next investigated whether IL-4 and IL-10 can modulate the sensitivity to chemotherapy-induced apoptosis and the expression of anti-apoptotic proteins in thyroid cells. Interestingly, both IL-4 and IL-10 drastically prevented death of normal thyrocytes exposed to cisplatinum, doxorubicin and taxol (Fig. 5a), suggesting that autocrine production of these cytokines in thyroid cancer cells is responsible for refractoriness to chemotherapy. Furthermore, both IL-4 and IL-10 upregulated Bcl-x_L and Bcl-2 after 48 hours of culture (Fig. 5b), while IFN-γ was not effective. Thus, it is likely that increased expression of anti-apoptotic proteins and subsequent protection of tumor cells from chemotherapy are mediated by the autocrine release of IL-4 and IL-10.

Example 6: Blocking autocrine IL-4 and IL-10 activity primes thyroid cancer cell for chemotherapy-mediated destruction

[0076] To test whether autocrine IL-4 and/or IL-10 release by thyroid tumors is responsible for upregulation of anti-apoptotic proteins, we treated tumor cells for two days with neutralizing Abs specific for IL-4 and/or IL-10 and measured Bcl-x_L and Bcl-2 expression. As shown in Fig. 6a, the levels of both proteins dramatically decreased in thyroid tumor cells exposed to neutralizing Abs against IL-4 and IL-10, while the blockade of a single cytokine had a very limited effect. To test whether cytokine-mediated increase in Bcl-x_L and Bcl-2 levels was responsible for thyroid tumor cell resistance to chemotherapy, PTC, FTC and UTC cells were treated for two days with neutralizing anti-IL-4 and anti-IL10 Abs and analyzed for viability and sensitivity to chemotherapeutic drugs. A significant percentage of thyroid tumor cells from all the histological variants underwent spontaneous apoptosis after 48-hour exposure to anti-IL-4 and anti-IL-10 Abs (Fig. 6a), indicating that these cytokines indeed act as survival factors for thyroid cancer cells. Moreover, these cells acquired sensitivity to chemotherapy-induced cytotoxicity and showed massive death after 24-hour treatment with cisplatinum, doxorubicin or taxol (Fig 6b). Thus, neutralization of IL-4 and IL-10 released by thyroid cancer cells allows their destruction through the use of chemotherapeutic drugs.

Example 7: Down-regulation of anti-apoptotic proteins sensitizes cells to TRAIL-induced cell death.

[0077] To determine the potential of TRAIL-mediated apoptosis *in vivo* TRAIL-Receptor (TR) expression in normal and thyroid carcinoma cells was documented. To determine the presence of TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 immunohistochemical stainings of paraffin embedded thyroid tissue sections from patients affected by PTC, FTC and UTC were performed and compared with sections from normal thyroid lobes contralateral to the cancerous lobe in patients with thyroid cancer. It was found that TRAIL-R1-TR4 were strongly expressed in all the papillary tumors analysed and completely absent in follicular and anaplastic tumors (data not shown). To test whether autocrine IL-4 and IL-10 release is responsible for TRAIL-induced apoptosis resistance in all the histological thyroid cancer variants examined carcinoma cells were treated for two days with IL-4- and IL-10-neutralizing antibodies and then tumor cell resistance to TRAIL-induced apoptosis was measured. A significant percentage of tumor cells was apoptotic after 48 hours' exposure to anti-IL-4 and anti-IL-10 Abs, indicating that these cytokines act as survival factors for these cells (data not shown). Thus, downregulation of anti-apoptotic proteins such as FLIP, Bcl-x_L and Bcl-2, through the inhibition of Th2 cytokines, sensitizes these cells to TRAIL-induced cell death.

MATERIALS AND METHODS

Specimens.

[0078] Thyroid tissues affected by eight PTC (aged 28 ± 5), eight FTC (aged 44 ± 3) and four UTC (aged 65 ± 4.5), were obtained at the time of thyroidectomy. Normal thyroid specimens were obtained from the uninvolved, controlateral lobes of thyroid glands with tumours. Histological diagnosis was based on the identification of papillary elements, on the behavioural characteristics of carcinoma cells (vascular and capsular invasion) and nuclear atypia (shape and chromatin pattern). Transgenic mouse hearts expressing human Bcl-2 and Bcl-x_L, provided by G.L. Condorelli (Thomas Jefferson University, Philadelphia, PA), were used as positive controls.

Thyroid cell purification and culture.

[0079] Thyroid tissues from normal, PTC, FTC and UTC were digested for 2 hours with collagenase (1.5 mg/ml) (Gibco BRL, Grand Island, NY) and hyaluronidase (20 μg/ml) (Sigma Chemical Co., St. Louis, MO) in DMEM. Thyrocytes were purified from the digested tissues by hematopoietic cell depletion with anti-CD45-coupled beads (Dynal, Wirral Merseyside, U. K.) and 12 hours of flask adherence, which allowed removal of other cells. After additional 12 hours of culture, thyroid cells were allowed to grow in monolayer for the immunocytochemistry or detached with trypsin+EDTA following exposure to cytokines or chemotherapeutic agents for functional and protein analyses. Thyrocytes were cultured in standard DMEM with 10% heat-inactivated FBS (Hyclone Laboratories, Logan, UK) in the presence or absence of human recombinant IL-4 (20 ng/ml), IL-10 (40 ng/ml) or IFN-γ (1000 IU/ml) (Euroclone, Paignton, UK) and cisplatin (300 ng/ml), doxorubicin (5

μM) and taxol (5 μM) (Sigma) or TRAIL (Alexis, San Diego, USA). For the IL-4 and IL-10 neutralization, thyroid cancer cells were pretreated, for 48 hours, with anti-human IL-4 and IL-10 neutralizing antibodies (1 μg/ml) (R&D systems, MN, USA).

Cell death quantitation.

[0080] Apoptotic events of neoplastic thyrocytes were evaluated by DNA staining and flow cytometry analysis. Thyroid cell pellets were resuspended in hypotonic fluorochrome solution containing propidium iodide (50 μg/ml), in 0.1 % sodium citrate and 0.1 % Triton X-100. The percentage of hypodiploid nuclei was evaluated as previously described. Alternatively, freshly purified thyrocytes were plated in 96-bottomed plates in triplicate at 15,000 cells/well and cultured. The number of viable cells was detected by CellTiter Aqueous Assay Kit (Promega Corporation, WI, USA) adding 20 μl of solution reagent directly to culture wells, incubating for 1 hours at 37°C and recording absorbance at 490 nm.

Immunostaining procedure.

[0081] Immunohistochemical stainings were performed on paraffin embedded thyroid sections 5 μm in thickness. Deparaffinised sections were pre-treated with 3% hydrogen peroxide fro 10 min at room temperature to inhibit endogenous peroxidase. Then slides were incubated for 10 min with Tris Bufferd Saline (TBS) containing 3% bovine serum albumin (BSA) to block the unspecific staining. Following elimination of excess serum, sections were exposed for 1 hour to specific antibodies against Bcl-x_L (H-5, mouse IgG_1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bcl-2 (124, mouse IgG1, Dako) IL-4 (B-S4 mouse IgG1, Caltag Laboratories, Burlingame, CA), IL-10 (B-N10 mouse IgG2a, Caltag), IFN-γ (B27, mouse IgG1, Caltag), TRAIL-R1 to R4 (Alexis, San Diego, USA) or isotype matched controls at appropriate dilutions. Prior to immunostaining for Bcl-2 and Bcl-x1, dewaxed sections were treated for 10 min in microwave oven in 0.1 M citrate buffer. After two washes in TBS, sections were treated with biotinylated anti-rabbit or anti-mouse immunoglobulins, washed in TBS and incubated with streptavidin peroxidase (Dako LSAB 2 Kit, Dako Corporation Carpinteria CA, USA). Staining was detected using 3-amino-9-ethylcarbazole (AEC) as a colorimetric substrate. Counterstaining of tissue sections was performed using aqueous hematoxylin.

Protein isolation and Western Blotting.

[0082] Cell pellets were resuspended in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40) containing 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml) and aprotinin (1 μg/ml). Each lysate (30 μg) was fractioned on 12% SDS-polyacrylamide gels and blotted to nitrocellulose (Hybond, Amersham, Little Chalfont Buckinghamshire England, UK). Membrane was blocked for 1 h with nonfat dry milk in TBS containing 0.05% Tween 20 and successively incubated for 2 h with Abs specific to actin (Ab-1, mouse IgM, Calbiochem, Darmstadt, Germany), Bcl-2 (124, mouse IgG1, Upstate Biotechnology Inc.), Bcl-x_L (H-5, mouse IgG1, Santa Cruz Biotechnology), IL-4 (3007.11, mouse IgG1, R&D Systems, Inc., Minneapolis, USA), IL-10 (23738.111, mouse IgG2b, R&D Systems), IFN-γ (25718.111, mouse IgG2a, R&D Systems). After washing, the blots were incubated for 1 hour with HRP-conjugated anti-mouse Abs (Amersham) and visualized using an enhanced chemiluminescence detection system (SuperSignal West Dura Extended duration Sustrate, Pierce, Illinois, USA). rhIL-4, rhIL-10 and rhIFN-γ (Euroclone) were used as positive control.

Production of retroviral particles and infection of thyrocytes.

[0083] Bcl-2 and Bcl-x_L cDNAs were cloned in PINCO vector. The amphotropic Phoenix packaging cell line was transiently transfected with PINCO using the calcium-phosphate/chloroquine method. Infection was performed by culturing $5x10^5$ thyrocytes in 1 ml of 0.45 mM filtered supernatant containing viral particles. Then, cells were centrifuged for 45 min at 1800 rpm and placed back in the CO_2 incubator for 2 hours. Three infection cycles were performed before the thyrocytes were placed back in supplemented medium. Sorted and enriched positive cells were plated and exposed to cisplatinum, doxorubicin and taxol for evaluation of cell death.

DESCRIPTION OF THE DRAWING

[0084]

Figure 1. Resistance to apoptotic cell death induced by chemotherapeutic drugs in thyroid cancer cells. Percentage of apoptotic cells in freshly purified thyrocytes from normal thyroid gland, PTC, FTC and UTC, exposed for 6, 12 and 24 h to cisplatinum (300 ng/ml), doxorubicin (5 μM) and taxol (5 μM). (Data are mean ± s.d. of four independent experiments).

Figure 2. Anti-apoptotic molecules expression on thyroid cancer. (a, b) Immunohistochemical analysis of Bcl-x_L and Bcl-2 on paraffin embedded normal thyroid gland, PTC, FTC and UTC sections revealed by AEC (red staining). **(b, c)** Immunoblot analysis of Bcl-x_L and Bcl-2 in freshly purified thyrocytes lysates from normal, PTC, FTC and UTC. Bcl-x1 and Bcl-2 transgenic hearts were used as positive controls (+ control). Loading controls were done by detecting β-actin in the same membrane blot (one of representative experiment of four is

shown).

Figure 3. Protection from chemotherapy-induced cell death in thyrocytes transduced with Bcl-x_L and Bcl-2. Immunoblot analysis of **(a)** Bcl-x_L and **(b)** Bcl-2 expression on flow cytometry sorted thyrocytes transduced with empty vector (Vector), Bcl-x_L and Bcl-2. Loading control was assessed by β-actin staining. **(c)** Percentage of apoptosis in normal thyrocytes transduced as in **a** and **b** following exposure to chemotherapeutic drugs. **(d)** GFP-positive cells stained with ethidium bromide and observed by immunofluorescence microscope. One representative experiments of three performed is shown.

Figure 4. IL4 and IL-10 expression on thyroid cancer cells. (a) Immunohistochemical analysis of IL-4, IL-10 and IFN-γ on paraffin embedded normal thyroid gland, PTC, FTC and UTC sections (red staining). **(b)** Immunostaining for IL-4, IL-10 and IFN-γ of purified thyrocytes from all histological variants of thyroid epithelial carcinoma. **(c)** Western analysis of IL-4, IL-10 and IFN-γ in freshly purified cancer thyrocytes. rhIL-4, rhIL-10 and rhIFN-γ (20ng/lane) were used as positive control. These experiments are representative of results from three independent experiments each using cultures from different patient specimens.

Figure 5. IL-4 and IL-10 rescue normal thyrocytes from chemotherapy-induced apoptotic cell death. (a) Percentage of apoptotic events of purified normal thyroid cells pre-treated for 48 h with control medium (left panel), rhIL-4 (20 ng/ml) or rhIL-10 (40 ng/ml) and then cultured with cisplatinum, doxorubicin and taxol for 12 additional hours. (b) Immunoblot analysis of normal thyrocytes cultured with IL-4 or IL-10 as in a or rhIFN-γ (1000 IU/ml).

Figure 6. Neutralizing antibodies against IL-4 and IL-10 sensitize thyroid carcinoma cells to chemotherapy. (a) Kinetics of viable cells on carcinoma thyrocytes cultivated with medium alone or with anti-IL-4 or with anti-IL-10 or with anti-IL-4+anti-IL-10. Percentage of viable purified thyroid carcinoma cells pre-treated for 48 h with control medium, anti-IL-4 (1 μg/ml) or anti-IL-10 (1 μg/ml) or anti-IL-4+anti IL-10 and then cultured with chemotherapeutic drugs for 24 additional hours (right panel). (Mean of one of representative experiment of four is shown). (b) Percentage of viable PTC, FTC and UTC cells pre-treated for 48 h with anti-IL-4+anti IL-10 and then cultured with cisplatinum, doxorubicin and taxol for 12 and 24 hours.

Claims

1. Use of a cytokine antagonist which modulates the expression and/or the function of a cytokine in a cell for the down-regulation of a cell death preventing protein in a cell.

2. The use according to claim 1, wherein the cell is sensitized for cell death.

3. The use according to claim 1 or 2, wherein the cell is a cancer cell.

4. The use according to any of claims 1 to 3, wherein the cytokine is selected from the group consisting of IL-4, IL-5, IL-6, IL-10 and IL-13, and combinations thereof, preferably IL-4, IL-10 and IL-13, and combinations thereof.

5. The use according to any of claims 1 to 3, wherein the cell death is caused by apoptosis and the cell death preventing protein is an anti-apoptotic protein selected from the group consisting of Bcl-2, Bcl-x_L, cFLIP, Mcl-1, A1, BOO, NR-13, sentrin, TOSO, CPAN, PED, DFF45, NAIP, XIAP, cIAP-1, cIAP-2, ML-IAP, KIAP, BIRC5, TIAP, Apollon and fortilin, preferably Bcl-2, Bcl-x_L, PED and cFLIP, and combinations thereof, most preferably Bcl-2 and/or Bcl-x_L.

6. The use according to any of claims 1 to 5, wherein the cytokine antagonist is selected from the group consisting of a transcriptional regulator of the cytokine/cytokine receptor gene, an antisense nucleic acid molecule that is complementary to a region of the cytokine/cytokine receptor gene, a dsRNA molecule that is complementary to the cytokine/cytokine receptor mRNA, a ribozyme that cleaves the cytokine/cytokine receptor mRNA, a translational regulator of the cytokine/cytokine receptor mRNA, an aptamer, which bind to the cytokine and/or cytokine receptor and prevents or disrupts the interaction between the cytokine and its receptor, an antibody that binds to the cytokine/cytokine receptor, a receptor, a fragment or derivative thereof, of the cytokine, preferably CD124, CD132, IL-13Rα-2 and IL-10Rα, a cytokine trap, and a cytokine mutein

7. The use according to claim 6, wherein the cytokine antagonist is an antibody that binds to the cytokine/cytokine receptor.

8. The use according to claim 7, wherein the antibody is an antibody that binds to IL-4, IL-10 or IL-13, and combinations thereof.

9. The use according to any of claims 1 to 8, wherein the cytokine antagonist is delivered to the proximity

of or into the target cell.

10. The use according to claim 9, wherein the cytokine antagonist is delivered via a retroviral vector.

11. A method for the down-regulation of a cell death preventing protein in a cell, the method comprising

(a) providing a sample of tissue or cells from a subject
(b) contacting the cell or the sample with a cytokine antagonist according to any of claims 6 to 8.

12. The method according to claim 11, wherein the cell is a cancer cell.

13. Use of a cytokine antagonist, optionally in combination with radiation therapy, for the manufacture of a medicament for the treatment of cancer.

14. Use of a cytokine antagonist, optionally in combination with at least one active compound, for the manufacture of a medicament for the treatment of cancer.

15. The use according to claim 14, wherein the active compound is selected from the group consisting of antimetabolites, preferably cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; DNA-fragmenting agents, preferably bleomycin, DNA-crosslinking agents, preferably chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; intercalating agents preferably adriamycin (doxorubicin) or mitoxantrone; protein synthesis inhibitors, preferably L-asparaginase, cycloheximide, puromycin or diphteria toxin; topoisomerase I poisons, preferably camptothecin or topotecan; topoisomerase II poisons, preferably etoposide (VP-16) or teniposide; microtubule-directed agents, preferably colcemid, colchicine, paclitaxel, vinblastine or vincristine; kinase inhibitors preferably flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); miscellaneous investigational agents, preferably PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols preferably quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid; hormones preferably glucocorticoids or fenretinide; hormone antagonists, preferably tamoxifen, finasteride or LHRH antagonists; plant-derived cytostatics (from Viscum and derivatives); alcaloids preferably vindesine; podophyllotoxins preferably vinorelbin; alkylants preferably nimustrine, carmustrine, lomustine, estramustrine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics preferably aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs preferably methotrexate, purine analogs preferably cladribin, mercaptopurin, tioguanine and pyrimidine analogs preferably cytarabine, fluorouracil, docetaxel; other antineoplastic, platinum compounds preferably thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecane, interferon-α, tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e. g. monoclonal antibodies preferably trastuzumab, rituximab, or derived from enyzmes preferably pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens preferably polyestradiol, fosfestriol, ethinylestradiol, gestagens preferably medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones preferably triptoreline, leuproreline, busereline, gosereline, other hormones preferably testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens preferably toremifen; antiandrogens preferably flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors preferably anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives preferably calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives preferably retinolpalmitate, thymus D9, amilomer.

16. The use according to claim 15, wherein the active compound is selected from the group consisting of paclitaxel, cisplatin, and doxorubicin.

17. The use according to claim 14, wherein the active compound is a death receptor agonist.

18. The use according to claim 17, wherein the death receptor agonist is a death receptor ligand selected from the group consisting of TNF-α, TNF-β, LT-β, TRAIL, CD95 ligand, TRAMP ligand, DR6 ligand, and fragments and derivatives thereof.

19. The use according to claim 17, wherein the death receptor agonist is an antibody against a death receptor, a derivative or fragment thereof, selected from the group consisting of anti-CD95 antibody, anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti-DR6 antibody, anti-TNF-R1 antibody and anti-TRAMP antibody.

20. The use according to claim 14, wherein the active compound is a negative regulator of anti-apoptotic

proteins, preferably IAPs.

21. The use according to any of claims 13 to 20, wherein the cancer to be treated is selected from the group consisting of neuroblastoma, intestine carcinoma preferablyrectum carcinoma, colon carcinoma, familiary adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tong carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, follicular thyroid carcinoma, anaplastic thyroid carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors preferablyglioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeolid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma.

22. The use according to any claim 21, wherein the cancer to be treated is selected from the group consisting of thyroid carcinoma, breast carcinoma, lung carcinoma, prostate carcinoma and colon carcinoma.

23. The use according to any claim 21, wherein the cancer to be treated is thyroid carcinoma.

24. A medicament for the treatment of cancer, comprising a cytokine antagonist, optionally in combination with at least one active compound, and a pharmaceutically acceptable carrier.

25. The use of a cytokine antagonist for diagnosing and monitoring the cancer disease of a subject, comprising

(a) providing a body fluid sample or a sample of tissue or cells from a tumor of a subject
(b) contacting the sample with a labeled probe that binds to a cytokine nucleic acid and/or with an antibody that binds to a cytokine
(c) determining the expression level of the cytokine in the tissue or cells and comparing the expression level with healthy control cells, and
(d) correlating a better prognosis for the subject with a low ratio of cytokine expression when compared to the expression level in healthy control cells.

26. A diagnostic kit containing at least one cytokine antagonist, optionally in combination with suitable buffers, enzymes and other compounds.

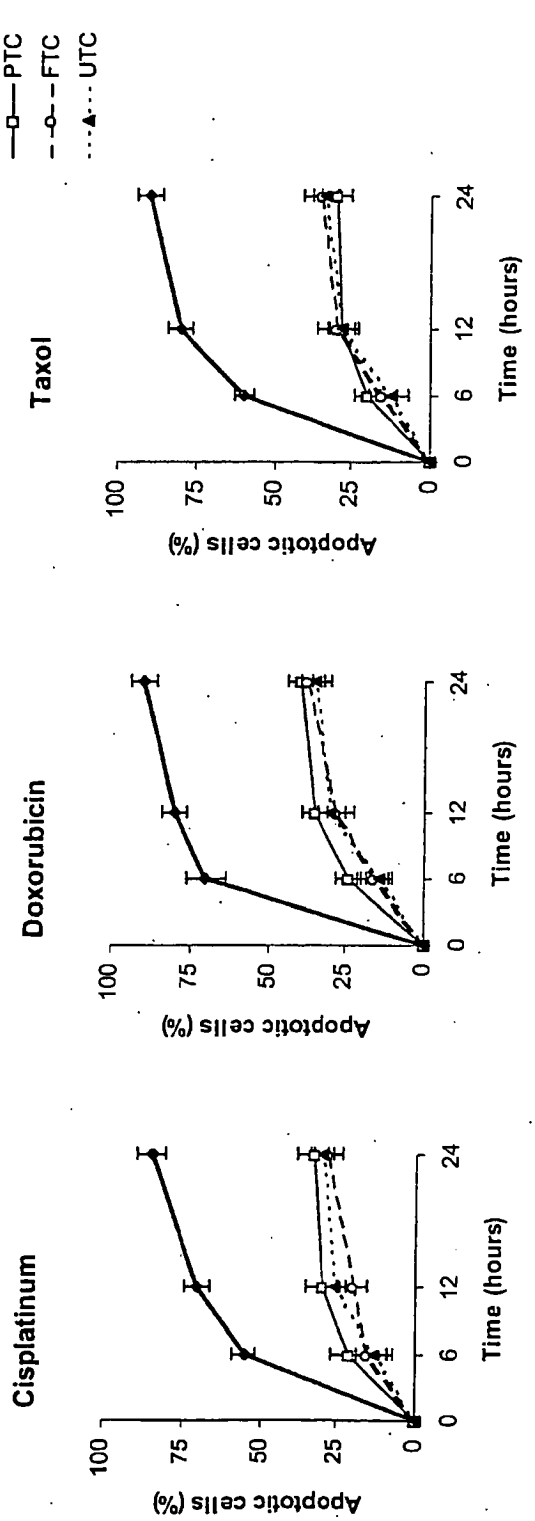


Fig. 1 Stassi et al.

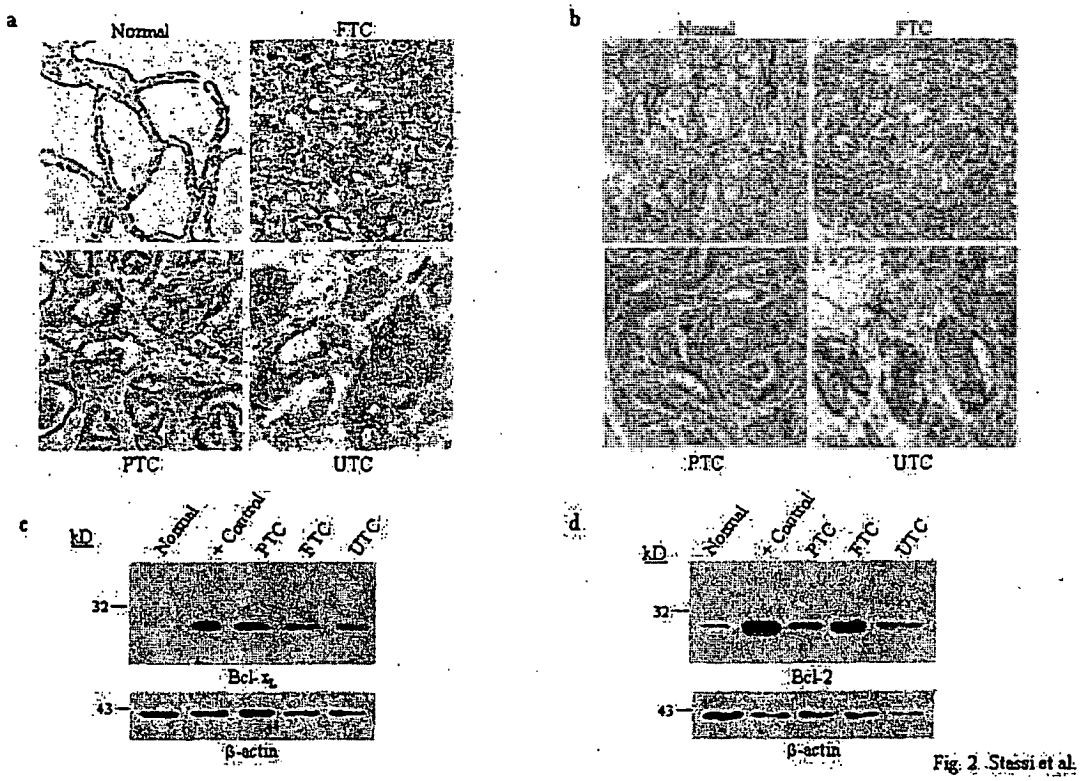


Fig. 2 Stassi et al.

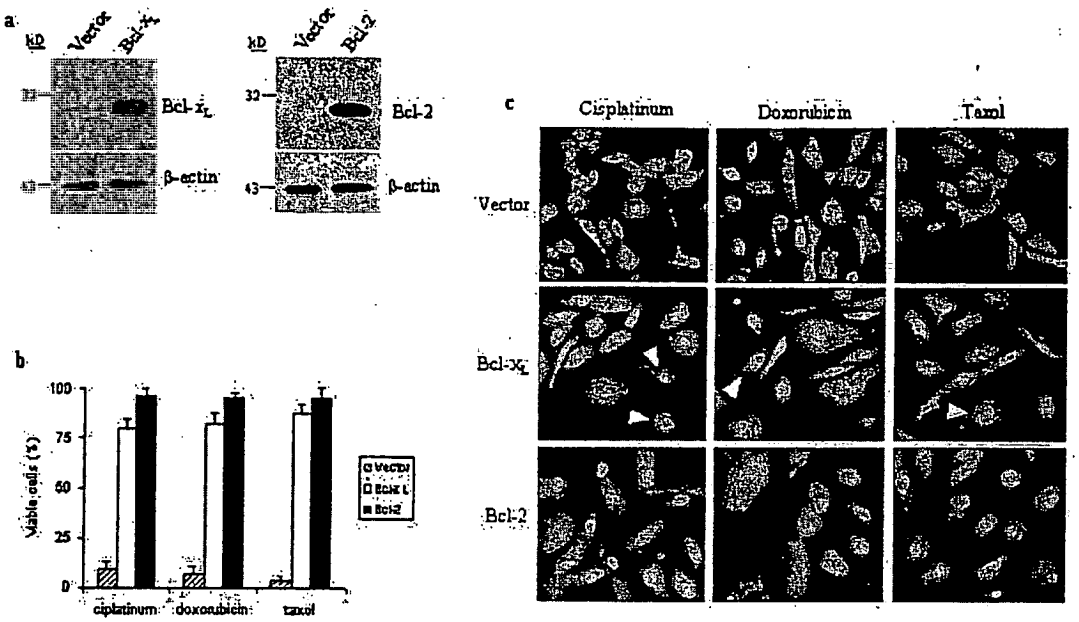


Fig. 3 Stassi et al.

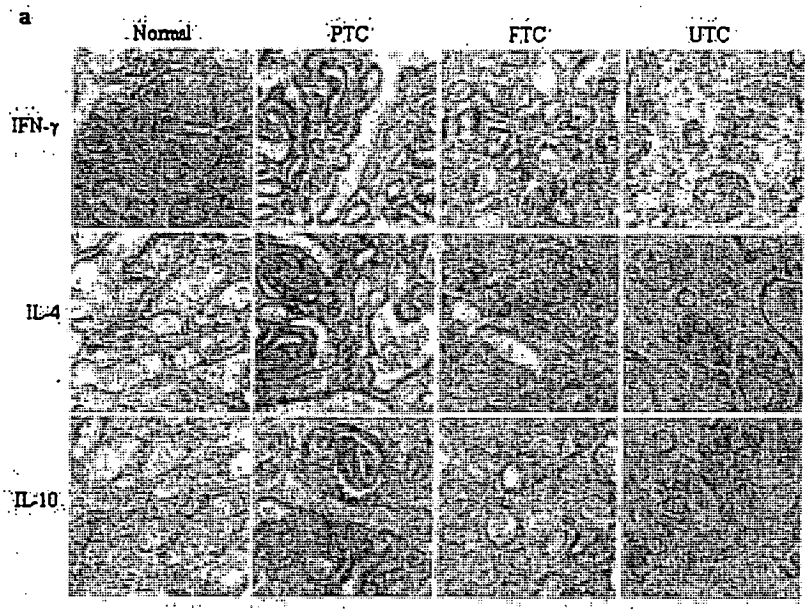


Fig. 4: Stassi et al.

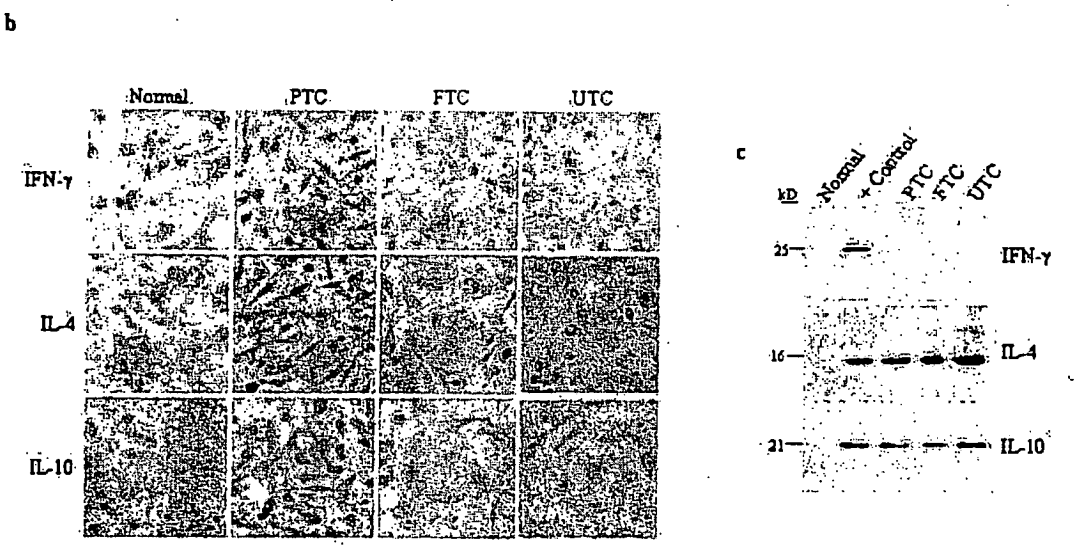


Fig. 4 Stassi et al

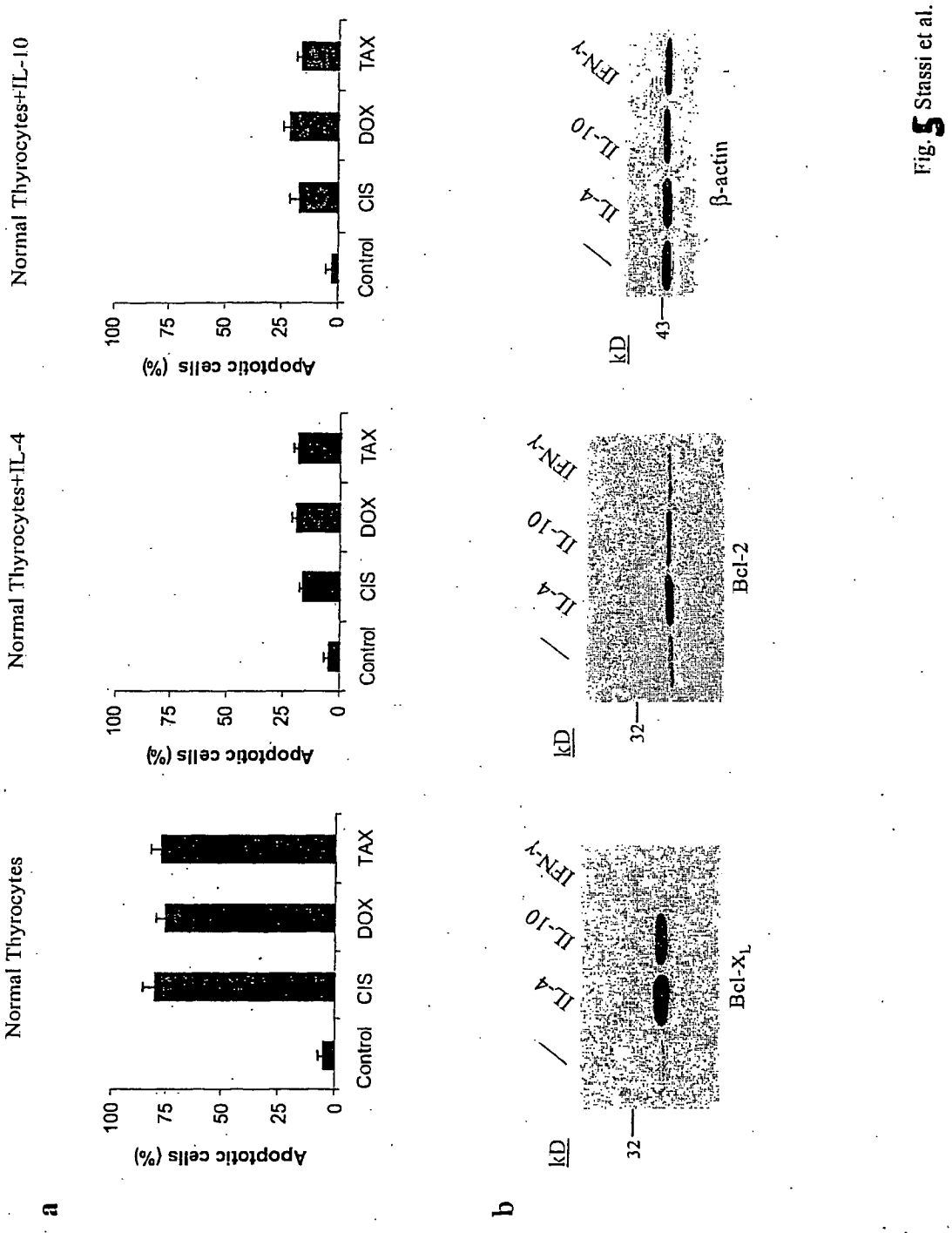


Fig. 5 Stassi et al.

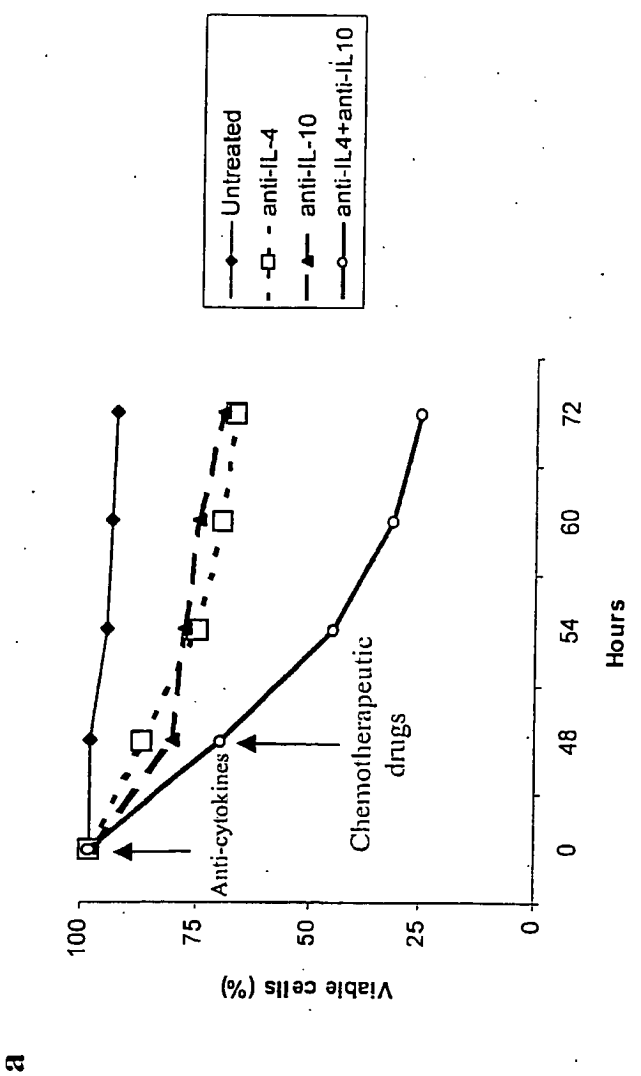


Fig. 6 Stassi et al.

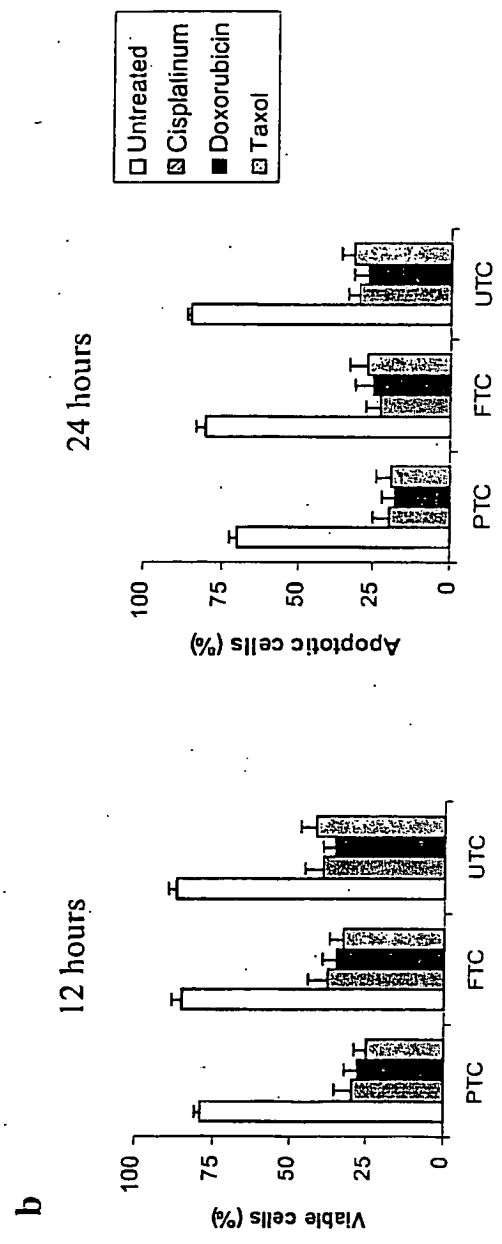


Fig. 6 Stassi et al.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 03 00 2603

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	LU ZHAO YANG ET AL: "Measurement of whole body interleukin-6 (IL-6) production: Prediction of the efficacy of anti-IL-6 treatments." BLOOD, vol. 86, no. 8, 1995, pages 3123-3131, XP002247617 ISSN: 0006-4971 * page 3123, column 1, paragraph 1 - column 2, paragraph 1 * ---	1-7,9, 11-26	A61K45/00 A61K39/395 A61K45/06 A61P35/00 A61P37/06
X	MAINOU-FOWLER T ET AL: "Modulation of apoptosis with cytokines in B-cell chronic lymphocytic leukaemia." LEUKEMIA & LYMPHOMA. SWITZERLAND MAY 1996, vol. 21, no. 5-6, May 1996 (1996-05), pages 369-377, XP009013850 ISSN: 1042-8194	1-12	
Y	Abstract * page 370, column 2, paragraph 1 * * page 371, column 1, paragraph 3 * --- -/--	13-26	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A61K
A61P

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
MUNICH	16 July 2003	Langer, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

INCOMPLETE SEARCH
SHEET C

Application Number
EP 03 00 2603

Although claims 1-12, 25 are directed to a method of treatment and/or diagnostic of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Claim(s) not searched:
1-12, 25

Reason for the limitation of the search (non-patentable invention(s)):

Article 52 (4) EPC - Method for treatment of the human or animal body by therapy

---

Further limitation of the search

Claim(s) searched incompletely:
1-26

Reason for the limitation of the search:

The present application refers to a vast amount of cytokine antagonists, acting on numerous cytokines, which in their turn influence numerous cell death preventing proteins in an extremely high amount of cells. The broad nature of the claims is further outlined in the comment on unity of the present application (see below). Technical support in terms of Art. 84 has however only been provided for neutralizing antibodies, the cytokines IL-4 and IL-10, the cell death preventing proteins Bcl-2 and Bcl-xL and thyrocytes. The limited examples provided to not support a generalisation of the subject-matter as in present claims 1-26, which therefore contravene Art. 84 EPC.

The unduly broad nature of the present claims is futher substantiated by the fact that even if technical support for the present claims was established, a problem of unity in terms of Art. 82 EPC would arise:
The common concept of the present application, which corresponds to the subject-matter of claim 1, is known in the art (see e.g. Lu et. all, 1995).
The theoretical number of inventions would correspond to all possible combinations of antagonists (see claim 6), cytokines (see claim 4), cell death preventing proteins (see claim 5) and cells/cancer disseases (see claim 21).

A lack of clarity (and conciseness) within the meaning of Article 84 EPC arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely the antagonists, cytokines cell death preventing proteins and cells of the examples (neutralizing antibodies, cytokines IL-4 and IL-10, the cell death proteins Bcl-2 and Bcl-xL and thyrocytes) as well as the combined

European Patent Office

INCOMPLETE SEARCH
SHEET C

Application Number
EP 03 00 2603

keywords "cytokine antagonist", "cell death/apoptosis" and "cancer".

It is emphasized that numerous compounds are likely to exist, which are known for the treatment of cancer or for promoting cell death, and which fall under the scope of present claims, even though their mechanism of action has not yet been discovered and/or published. The relevant documents would not have been found by the present search, but would nevertheless be highly relevant for the evaluation of novelty and inventive step of present claims.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 03 00 2603

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	LOMO JON ET AL: "Interleukin-13 in combination with CD40 ligand potently inhibits apoptosis in human B lymphocytes: Upregulation of Bcl-xL and McL-1." BLOOD, vol. 89, no. 12, 1997, pages 4415-4424, XP002247618 ISSN: 0006-4971 Abstract ---	1-26	
Y	NING ZHI-QIANG ET AL: "Distinct mechanisms for rescue from apoptosis in Ramos human B cells by signaling through CD40 and interleukin-4 receptor: Role for inhibition of an early response gene, Berg36." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 26, no. 10, 1996, pages 2356-2363, XP009013662 ISSN: 0014-2980 Abstract * page 2356, column 2, line 17 - line 18 * ---	1-26	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Y	STASSI GIORGIO ET AL: "Autoimmune thyroid disease: new models of cell death in autoimmunity." NATURE REVIEWS. IMMUNOLOGY. ENGLAND MAR 2002, vol. 2, no. 3, March 2002 (2002-03), pages 195-204, XP009013434 ISSN: 1474-1733 * page 202, column 1, paragraph 2 * ---	1-26	
Y	WO 00 04901 A (THOMAS JEFFERSON UNIVERSITY) 3 February 2000 (2000-02-03) * page 5, line 15 - page 6, line 25; claims 44-54 * --- -/--	1-26	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 03 00 2603

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	OPPENHEIM JOOST J: "Cytokines: Past, present, and future." INTERNATIONAL JOURNAL OF HEMATOLOGY, vol. 74, no. 1, July 2001 (2001-07), pages 3-8, XP009013664 ISSN: 0925-5710 * the whole document * * page 7, column 1, paragraph 2 *	1-26	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

EPO FORM 1503 03.82 (P04C10)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 03 00 2603

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

16-07-2003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0004901 A	03-02-2000	CA 2338328 A1	03-02-2000
		EP 1100496 A1	23-05-2001
		WO 0004901 A1	03-02-2000
		US 6492389 B1	10-12-2002

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 354 952 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
22.10.2003 Bulletin 2003/43

(21) Application number: **02008199.8**

(22) Date of filing: **17.04.2002**

(51) Int Cl.[7]: **C12N 15/12**, C12N 15/62, A61K 47/48, C07K 5/103, C07K 19/00, C07K 14/47, A61K 38/17

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(71) Applicant: **Deutsches Krebsforschungszentrum Stiftung des öffentlichen Rechts**
69120 Heidelberg (DE)

(72) Inventors:
- **Debatin, Klaus, Michael**
  89075 Ulm (DE)
- **Fulda, Simone**
  89075 Ulm (DE)

(74) Representative: **Isenbruck, Günter, Dr.**
Isenbruck, Bösl, Hörschler, Wichmann, Huhn, Patentanwälte
Theodor-Heuss-Anlage 12
68165 Mannheim (DE)

Remarks:
A request for correction - to delete any reference to drawings in the application - has been filed pursuant to Rule 88 EPC. A decision on the request will be taken during the proceedings before the Examining Division (Guidelines for Examination in the EPO, A-V, 3.).

(54) Smac-peptides as therapeutics against cancer and autoimmune diseases

(57) The invention is directed to the use of Smac to sensitize different tumors and self-reactive immune cells to various pro-apoptotic stimuli, in that the cells subsequently undergo apoptosis. Therefore, Smac can be used as a compound for the manufacture of a medicament for the treatment of cancer and autoimmune diseases. Sensitization of the cells is achieved either by applying a cell-permeable form of Smac combined with known anticancer agents or by overexpression of the protein. It is an object of the invention to provide a new method in cancer and autoimmune disease therapy by using Smac agonists for apoptosis regulation. Thus, Smac agonists represent novel promising cancer and autoimmune disease therapeutics to potentiate the efficacy of cytotoxic therapies even in resistant tumors and immune cells.

EP 1 354 952 A1

Printed by Jouve, 75001 PARIS (FR)

Description

[0001] The present invention relates to the use of the so-called Smac protein and derivatives thereof to cause apoptosis in cancer cells and self-reactive cells of the immune system.

[0002] Cancer constitutes the fourth leading cause of death in Western countries. As the average age in the Western population steadily rises, so do cancer-related deaths indicating that cancer will be one of the most common causes of death in the 21st century. The aggressive cancer cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways. Cancer cells commonly fail to undergo so-called "programmed cell death" or "apoptosis", a signaling process that plays a key role in preventing cell tissues from abnormal growth. Thus, apoptosis defects appear to be a major problem in cancer therapy as they confer resistance to many tumors against current treatment protocols, leading to tumor progression.

[0003] In addition to apoptosis defects found in tumors, defects in the ability to eliminate self-reactive cells of the immune system due to apoptosis resistance are considered to play a key role in the pathogenesis of autoimmune diseases. Autoimmune diseases are characterized in that the cells of the immune system produce antibodies against own organs and molecules or directly attack tissues resulting in the destruction of the latter. A failure of those self-reactive cells to undergo apoptosis leads to the manifestation of the disease. Defects in apoptosis regulation have been identified in autoimmune diseases such as Lupus erythematodes disseminatus or rheumatoid arthritis.

[0004] Apoptosis pathways involve diverse groups of molecules. One set of mediators implicated in apoptosis are so-called caspases, cysteine proteases that cleave their substrates specifically at aspartate residues. Caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases which subsequently degrade other cellular targets eventually resulting in cellular breakdown. If one or more steps in this cascade is inhibited in tumor cells, these cells fail to accomplish apoptosis and, thus, continue to grow. Caspase activation itself can be triggered by external stimuli affecting certain cell surface receptors, known to the person skilled in the art as so-called death receptors, or by intracellular stress response via the mitochondria leading to the release of mitochondrial proteins. Known death receptors mediating apoptosis include members of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL (TNF-related apoptosis inducing ligand) receptors 1 and 2. Stimulation of death receptors with apoptosis-inducing substances leads, among others, to the activation of caspase-8, which in turn activates other caspases and members of another group of apoptosis mediators. This group is called the Bcl-2 family and is thought to regulate the release of the mitochondrial proteins and, thus, link both pathways together, in order to regulate the downstream acting proteolytic caspase cascade.

[0005] A failure in activating the caspase cascade is caused by the action of so-called Inhibitors of Apoptosis Proteins (TAPS). IAPs bind to early active caspases, thereby preventing the ongoing of the apoptosis process. They are expressed at high levels in many tumors and, by inhibition of caspases, contribute to the resistance of cancers against apoptosis induction.

[0006] A major role in activating the caspase cascade is ascribed to a mammalian protein called Smac in humans (or DIABLO in mice). As disclosed, among others, by Du et al. (Cell 102, 2000, 33-42), Smac is a mitochondrial protein of 239 aminoacids possessing a molecular weight of approximately 25000 Dalton (GenBank accession number AAF87716). In the course of an apoptotic response e.g. upon stimulating CD95- or TRAIL death receptors, Smac is released from mitochondria along with other proteins, e.g. cytochrome c. It has been demonstrated earlier that Smac, once released into the cytosol, can bind to IAPs (Du et al. 2000; Verhagen et al. 2000; Srinivasula et al. 2001), particularly to the so-called X-linked IAP (XIAP), the most potent inhibitor of caspases. Binding of Smac to XIAP promotes the proteolytic activation of caspases resulting in apoptosis.

[0007] Similar to cancer cells in which activation of caspases is inhibited by IAP-dependent mechanisms, failure to eliminate autoreactive T-cells may be due to a blockade in apoptosis signalling. For physiological elimination of activated lymphocytes death receptor systems such as CD95 play a key role. Increased expression of TAPS or members of the Bcl-2 family in activated T-cells prevents the release of Smac from mitochondria and inhibits the function of the latter.

[0008] From the foregoing, it becomes evident that impaired release of Smac and other proteins from mitochondria into the cytosol can cause resistance of tumor cells and cells of the immune system to apoptosis. Overexpression of Smac by transfecting the cells with an expression plasmid carrying the Smac gene is one way to overcome the IAP-caused inhibition of caspases, resulting in an enhanced apoptosis rate. This approach was followed by different research groups, which have found that various types of cancer can thus be treated, e.g. melanoma, breast carcinoma or prostate cancer. However, previous studies do not mention or give any hint to treat neuroblastoma or glioblastoma by overexpressing Smac or related proteins.

[0009] A direct delivery of proteins into cells is often limited by the poor permeability of the cell membrane. Recently, Carson et al. (Cancer Research 62 (2002) 18-23) have used purified Smac which was microinjected alone or together with cytochrome c into the cytosol of prostate cancer cells which were initially resistant to apoptosis. However, various problems can be encountered when using microinjection for the delivery of biologically active compounds into cells. Problems include

low transfer efficiency or complex manipulation, which would preclude their routine use *in vivo*.

[0010] The object of the present invention is to provide a form of Smac that is rapidly internalized into tumor cells and cells of the immune system, e.g. T-cells, by cellular uptake.

[0011] This object is attained by a Smac protein / carrier entity comprising

(i) a Smac protein, as disclosed by the GenBank accession number AAF87716, or a derivative or fragment thereof,

(ii) a carrier

and wherein the Smac protein, fragment or derivative thereof and the carrier are linked together enabling the penetration of the Smac/carrier entity through the cell membrane into the cell.

[0012] Said entity will be referred to as Smac/carrier entity hereinafter.

[0013] A further object of the invention is the therapy of cancers and autoimmune diseases which, until now, could not be treated using Smac proteins.

[0014] In the context of the present invention, the term derivative or fragment of the Smac protein refers to peptides in which one or more aminoacids of the sequence of 239 aminoacids, as disclosed in GenBank number AAF87716, can be substituted by one or more aminoacids different from the original one(s), or peptides the aminoacid sequence of which is either extended, shortened, or both, on either the aminoterminal, or the carboxyterminal or both ends with respect to the original Smac proteins, provided that the function of the Smac protein remains unaffected.

[0015] In a further embodiment, the present invention includes preferably a peptide comprising aminoacids 56 to 70 of Smac. An even more preferred peptide comprises aminoacids 56 to 62 of Smac. Hereinafter, the latter will be referred to as Smac peptide.

[0016] Most preferably, said derivatives or fragments contain the 4 aminoterminal aminoacids 56 to 59 of Smac. This region mediates the interaction of the Smac protein with IAPs.

[0017] The carrier, which is preferably a protein, a fragment or derivative thereof, serves as a vehicle the attachment of which to the Smac protein, fragment or derivative thereof enables the penetration of the Smac/carrier entity through the cell membrane into the cell. Appropriate carriers, in particular proteins, are known to the person skilled in the art and include TAT, influenza virus hemagglutinin, the VP22 protein from herpes simplex virus, Antennapedia, fibroblast growth factor, Galparan (transportan), poly-arginine, Pep-1. Other carriers known to a person skilled in the art which do not belong to proteins, but mediate the internalization of molecules into cells include lipids and cationic lipids.

[0018] When a protein is used as a carrier, the term derivative or fragment of a protein refers to peptides in which one or more aminoacids can be substituted by other aminoacids different from the original one(s), or peptides the aminoacid sequence of which is either extended, shortened, or both, on either the aminoterminal, or the carboxyterminal or both ends, with respect to the original one(s), provided that the function as a carrier for the cellular uptake of Smac remains unaffected. The above definition relates to TAT, influenza virus hemagglutinin, the VP22 protein from herpes simplex virus, Antennapedia, fibroblast growth factor, Galparan (transportan), poly-arginine and Pep-1.

[0019] The Smac protein, fragment or derivative thereof is linked to the carrier. This can occur by any chemical interaction known to the person skilled in the art, like coordinative bonds, chemical adsorption, dipole-dipole interaction or the like. Preferably, the carrier is linked to the Smac protein by a chemical bond, in particular a covalent bond, in case the carrier is a protein. This bond must be such that it remains unaffected before and while penetrating the cell membrane and, if necessary for the interaction of the Smac protein with IAPs, can be cleaved. In general, the Smac/carrier entity can interact with IAPs to the necessary extent, a cleavage being not necessary.

[0020] In a preferred embodiment of the present invention, the carrier is TAT or a derivative or a fragment thereof. TAT is the human immunodeficiency virus-1 (HIV-1) trans-activating protein consisting of 86 aminoacids. More preferably, the fragment or derivative of TAT comprises the aminoacids 37 to 72 of TAT, as disclosed in GenBank accession number M15654. It is even more preferred to use, as a carrier, the so-called protein transduction domain of TAT (PTD) which comprises a region on the protein extending from aminoacid residues 47 to 58, according to the disclosed sequence. In this preferred embodiment of the invention, PTD is linked to Smac, or a fragment or derivative thereof.

[0021] The Smac/carrier entity as disclosed in the present invention can be used as a pharmaceutical, optionally in combination with at least one active compound. This is a further embodiment of the present invention. The term "active compound" refers to a compound other than Smac, a fragment or derivative thereof, which is able to induce apoptosis or which inhibits cell proliferation.

[0022] Active compounds which are able to induce apoptosis are known to the person skilled in the art. One class of active compounds are chemical compounds having a cytostatic or antineoplastic effect ("cytostatic compound"). Cytostatic compounds included in the present invention comprise, but are not restricted to (i) antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, such as bleomycin, (iii) DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; (iv) intercalating agents such as adriamycin (doxoru-

bicin) or mitoxantrone; (v) protein synthesis inhibitors, such as L-asparaginase, cycloheximide, puromycin or diphteria toxin; (vi) topoisomerase I poisons, such as camptothecin or topotecan; (vii) topoisomerase II poisons, such as etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; (ix) kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents such as PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; (xi) hormones such as glucocorticoids or fenretinide; (xii) hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists.

[0023] Other cytostatic compounds, which are included in the present invention, include plant-derived cytostatics (from Viscum and derivatives); alcaloids such as vindesine; podophyllotoxins such as vinorelbin; alkylants such as nimustrine, carmustrine, lomustine, estramustrine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics such as aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs such as methotrexate, purine analogs such as cladribin, mercaptopurin, tioguanine and pyrimidine analogs such as cytarabine, fluorouracil, docetaxel; platinum compounds such as thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecane, interferon-α, tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies such as trastuzumab, rituximab, or derived from enyzmes such as pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens such as polyestradiol, fosfestriol, ethinylestradiol, gestagens such as medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones such as triptoreline, leuproreline, busereline, gosereline, other hormones such as testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens such as toremifen; antiandrogens such as flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors such as anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives such as calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives such as retinolpalmitate, thymus D9, amilomer.

[0024] Another class of active compounds which can be used in the present invention are those which are able to induce apoptosis by binding to death receptors ("death receptor ligands"). They include tumor necrosis factor α (TNF-α), tumor necrosis factor β (TNF-β, lymphotoxin-α), LT-β (lymphotoxin-β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands. Preferably, the death receptor ligand is selected from the group consisting of TNF-α, a fragment or derivative thereof, and TRAIL, a fragment and derivative thereof.

[0025] Other active compounds include agonistic antibodies to death receptors such as anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies. Preferably, the agonistic antibodies are selected from the group consisting of anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti TNF-R antibody and fragments and derivatives of any of said antibodies.

[0026] The preferred Smac/carrier entity of the present invention is the Smac peptide linked to PTD, and will be referred to as Smac peptide/PTD hereafter.

[0027] In the present invention, the cytostatic compound used in combination with the Smac/carrier entity is preferably selected from the group consisting of doxorubicin, cisplatin and etoposide (VP-16). Further preferred active compounds of the present invention used in combination with the Smac/carrier entity are selected from the group of death receptor agonists consisting of TRAIL, anti-CD95 antibody and derivatives and fragments of any of said agonists.

[0028] The Smac/carrier entity can be administered alone or in combination with one or more active compounds. The latter can be administered before, after or simultaneously with the administration of the Smac/carrier entity. The dose of either the Smac/carrier entity or the active compound as well as the duration and the temperature of incubation can be variable and depends on the target that is to be treated.

[0029] A further object of the present invention are pharmaceutical preparations which comprise an effective dose of at least one Smac/carrier entity and/or at least one active compound and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

[0030] The pharmaceutical according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for ex-

ample, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

[0031] The preparation of the pharmaceutical compositions can be carried out in a manner known per se. To this end, the Smac/carrier entity and/or the active compound, together with one or more solid or liquid pharmaceutical carrier substances and/or additives (or auxiliary substances) and, if desired, in combination with other pharmaceutically active compounds having therapeutic or prophylactic action, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human or veterinary medicine.

[0032] For the production of pills, tablets, sugar-coated tablets and hard gelatin capsules it is possible to use, for example, lactose, starch, for example maize starch, or starch derivatives, talc, stearic acid or its salts, etc. Carriers for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, etc. Suitable carriers for the preparation of solutions, for example of solutions for injection, or of emulsions or syrups are, for example, water, physiological sodium chloride solution, alcohols such as ethanol, glycerol, polyols, sucrose, invert sugar, glucose, mannitol, vegetable oils, etc. It is also possible to lyophilize the Smac/carrier entity and/or the active compound and to use the resulting lyophilisates, for example, for preparing preparations for injection or infusion. Suitable carriers for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid.

[0033] The pharmaceutical preparations can also contain additives, for example fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, dispersants, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, agents for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants.

[0034] The dosage of the Smac/carrier entity, in combination with one or more active compounds to be administered, depends on the individual case and is, as is customary, to be adapted to the individual circumstances to achieve an optimum effect. Thus, it depends on the nature and the severity of the disorder to be treated, and also on the sex, age, weight and individual responsiveness of the human or animal to be treated, on the efficacy and duration of action of the compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to the Smac/carrier entity.

[0035] The Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, can be used for the treatment of all cancer types which are resistant to apoptosis due to the expression of IAPs. Examples of such cancer types comprise neuroblastoma, intestine carcinoma such as rectum carcinoma, colon carcinoma, familiary adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tong carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroidea carcinoma, papillary thyroidea carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeolid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basaliorna, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma.

[0036] Examples of cancer types where the use of the Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, is particularly advantageous include neuroblastoma, glioblastoma, breast carcinoma, melanoma, prostate carcinoma, pancreatic carcinoma, hepatocellular carcinoma, colon carcinoma, small cell and non-small cell lung carcinoma.

[0037] The Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, can furthermore be used for the treatment of all autoimmune diseases which are resistant to apoptosis due to the expression of IAPs or members of the Bcl-2 family. Examples of such autoimmune diseases are collagen diseases such as rheumatoid arthritis, Lupus erythematodes disseminatus, Sharp syndrome, CREST syndrome (calcinosis, Raynaud syndrome, esophageal dysmotility, teleangiectasia), dermatomyositis, vasculitis (Morbus Wegener) and Sjögren syndrome, renal diseases such as Goodpasture syndrome, rapidly-progressing glomerulonephritis and membrane-proliferative glomerulonephritis type II, endocrine diseases such as type-I diabetes, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), autoimmune parathyreoidism, pemicious anemia, gonad insufficiency, idiopathic Morbus Addison, hyperthyreosis, Hashimoto thyreoiditis and primary myxedemia, skin diseases such as Pemphigus vulgaris, bullous pemphigoid, Herpes gestationis, Epidermolysis bullosa and Erythema multiforme major, liver diseases such as primary biliary cirrhosis, autoimmune cholangitis, autoimmune hepatitis type-1, autoimmune hepatitis

type-2, primary sclerosing cholangitis, neuronal diseases such as multiple sclerosis, Myastenia gravis, myasthenic Lambert-Eaton syndrome, acquired neuromyotony, Guillain-Barré syndrome (Müller-Fischer syndrome), Stiff-man syndrome, cerebellar degeneration, ataxia, opsoklonus, sensoric neuropathy and achalasia, blood diseases such as autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura (Morbus Werlhof), infectious diseases with associated autoimmune reactions such as AIDS, Malaria and Chagas disease.

[0038] In a further embodiment of the present invention neuroblastoma and glioblastoma cells or self-reactive cells of the immune system are treated by administering an active compound in combination with the overexpression of Smac in the cells. The latter is achieved by methods known to persons skilled in the art, preferably by transfecting the cells with an expression plasmid carrying the full length Smac gene, as disclosed in GenBank number AF262240, or a derivative or a fragment thereof.

[0039] Active compounds which can be used in the above treatment include cytostatic compounds from the group of antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; DNA-fragmenting agents, such as bleomycin, DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; intercalating agents such as adriamycin (doxorubicin) or mitoxantrone; protein synthesis inhibitors, such as L-asparaginase, cycloheximide, puromycin or diphteria toxin; topoisomerase I poisons, such as camptothecin or topotecan; topoisomerase II poisons, such as etoposide (VP-16) or teniposide; microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); miscellaneous investigational agents such as PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; hormones such as glucocorticoids or fenretinide; hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists; plant-derived cytostatics (from Viscum and derivatives); alcaloids such as vindesine; podophyllotoxins such as vinorelbin; alkylants such as nimustrine, carmustrine, lomustine, estramustrine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics such as aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs such as methotrexate, purine analogs such as cladribin, mercaptopurin, tioguanine and pyrimidine analogs such as cytarabine, fluorouracil, docetaxel; other antineoplastic, platinum compounds such as thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecane, interferon-α, tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies such as trastuzumab, rituximab, or derived from enyzmes such as pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens such as polyestradiol, fosfestriol, ethinylestradiol, gestagens such as medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones such as triptoreline, leuproreline, busereline, goserereline, other hormones such as testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens such as toremifen; antiandrogens such as flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors such as anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives such as calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives such as retinolpalmitate, thymus D9, amilomer.

[0040] Preferred active compounds are selected from the group consisting of cisplatin, doxorubicin, and VP-16.

[0041] Other active compounds, which can be used for the treatment of tumor cells and self-reactive cells of the immune system overexpressing Smac include death receptor ligands, such as tumor necrosis factor α (TNF-α), tumor necrosis factor β (TNF-β, lymphotoxin-α), LT-β (lymphotoxin-β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands. Preferably, the death receptor ligand is selected from the group consisting of TNF-α, a fragment or derivative thereof, and TRAIL, a fragment and derivative thereof.

[0042] For the treatment of tumor cells overexpressing Smac there can also be used agonistic antibodies to death receptors such as anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies. Preferably, the agonistic antibodies are selected from the group consisting of anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti TNF-R antibody and fragments and derivatives of any of said antibodies.

[0043] The term derivative or fragment of the Smac gene refers to DNA sequences in which one or more nucleotides of the coding sequence of 1358 nucleotides, as disclosed in GenBank number AF262240, can be substituted by one or more nucleotides different from the original one(s), or Smac DNA sequences the nucleotide sequence of which is either extended, shortened, or both, on either the 5'-, or the 3'- or both ends, provided that the function of the encoded Smac protein remains unaffected.

[0044] A preferred fragment of the Smac gene in the present invention to be overexpressed in tumor cells include the Smac cDNA lacking the nucleotides 20-184 of the disclosed coding sequence, which codes for the so-called mitochondrial targeting sequence (aminoacids 1-55 of the corresponding Smac protein), thus enabling the overexpression of Smac directly in the cytosol, which is the preferred site of Smac action.

[0045] By the administration of an active compound combined with the overexpression of Smac in the cells to be treated, as described beforehand, neuroblastoma and glioblastoma and related types of cancer, like colon carcinoma, hepatocelluar carcinoma or small cell and non-small cell lung carcinoma, can be treated successfully. Thus, a further object of the present invention are kits comprising at least one active compound, as described above, and expression plasmids carrying the full length Smac gene, as disclosed in GenBank number AF262240, or a derivative or fragment thereof. The said kits can be used as a medicament for the treatment of neuroblastoma, glioblastoma and related cancers.

EXAMPLES

[0046] Overexpression of Smac sensitizes for death receptor or drug-induced apoptosis. A full length Smac construct was used to transfect SHEP neuroblastoma cells, which exhibit intermediate sensitivity to various pro-apoptotic stimuli. Representative experiments performed with clone #28 which overexpressed high levels of Smac are subsequently shown (Fig. 1*a*). Overexpression of Smac potentiated TRAIL-induced apoptosis in a dose- and time-dependent manner compared to vector control cells (Fig. 1*b*) and also markedly increased apoptosis induced by anti-CD95 antibody or cytotoxic drugs (Fig. 1*c*). Because overexpression of Smac enhanced both death receptor and drug-induced apoptosis, Smac acts at a common point where these two pathways converge, e.g. at the level of postmitochondrial activation of caspases.

[0047] Smac sensitizes for apoptosis by antagonizing XIAP. It was investigated whether the apoptosis promoting effect of Smac was mediated by antagonizing XIAP, a prominent caspase inhibitor. Treatment with TRAIL resulted in enhanced release of Smac from mitochondria into the cytosol in cells transfected with Smac compared to vector control cells (Fig. 2*a*). Immunoprecipitation of Flag-tagged Smac showed binding of Smac to XIAP upon treatment with TRAIL (Fig. 2b). Also, immunoprecipitation of endogenous XIAP revealed enhanced binding of Smac to XIAP in Smac transfected cells upon TRAIL treatment compared to vector control cells resulting in complete dissociation of XIAP from caspase-9 (Fig. 2*c*). Furthermore, overexpression of Smac enhanced activation of caspase-8, -9, -3, cleavage of the caspase substrates PARP and DFF45 and cleavage of Bid and XIAP upon treatment with TRAIL or doxorubicin (Fig. 2*d* and 2*e*). These findings indicate that overexpression of Smac promoted apoptosis through antagonizing the inhibition of XIAP of both distal and proximal events in the caspase cascade.

[0048] Cytosolic Smac bypasses the Bcl-2 inhibition. Since Bcl-2 may prevent Smac release from mitochondria, Smac function was analyzed in SHEP neuroblastoma cells transfected with Bcl-2. Overexpression of Bcl-2 prevented the release of Smac and cytochrome c from mitochondria upon TRAIL treatment (Fig. 3*a*). Also, Bcl-2 inhibited activation of caspase-3 into active fragments and cleavage of the caspase-3 substrates PARP and DFF45 (Fig. 3*b*). Interestingly however, Bcl-2 reduced, but did not prevent the initial cleavage of caspase-3 into the p24 intermediate fragment or cleavage of caspase-8 (Fig. 3*b*) consistent with a block at the postmitochondrial level, e.g. by XIAP. It was investigated whether cytosolic Smac without the mitochondrial targeting sequence can bypass the Bcl-2 block. Ectopic expression of GFP-tagged Smac in the cytosol was controlled by fluorescence microscopy (data not shown). Importantly, ectopic expression of cytosolic Smac sensitized SHEP neuroblastoma cells overexpressing Bcl-2 for apoptosis induction (Fig. 3c). Also, cytosolic Smac further enhanced treatment-induced apoptosis in SHEP vector control cells (Fig. 3c), consistent with high XIAP expression in these cells (Fig. 2*d*). Expression of cytosolic Smac per se showed no cytotoxic effect (Fig. 3c) indicating that the release from IAP inhibition by Smac only becomes relevant upon apoptosis induction. The studies were further extended to different cell lines with Bcl-2 overexpression. Ectopic expression of cytosolic Smac sensitized Bcl-2 transfected glioblastoma (U87MG/Bcl-2, LN18/Bcl-2, LN229/Bcl-2) and breast carcinoma (MCF7/Bcl-2) cells for treatment with TRAIL, anti-CD95 antibody or doxorubicin (Fig. 3*d*). Thus, cytosolic Smac may bypass Bcl-2 inhibition in several cell types and in response to different pro-apoptotic stimuli.

[0049] Smac peptides sensitize resistant tumor cells for death receptor or drug-induced apoptosis. The N-terminal 4 residues of Smac that are essential for inactivation of XIAP and thus for apoptosis induction, together with the 3 following residues, were linked to the protein transduction domain of the TAT protein to facilitate intracellular delivery (Smac peptide / PTD). Cellular uptake of Smac peptides was controlled by flow cytometry (Fig. 4*a*) and fluorescence microscopy (data not shown). Smac peptides markedly enhanced TRAIL-induced apoptosis and also sensitized for treatment with anti-CD95 antibody or cytotoxic drugs. Furthermore, Smac peptides sensitized several resistant cell lines with defects in apoptosis signaling for treatment with TRAIL or doxorubicin, including neuroblastoma cells with Bcl-2 overexpression (SHEP/Bcl-2), neuroblastoma cells with absent caspase-8 expression (SH-SY5Y), melanoma cells with impaired Apaf-1 expression (Mel-HO) or pancreatic carcinoma cells with defective Ras/PI3 Kinase/Akt signaling (Panc-1) (Fig. 4c).

To exclude that the observations were restricted to cell

lines maintained in long-term culture, primary tumor cells derived from a malignant pleural effusion of a patient with neuroblastoma at tumor relapse with refractory disease were examined. Importantly, Smac peptides sensitized these patient's derived resistant neuroblastoma cells with high levels of XIAP and Bcl-2 (Fig. 4*d*, insert), for apoptosis induced *ex vivo* by TRAIL or anticancer drugs (Fig. 4*d*).

[0050] Smac peptides enhance the antitumor effect of TRAIL in glioblastoma *in vivo* and induce eradication of tumors. The effect of Smac was examined in a glioblastoma tumor model *in vivo*. Glioma cells were implanted into the right striatum of athymic mice and Smac peptides and /or TRAIL were locally administered at day 7 and day 9 after tumor inoculation. Importantly, Smac peptides significantly sensitized glioblastoma cells for TRAIL-induced apoptosis, while treatment with Smac peptides alone showed no antitumor effect (Fig. 5*a* and 5*b*). Complete eradication of preestablished glioblastoma tumors was only found in mice treated with the combination of Smac peptides and TRAIL in 33% (2 of 6) or 50% (3 of 6) of tumors (Fig. 5*a*). Combined administration of Smac peptides and TRAIL showed no acute or delayed neurotoxicity as assessed by a compound neurological score, whereas 2 of 6 mice treated with TRAIL alone developed neurological deficits (data not shown) indicating that the combination of Smac peptides and TRAIL may also improve neurological outcome.

MATERIALS AND METHODS

[0051] *Cell culture.* Neuroblastoma (SHEP, SH-SY5Y), glioblastoma (U87MG, LN18, LN229), Panc-1 pancreatic carcinoma or MCF-7 breast carcinoma were maintained in RPMI 1640 medium (Life Technologies, Inc., Eggenstein, Germany) as previously described. 0.5 x 10^5 cells/ml were cultured in 24-well-plates for determination of apoptosis or in 75 cm^2 flasks (Falcon, Heidelberg, Germany) for protein isolation.

[0052] *Determination of apoptosis.* Cells were incubated with recombinant human TRAIL (PeproTech Inc., Rocky Hill, NJ), cisplatin (Sigma, Deisenhofen, Germany), doxorubicin (Amersham Pharmacia, Freiburg, Germany) VP-16 (Bristol Myers, Erlangen, Germany) or anti-CD95 (APO1) monoclonal antibody. Smac peptides corresponding to aa 56-62 were linked to the protein transduction domain of Tat protein (Interactiva GmbH, Ulm, Germany). For assessment of cellular uptake, FITC-labelled peptides were used. Quantification of DNA fragmentation was performed by fluorescence-activated cell-sorting (FACS) analysis of propidium iodide stained nuclei as previously described.

[0053] *Western blot analysis and immunoprecipitation.* Western blot analysis and immunoprecipitation were performed as previously described using mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant), mouse anti-caspase-3 monoclonal antibody (1:1000, Transduction Laboratories, Lexington, KY), rabbit anti-caspase-9 polyclonal antibody (1:1000, PharMingen, San Diego, CA), mouse anti-XIAP monoclonal antibody (1:1000, H62120, Transduction Laboratories), mouse anti-DFF45 monoclonal antibody (1:1000, Transduction Laboratories), rabbit anti-AIF polyclonal antibody (1:5000, kindly provided by G. Kroemer), rabbit anti-Smac polyclonal antibody (1:5000, kindly provided by X. Wang), mouse anti-COX4 monoclonal antibody (1:1000, Clontech Laboratories, Inc., Palo Alto, CA), mouse anti-Flag monoclonal antibody (1:1000, Sigma) or mouse anti-β-actin monoclonal antibody (1:5000, Sigma) followed by goat anti-mouse IgG or goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence (ECL, Amersham Pharmacia) was used for detection. Expression of β-actin was used to control for equal gel loading.

[0054] *Transfection experiments.* SHEP neuroblastoma cells were transfected with expression plasmid pcDNA3.1 vector containing full length Smac cDNA or empty vector using lipofectamine transfection reagent (Life Technologies, Inc.) and cultured in 0.5 mg/ml G418 (Life Technologies, Inc.). Transient transfections with pEGFPC1 vector containing GFP-tagged Smac without the mitochondrial targeting sequence (aa 1-55) [26] were performed using gene porter transfection reagent.

[0055] *Preparation of mitochondria or cytosolic extracts.* Preparation of mitochondria or cytosolic extracts was performed using the ApoAlert cell fractionation kit (Clontech Laboratories) according to the manufacturer's instructions.

[0056] *Animal studies.* 5 x 10^4 U87MG human glioblastoma cells were stereotactically implanted into the right striatum of athymic mice (CD1 nu/nu, Charles River, Sulzfeld, Germany). At day 7 or at day 7 and day 9, mice were locally treated with Apo2L/TRAIL (2 µg/4 µl buffer) and/or Smac (1 mg/4 µl buffer) or buffer only. Tumor cell volumes were measured at day 21 or 35 after tumor cell implantation as previously described. Neurological symptoms (alertness, behaviour, weight loss, focal neurological deficits) were evaluated daily and a compound score of all categories was formed (++: severe deficits, +: deficits, -: no relevant deficits). Statistical significance was assessed using ANOVA.

DESCRIPTION OF THE DRAWINGS

Fig. 1. Overexpression of Smac sensitizes for death receptor or drug-induced apoptosis.

[0057]

***a*, Overexpression of Smac.** SHEP neuroblastoma cells were transfected with vector control or Smac cDNA. Expression of Smac protein in vector control (V) or single cell clones was determined by Western blot analysis.

***b*, Effect of Smac overexpression on TRAIL-induced DNA fragmentation.** SHEP neuroblastoma cells transfected with vector control (white bars) or Smac (black bars) were treated with TRAIL. Apoptosis was determined by FACS analysis of propidium iodide stained DNA content. Percentage of specific apoptosis was calculated as follows: 100 x [experimental apoptosis (%) - spontaneous apoptosis (%) / 100% - spontaneous apoptosis (%)]. Mean and SD of triplicates are shown, similar results were obtained in three independent experiments.

***c*, Effect of Smac overexpression on anti-CD95- or drug-induced DNA fragmentation.** SHEP neuroblastoma cells transfected with vector control (white bars) or Smac (black bars) were treated with anti-CD95 monoclonal antibody, doxorubicin, cisplatin or VP-16. Apoptosis was determined as described above.

Fig. 2. Effect of Smac overexpression on apoptosis pathways.

[0058]

***a*, Effect of Smac overexpression on Smac release from mitochondria.** SHEP neuroblastoma cells transfected with vector control (Neo) or Smac were treated with TRAIL. Expression of Smac in mitochondrial or cytosolic extracts was determined by Western blot analysis.

***b* and *c*, Effect of Smac overexpression on interaction with XIAP.** SHEP neuroblastoma cells transfected with vector control (Neo) or Smac were subjected to immunoprecipitation of Smac using Flag antibody (b) or to immunoprecipitation of XIAP using monoclonal XIAP antibody (c) and analyzed for XIAP, Smac or caspase-9 by Western blot analysis.

***d* and *e*, Effect of Smac overexpression on TRAIL (d) or doxorubicin (e) induced caspase activation.** SHEP neuroblastoma cells transfected with vector control (Neo) or Smac were treated for indicated times with 10 ng/ml TRAIL or 0.03 μg/ml doxorubicin. Expression of caspase-8, -9, -3, Bid. XIAP. PARP. DFF45 and β-actin was determined by Western blot analysis.

Fig. 3. Overexpression of Smac bypasses the Bcl-2 inhibition.

[0059]

***a*, Effect of Bcl-2 overexpression on mitochondrial release of Smac, cytochrome c and AIF.** SHEP neuroblastoma cells transfected with vector control (Neo) or Bcl-2 were treated for 24 h with 100 ng/ml TRAIL. Expression of cytochrome c, AIF or Smac in mitochondrial, cytosolic or nuclear extracts was determined by Western blot analysis.

***b*, Effect of Bcl-2 overexpression on caspase activation.** SHEP neuroblastoma cells transfected with vector control (Neo) or Bcl-2 were treated with TRAIL. Protein expression was determined by Western blot analysis.

***c*, Effect of cytosolic Smac on death receptor or drug-induced apoptosis.** SHEP neuroblastoma cells stably transfected with vector control (Neo) or Bcl-2 were transiently transfected with GFP-tagged Smac and treated with TRAIL, anti-CD95 antibody, doxorubicin, cisplatin or /ml VP-16. Apoptosis was determined by flow cytometry.

***d*, Effect of cytosolic Smac on death receptor or drug-induced apoptosis in different Bcl-2 overexpressing cells.** Glioblastoma (U87MG, LN18, LN229) or breast carcinoma (MCF7) cells stably transfected with vector control (Neo) or Bcl-2 were transiently transfected with GFP-tagged Smac and treated with TRAIL, anti-CD95 monoclonal antibody or doxorubicin. Apoptosis was determined by flow cytometry.

Fig. 4. Smac peptides sensitizes for death receptor or drug-induced apoptosis.

[0060]

***a*, Uptake of Smac peptides.** SHEP neuroblastoma cells were treated with FITC-labelled Smac peptides. Cellular uptake was determined by flow cytometry.

***b*, Effect of Smac peptides on TRAIL-, CD95- or drug-induced apoptosis.** SHEP neuroblastoma cells were treated with TRAIL, anti-CD95 monoclonal antibody, doxorubicin, cisplatin or VP-16 in the absence or presence of Smac peptides (S) or control peptides corresponding to the reversed version of Smac (Sr). Apoptosis was determined by flow cytometry.

***c*, Effect of cytosolic Smac on death receptor or drug-induced apoptosis in cells with defects in apoptosis signaling.** SHEP neuroblastoma cells stably transfected with Bcl-2, SH-SY5Y neuroblastoma cells, Mel-HO melanoma cells or Panc-1 pancreatic carcinoma cells were treated with TRAIL or doxorubicin in the absence or presence of Smac peptides or control peptides. Apoptosis was determined by flow cytometry.

***d*, Effect of Smac peptides on death receptor or drug-induced apoptosis in primary neuroblastoma cells.** Primary neuroblastoma cells were collected from a malignant pleural effusion, identified by anti-GD2 staining (data not shown) and treated with TRAIL, anti-CD95 monoclonal antibody, doxorubicin, cisplatin or VP-16. Apoptosis was determined by flow cytometry; insert: Expression of XIAP and Bcl-2 in primary neuroblastoma cells was determined by Western blot analysis..

Fig. 5. Smac peptides sensitizes for TRAIL-induced apoptosis in glioblastoma in vivo.

[0061]

***a* and *b*, Effect of Smac peptides on TRAIL-induced apoptosis *In vivo*.** U87MG human glioblastoma cells were stereotactically implanted into the right striatum of athymic mice and locally treated with Apo2L/TRAIL and/or Smac or buffer. In the first experiment (white dots), mice were treated twice at day 7 and day 9 and tumor cell volumes were measured at day 21, in the second experiment (black dots), mice were treated once at day 7 and tumor cell volumes were measured at day 35 after tumor cell implantation (a). Statistical significance was assessed using ANOVA (1. experiment: $P< 0.026$, 2. experiment: $P< 0.028$). Representative histological sections are shown (b) (A: vehicle buffer, B: Smac, C, Apo2L/TRAIL, D: Smac + Apo2L/TRAIL).

Claims

1. A Smac protein / carrier entity comprising

   (i) a Smac protein, as disclosed by the GenBank accession number AAF87716, or a derivative or fragment thereof,

   (ii) a carrier

   and wherein the Smac protein, fragment or derivative thereof and the carrier are linked together enabling the penetration of the Smac/carrier entity through the cell membrane into the cell.

2. The entity according to claim 1, wherein the fragment or derivative of Smac is a peptide comprising the aminoacid sequence 56 to 70.

3. The entity according to claim 1 or 2, wherein the fragment or derivative of Smac is a peptide comprising aminoacids 56 to 62 of Smac.

4. The entity according to any of claims 1 to 3, wherein the fragment or derivative of Smac comprises the aminoacids 56 to 59 of Smac.

5. The entity according to any of claims 1 to 4, wherein said carrier is a protein, a fragment or derivative thereof.

6. The entity according to any of claims 1 to 5, wherein said carrier is selected from the group consisting of TAT, influenza virus hemagglutinin, the VP22 protein from herpes simplex virus, Antennapedia, fibroblast growth factor, Galparan (transportan), poly-arginine, and Pep-1, and fragments and derivatives thereof, and lipids and cationic lipids.

7. The entity according to any of claims 1 to 6, wherein said protein is the TAT protein or a fragment or derivative thereof, as disclosed by GenBank accession number M15654.

8. The entity according to any of claims 1 to 7, wherein the fragment or derivative of the TAT protein comprises the aminoacids 37 to 72 of TAT.

9. The entity according to any of claims 1 to 8, wherein said carrier is the protein transduction domain of TAT comprising the aminoacids 47 to 58 of TAT.

10. The entity according to any of claims 1 to 9, optionally in combination with at least one active apoptosis-inducing or proliferation-inhibiting compound for use as pharmaceutical.

11. The entity for use as pharmaceutical according to claim 10, wherein the active compound is a cytostatic compound.

12. The entity for use as a pharmaceutical according to claims 10 or 11, wherein the cytostatic compound is selected from the group consisting of antimetabolites, preferably cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; DNA-fragmenting agents, preferably bleomycin, DNA-crosslinking agents, preferably chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; intercalating agents preferably adriamycin (doxorubicin) or mitoxantrone; protein synthesis inhibitors, preferably L-asparaginase, cycloheximide, puromycin or diphteria toxin; topoisomerase I poisons, preferably camptothecin or topotecan; topoisomerase II poisons, preferably etoposide (VP-16) or teniposide; microtubule-directed agents, preferably colcemid, colchicine, paclitaxel, vinblastine or vincristine; kinase inhibitors preferably flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); miscellaneous investigational agents, preferably PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832);

polyphenols preferably quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid; hormones preferably glucocorticoids or fenretinide; hormone antagonists, preferably tamoxifen, finasteride or LHRH antagonists; plant-derived cytostatics (from Viscum and derivatives); alcaloids preferably vindesine; podophyllotoxins preferably vinorelbin; alkylants preferably nimustrine, carmustrine, lomustine, estramustrine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics preferably aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs preferably methotrexate, purine analogs preferably cladribin, mercaptopurin, tioguanine and pyrimidine analogs preferably cytarabine, fluorouracil, docetaxel; other antineoplastic, platinum compounds preferably thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecane, interferon-α, tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies preferably trastuzumab, rituximab, or derived from enyzmes preferably pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens preferably polyestradiol, fosfestriol, ethinylestradiol, gestagens preferably medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones preferably triptoreline, leuproreline, busereline, gosereline, other hormones preferably testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens preferably toremifen; antiandrogens preferably flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors preferably anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives preferably calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives preferably retinolpalmitate, thymus D9, amilomer.

13. The entity for use as a pharmaceutical according to any of claims 10 to 12, wherein the cytostatic compound is selected is from the group consisting of doxorubicin, cisplatin and etoposide (VP-16).

14. The entity for use as a pharmaceutical according to claim 10, wherein the active compound is a death receptor ligand, derivative or fragment thereof.

15. The entity for use as a pharmaceutical according to claim 14, wherein the death receptor ligand is selected from the group consisting of tumor necrosis factor α (TNF-α), tumor necrosis factor (3 (TNF-β, lymphotoxin-α), LT-β (lymphotoxin-β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands.

16. The entity for use as a pharmaceutical according to claims 14 or 15, wherein the death receptor ligand is TRAIL.

17. The entity for use as a pharmaceutical according to claim 10, wherein the active compound is an antibody against a death receptor, a derivative or fragment thereof.

18. The entity for use as a pharmaceutical according to claim 17, wherein the antibody against the death receptor ligand is selected from the group consisting of anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies.

19. The entity for use as a pharmaceutical according to claims 17 or 18, wherein the antibody against the death receptor is the anti-CD95 antibody.

20. The use of Smac/carrier entity according to any of claims 1 to 9, optionally in combination with at least one active apoptosis-inducing compound for the manufacture of a medicament for the treatment of cancer.

21. The use according to claim 20, wherein the cancer to be treated is selected from a group consisting of neuroblastoma, rectum carcinoma, colon carcinoma, familiary adenomatous polyposis carcinoma, hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tong carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroidea carcinoma, papillary thyroidea carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors preferably glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeolid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellualar carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung

carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma.

22. The use according to claim 20 or 21, wherein the cancer to be treated is selected from the group consisting of neuroblastoma, glioblastoma, breast carcinoma, melanoma, prostate cancer and pancreatic carcinoma.

23. A medicament for the treatment of cancer, comprising a Smac/carrier entity as claimed in any of the claims 1 to 9 and a pharmaceutically acceptable carrier.

24. The use of Smac/carrier entity according to any of claims 1 to 9, optionally in combination with at least one active apoptosis-inducing compound for the manufacture of a medicament for the treatment of autoimmune diseases.

25. The use according to claim 24, wherein the autoimmune disease to be treated is selected from a group consisting of collagen diseases particularly rheumatoid arthritis, Lupus erythematodes disseminatus, Sharp syndrome, CREST syndrome (calcinosis, Raynaud syndrome, esophageal dysmotility, teleangiectasia), dermatomyositis, vasculitis (Morbus Wegener) and Sjögren syndrome, renal diseases particularly Goodpasture syndrome, rapidly-progressing glomerulonephritis and membrane-proliferative glomerulonephritis type II, endocrine diseases particularly type-I diabetes, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), autoimmune parathyreoidism, pernicious anemia, gonad insufficiency, idiopathic Morbus Addison, hyperthyreosis, Hashimoto thyreoiditis and primary myxedemia, skin diseases particularly Pemphigus vulgaris, bullous pemphigoid, Herpes gestationis, Epidermolysis bullosa and Erythema multiforme major, liver diseases particularly primary biliary cirrhosis, autoimmune cholangitis, autoimmune hepatitis type-1, autoimmune hepatitis type-2, primary sclerosing cholangitis, neuronal diseases particularly multiple sclerosis, Myastenia gravis, myasthenic Lambert-Eaton syndrome, acquired neuromyotony, Guillain-Barré syndrome (Müller-Fischer syndrome), Stiff-man syndrome, cerebellar degeneration, ataxia, opsoklonus, sensoric neuropathy and achalasia, blood diseases particularly autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura (Morbus Werlhof), infectious diseases particularly AIDS, Malaria and Chagas disease.

26. A medicament for the treatment of autoimmune diseases, comprising a Smac/carrier entity as claimed in any of the claims 1 to 9 and a pharmaceutically acceptable carrier.

27. The use of an expression plamid carrying the full length Smac gene, as disclosed by GenBank accession number AF262240, or a derivative or a fragment thereof, in combination with an active compound for the manufacture of a medicament for the treatment of neuroblastoma, glioblastoma, prostate carcinoma, colon carcinoma, small cell and non-small cell lung carcinoma.

28. The use according to claim 27, wherein the full length Smac gene as disclosed is substituted by a Smac DNA fragment lacking the nucleotides 20 to 184 of the disclosed coding sequence.

29. The use according to claim 27 or 28, wherein the active compound is selected from the group of cytostatic compounds consisting of cisplatin, doxorubicin, and VP-16.

30. The use according to claim 27 or 28, wherein the active compound is selected from the group of death receptor ligands consisting of tumor necrosis factor α (TNF-α), tumor necrosis factor (3 (TNF-β, lymphotoxin-α), LT-β (lymphotoxin-β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands.

31. The use according to claim 30, wherein the death receptor ligand is TRAIL.

32. The use according to claim 27 or 28, wherein the active compound is an antibody against a death repector.

33. The use according to claim 32, wherein the antibody against a death receptor is the anti-CD95 antibody.

34. A kit, comprising at least one active compound, as described above, and expression plasmids carrying the full length Smac gene, as disclosed in GenBank number AF262240, or a derivative or fragment thereof.

35. The use of the kit according to claim 34 for the manufacture of a medicament for the treatment of neuroblastoma, glioblastoma, prostate cancer, colon cancer, hepatocellular carcinoma, small cell lung cancer and non-small cell lung cancer and related cancers.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 02 00 8199

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 02 016418 A (THOMAS JEFFERSON UNIVERSITY, USA) 28 February 2002 (2002-02-28) * examples * * claims * ---	1-35	C12N15/12 C12N15/62 A61K47/48 C07K5/103 C07K19/00 C07K14/47
X	WO 02 016402 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM, USA) 28 February 2002 (2002-02-28) * examples * * claims * ---	1-35	A61K38/17
X	WO 02 026775 A (TRUSTEES OF PRINCETON UNIVERSITY, USA) 4 April 2002 (2002-04-04) * examples * * claims * ---	1-35	
X	WO 01 49719 A (UNIV TEXAS SYSTEM) 12 July 2001 (2001-07-12) * examples * --- -/--	1-35	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A61K
C07K

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	5 July 2002	Dullaart, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

INCOMPLETE SEARCH
SHEET C

Application Number
EP 02 00 8199

Claim(s) searched incompletely:
1-35

Reason for the limitation of the search:

Present claims 1-35 relate to an extremely large number of possible compounds, as well as their use. Support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC is to be found, however, for only the expression of the Smac protein. For the protein-carrier combination, no actual example is given. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.
Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds specifically prepared in the examples.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 02 00 8199

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; ENDO, HITOSHI ET AL: "Cysteine, basic and neutral amino acid transporter BAT1 from rat and human activated by rBAT, cDNA, and recombinant expression" retrieved from STN Database accession no. 134:174559 HCA XP002204777 * abstract * -& JP 2001 046070 A (FOUNDATION FOR SCIENTIFIC TECHNOLOGY PROMOTION, JAPAN) 20 February 2001 (2001-02-20) ---	1-35	
Y	WO 00 58488 A (DALBY BRIAN ;INVITROGEN CORP (US); BENNETT ROBERT P (US)) 5 October 2000 (2000-10-05) * examples * * claims * ---	1-35	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Y	WO 01 38547 A (ROSENECKER JOSEPH ;PLANK CHRISTIAN (DE); RITTER WOLFGANG (DE); RUD) 31 May 2001 (2001-05-31) * examples * * claims * ---	1-35	
Y	WO 00 29427 A (CYCLACEL LTD ;FISCHER M PETER (GB); ZHELEV NIKOLAI (GB)) 25 May 2000 (2000-05-25) * examples * * claims * ---	1-35	
Y	WO 99 05302 A (PERKIN ELMER CORP) 4 February 1999 (1999-02-04) * examples * * claims * --- -/--	1-35	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 02 00 8199

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	DEROSSI D ET AL: "TROJAN PEPTIDES: THE PENETRATIN SYSTEM FOR INTRACELLULAR DELIVERY" TRENDS IN CELL BIOLOGY, ELSEVIER SCIENCE LTD, XX, vol. 8, February 1998 (1998-02), pages 84-87, XP002940006 ISSN: 0962-8924 * abstract * * figure 2 * * page 86, right-hand column, last paragraph - page 87, left-hand column, line 4 *	1-35	
Y	SCHWARZE S ET AL: "In vivo protein transduction: delivery of a biologically active protein into the mouse" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 285, no. 5433, 3 September 1999 (1999-09-03), pages 1569-1572, XP002140133 ISSN: 0036-8075 * abstract * * page 1571 *	1-35	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Y	FISCHER P M ET AL: "STRUCTURE-ACTIVITY RELATIONSHIP OF TRUNCATED AND SUBSTITUTED ANALOGUES OF THE INTRACELLULAR DELIVERY VECTOR PENETRATIN" JOURNAL OF PEPTIDE RESEARCH, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, DK, vol. 55, no. 2, February 2000 (2000-02), pages 163-172, XP000899124 ISSN: 1397-002X * the whole document *	1-35	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

Application Number
EP 02 00 8199

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☒ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 02 00 8199

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-9, 23

   The Smac/carrier entity as claimed, and a medicament for the treatment of cancer containing it.

2. Claims: 10-22

   Use of the Smac/carrier entity as claimed in combination with another anticancer agent, in the treatment of cancer

3. Claims: 24-26

   Use of the Smac/carrier entity as claimed in the treatment of autoimmune diseases

4. Claims: 27-35

   Use of an expression plasmid carrying the gene of the Smac protein as claimed for the treatment of cancer

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 02 00 8199

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

05-07-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02016418 A		NONE	
WO 02016402 A		NONE	
WO 02026775 A		NONE	
WO 0149719 A	12-07-2001	US 6110691 A	29-08-2000
		AU 2630701 A	16-07-2001
		WO 0149719 A2	12-07-2001
JP 2001046070 A	20-02-2001	NONE	
WO 0058488 A	05-10-2000	AU 4055500 A	16-10-2000
		EP 1165819 A2	02-01-2002
		WO 0058488 A2	05-10-2000
WO 0138547 A	31-05-2001	AU 2508501 A	04-06-2001
		WO 0138547 A2	31-05-2001
WO 0029427 A	25-05-2000	AU 1063000 A	05-06-2000
		CZ 20011671 A3	17-10-2001
		EP 1135410 A2	26-09-2001
		WO 0029427 A2	25-05-2000
		GB 2346616 A	16-08-2000
WO 9905302 A	04-02-1999	AU 741546 B2	06-12-2001
		AU 8408098 A	16-02-1999
		EP 0998577 A1	10-05-2000
		JP 2002511885 T	16-04-2002
		WO 9905302 A1	04-02-1999
		US 6025140 A	15-02-2000

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

(19) BUNDESREPUBLIK DEUTSCHLAND

DEUTSCHES PATENT- UND MARKENAMT

(12) **Offenlegungsschrift**

(10) **DE 103 06 724 A 1**

(51) Int. Cl.[7]:
A 61 K 31/4184
A 61 K 9/127
A 61 P 35/00

(21) Aktenzeichen: 103 06 724.8
(22) Anmeldetag: 17. 2. 2003
(43) Offenlegungstag: 18. 9. 2003

DE 103 06 724 A 1

(66) Innere Priorität:
102 08 622. 2 28. 02. 2002

(71) Anmelder:
G.O.T. Therapeutics GmbH, 13125 Berlin, DE

(74) Vertreter:
Baumbach, F., Dr.rer.nat. Pat.-Ing., Pat.-Ass., 13125 Berlin

(72) Erfinder:
Reszka, Regina, Dr., 16341 Schwanebeck, DE;
Scherrer, Peter, Dr., 14197 Berlin, DE

Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

(54) Vesikuläre Verkapselung von Bendamustin

(57) Die Erfindung betrifft vesikulär verkapseltes Bendamustin oder Derivate des Bendamustins, wobei die Vesikel einen hohen Wirkstoffanteil von ≥ 50% aufweisen. Sie werden u. a. durch remote loading von Lipid Vesikeln, durch Hochdruckhomogenisation, bevorzugt unter vorheriger Bildung eines Vesikelgels, in das der Wirkstoff in Lösung diffundiert, durch Ethanol-Injection Methode oder unter Aerosolbildung hergestellt. Alternativ kann auch ein Derivat von Bendamustin mit einem gekoppelten hydrophoben Anker (z. B. einem Fettsäurederivat) zur hohen Beladung von Vesikeln verwendet werden.

DE 103 06 724 A 1

Beschreibung

[0001] Die Erfindung betrifft vesikulär verkapseltes Bendamustin oder Derivate des Bendamustins, wobei die Vesikel einen hohen Wirkstoffanteil von ≥ 50% aufweisen. Sie werden u. a. durch remote loading von Lipid Vesikeln, durch Hochdruckhomogenisation, bevorzugt unter vorheriger Bildung eines Vesikelgels, in das der Wirkstoff in Lösung diffundiert, durch Ethanol-Injection Methode oder unter Aerosolbildung hergestellt. Alternativ kann auch ein Derivat von Bendamustin mit einem gekoppelten hydrophoben Anker (z. B. einem Fettsäurederivat) zur hohen Beladung von Vesikeln verwendet werden.

[0002] Darüber hinaus betrifft die Erfindung pharmazeutische Zubereitungen, die vesikulär verkapseltes Bendamustin oder Derivate des Bendamustins, ggf. in Kombination mit weiteren zytostatischen Mitteln, umfassen und zur Behandlung maligner Erkrankungen geeignet sind.

[0003] Bendamustin ist ein seit langem bekanntes bifunktionelles Alkylans, das über eine breite zytostatische Aktivität bei einer Vielzahl maligner Erkrankungen verfügt. Es ein Stickstoff-Lost-Derivat aus der Gruppe der Alkylantien. Seine antineoplastische Wirkung beruht vor allem auf der Quervernetzung von DNA-Einzel- und Doppelsträngen durch Alkylierung, so dass die Erbinformation nicht mehr weitergegeben werden kann. Dann teilt sich die Zelle nicht mehr und stirbt ab. Bendamustin wird in der Leber zu der eigentlich aktiven Wirksubstanz abgebaut und über die Nieren ausgeschieden.

[0004] In der Regel erfolgt die Behandlung mit Bendamustin, indem wässrige Lösungen des Wirkstoffs in die Vene gespritzt bzw. infundiert werden. Je nach Krebsart und -größe erfolgt die Behandlung an 5 Tagen hintereinander, wobei zwischen jedem Therapiezyklus ein Abstand von 3–4 Wochen liegen sollte. Aufgrund von Diffusionsvorgängen in die Gewebestrukturen des Körpers und einer i. d. R. gleichmäßigen Bioverteilung, erreichen nur geringe Mengen den Wirkort und das Pharmakon ruft wegen der gleichmäßigen Verteilung auf das gesunde Gewebe zahlreiche Nebenwirkungen hervor. Die Halbwertszeit des Bendamustins ist sehr gering, sie beträgt nur ca. 6 bis 10 Minuten.

[0005] Eine Hochdosis-Therapie mit Bendamustin ist derzeit nicht möglich. Insbesondere anticholinerge Nebenwirkungen wie extreme Mundtrockenheit und Durstgefühl begrenzen die Dosis.

[0006] Deshalb wird nach neuen Formulierungen gesucht, die eine selektive Therapie ermöglichen. Es wird aus diesem Grund insbesondere nach Trägern für die tumoraktive Verbindung gesucht, die eine verlängerte Halbwertszeit im systemischen Kreislauf aufweisen.

[0007] Die Aufgabe der Erfindung bestand deshalb darin, den Wirkstoff Bendamustin oder seine Derivate in einer pharmazeutischen Darreichungsform bereitzustellen, die eine effektive wirksame Applikation ermöglicht.

[0008] Die Erfindung wird gemäß den Ansprüchen realisiert. Unter Anwendung an sich bekannter Techniken, wie z. B. "remote loading" über einen pH-Gradienten, Hochdruckhomogenisierung oder Ethanol-injection method" (Injektion einer Ethanol-Lipid Lösung in die wässrige Bendamustinlösung) ist es möglich, das Bendamustin oder seine Derivate in eine vesikuläre Schicht eindringen zu lassen und somit entsprechend zu verkapseln. Hydrophobe Derivate von Bendamustin lagern sich spontan in die Lipidschicht von Liposomen ein.

[0009] Überraschend weisen diese Vesikel einen hohen Wirkstoffgehalt mit einer Einschlussrate von ≥ 50%, bevorzugt ≥ 70% oder insbesondere sogar ≥ 80%, auf und verlängern die Halbwertszeit von Bendamustin im Blut um mehr als das Zehnfache.

[0010] Die bevorzugte Beladung der Vesikel erfolgt durch das remote loading. Es werden Vesikel unter Verwendung hoher Pufferkonzentrationen und tiefem pH (stark sauer) hergestellt. Anschließend wird der Puffer im Außenmedium geändert, was zu einem pH-Gradienten zwischen dem Vesikelinneren und dem Außenmedium führt. Bendamustin oder ein Derivat davon wird außen zugegeben, diffundiert durch die Membran und wird im Inneren in eine Membran undurchlässige Form überführt und entsprechend konzentriert. Diese Methode erlaubt die Aufnahme von hohen Konzentrationen an Bendamustin bzw. Derivaten, nämlich > 90% des außen zugegebenen Derivats.

[0011] Alternativ erfolgt die Herstellung bevorzugt mittels Hochdruckhomogenisation, wobei zuerst Vesikelgele hergestellt werden. Anschließend lässt man die Wirkstoffe in wässriger oder alkoholischer Lösung in die Gele diffundieren.

[0012] Es wurde eine hohe Effizienz bei der Ladung von Vesikelgelen erreicht, wobei die Einschlussrate > 90% beträgt. Die Ladung der Gele kann durch Temperatursteigerung noch erhöht werden.

[0013] In einer weiteren Ausführungsvariante der Erfindung enthält das gebildete Vesikelgel in oder an der Membran der Vesikel ein Polymer, vorzugsweise Polyethylenglycol (MG 2000 – 10 000), das den Wirkstoff an der Vesikeloberfläche vor Inaktionen mit Blutkomponenten schützt.

[0014] Die vorherige Bildung eines Vesikelgels hat verschiedene Vorteile:

[0015] Das Vesikelgel kann (z. B. im Autoklaven) in Abwesenheit des Wirkstoffs sterilisiert werden, wodurch der Wirkstoff geschützt ist und auch eine Wirkstoff/Lipid-Degradation vermieden wird. Der Wirkstoff wird keinen extremen Belastungen (physikalischen oder hohen Temperaturen) während der Hochdruckhomogenisierung ausgesetzt.

[0016] Kein Verlust von möglicherweise teuren Substanzen während der Herstellung (bei Zugabe während Hochdruckhomogenisierung ca. 10–20% Verlust).

[0017] Eine Depot-Anwendung mit langsamer Freisetzung an Vesikel ist möglich. Die Wirkstoffdosierung kann variiert bei konstanter Lipiddosis werden. Die Bioverfügbarkeit sollte deshalb unabhängig von der Wirkstoffdosierung sein. Das Geldepot ermöglicht eine langsame Freisetzung des Wirkstoffs (hohe Durchlässigkeit).
Kontrollierte Freisetzung wird möglich
Die Wirkstofffreisetzungsrate kann durch Anpassung der Lipidzusammensetzung in den Vesikel reguliert werden.

[0018] Weiterhin betrifft die Erfindung pharmazeutische Zusammensetzungen zur Therapie zahlreicher maligner Erkrankungen, insbesondere solider Tumore, die erfindungsgemäß verkapseltes Bendamustin und seine Derivate umfassen. Sie werden bevorzugt bei Melanomen, Non Hodgkin, small, cell & non-small cell Lungenkrebs, kleinzelliges Bronchialkarzinom, Glioblastom, chronischer lymphatischer Leukämie, Brustkrebs, aber auch anderen appliziert. Insbesondere sind sie für die Behandlung von Non-Hodgkin-Lymphomen und chronischer lymphatischer Leukämie geeignet.

[0019] Die Herstellung von pharmazeutischen Formulierungen, die die Vesikel mit dem Wirkstoff enthalten, erfolgt

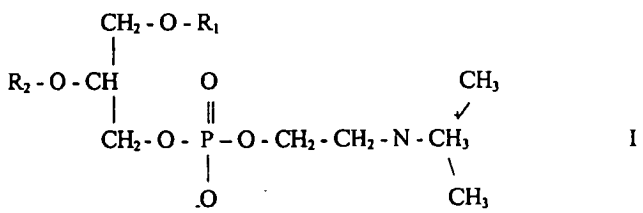
nach an sich bekannten Verfahren zur Herstellung pharmazeutischer Formulierungen, die für eine orale, paranterale (i.v., s.c., i.m.), rektale, nasale und inhalatorische Applikation geeignet sind.

[0020] Mit der vorliegenden Erfindung werden hoch stabile, pharmazeutisch aktive Präparate von Bendamustin und seinen Derivaten bereitgestellt, die nach ihrer vesikulären Verkapselung eine verlängerte Verweilzeit im Körper des Patienten aufweisen und die für die Behandlung unterschiedlichster Formen von Krebserkrankungen geeignet sind.

[0021] Bevorzugt liegen die Pufferlösungen (pH, Salze, Ionen, Ionenlänge), die eine optimale Stabilität von Bendamustin gewährleisten, im Inneren der Vesikel im stark sauren Bereich (bevorzugter pH 3,15). Nach bevorzugter Verabreichung der Bendamustin enthaltenden Vesikeln ins Blut wird der Innen-pH langsam angehoben und Bendamustin oder seine Derivate werden über einen längeren Zeitraum freigesetzt.

Patentansprüche

1. Vesikulär verkapseltes Bendamustin oder Derivate des Bendamustins, wobei die Vesikel einen hohen Wirkstoffgehalt von ≥ 50%, bevorzugt ≥ 70 oder ≥ 80%, aufweisen.
2. Vesikel nach Anspruch 1, dadurch gekennzeichnet, dass sie zusätzlich weitere Zytostatika beinhalten.
3. Verfahren zur Herstellung von Bendamustin oder Derivaten des Bendamustins enthaltenden Vesikeln mit einem Anteil ≥ 50%, bevorzugt ≥ 70 oder ≥ 80% in den Vesikeln, dadurch gekennzeichnet, dass die Verkapselung durch Remote loading erfolgt, indem Vesikel unter Verwendung hoher Pufferkonzentrationen und tiefem pH hergestellt werden, anschließend der Puffer im Außenmedium geändert, und Bendamustin oder ein Derivat davon außen zugegeben wird, durch die Membran diffundiert, im Inneren in eine Membran undurchlässige Form überführt und konzentriert wird.
4. Verfahren zur Herstellung von Bendamustin oder Derivaten des Bendamustins enthaltenden Vesikeln mit einem Anteil ≥ 50%, bevorzugt ≥ 70 oder ≥ 80% in den Vesikeln, dadurch gekennzeichnet, dass zur Verkapselung ein Gemisch von membranbildenden Amphiphilen und eine wässrige Phase ein- bis fünfzigmal einer Hochdruckhomogenisation mit Drucken von 50 bis 1600 bar (5–160 Mpa) unterzogen wird und der Wirkstoff nach Vesikelgelbildung in wässriger oder alkoholischer Lösung zugegeben wird und in das Vesikelgel diffundiert.
5. Verfahren zur Herstellung von Bendamustin oder Derivaten des Bendamustins enthaltenden Vesikeln mit einem Anteil ≥ 50%, bevorzugt ≥ 70 oder ≥ 80% in den Vesikeln, dadurch gekennzeichnet, dass zur Verkapselung ein Gemisch von membranbildenden Amphiphilen, in welchem der Wirkstoff gelöst vorliegt, und eine wässrige Phase ein- bis fünfzigmal, einer Hochdruckhomogenisation mit Drucken von 50 bis 1600 bar (5–160 Mpa) unterzogen wird.
6. Verfahren nach einem der Ansprüche 4 bis 5, dadurch gekennzeichnet, dass vor der Hochdruckhomogenisation ein dünner, trockener Lipidfilm unter Entfernung der Lösungsmittel durch Evaporation oder durch Sprühtrocknung hergestellt und der Lipidfilm in Wasser dispergiert wird.
7. Verfahren nach nach einem der Ansprüche 4 bis 6, dadurch gekennzeichnet, dass zwischen oder nach der Hochdruckhomogenisation eine Gefrier/Tau-Behandlung oder eine Gefriertrocknung/Redispersions-Behandlung erfolgt.
8. Verfahren nach einem der Ansprüche 4 bis 7, dadurch gekennzeichnet, dass eine Überführung in eine freifließende Dispersion erfolgt.
9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, dass die Dispersion durch Filter mit einer Porenweite von 0,1 bis 1 µm filtriert wird.
10. Verfahren zur Herstellung von Bendamustin oder Derivaten des Bendamustins enthaltenden Vesikeln mit einem Anteil ≥ 50%, bevorzugt ≥ 70 oder ≥ 80% in den Vesikeln, dadurch gekennzeichnet, dass zur Verkapselung des Wirkstoffs eine vorgefertigte Vesikel-Mischung aus Verkapselungsmitteln in fester oder flüssiger Form mit dem Wirkstoff vereinigt und nachfolgend in Aerosol-bildende Vorrichtungen überführt wird.
11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, dass der Wirkstoff und Verkapselungsmittel in einem druckverflüssigten Treibgas gelöst vorliegen und nach Verdampfen des Treibgases in verkapselte Wirkstoff-Vesikel überführt werden.
12. Verfahren nach einem der Ansprüche 3 bis 11, dadurch gekennzeichnet, dass als Verkapselungsmittel
    a) ein natürliches, halbsynthetisches oder vollsynthetisches Amphiphil
    b) eine geladene Lipidkomponente und/oder eine gesättigte Lipidkomponente und/oder eine Etherlipidkomponente,
    c) ein Polymer
    d) eine Trägerflüssigkeit eingesetzt werden.
13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass das Amphiphil ein Lipid, ein Tensid oder ein Emulgator ist.
14. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass ein natürliches, halbsynthetisches oder vollsynthetisches Amphiphil der allgemeinen Formel I,



(I)

worin R_1 und R_2 C_{10}-C_{20}-Alkanoyl, -Alkenoyl, -Alkyl, -Alkenyl bedeuten, eingesetzt wird.
15. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass als geladene Lipidkomponente das Anion des Di-

cethylphosphats, der Palmitinsäure, der Stearinsäure, das Anion eines Phospholipids, das Anion eines Sphingolipids, eingesetzt wird oder dass ein chemisch modifiziertes Phosphatidylethanolamin, über das Proteine angekoppelt werden können, oder Etherlipide, eingesetzt werden.

16. Verfahren nach Anspruch 15, dadurch gekennzeichnet, dass, Phosphatidylserin, Phosphatidsäure, Phosphatidylglycerol oder Sulfatid eingesetzt wird.

17. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass als neutrale Lipidkomponente Phosphatidylcholin eingesetzt wird.

18. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass als gesättigte Lipidkomponenten Dipalmitoylphosphatidylcholin oder Dimyrestoylphosphatidylcholin eingesetzt wird.

19. Verfahren nach einem der Ansprüche 12 bis 18 dadurch gekennzeichnet, dass Nanopartikel als zusätzliche Hilfsstoffe eingesetzt werden.

20. Verfahren nach einem der Ansprüche 12 bis 19, dadurch gekennzeichnet, dass in oder an der Membran der Vesikel als Polymer Polyethylenglycol (MG 2000–10 000) enthalten ist.

21. Pharmazeutische Zubereitungen zur Therapie maligner Erkrankungen, insbesondere solider Tumore, dadurch gekennzeichnet, dass sie vesikulär verkapseltes Bendamustin oder Derivate des Bendamustins umfassen.

22. Zubereitung nach Anspruch 21, dadurch gekennzeichnet, dass sie zusätzlich weitere zytostatische Mittel, ggf. mindestens einen Immunmodulator, vorzugsweise Cyclosporine, und/oder mindestens ein Cytokin, vorzugsweise PEG-Cytokine, umfassen.

23. Pharmazeutische Zubereitung nach Anspruch 21 oder 22, dadurch gekennzeichnet, dass sie zusätzlich an sich übliche pharmazeutische Hilfs- und Zusatzstoffe umfassen und zur oralen, parenteralen, rektalen, nasalen oder inhalatorischen Applikation geeignet sind.

24. Verwendung einer Zubereitung nach einem der Ansprüche 21 bis 23 zur Behandlung von Non-Hodgkin-Lymphomen und chronischer lymphatischer Leukämie.

(19) Bundesrepublik Deutschland
Deutsches Patent- und Markenamt

(10) **DE 103 04 403 A1** 2004.08.05

(12) # Offenlegungsschrift

(21) Aktenzeichen: **103 04 403.5**
(22) Anmeldetag: **28.01.2003**
(43) Offenlegungstag: **05.08.2004**

(51) Int Cl.[7]: **A61K 9/14**
A61K 9/20, A61K 47/12

(71) Anmelder:
Röhm GmbH & Co. KG, 64293 Darmstadt, DE

(72) Erfinder:
Petereit, Hans-Ulrich, 64291 Darmstadt, DE; Meier, Christian, Dr., 64295 Darmstadt, DE; Gryczke, Andreas, 64347 Griesheim, DE

Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

(54) Bezeichnung: **Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung**

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, durch intensives Mischen
(a) eines anionischen pharmazeutischen Wirkstoffs mit
(b) einem Copolymer, bestehend aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren, die funktionelle tertiäre Aminogruppen aufweisen, sowie
(c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure
in der Schmelze, Erstarren der Mischung und Mahlen zu einem wirkstoffhaltigen Pulver mit einer mittleren Korngröße von 200 µm oder weniger, Einbetten des Pulvers in eine wasserlösliche Matrix aus pharmazeutisch üblichen Hilfsstoffen, mit der Maßgabe, daß nicht mehr als 3 Gew.-%, bezogen auf das Copolymer, an Emulgatoren mit einem HLB-Wert von mindestens 14 enthalten sein dürfen. Die Erfindung betrifft weiterhin das wirkstoffhaltige Pulver und dessen Verwendungen.

Beschreibung

Gebiet der Erfindung

[0001] Die Erfindung betrifft ein Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund. Die Erfindung betrifft weiterhin ein wirkstoffhaltiges Pulvers und dessen Verwendung.

Stand der Technik

[0002] EP-A 0 417 588 beschreibt ein Verfahren zur Herstellung eines komplexierten Arzneimittels aus einem ionogenen Wirkstoff durch Umsetzung des Wirkstoffs mit einem komplementär ionogenen, partikelförmigen Polymer in Gegenwart einer zum Anfeuchten der Mischung ausreichenden Wassermenge. Bei Wirkstoffssalzen ist es erforderlich, der Mischung eine Säure oder Base zur Neutralisation des Gegenions des Wirkstoffs zuzusetzen. Bei der Umsetzung von Wirkstoffsalzen wie Propranolol-HCl, Verapamil-HCl oder Metoclopramid-HCl mit anionischen (Meth)acrylat-Copolymeren wie EUDRAGIT® L oder EUDRAGIT® L100-55 wird der Mischung beispielsweise Natriumcarbonat zugesetzt. In diesem Fall kann eine Geschmacksisolierung der bitter schmeckenden Wirkstoffe erreicht werden. Weiterhin ist erwähnt, daß im Falle, daß der ionogene Wirkstoff eine Säure ist, ein partikelförmiges Polymer eingesetzt werden kann, welches als komplementär ionogene Gruppen seitenständige Aminogruppen aufweist. Die seitenständige Aminogruppen kann z. B. eine tertiäre Aminogruppe, stammend aus polymerisierten Monomeren wie z. B. 2-Dimethylamino-ethyl-methacrylat sein.

[0003] WO 01/39751 beschreibt ein Verfahren zur Herstellung von Formkörpern mittels Spritzguß mit den Verfahrensschritten

a) Aufschmelzen eines (Meth)acrylat-Copolymeren, das sich aus 30 bis 80 Gew.-% radikalisch polymerisierten C1- bis C4-Alkylestern der Acryl- oder der Methacrylsäure und 70 bis 20 Gew.-% (Meth)acrylat-Monomeren mit einer tertiären Ammonium- bzw. Aminogruppe im Alkylrest zusammensetzt,
wobei das (Meth)acrylat-Copolymere in Mischung mit 1 bis 70 Gew.-% von einem Weichmacher und einem Trockenstellmittel im Verhältnis 1: 1 bis 1 : 20 vorliegt,
wobei mindestens 1 Gew.-% Weichmacher enthalten ist,
sowie 0,05 bis 5 Gew.-% eines Trennmittels enthalten sind und
zusätzlich weitere übliche Additive oder Hilfsstoffe und gegebenenfalls ein pharmazeutischer Wirkstoff in der Mischung enthalten sein können und die Mischung vor dem Aufschmelzen einen Gehalt an niedrigsiedenden Bestandteilen mit einem Dampfdruck von mindestens 1,9 bar bei 120°C von über 0,5 Gew.-% aufweist,
b) Entgasen der Mischung im thermoplastischen Zustand bei Temperaturen von mindestens 120 °C, wodurch der Gehalt der niedrigsiedenden Bestandteile mit einem Dampfdruck von mindestens 1,9 bar bei 120°C auf höchstens 0,5 Gew.-% gesenkt wird
c) Einspritzen der aufgeschmolzenen und entgasten Mischung in den Formhohlraum eines Spritzgießwerkzeugs, wobei der Formhohlraum eine Temperatur aufweist, die mindestens 10 °C unterhalb der Glastemperatur des (Meth)acrylat-Copolymeren liegt, Abkühlen der Schmelzemischung und Entnahme des erhaltenen Formkörpers aus der Form.

[0004] WO 02/67906 beschreibt ein Verfahren zur Herstellung eines Überzugs- und Bindemittels für orale oder dermale Arzneiformen bestehend im wesentlichen aus (a) einem Copolymer, bestehend aus radikalisch polymerisierten C1- bis C4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren, die funktionelle tertiäre Ammoniumgruppen aufweisen, wobei das Copolymer in Pulverform mit einer mittleren Teilchengröße von 1 – 40 µm vorliegt, (b) 3 bis 15 Gew.-%, bezogen auf (a), eines Emulgators mit einem HLB-Wert von mindestens 14 und (c) 5 bis 50 Gew.-%, bezogen auf (a), einer C_{12}- bis C_{18}-Monocarbonsäure oder einer C_{12}- bis C_{18}-Hydroxylverbindung, wobei die Komponenten (a), (b) und (c) mit oder ohne Zusatz von Wasser und gegebenenfalls unter Zusatz eines pharmazeutischen Wirkstoffs und weiterer üblicher Zuschlagstoffe miteinander vermengt oder vermischt werden und das Überzugs- und Bindemittel aus der Mischung durch Schmelzen, Gießen, Ausstreichen, Aufsprühen oder Granulieren hergestellt wird.

[0005] Gemäß der WO 02/67906 sind besonders lagerstabile Arzneiformen erhältlich, die insbesondere feuchteempfindliche Wirkstoffe wie Acetylsalicyläure, Carbenoxolon, Cefalotin, Epinefrin, Imipramin, Kaliumjodid, Ketoprofen, Levodopa, Nitrazepam, Nitroprussid, Oxitetracyclin-HCl, Promethazin, Omeprazol oder andere Benzimidazolderivate oder Streptomycin enthalten können.

[0006] Wirkstoffklassen und Substanzen, die oftmals bitteren Geschmack hervorrufen können und sich mit den Überzugs- und Bindemittel gemäß der WO 02/67906 vorteilhafterweise auch geschmacksisolierend formulieren lassen sind z. B.: Analgetika und Antirheumatika: Paracetamol, Diclofenac, Aceclofenac,
Ibuprofen, Ketoprofen, Flubiprofen, Levacetylmethadol, Oxycodon
Psychopharmaka: Prometazine, Donepezil, Modafinil, Nefazodon, Reboxetin, Sertindol, Sertralin
Antibiotika: Erythromicyn, Roxithromycin, Clarithromycin, Grepafloxacin, Ciprofloxacin, Levofloxacin, Sparfloxacin, Trovafloxacin, Nevirapin
Betablocker: Propanolol, Metoprolol, Bisoprolol, Ne-

bivolol
Antidiabetika: Metformin, Miglitol, Repaglinid
H1 Antihistaminika: Diphenhydramin, Fexofenadin, Mizolastin
H2 Antihistaminika: Cimetidin, Nizatidin, Ticlopidin, Cetridin, Ranitidin, Vitamine: Thiaminenitrate;
sowie weitere Wirkstoffe: Chinidin-Sulfat, Amiloprilose-HCl, Pseudoephedrin-HCl, Sildenafil, Topiramat, Granisetron, Rebamipide, Chinin-HCl

Aufgabenstellung

[0007] Ein Problem bei vielen oralen Arzneiformen, ist daß das Herunterschlucken oftmals die Zuhilfenahme von Flüssigkeit, z. B. einem Schluck Wasser, erfordert. Dies ist ungünstig, wenn im Bedarfsfall kein Getränk zur Verfügung steht oder etwa die momentane berufliche Tätigkeit unterbrochen werden muß, um das Medikament einnehmen zu können. Für viele Patienten ist es zudem unangenehm in Gegenwart anderer Personen quasi beobachtet und Aufmerksamkeit erregend ihr Medikament einzunehmen, was umso auffälliger ist, wenn nach einem Getränk benutzt werden muß oder für diesen Zweck gar erbeten werden muß.

[0008] Viele Patienten, insbesondere zu nennen ältere Menschen und Kinder, wünschen daher orale Arzneiformen, die einfach und unauffällig praktisch an beliebigen Orten eingenommen werden können. Dies ist insbesondere bei Krankheiten der Fall, die sehr pünktlich oder bei Bedarf unverzüglich eingenommen werden sollen oder müssen, wie z. B. bei Schmerzmitteln.

[0009] Es besteht zusätzlich ein Bedarf an Arzneiformen, die den enthalten Wirkstoff z. B. Schmerzmittel bei oraler Einnahme bereits im Mund freisetzen und auf diese Weise rasch wirken können. Bekannte Applikationsformen sind z. B. verpreßten Tabletten oder Lutschtabletten, gefriergetrockneten Tabletten, gegossenen Tabletten oder Pastillen, Sachets, Kautabletten, Trockensäften und/oder flüssigkeitsgefüllten Bonbons.

[0010] Viele dieser schnell zerfallenden Arzneiformen haben jedoch den Nachteil, daß sie einen sandigen Mundgeschmack bewirken, der einige Minuten andauern kann, bis sich die Tablettenbestanteile völlig aufgelöst haben. Das sandige Mundgeschmack wird als unangenehm empfunden wird und kann einen Hustenreiz bewirken. Ein weiteres Problem ist dabei die Geschmacksisolierung von bitter schmeckenden Wirkstoffen. Wegen der Anforderung der Wirkstoffreisetzung im Mund können die bekannten geschmacksisolierenden Überzüge nicht verwendet werden.

[0011] Zur Lösung dieser Probleme sollte eine Arzneiform bereitgestellt werden, die ohne Flüssigkeit einnehmbar ist und den Wirkstoff ummittelbar freisetzt. Dabei soll ein sandiger Mundgeschmack ausbleiben. Die Arzneiform soll für eine Vielzahl von Wirkstoffen, insbesondere jedoch für Schmerzmittel der Klasse der Antirheumatika oder für Antibiotika geeignet sein.

[0012] Die Aufgabe wird gelöst durch ein Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, durch intensives Mischen

(a) eines anionischen pharmazeutischen Wirkstoffs mit

(b) einem Copolymer, bestehend aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren die funktionelle tertiäre Aminogruppen aufweisen, sowie

(c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure

in der Schmelze, Erstarren der Mischung und Mahlen zum einem wirkstoffhaltigen Pulver mit einer mittleren Korngröße von 200 µm oder weniger, Einbetten des Pulvers in eine wasserlösliche Matrix aus pharmazeutisch üblichen Hilfsstoffen, mit der Maßgabe, daß nicht mehr als 3 Gew.-%, bezogen auf das Copolymer, an Emulgatoren mit einem HLB-Wert von mindestens 14 enthalten sein dürfen.

[0013] In bisher nicht verstandener Weise ergeben sich die Vorteile der Erfindung anders als bei der WO 02/67906 nur bei anionischen Wirkstoffen. Möglicherweise ergibt sich eine thermisch induzierte Wechselwirkung der anspruchsgemäßen Bestandteile (a), (b) und (c), die in dieser Weise nicht aus der WO 02/67906 ableitbar ist. Die erfindungsgemäß erhältlichen Arzneiformen sind gut ohne zusätzliche Flüssigkeit einnehmbar und verursachen nach Wirkstofffreisetzung im Mund keinen sandigen Geschmack.

Ausführung der Erfindung

[0014] Die Erfindung betrifft ein Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, durch intensives Mischen

(a) eines anionischen pharmazeutischen Wirkstoffs mit (b) einem Copolymer, bestehend aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren die funktionelle tertiäre Aminogruppen aufweisen, sowie (c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure

in der Schmelze, Erstarren der Mischung und Mahlen zum einem wirkstoffhaltigen Pulver mit einer mittleren Korngröße von 200 µm oder weniger, Einbetten des Pulvers in eine wasserlösliche Matrix aus pharmazeutisch üblichen Hilfsstoffen, mit der Maßgabe, daß nicht mehr als 3 Gew.-%, bezogen auf das Copolymer, an Emulgatoren mit einem HLB-Wert von mindestens 14 enthalten sein dürfen.

Pharmazeutischer Wirkstoff (a)

[0015] Der anionische pharmazeutischen Wirkstoff, liegt bedingt durch die Herstellung in der Schmelze eingebettet im Copolymer in Form einer „solid solution" vor. Der Zustand der „solid solution" kann z. B. im Polarisationsmikroskop, thermoanalytisch (Differential Scanning Calorimetry (DSC)) oder im Röntgenbeugungsspektrum nachgewiesen werden.

[0016] Das Mengenverhältnis bezogen auf Gew.-% von Wirkstoff zu Copolymer liegt günstigerweise bei 2 zu 1 bis 1 zu 2. Bevorzugt ist das Copolymer in gleichen Mengen oder im Überschuß vorhanden.

[0017] Der anionischer Wirkstoff (a) ist bevorzugt ein anionisches Schmerzmittel ein anionisches Antirheumatikum oder ein anionisches Antibiotikum.

[0018] Das wirkstoffhaltige Pulver kann z. B. die folgenden anionischer Wirkstoff enthalten:

Acamprosat, Aceclofenac, Acemetacin, Acetylcystein, Acetylsalicylsäure, Acetyltyrosin, Acipimox, Acitretin, Alanin, Alendronsäure, Amethopterin, Aminosäuren, Amoxicillin, Ampicillin , Ascorbinsäure, Atorvastatin, Azidocillin, Aztreonam, Bacampicillin, Baclofen, Benazepril, Bendamustin, Benzylpenicillin, Bezafibrat, Biotin, Bornaprin, Bumetanid, Cabastin, Canrenoinsäure, Carbamoylphenoxyessigsäure, Carbidopa, Carbimazol, Carbocistein, Carisoprodol, Cefaclor, Cefadroxil, Cefalexin, Cefazolin, Cefepim, Cefetamet, Cefixim, Cefotaxim, Cefotiam, Cefoxitin, Cefpodoxim, Ceftazidim, Ceftibuten, Ceftriaxon, Cefuroxim, Cetirizin, Chenodeoxycholsäure, Chlorambucil, Cidofovir, Cilastatin, Cilazapril, Cinoxacin, Ciprofloxacin, Cisatracurium besilat, Clavulansäure, Clodronsäure, Clorazepat, Cromoglicinsäure, Desmeninol, Diclofenac, Dicloxacillin, Enoxacin, Eprosartan, Etacrynsäure, Etidronsäure, Etofyllin, Etomidat, Felbinac, Felodipin, Fenofibrat, Fexofenadin, Flavoxat, Fleroxacin, Flucloxacillin, Flufenaminsäure, Flumazenil, Flupirtin, Flurbiprofen, Fluvastatin, Fosfomycin, Fosinopril, Furosemid, Fusidinsäure, Gabapentin, Gemfibrozil, Ibandronsäure, Ibuprofen, Iloprost, Imidapril, Imipenem, Indomethacin, Irinotecan, Isradipin, Ketoprofen, Lercanidipin, Levodopa, Levofloxacin, Liothyronin, Liponsäure, Lisinopril, Lodoxamid, Lomefloxacin, Lonazolac, Loracarbef, Loratadin, Lovastatin, Mefenaminsäure, Meropenem, Mesalazin, Metamizol, Methotrexat, Methyldopa, Mezlocillin, Moexipril, Montelukast, Moxifloxacin, Mupirocin, Naproxen, Natamycin, Nateglinid, Nedocromil, Nicotinsäure, Nifedipin, Nilvadipin, Nimodipin, Nisoldipin, Nitrendipin, Norfloxacin, Ofloxacin, Olsalazin, Orotsäure, Oxacillin, Pamidronsäure, Pangamsäure, Penicillamin, Phenoxymethylpenicillin, Pentosanpolysulfat, Perindopril, Pethidin, Pipemidsäure, Piperacillin, Pirenoxin, Piretanid, Probenecid, Proglumid, Propicillin, Prostaglandine, Quinapril, Quinaprilat, Ramipril, Repaglinid, Reserpin, Risedronsäure, Salicylsäure, Sulfasalazin, Spirapril, Sulbactam, Sulfasalazin, Sultamicillin, Tazaroten, Tazobactam, Telmisartan, Tiagabin, Tiaprofensäure, Tilidin, Tiludronsäure, Trandolapril, Tranexamsäure, Valproinsäure Vigabatrin, Vincamin, Vinpocetin, Zanamivir, Zoledronsäure, Zopiclon und/oder deren Salze, Isomere und/oder Kombinationen enthalten sind.

Copolymer (b)

[0019] Die Copolymere (a) bestehen im wesentlichen oder ganz aus radikalisch polymerisierten C1- bis C4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren, die funktionelle tertiäre Aminogruppen aufweisen.

[0020] Geeignete Monomere mit funktionellen tertiären Aminogruppen sind in US 4 705 695, Spalte 3, Zeile 64 bis Spalte 4, Zeile 13 aufgeführt. Insbesondere zu nennen sind Dimethylaminoethylacrylat, 2-Dimethylaminopropylacrylat, Dimethylaminopropylmethacrylat, Dimethylaminobenzylacrylat, Dimethylaminobenzylmethacrylat, (3-Dimethylamino-2,2-dimethly)propylacrylat, Dimethylamino-2,2-dimethly)propylmethacrylat, (3-Diethylamino-2,2-dimethly)propylacrylat und Diethylamino-2,2-dimethly)propylmethacrylat. Besonders bevorzugt ist Dimethylaminoethylmethacrylat.

[0021] Der Gehalt der Monomere mit tertiären Aminogruppen im Copolymeren kann vorteilhafterweise zwischen 30 und 70 Gew.-%, bevorzugt zwischen 40 und 60 Gew.-% liegen. Der Anteile der C1- bis C4-Estern der Acryl- oder Methacrylsäure beträgt 70 – 30 Gew.-%. Zu nennen sind Methylmethacrylat, Ethylmethacrylat, Butylmethacrylat, Methylacrylat, Ethylacrylat, Butylacrylat.

[0022] Ein der Komponente (b) entsprechendes (Meth)acrylatcopolymer mit tertiären Aminogruppen kann z. B. aus 20 – 30 Gew.-% Methylmethacrylat, 20 – 30 Gew.-% Butylmethacrylat und 60 – 40 Gew.-% Dimethylaminoethylmethacrylat aufgebaut sein. Der Anteil der Komponente (a) an der Formulierung beträgt bevorzugt 50 – 90 Gew.-%.

[0023] Die Copolymere (b) werden in an sich bekannter Weise durch radikalische Substanz-, Lösungs-, Perl- oder Emulsionspolymerisation erhalten. Sie müssen vor der Verarbeitung durch geeignete Mahl-, Trocken- oder Sprühprozesse in geeignete Teilchengrößenbereich gebracht werden. Geeignet sind Granulate und Pulver. Geeignete Handelsprodukte sind z. B. EUDRAGIT® E 100 (Granulat) oder EUDRAGIT® E PO (Pulver).

Komponente (c)

[0024] Komponente (c): 5 bis 50, bevorzugt 10 bis 20 Gew. % (bezogen auf die Copolymer-Komponente (b) einer C_{12}- bis C_{22}-Carbonsäure. Die Komponente (c) ist wichtig für die Verarbeitbarkeit. Bevorzugt sind unverzweigte C_{12}- bis C_{22}-Monocarbonsäuren. Es können gegebenenfalls auch verzweigte Derivate der genannten Substanzen geeignet sein.

[0025] C_{12}- bis C_{22}-Monocarbonsäuren sind z.B insbesondere Laurinsäure und Myristinsäure. Bevorzugt

sind Palmitinsäure und Stearinsäure.

Emulgatoren mit einem HLB-Wert von mindestens 14

[0026] Emulgatoren mit einem HLB-Wert von mindestens 14 sollen zu weniger als 3 Gew.-%, bevorzugt weniger als 2 oder 1 Gew. %, insbesondere soll kein solcher Emulgator enthalten sein. Der Grund dafür liegt in der Pulverstruktur des Ausgangsmaterials, in dem der Eigengeschmack solcher Emulgatoren besonders hervortritt. Überraschenderweise ist im Gegensatz zur Lehre der WO 02/67906 unter Anwendung des speziellen erfindungsgemäßen Verfahrens ein Verzicht auf den Emulgatoreinsatz möglich.

[0027] Emulgatoren oder Tenside sind grenzflächenaktive Substanzen mit lyobipolarem Charakter, d.h. in ihrem Molekül müssen unpolare, lipophile und polare, hydrophile Zentren vorliegen (P.H. List, Arzneiformenlehre, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1982,Kap. 6.2.). Je nach molekularem Aufbau unterscheidet man zwischen ionogenen und nichtionogen Emulgatoren.

[0028] Der HLB-Wert ist ein 1950 von Griffin eingeführtes Maß der Hydrophilie bzw. Lipophilie von nichtionischen Tensiden. Er läßt sich experimentell durch die Phenol-Titrationsmethode nach Marszall bestimmen; vgl. "Parfümerie, Kosmetik", Band 60, 1979, S. 444 – 448; weitere Literaturhinweise in Römpp, Chemie-Lexikon, 8.Aufl. 1983, S.1750. Siehe weiterhin z. B. US 4 795 643 (Seth)).

[0029] Ein HLB-Wert (Hydrophile/Lipophile Balance) läßt sich nur bei nicht ionischen Emulgatoren exakt bestimmen. Bei anionischen Emulgatoren kann dieser Wert rechnerisch ermittelt werden, liegt jedoch praktisch immer über oder weit über 14.

[0030] Unter Emulgatoren mit einem HLB-Wert über 14 werden hydrophile, nicht ionische Emulgatoren mit HLB – Bereich von mindestens 14 sowie ebenfalls hydrophile, anionische Emulgatoren und deren Salze, die einen rechnerischen HLB-Wert über 14 aufweisen, verstanden. Beispiele für Emulgatoren mit einem HLB-Wert über 14 sind z. B. Natriumlaurylsulfat und Natriumcetylstearylsulfat, Saccharosestearat und Polysorbat 80.

[0031] Emulgatoren mit HLB-Werten von weniger als 14, wie z. B. Glycerolmonostearat können hingegen auch in Mengen von mehr als 3 Gew.-% enthalten sein.

Pharmazeutisch übliche Hilfsstoffe

[0032] Das Pulvers wird in eine wasserlösliche Matrix aus pharmazeutisch üblichen Hilfsstoffen eingebettet.

Füll- und Bindemittel

[0033] Die wasserlösliche Matrix wird überwiegend gebildet aus Füll- und Bindemitteln. Bevorzugt sind dies z. B. wasserlösliche Mono-, Di, Oligo- oder Polysaccharide oder deren Derivate, weiterhin Peptide, Proteine etc.. Beispiele sind z. B. Lactose, Fructose, Glucose, Dextrose, Galaktose, Mannit, Rhamnose, Tragant, Dextrin, Guar Gum, Sorbitol, Xylitol, Isomatose, Saccharose, Maltose, Hydroxypropylmethylcellulose (HPMC), Stärkehydrolysate, Gelatine.

[0034] Einsatzmengen und Verwendung der üblichen Zuschlagstoffe in Arzneimittelüberzügen oder Beschichtungen sind dem Fachmann geläufig. Übliche Zuschlagstoffe können z. B. Trennmittel, Pigmente, Stabilisatoren, Antioxidantien, Porenbildner, Penetrationsförderer, Aromastoffe oder Geschmacksmittel sein. Sie dienen als Verarbeitungshilfsmittel und sollen ein sicheres und reproduzierbares Herstellungsverfahren sowie gute Langzeitlagerstabilität gewährleisten oder sie erreichen in der Arzneiform zusätzliche vorteilhafte Eigenschaften.

Trennmittel:

[0035] Trennmittel besitzen in der Regel lipophile Eigenschaften und werden in der Regel den Sprühsuspensionen zugesetzt. Sie verhindern eine Agglomeration der Kerne während der Befilmung. Bevorzugt werden Talkum, Mg- oder Ca- Stearat, gemahlene Kieselsäure, Kaolin oder nicht ionische Emulgatoren mit einem HLB – Wert zwischen 3 und 8 eingesetzt. Übliche Einsatzmengen für Trennmittel in den erfindungsgemäßen Überzugs- und Bindemitteln liegen zwischen 0,1 bis 10 Gew.-% bezogen auf die Arzneiform.

Pigmente:

[0036] Der Zusatz erfolgt nur selten in Form des löslichen Farbstoffs. In der Regel dispergiert man Aluminium- oder Eisenoxidpigmente. Übliche Einsatzmengen für Pigmente in den erfindungsgemäßen Überzugs- und Bindemitteln zwischen 1 und 10 Gew.-%, bezogen auf die Arzneiform.

[0037] Grundsätzlich müssen natürlich alle eingesetzten Substanzen toxikologisch unbedenklich und in Arzneimitteln ohne Risiko für Patienten zu verwenden sein.

[0038] Weitere Zuschlagstoffe können auch Weichmacher sein. Übliche Mengen liegen zwischen 0 und 50, bevorzugt 0 bis 20, insbesondere 0 bis 10 Gew.-%. Besonders bevorzugt sind allerdings höchstens 5 Gew.-% oder kein Weichmacher enthalten, da die Formulierungen durch die Anwesenheit der Komponenten (c) häufig bereits elastisch genug sind und zusätzlicher Weichmacher zu unerwünschter Klebrigkeit führen kann.

Weichmacher:

[0039] Weichmacher können je nach Typ (lipophil oder hydrophil) und zugesetzter Menge die Funktionalität der Polymerschicht beeinflussen. Weichmacher erreichen durch physikalische Wechselwirkung

mit dem Polymeren eine Absenkung der Glasübergangstemperatur und fördern in Abhängigkeit von der zugesetzten Menge die Verfilmung. Geeignete Stoffe haben in der Regel ein Molekulargewicht zwischen 100 und 20.000 und enthalten eine oder mehrere hydrophile Gruppen im Molekül, z. B. Hydroxyl-, Ester- oder Aminogruppen.

[0040] Beispiele geeigneter Weichmacher sind Citronensäurealkylester, Glycerinester, Phthalsäurealkylester, Sebacinsäurealkylester, Succroseester, Sorbitanester, Diethylsebacat, Dibutylsebacat und Polyethylenglykole 200 bis 12.000. Bevorzugte Weichmacher sind Triethylcitrat (TEC), Acetyltriethylcitrat (ATEC) und Dibutylsebacat (DBS). Weiterhin zu nennen sind in der Regel bei Raumtemperatur flüssige Ester wie Citrate, Phthalate, Sebacate oder Rizinusöl. Bevorzugt werden Zitronensäure- und Sebacinsäureester verwendet.

[0041] Auch können Mischungen von Weichmachern eingesetzt werden.

Das Herstellungsverfahren

[0042] Das erfindungsgemäße Verfahren sieht die Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, durch intensives Mischen der Komponenten (a), (b) und (c) im der Schmelze vor. Geeignet sind Verarbeitungstemperaturen im Bereich von 80 bis 200 °C, bevorzugt von 100 bis 180 °C. Bevorzugt setzt man zum Zweck des intensiven Mischens in der Schmelze einen Doppelschneckenextruder ein. Nach dem Erstarren wird die Mischung zum einem wirkstoffhaltigen Pulver gemahlen. Die mittleren Korngröße des Pulvers soll 200 µm oder weniger, bevorzugt 50 bis 150 µm betragen.

[0043] Die mittlere Korn- bzw. Teilchengröße der Pulver kann wie folgt bestimmt werden: Durch Luftstrahlsiebung zur einfachen Aufteilung des Mahlproduktes in wenige Fraktionen. Diese Methode ist in diesem Meßbereich etwas ungenauer als die Alternativen. Mindestens 70, bevorzugt 90 % der Teilchen bezogen auf die Masse (Masseverteilung) sollen jedoch in dem erfindungsgemäßen Größenbereich von 200 µm oder weniger, bevorzugt von 50 bis 150 µm liegen. Eine gut geeignete Meßmethode ist die Laserbeugung zur Bestimmung der Korngrößenverteilung. Handelsübliche Geräte erlauben die Messung in Luft (Fa. Malvern S3.01 Partikelsizer) oder bevorzugt in flüssigen Medien (Fa. LOT, Galai CIS 1). Voraussetzung für die Messung in Flüssigkeiten ist, das sich das Polymer darin nicht löst oder die Teilchen auf eine andere Weise während der Messung verändern. Ein geeignetes Medium ist z. B. eine stark verdünnte (ca. 0,02%ige) wäßrige Polysorbat 80 Lösung.

[0044] Das wirkstoffhaltige Pulver kann zu einer Tablette, Lutschtablette, gefriergetrockneten Tabletten, gegossenen Tabletten oder Pastillen, Sachets, Kautabletten, Trockensäften, Bonbons und/oder flüssigkeitsgefüllten Bonbons verarbeitet werden.

[0045] Diese Verarbeitung erfolgt in der Regel in mehreren Schritten. Zunächst wird das wirkstoffhaltige Copolymerpulver mit pharmazeutischen Hilfsstoffen gemischt und kann z. B. direkt zu Tabletten, Lutschtabletten oder Kautabletten verpreßt werden. Das Gemisch kann auch mit Wasser angeteigt, in eine Form gefüllt und gefriergetrocknet werden, so daß man gefriergetrocknete Tabletten erhält. Gegossene Tabletten oder Pastillen können erhalten werden, indem man das wirkstoffhaltige Copolymerpulver, z. B. mit einer Sacchandlösung bei erhöhter Temperatur mischt, in eine Form, Z. B. für Tabletten oder Bonbons gießt und durch Abkühlen erstarren läßt. Flüssigkeitsgefüllte Bonbons können erzeugt werden, indem man ein flüssiges Gemisch, enthaltend das wirkstoffhaltige Copolymerpulver z. B. in einer Zuckerlösung in eine feste Hülle aus z. B. einem Zucker einspritzt und diese anschließend verschließt.

Wirkstoffhaltiges Pulver

[0046] Das wirkstoffhaltige Pulver hat eine mittlere Korngröße von 200 µm oder darunter, bevorzugt 50 bis 150 µm und enthält

(a) einen anionischen pharmazeutischen Wirkstoff, der in Form einer solid solution vorliegt und eingebettet ist in

(b) ein Copolymer, welches aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren die funktionelle tertiäre Aminogruppen aufweisen, besteht, sowie

(c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure,

(d) mit der Maßgabe, daß kein oder weniger als 3 Gew.-%, bezogen auf das Copolymer, eines Emulgators mit einem HLB-Wert von mindestens 14 enthalten ist.

Verwendungen

[0047] Das wirkstoffhaltiges Pulvers kann zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, die nach Freisetzung für mindestens 30 Sekunden keinen bitteren Geschmack hervorruft, verwendet werden. Die Arzneiform kann in Form von verpreßten Tabletten oder Lutschtabletten, gefriergetrockneten Tabletten, gegossenen Tabletten oder Pastillen, Sachets, Kautabletten, Trockensäften, Bonbons und/oder flüssigkeitsgefüllten Bonbons vorliegen.

Bitterwerte

[0048] Die Überprüfung der Geschmacksisolierung kann auf einfache Weise organoleptisch durch Verkosten erfolgen. Bei dieser Prüfung soll nach Wirkstoffreisetzung für mindestens 30 Sekunden noch kein oder leicht bitterer Geschmack wahrnehmbar sein. Genauer ist die Bestimmung von Bitterwerten.

Kein oder leicht bitterer Geschmack entspricht Bitterwerten unter 1000.

[0049] Bitterwerte können nach DAB 1999 Methode 2.8.N8 (Bestimmung des Bitterwertes) bestimmt werden.

[0050] Während z. B. Ibuprofen einen Bitterwert um die 100.000 hat, liegt der erfindungsgemäße Wert für einen eingebetteten anionischen Wirkstoff in der Regel unter 1000, bevorzugt unter 100. Ein Bitterwert von 1000 ist für pharmazeutische Praxis in der Regel ausreichend.

Ausführungsbeispiel

[0051] In den Beispielen verwendete Copolymere:
EUDRAGIT® E PO: Copolymerpulver aus Methylmethacrylat, Butylmethacrylat, und Dimethylaminoethylmethacrylat in Verhältnis 25 : 25 : 50 mit einer mittleren Teilchengröße von 15 µm.
EUDRAGIT® E 100 : Copolymer aus Methylmethacrylat, Butylmethacrylat, und Dimethylaminoethylmethacrylat in Verhältnis 25 : 25 : 50 im Granulatform.

[0052] Die Wirksamkeit der Geschmacksisolierung wurde organoleptisch durch Verkosten geprüft. Dabei wurde die Zeit zwischen Aufnahme in den Mund und Auftreten des bitteren Geschmacks ermittelt.

Beispiel 1:

[0053] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 1 mol Stearinsäure : 0,66 mol Ibuprofen : 0,18 mol Talk.

[0054] Es wurden 39,42 g EUDRAGIT® E PO, 35,2 g Stearinsäure, 16,9 g Ibuprofen und 8,4 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0055] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 2 min nicht bitter.

Beispiel 2:

[0056] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO : 0,5 mol Stearinsäure : 0,66 mol Ibuprofen 0,18 mol Talk.

[0057] Es wurden 47,85 g EUDRAGIT® E PO, 21,38 g Stearinsäure, 20,5 g Ibuprofen und 10,25 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0058] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 2 min nicht bitter bis leicht bitter.

Beispiel 3:

[0059] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO : 0,65 mol Stearinsäure : 0,65 mol Ibuprofen 0,18 mol Talk

[0060] Es wurden 44,8 g EUDRAGIT® E PO, 26,4 g Stearinsäure, 19,2 g Ibuprofen und 9,6 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0061] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 2 min nicht bitter.

Beispiel 4:

[0062] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,33 mol Stearinsäure : 0,66 mol Ibuprofen 0,18 mol Talk

[0063] Es wurden 51,6 g EUDRAGIT® E PO, 15,23 g Stearinsäure, 22,1 g Ibuprofen und 11 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0064] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 1 min leicht bitter.

Beispiel 5:

[0065] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,34 mol Stearinsäure : 1 mol Ibuprofen : 0,27 mol Talk.

[0066] Es wurden 34,73 g EUDRAGIT® E PO, 15,52 g Stearinsäure, 33,1 g Ibuprofen und 16,58 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0067] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 1 min bitter.

Vergleichsbeispiel 6: (Wirkstoff Coffein nicht erfindungsgemäß)

[0068] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO : 0,5 mol Stearinsäure : 1,58 mol Cof-

fein (F_p: 234 – 239 °C).

[0069] Es wurden 41,47 g EUDRAGIT® E PO, 18,53 g Stearinsäure, 40 g Coffein eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0070] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 10 s bitter.

Vergleichsbeispiel 7: (ohne Stearinsäure)

[0071] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,67 mol Ibuprofen : 0,18 mol Talk.

[0072] Es wurden 60 g EUDRAGIT® E PO, 26,4 g Ibuprofen und 13,2 g Talk eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0073] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 10 s bitter.

Vergleichsbeispiel 8: (Wirkstoff Paracetamol nicht erfindungsgemäß)

[0074] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,5 mol Stearinsäure : 2,03 mol Paracetamol (F_p: 168 – 172 °C).

[0075] Es wurden 41,47 g EUDRAGIT® E PO, 18,53 g Stearinsäure, 40 g Paracetamol eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0076] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er sofort bitter.

Vergleichsbeispiel 9: (Wirkstoff Paracetamol nicht erfindungsgemäß)

[0077] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E : 0,5 mol Stearinsäure : 1 mol Paracetamol.

[0078] Es wurden 41,5 % EUDRAGIT® E 100, 18,53 % Stearinsäure und 40 % Paracetamol zusammen in einem 18 mm Doppelschneckenextruder in einem Temperaturbereich von 100 °C bis 172 °C extrudiert. In dem Bereich des Extruders, wo 172 °C bestanden, war die Schnecke besonders mischintensiv ausgelegt um eine homogene Schmelze zu erreichen.

[0079] Der so entstandene Compound schmeckt sofort bitter.

Beispiel 10:

[0080] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,06 mol Stearinsäure : 0,77 mol Ibuprofen.

[0081] Es wurden 100 g EUDRAGIT® E PO, 5 g Stearinsäure und 50 g Ibuprofen eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0082] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 30 – 60 s min bitter.

Beispiel 11:

[0083] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,12 mol Stearinsäure : 0,77 mol Ibuprofen.

[0084] Es wurden 100 g EUDRAGIT® E PO, 10 g Stearinsäure und 50 g Ibuprofen eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0085] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 1 min bitter.

Vergleichsbeispiel 12 (C_{12}-Alkohol-Verbindung anstelle von Stearinsäure)

[0086] Compound mit 1 mol Dimethylaminoethylmethacrylat-Einheiten enthalten im Copolymer EUDRAGIT® E PO: 0,34 mol Dodecanol : 0,77 mol Ibuprofen.

[0087] Es wurden 100 g EUDRAGIT® E PO, 20 g Dodecanol und 50 g Ibuprofen eingewogen und zusammen in den auf 100 °C vorgewärmten IKA Messkneter gegeben, wo die Mischung bei 100 °C Produkttemperatur für 20 min mit 60 U/min (2 Knetschaufeln) geknetet wurde. Die Mischung wurde dem Messkneter entnommen und mit Trockeneis abgekühlt.

[0088] Nimmt man 1 g dieses Compounds in den Mund, schmeckt er nach 20 s bitter und weist den unangenehmen Geschmack von Dodecanol auf.

Patentansprüche

1. Verfahren zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofffreisetzung bereits im Mund, durch intensives Mischen

(a) eines anionischen pharmazeutischen Wirkstoffs mit

(b) einem Copolymer, bestehend aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren die funktionelle tertiäre Aminogruppen aufweisen, sowie
(c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure
in der Schmelze, Erstarren der Mischung und Mahlen zum einem wirkstoffhaltigen Pulver mit einer mittleren Korngröße von 200 µm oder weniger, Einbetten des Pulvers in eine wasserlösliche Matrix aus pharmazeutisch üblichen Hilfsstoffen, mit der Maßgabe, daß nicht mehr als 3 Gew.-%, bezogen auf das Copolymer, an Emulgatoren mit einem HLB-Wert von mindestens 14 enthalten sein dürfen.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man zum Zweck des intensiven Mischens in der Schmelze einen Doppelschneckenextruder einsetzt.

3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß man bei Temperaturen im Bereich von 80 bis 200 °C extrudiert.

4. Verfahren nach einem oder mehreren der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß das Einbetten des Pulvers in die wasserlösliche Matrix durch Verpressen, Gießen, Granulieren oder Gefriertrocknen erfolgt.

5. Wirkstoffhaltiges Pulver mit einer mittleren Korngröße von 200 µm oder darunter, enthaltend
(a) einen anionischen pharmazeutischen Wirkstoff, welcher in Form einer solid solution vorliegt und eingebettet ist in
(b) ein Copolymer, welches aus radikalisch polymerisierten C_1- bis C_4-Estern der Acryl- oder Methacrylsäure und weiteren (Meth)acrylat-Monomeren die funktionelle tertiäre Aminogruppen aufweisen, besteht, sowie
(c) 5 bis 50 Gew.-%, bezogen auf (b), einer C_{12}- bis C_{22}-Carbonsäure,
(d) mit der Maßgabe, daß kein oder weniger als 3 Gew.-%, bezogen auf das Copolymer, eines Emulgators mit einem HLB-Wert von mindestens 14 enthalten ist.

6. Wirkstoffhaltiges Pulver nach Anspruch 5, dadurch gekennzeichnet, daß als anionischer Wirkstoff (a) ein anionisches Schmerzmittel bzw. ein anionisches Antirheumatikum oder ein anionisches Antibiotikum enthalten ist.

7. Wirkstoffhaltiges Pulver nach Anspruch 5 oder 6, dadurch gekennzeichnet, daß als anionischer Wirkstoff (a) Acamprosat, Aceclofenac, Acemetacin, Acetylcystein, Acetylsalicylsäure, Acetyltyrosin, Acipimox, Acitretin, Alanin, Alendronsäure, Amethopterin, Aminosäuren, Amoxicillin, Ampicillin, Ascorbinsäure, Atorvastatin, Azidocillin, Aztreonam, Bacampicillin, Baclofen, Benazepril, Bendamustin, Benzylpenicillin, Bezafibrat, Biotin, Bornaprin, Bumetanid, Cabastin, Canrenoinsäure, Carbamoylphenoxyessigsäure, Carbidopa, Carbimazol, Carbocistein, Carisoprodol, Cefaclor, Cefadroxil, Cefalexin, Cefazolin, Cefepim, Cefetamet, Cefixim, Cefotaxim, Cefotiam, Cefoxitin, Cefpodoxim, Ceftazidim, Ceftibuten , Ceftriaxon, Cefuroxim, Cetirizin, Chenodeoxycholsäure, Chlorambucil, Cidofovir, Cilastatin, Cilazapril, Cinoxacin, Ciprofloxacin, Cisatracurium besilat, Clavulansäure, Clodronsäure, Clorazepat, Cromoglicinsäure, Desmeninol, Diclofenac, Dicloxacillin, Enoxacin, Eprosartan, Etacrynsäure, Etidronsäure, Etofyllin, Etomidat, Felbinac, Felodipin, Fenofibrat, Fexofenadin, Flavoxat, Fleroxacin, Flucloxacillin, Flufenaminsäure, Flumazenil, Flupirtin, Flurbiprofen, Fluvastatin, Fosfomycin, Fosinopril, Furosemid, Fusidinsäure, Gabapentin, Gemfibrozil, Ibandronsäure, Ibuprofen, Iloprost, Imidapril, Imipenem, Indomethacin, Irinotecan, Isradipin, Ketoprofen, Lercanidipin , Levodopa, Levofloxacin, Liothyronin, Liponsäure, Lisinopril, Lodoxamid, Lomefloxacin, Lonazolac, Loracarbef, Loratadin, Lovastatin, Mefenaminsäure, Meropenem, Mesalazin, Metamizol, Methotrexat, Methyldopa, Mezlocillin, Moexipril, Montelukast, Moxifloxacin, Mupirocin, Naproxen, Natamycin, Nateglinid, Nedocromil, Nicotinsäure, Nifedipin, Nilvadipin, Nimodipin, Nisoldipin, Nitrendipin, Norfloxacin, Ofloxacin, Olsalazin, Orotsäure, Oxacillin, Pamidronsäure, Pangamsäure, Penicillamin, Phenoxymethylpenicillin, Pentosanpolysulfat, Perindopril, Pethidin, Pipemidsäure, Piperacillin, Pirenoxin, Piretanid, Probenecid, Proglumid, Propicillin, Prostaglandine, Quinapril, Quinaprilat, Ramipril, Repaglinid, Reserpin, Risedronsäure, Salicylsäure, Sulfasalazin, Spirapril, Sulbactam, Sulfasalazin, Sultamicillin, Tazaroten, Tazobactam, Telmisartan, Tiagabin, Tiaprofensäure, Tilidin, Tiludronsäure, Trandolapril, Tranexamsäure, Valproinsäure Vigabatrin, Vincamin, Vinpocetin, Zanamivir, Zoledronsäure, Zopiclon und/oder deren Salze, Isomere und/oder Kombinationen enthalten sind.

8. Verwendung eines wirkstoffhaltiges Pulvers nach einem oder mehreren der Ansprüche 5 bis 7 zur Herstellung einer oralen Arzneiform mit unmittelbarem Zerfall und Wirkstofftreisetzung bereits im Mund, die nach Freisetzung für mindestens 30 Sekunden keinen oder nur einen leicht bitteren Geschmack hervorruft.

9. Verwendung des wirkstoffhaltiges Pulvers nach Anspruch 8 zur Herstellung von Arzneiformen wie verpreßten Tabletten oder Lutschtabletten, gefriergetrockneten Tabletten, gegossenen Tabletten oder Pastillen, Sachets, Kautabletten, Trockensäften, Bonbons und/oder flüssigkeitsgefüllten Bonbons.

Es folgt kein Blatt Zeichnungen

(19) BUNDESREPUBLIK DEUTSCHLAND

DEUTSCHES PATENT- UND MARKENAMT

(12) **Offenlegungsschrift**

(10) **DE 100 16 077 A 1**

(51) Int. Cl.[7]:
C 12 Q 1/02
C 12 M 1/34
// (G06F 19/00, 159:00)

(21) Aktenzeichen: 100 16 077.8
(22) Anmeldetag: 31. 3. 2000
(43) Offenlegungstag: 13. 12. 2001

(71) Anmelder:
CellControl Biomedical Laboratories GmbH, 82152 Planegg, DE

(74) Vertreter:
Vossius & Partner, 81675 München

(72) Erfinder:
Dietel, Alfred, 84079 Bruckberg, DE

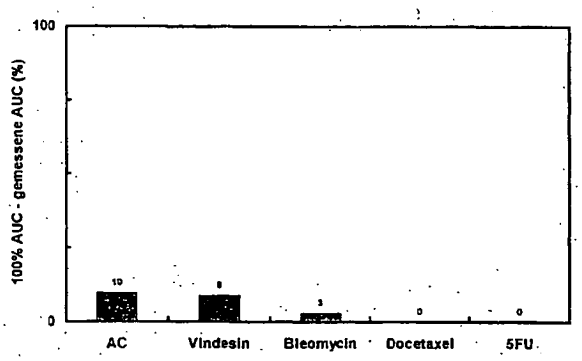
(56) Entgegenhaltungen:
EP 09 74 232 A2

Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

Prüfungsantrag gem. § 44 PatG ist gestellt

(54) Verfahren und Vorrichtung zum automatischen Nachweisen einer Wirkung eines zellbeeinflussenden Mittels auf lebende Zellen

(57) Die Erfindung betrifft ein Verfahren und eine Vorrichtung zum automatischen Nachweisen einer Wirkung eines zellbeeinflussenden Mittels, insbesondere eines Zytostatikums, auf lebende Zellen zum prätherapeutischen Evaluieren eines wirksamen Zytostatikums für die Chemotherapie.



DE 100 16 077 A 1

DE 100 16 077 A 1

Beschreibung

[0001] Die Erfindung betrifft ein Verfahren und eine Vorrichtung zum automatischen Nachweisen einer Wirkung eines zellbeeinflussenden Mittels, insbesondere eines Zytostatikums, auf lebende Zellen.

[0002] In der Bundesrepublik Deutschland treten jährlich etwa 350.000 neue maligne Krebserkrankungen auf, wobei 1994 insgesamt 210.000 Todesfälle registriert wurden. Bösartige Krebserkrankungen sind die mit Abstand zweithäufigste Todesursache nach Herz- und Kreislauferkrankungen. Bislang stehen drei Behandlungsmethoden zur Verfügung: die chirurgische Tumorentfernung, die Strahlentherapie und die Chemotherapie. Letztere wird vor allem bei der systematischen Therapie oder inoperablen Tumoren oder metastasierenden Karzinomen eingesetzt, so z. B. bei der Behandlung von Leukämien, Lymphomen, Hodentumoren, Chorionkarzinom, Brustkrebs oder dem Lungenkarzinom. Allerdings liegen die Heilungschancen bei der Chemotherapie nur bei etwa 10%.

[0003] Bei Chemotherapeutika unterscheidet man zwischen mehreren Stoffklassen: alkylierende Substanzen, Antimetabolite, Antibiotika, Mitosehemmstoffe, Hormone und -antagonisten. Bei der kurativen Therapie macht man sich die Zellkinetik zunutze. Da sich Tumorzellen schneller teilen als gesunde, reagieren sie auf Zytostatika wesentlich empfindlicher als normale Körperzellen. Die Selektivität der Zytostatikawirkung ist jedoch eng begrenzt, da ein gezielter Einsatz an der Tumorzelle kaum möglich ist. Als Folge kommt es meist zu Belastungen durch Nebenwirkungen, und bei wiederholten Behandlungszyklen durch Zytostatika besteht die Gefahr der Resistenzentwicklung von Tumorzellen. Um dies zu vermeiden, wird oft eine Kombinationstherapie aus unterschiedlichen Chemotherapeutika angewendet.

[0004] Die Mehrzahl der Tumorarten verhält sich jedoch heterogen und spricht unterschiedlich auf Zytostatika an.

[0005] Der Erfindung liegt die Aufgabe zugrunde, ein Verfahren und eine Vorrichtung bereitzustellen, mit denen prätherapeutisch ein wirksames Zytostatikum evaluiert wird und somit eine effiziente Behandlung des Patienten erlauben. Diese Aufgabe wird mit den Merkmalen der Ansprüche gelöst.

[0006] Die Erfindung geht von dem Grundgedanken aus, die zu untersuchenden lebenden Zellen mit mindestens einem zellbeeinflussenden Mittel, wie z. B. einem Zytostatikum in Kontakt zu bringen und die Zellaktivität bzw. Zellvitalität zu erfassen. Insbesondere wird hierbei die pH-Wert Veränderung erfaßt. Parallel dazu wird die Zellaktivität bzw. Zellvitalität der lebenden Zellen in unbehandelter Form und/oder die Zellaktivität bzw. Zellvitalität der mit dem Lösungsmittel des zellbeeinflussenden Mittels behandelten Zellen als Referenzwerte ermittelt. Die Meßdaten der mit dem mindestens einen zellbeeinflussenden Mittel behandelten lebenden Zellen werden in Beziehung zu den Referenzwerten gesetzt. Aus diesem Vergleich kann auf die Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen geschlossen werden. Vorzugsweise werden die lebenden Zellen mit mehreren unterschiedlichen zellbeeinflussenden Mitteln parallel behandelt, so daß anhand der Vergleiche zwischen den jeweiligen Meßdaten und den Referenzwerten die unterschiedliche Wirkungsweise bzw. Wirksamkeit des zellbeeinflussenden Mittels in Form einer Rangordnung angegeben werden kann. Somit kann prätherapeutisch aus einer Anzahl möglicher Wirkstoffe der wirksamste für den konkreten Patienten ermittelt werden. Dazu wird eine Biopsieprobe von vitalen Gewebe- bzw. Zellmaterialien, die dem Patienten entnommen werden, verwendet.

[0007] Bevorzugte Ausführungsformen der Erfindung sind in den abhängigen Ansprüchen angegeben.

[0008] Der Vorteil des erfindungsgemäßen Verfahrens bzw. der erfindungsgemäßen Vorrichtung liegt darin, daß für eine Therapie in Frage kommende Zytostatika eine Vorhersage getroffen werden kann, welches Medikament die höchste Wirksamkeit für eine erfolgreiche Behandlung des Tumors erzielen wird. Erfindungsgemäß wird dazu automatisch eine Rangfolge der getesteten Zytostatika sowie eine Aussage über die Höhe der Wirksamkeit jedes einzelnen Zytostatikums bereitgestellt. Entsprechend kann dies für Substanzkombinationen erstellt werden. Die Erfindung zeigt weiterhin Resistenzeigenschaften der Zellen auf das verwendete Medikament auf. Dadurch können unwirksame Medikamente bereits vor Beginn einer Therapie erkannt und von der Behandlung ausgeschlossen werden. Dies reduziert zudem die Belastungen des Patienten durch Nebenwirkungen der verwendeten und eventuell unwirksamen Wirkstoffe. Zudem kann eine weitere Metastierung des Tumors eingeschränkt werden. Neben den gesundheitlichen Aspekten für den Patient sind auch finanzielle Aspekte für den Kostenträger der Therapie zu nennen. Der Kostenträger "spart" sich eine unwirksame Therapie und kann individuell und gezielt seine Mittelverwendung gegenüber dem Patienten einsetzen. Die Effektivität und Rentabilität der eingesetzten Mittel steigt somit enorm, da die Kosten der eventuell in Frage kommenden Chemotherapie signifikant differieren.

[0009] Ferner erlaubt das erfindungsgemäße Verfahren, zwischen Effekten von metabolisierenden Zellen und Tumorzellen zu unterscheiden.

[0010] Das erfindungsgemäße Verfahren bzw. die erfindungsgemäße Vorrichtung wird erfindungsgemäß zur Untersuchung von vitalen Zellen, beispielsweise Tumorzellen, frischen Biopsieproben und Blutzellen, von Körperflüssigkeiten jeder Art, von Wirkstoffen und Medikamenten, von Antikrebsmitteln, von Zytostatika, von Antikörpern, von Hormonen und Antihormonen und von Gentherapeutika verwendet. Insbesondere wird das Verfahren/die Vorrichtung zur Untersuchung der Wirksamkeit von Zytostatika wie etwa Carboplatin, Cylophosphamid, Cisplatin, Docetaxel, Lomustin, Mitomycin, Treosulfan, Epirubicin, 5-Fluorouracil (5FU), Paclitaxel, Methotrexat, Bendamustin, Vinorelbin, Vindesin, Bleomycin Gemcitabin, Adriamycin, Mitoxantron, Irinotecan, Etoposit und jegliche Art von Kombinationstherapeutika (z. B. CMF, AC, FEM, usw.) daraus verwendet.

[0011] Die Erfindung wird nachstehend mit Bezug auf die beigefügten Zeichnungen näher erläutert. Es zeigen:

[0012] **Fig.** 1 den zeitlichen Verlauf der Tumorzellenaktivität für fünf ausgewählte Zytostatika;

[0013] **Fig.** 2 ein Diagramm, das die Wirksamkeit der verwendeten Zytostatika angibt;

[0014] **Fig.** 3 ein Diagramm, das die gemessene Tumorzellenaktivität für die verwendeten Zytostatika angibt;

[0015] **Fig.** 4 ein Diagramm, das die Tumorzellenaktivität für die verwendeten Zytostatika anhand eines Teils der Meßdaten angibt; und

[0016] **Fig.** 5 ein Flußdiagramm einer bevorzugten Ausführungsform des erfindungsgemäßen Verfahrens.

[0017] **Fig.** 1 zeigt den zeitlichen Verlauf der gemessenen Tumorzellaktivität für fünf verschiedene Zytostatika (Docetaxel, AC (Adriamycin-Cyclophosphamid-Kombination), Bleomycin, Vindesin, 5FU). Es ist zu erkennen, daß für die Zytostatika Docetaxel und 5FU die Tumorzellaktivität während des Meßzeitraumes sogar zunimmt, während für die übrigen Zytostatika eine Abnahme der Tumorzellaktivität ersichtlich ist. Die gemessenen Verläufe der Tumorzellaktivitäten werden anhand einer Kontrollzelle normiert. Hierzu

wird die Aktivität von unbehandelten Zellen gemessen. Alternativ dazu werden die lebenden Zellen lediglich mit dem Lösungsmittel des Zytostatikums versetzt und die Zellaktivität ermittelt.

[0018] Die gemessenen Tumorzellaktivitäten der mit den unterschiedlichen Wirkstoffen versetzten Zellen werden anschließend mit den Referenzwerten der Kontrollzellen bzw. der Zellen mit Lösungsmittelzusatz verglichen. Dazu wird gemäß einer ersten Ausführungsform die Fläche der jeweiligen Meßkurve (AUC – area under curve) für den spezifischen Wirkstoff von der Fläche der Referenzkurve (100%-Kurve) subtrahiert. Die Größe der Differenz gibt die Wirksamkeit des verabreichten Wirkstoffes an, wobei eine größere Differenz einer höheren Wirksamkeit entspricht. Dies ist in **Fig.** 2 dargestellt, wobei hier die Wirkstoffe bereits nach ihrer Wirksamkeit sortiert aufgeführt sind.

[0019] Alternativ dazu wird entweder über den gesamten Meßzeitraum (**Fig.** 3) oder alternativ lediglich über einen Teilbereich (**Fig.** 4) die prozentuale Tumorzellaktivität ermittelt (wobei wiederum die Referenzwerte als 100%-Marke dienen). Im zweiten Fall wird dabei vorzugsweise auf die letzten 20 bis 60, mehr bevorzugt 40 Meßwerte zurückgegriffen. Bevorzugt wird beispielsweise alle zwei Minuten ein neuer Meßwert aufgenommen, so daß bei 40 Meßwerten die letzten 80 Minuten ausgewertet werden.

[0020] **Fig.** 5 zeigt den Ablauf einer bevorzugten Ausführungsform des erfindungsgemäßen Verfahrens. Im ersten Schritt S1 werden die ermittelten Meßdaten bereit gestellt. Diese Meßdaten werden zunächst mit entsprechenden Minimal- und Maximal-Schwellwerten verglichen, um eventuelle Ausreißer bzw. Spitzen aus dem gesamten Meßverlauf zu entfernen (Schritt S2). In der bevorzugten Ausführungsform werden zwei Messungen parallel mit der gleichen Substanz durchgeführt oder die Aktivität der Kontrollzellen (d. h. die unbehandelten Zellen) wird parallel in zwei Kanälen erfaßt. Die dabei ermittelten Daten werden einer Mittelwertbildung unterzogen (Schritt S3). Anschließend werden bei der in **Fig.** 5 dargestellten Ausführungsform die Meßkurven mittels eines Glättfaktors geglättet (Schritt S4). Anschließend erfolgt eine Normierung (Schritt S5) und eine Nivellierung der Meßwerte zu Beginn auf 100%, so daß alle Meßwerte von einem gemeinsamen Startpunkt ausgehen (Schritt S6). Hierbei geben entweder die Kontrollzellen (d. h., die unbehandelten Zellen) und/oder die mit Lösungsmittel versetzten Zellen den Ausgangspunkt vor. Zur besseren Darstellbarkeit werden anschließend die Daten auf die Einheit "Stunden" umgerechnet (Schritt S7). Alternativ dazu könnten die Daten auch mit kleineren bzw. größeren Zeiteinheiten angegeben werden, wie etwa halbstündig oder viertelstündig. Weiter bevorzugt ist die graphische Darstellung der Meßkurven in Schritt S8. Dies erlaubt beispielsweise frühzeitig Störungen, wie etwa Bakterienbildung zu erkennen. Anschließend erfolgt in Schritt S9 die Berechnung der einzelnen Flächen für die verwendeten Wirkstoffe und die Vergleiche mit den Referenzwerten bzw. Referenzflächen. Anhand dieser Vergleichsergebnisse wird in Schritt S10 ein Diagramm mit der Rangfolge der getesteten Wirkstoffe basierend auf allen Meßwerten ausgegeben. In Schritt S11 und Schritt S12 wird ein Diagramm mit den tatsächlichen Tumorzellaktivitätswerten ausgegeben, in Schritt S11 nur mit den letzten 40 Meßwerten.

[0021] Erfindungsgemäß wird bevorzugt die Flächenberechnung eingesetzt, um die Gesamtzellaktivität zu bestimmen und für die verwendeten Wirkstoffe in Korrelation zu setzen. Ergänzend wird dazu gemäß einer mehr bevorzugten Ausführungsform eine polynomische Regression verwendet, um bakterielle Kontamination automatisiert über Referenzkurven-Schemata erkennen zu können. Des weiteren werden alternativ lineare, logarithmische, polynomische, potentielle, exponentielle Regression, dynamische Wachstumssysteme und dynamische Zerfallsysteme verwendet, um die Messung generell zu beschleunigen, indem das Meßfenster limitiert bzw. die Berechnungszeit/Meßzeit minimiert und das Meßergebnis genauer spezifiziert wird. Alternativ dazu werden lineare oder logarithmische Trendlinien ermittelt. Weiter alternativ werden anhand der bereitgestellten Daten Mittelwert-Geraden ermittelt, um die Entwicklung bzw. Veränderung der Zellaktivitäten zu erfassen.

Patentansprüche

1. Verfahren zum automatischen Nachweisen einer Wirkung eines zellbeeinflussenden Mittels, insbesondere eines Zytostatikums, auf lebende Zellen, mit den Schritten:
   a) Bereitstellen von Meßdaten, die die Zellaktivität der mit dem zellbeeinflussenden Mittel kontaktierten lebenden Zellen angeben;
   b) Normieren der Meßdaten, die die Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen angeben, mit Meßdaten, die die Zellaktivität von unbehandelten lebenden Zellen und/oder von mit Lösungsmittel des zellbeeinflussenden Mittels behandelten Zellen angeben;
   c) Berechnen der Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen durch Vergleichen der normierten Meßdaten mit Referenzwerten, die den unbehandelten, lebenden Zellen und/oder den mit Lösungsmittel des zellbeeinflussenden Mittels behandelten Zellen entsprechen; und
   d) Ausgeben von Daten, die die Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen angeben.
2. Verfahren nach Anspruch 1, wobei in Schritt c) die jeweiligen Flächenintegrale zueinander in Beziehung gesetzt werden.
3. Verfahren nach Anspruch 2, wobei das Flächenintegral der Meßdaten von dem Flächenintegral der Referenzwerte subtrahiert wird.
4. Verfahren nach Anspruch 1, 2 oder 3, wobei in Schritt c) nur ein Teil der Meßdaten verwendet wird.
5. Verfahren nach Anspruch 4, wobei die letzten 20 bis 60 Meßdaten, vorzugsweise die letzten 40 Meßdaten verwendet werden.
6. Verfahren nach einem der Ansprüche 1 bis 5, wobei in Schritt c) lineare und/oder logarithmische Trendlinien anhand der Meßdaten berechnet werden.
7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die Meßdaten mittels Polynome n-ten Grades, lineare, logarithmische, potentielle, exponentielle Regression, dynamische Wachstumssysteme oder dynamische Zerfallsysteme interpoliert werden.
8. Verfahren nach einem der Ansprüche 1 bis 7, wobei es sich bei der gemessenen Zellaktivität um die pH-Wert Veränderung handelt.
9. Verfahren nach einem der Ansprüche 1 bis 8, wobei das zellbeeinflussende Mittel ein Zytostatikum aufweist.
10. Verfahren nach einem der Ansprüche 1 bis 9, wobei das zellbeeinflussende Mittel ausgewählt ist aus der Gruppe: Carboplatin, Cylophosphamid, Cisplatin, Docetaxel, Lomustin, Mitomycin, Treosulfan, Epirubicin, 5-Fluorouracil (5FU), Paclitaxel, Methotrexat, Bendamustin, Vinorelbin, Vindesin, Bleomycin, Gemcitabin, Adriamycin, Mitoxantron, Irinotecan, Etoposit und Kombinationen daraus.

11. Verfahren nach einem der Ansprüche 1 bis 10, wobei die Schritte a) bis d) an den bereitgestellten Zeilen mit mehreren zellbeeinflussenden Mitteln parallel durchgeführt werden.

12. Verfahren nach einem der Ansprüche 1 bis 11, wobei vor Schritt b) die bereitgestellten Meßdaten mit Schwellwerten verglichen werden, um Extrema herauszufiltern.

13. Verfahren nach einem der Ansprüche 1 bis 12, wobei die Zellaktivität der unbehandelten lebenden Zellen oder der mit Lösungsmittel des zellbeeinflussenden Mittels behandelten lebenden Zellen parallel gemessen wird und die erhaltenen Meßdaten gemittelt werden.

14. Verfahren nach einem der Ansprüche 1 bis 13, wobei die bereitgestellten Meßdaten mittels eines Glättfaktors geglättet werden.

15. Verfahren nach einem der Ansprüche 1 bis 14, ferner mit dem Schritt grafisches Darstellen der Meßdaten.

16. Verfahren nach einem der Ansprüche 1 bis 15, wobei in Schritt d) das Ergebnis des in Schritt c) vorgenommenen Vergleiches ausgegeben wird.

17. Verfahren nach einem der Ansprüche 3 bis 16, wobei die ermittelte Differenz die Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen angibt.

18. Verfahren nach einem der Ansprüche 1 bis 17, wobei in Schritt d) die tatsächliche Zellaktivität ausgegeben wird.

19. Verfahren nach einem der Ansprüche 11 bis 18, wobei anhand der ermittelten Differenzen eine Rangfolge entsprechend der Wirkung der zellbeeinflussenden Mittel auf die lebenden Zellen ausgegeben wird.

20. Vorrichtung zum automatischen Nachweisen einer Wirkung eines zellbeeinflussenden Mittels, insbesondere eines Zytostatikums, auf lebende Zellen, mit:
einer Einrichtung zum Bereitstellen von Meßdaten, die die Zellaktivität der mit einem zellbeeinflussenden Mittel kontaktierten lebenden Zellen angibt;
einer Einrichtung zum Normieren der Meßdaten, die die Zellaktivität angeben, mit Meßdaten, die die Zellaktivität von unbehandelten lebenden Zellen und/oder mit Lösungsmittel des zellbeeinflussenden Mittels behandelten Zellen angeben;
einer Berechnungseinrichtung zum Berechnen der Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen durch Vergleichen der normierten Meßdaten mit Referenzwerten, die den unbehandelten lebenden Zellen und/oder den mit Lösungsmittel des zellbeeinflussenden Mittels behandelten Zellen entsprechen; und
einer Ausgabeeinrichtung zum Ausgeben von Daten, die die Wirkung des zellbeeinflussenden Mittels auf die lebenden Zellen angeben.

21. Computerprogrammprodukt mit Programmcodemitteln, die auf einem computerlesbaren Datenträger gespeichert sind, um das Verfahren nach einem der Ansprüche 1 bis 19 durchzuführen, wenn das Programmprodukt auf einem Computer ausgeführt wird.

Hierzu 3 Seite(n) Zeichnungen

- Leerseite -

Nummer: DE 100 16 077 A1
Int. Cl.[7]: C 12 Q 1/02
Offenlegungstag: 13. Dezember 2001

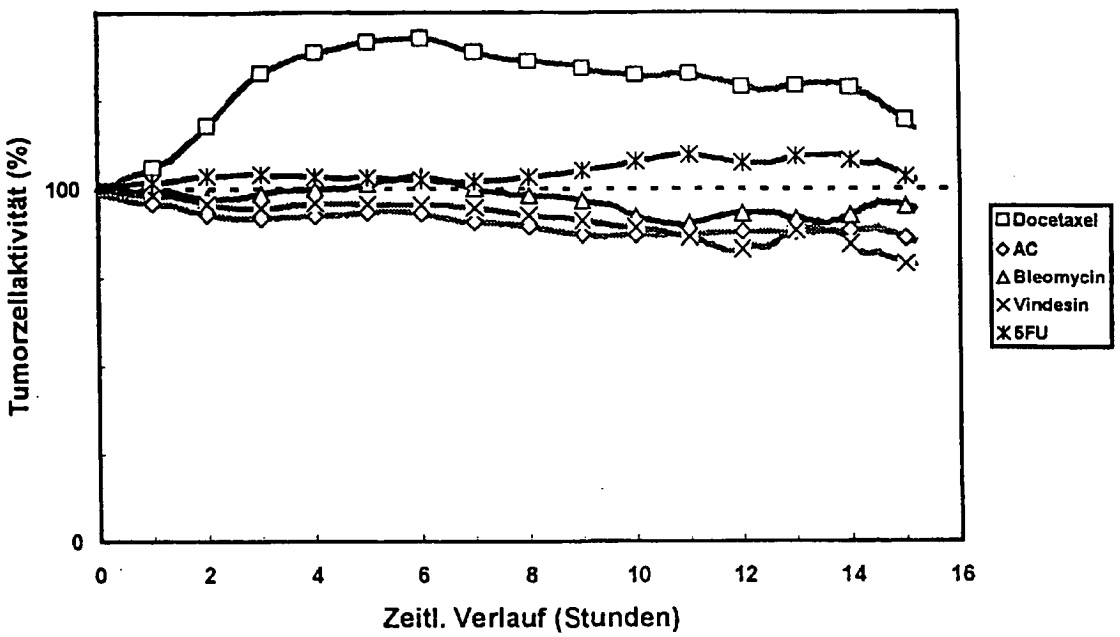


Fig. 1

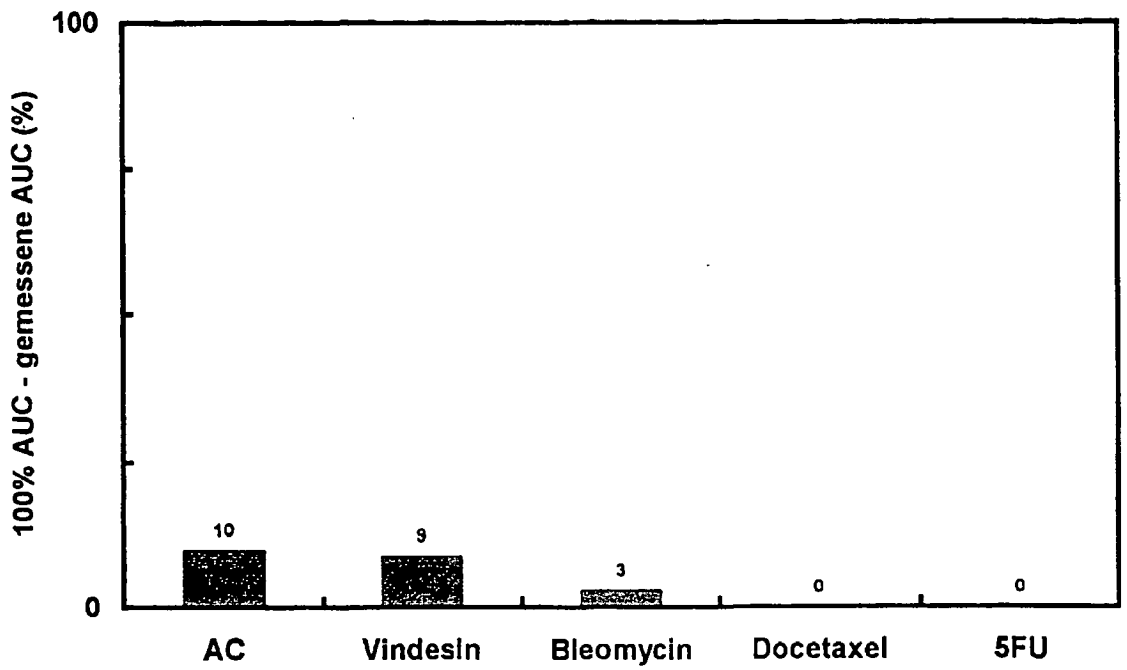


Fig. 2

Nummer: DE 100 16 077 A1
Int. Cl.[7]: C 12 Q 1/02
Offenlegungstag: 13. Dezember 2001

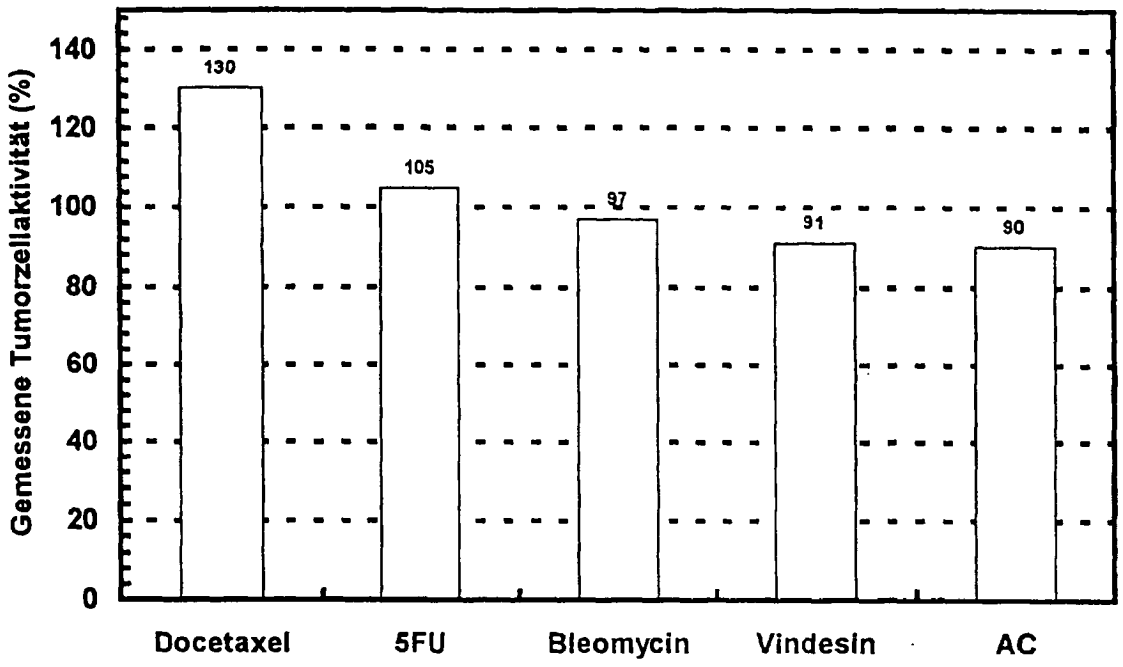


Fig. 3

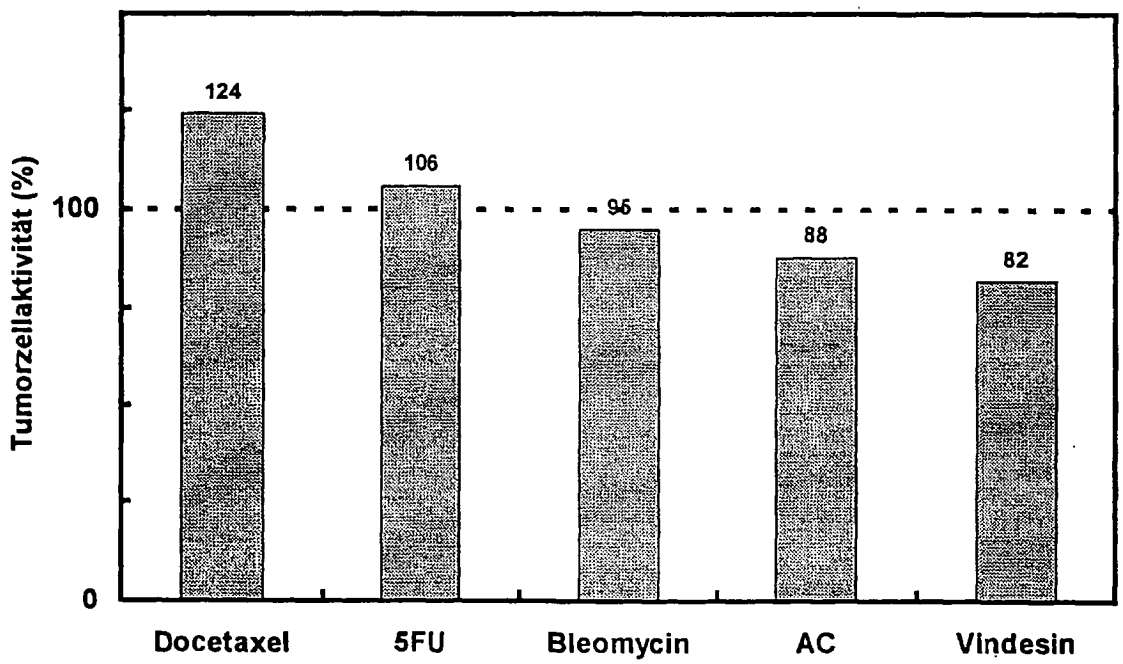


Fig. 4

Nummer: DE 100 16 077 A1
Int. Cl.[7]: C 12 Q 1/02
Offenlegungstag: 13. Dezember 2001

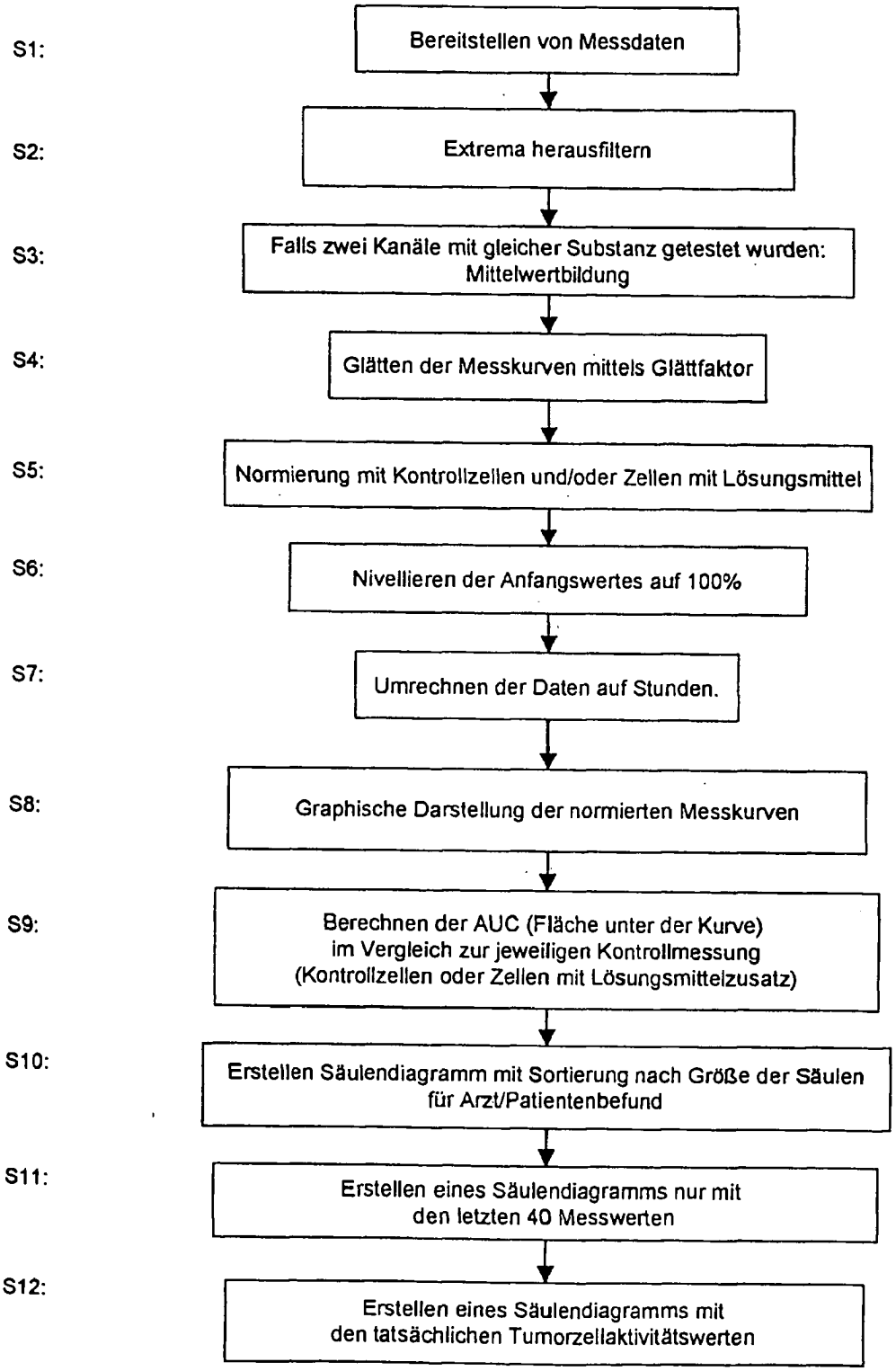


Fig. 5

(19)
Bundesrepublik Deutschland

Deutsche Demokratische Republik

Amt für Erfindungs- und Patentwesen

PATENTSCHRIFT

Wirtschaftspatent

Erteilt gemäß § 5 Absatz 1 des Änderungsgesetzes zum Patentgesetz

80 967

Zusatzpatent zum Patent: —

Anmeldetag: 19. I. 1970 (WP 30 h / 145 031)

Priorität: —

Ausgabetag: 05. IV. 1971

Kl.: 30 h, 2/30

Int. Cl.: A 61 k, 27/10

Erfinder zugleich Inhaber:

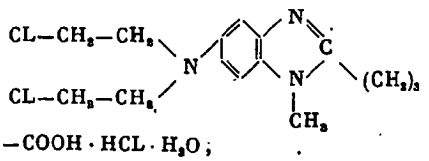
Prof. Dr. habil. Joachim Richter
Udo Reichenberger

Verfahren zur Herstellung eines stabilen injizierbaren Stickstofflostderivat-Präparates

1

Die Erfindung betrifft ein Verfahren zur Gewinnung eines stabilen injizierbaren Stickstofflostderivat-Präparates.

Stickstofflostderivate, insbesondere das γ-[1-Methyl-5-bis-(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid



$$CL-CH_2-CH_2 \quad CL-CH_2-CH_2 \quad -COOH \cdot HCL \cdot H_2O;$$

das die Bezeichnung IMET 3393 trägt, werden zur Rezidiv- und Metastasepropylaxe nach Krebsoperationen sowie bei myeloischer Leukämie und Lymphogranulomatose angewandt. Diese Stoffe sind lokal äußerst unverträglich, so daß nur die streng intravenöse Applikation in Frage kommt. Der Wirkstoff muß in sehr geringen Dosen verabreicht werden.

Es ist bereits bekannt, daß Stickstofflost-Verbindungen durch Lyophilisierung zu injizierbaren Präparaten verarbeitet werden können.

Der Nachteil des Verfahrens besteht darin, daß nur Stickstofflostderivate verarbeitet werden können, die genügend stabil sind. Außerdem muß für einige Stickstofflostderivate ein bestimmter pH-Wert-Bereich garantiert werden, damit die Injektionslösungen bis zur Applikation unzersetzt bleiben.

2

Die Erfindung bezweckt, die Herstellung eines stabilen injizierbaren Stickstofflostderivat-Präparates zu ermöglichen.

Das technische Problem besteht darin, für Stickstofflostderivate, die unter Produktionsbedingungen nicht lyophilisierbar sind, einen geeigneten Hilfsstoff zu finden, der nach Zusatz des Lösungsmittels einen bestimmten pH-Wert-Bereich garantiert, keine Inkompatibilitäten mit dem Wirkstoff zeigt und physiologisch verträglich ist. Außerdem muß eine hohe Dosiergenauigkeit garantiert werden.

Erfindungsgemäß läßt sich ein wirksames, stabiles injizierbares Präparat dadurch herstellen, daß Stickstofflostderivate mit Askorbinsäure gemischt werden. Dadurch wird erreicht, daß

1. mit Wasser für eine bestimmte Zeit eine stabile injizierbare Lösung hergestellt werden kann,
2. der erreichte pH-Wert der Lösung die Stabilität garantiert,
3. durch den Zusatz der Askorbinsäure die Dosiergenauigkeit beim Abfüllprozeß erhöht wird,
4. keine Inkompatibilitäten mit dem Wirkstoff auftreten,
5. die Mischung mit Askorbinsäure lagerfähig ist.

Ausführungsbeispiel:

γ-[1-Methyl-5-bis(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid	0,025 g
Askorbinsäure	0,175 g

Anwendung: Der Inhalt der Ampulle ist in 10 ml Wasser zur Injektion (DAB 7) zu lösen.

Deutsche Demokratische Republik

Amt für Erfindungs- und Patentwesen

PATENTSCHRIFT

Wirtschaftspatent

Erteilt gemäß § 5 Absatz 1 des Änderungsgesetzes zum Patentgesetz

80 967

Zusatzpatent zum Patent: —

Anmeldetag: 19. I. 1970 (WP 30 h / 145 031)

Priorität: —

Ausgabetag: 05. IV. 1971

Kl.: 30 h, 2/30

Int. Cl.: A 61 k, 27/10

Zur PS Nr. 80 967

ist eine Zweitschrift erschienen.

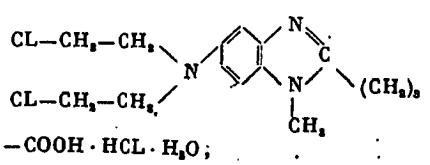
(Teilweise aufgehoben gem. § 6 Abs. 1 d. Änd. Ges. z. Pat. Ges.)

Verfahren zur Herstellung eines stabilen injizierbaren Stickstofflostderivat-Präparates

1

Die Erfindung betrifft ein Verfahren zur Gewinnung eines stabilen injizierbaren Stickstofflostderivat-Präparates.

Stickstofflostderivate, insbesondere das γ-[1-Methyl-5-bis-(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid



$$CL-CH_2-CH_2 \quad CL-CH_2-CH_2 \quad -COOH \cdot HCL \cdot H_2O;$$

das die Bezeichnung IMET 3393 trägt, werden zur Rezidiv- und Metastasepropylaxe nach Krebsoperationen sowie bei myeloischer Leukämie und Lymphogranulomatose angewandt. Diese Stoffe sind lokal äußerst unverträglich, so daß nur die streng intravenöse Applikation in Frage kommt. Der Wirkstoff muß in sehr geringen Dosen verabreicht werden.

Es ist bereits bekannt, daß Stickstofflost-Verbindungen durch Lyophilisierung zu injizierbaren Präparaten verarbeitet werden können.

Der Nachteil des Verfahrens besteht darin, daß nur Stickstofflostderivate verarbeitet werden können, die genügend stabil sind. Außerdem muß für einige Stickstofflostderivate ein bestimmter pH-Wert-Bereich garantiert werden, damit die Injektionslösungen bis zur Applikation unzersetzt bleiben.

2

Die Erfindung bezweckt, die Herstellung eines stabilen injizierbaren Stickstofflostderivat-Präparates zu ermöglichen.

Das technische Problem besteht darin, für Stickstofflostderivate, die unter Produktionsbedingungen nicht lyophilisierbar sind, einen geeigneten Hilfsstoff zu finden, der nach Zusatz des Lösungsmittels einen bestimmten pH-Wert-Bereich garantiert, keine Inkompatibilitäten mit dem Wirkstoff zeigt und physiologisch verträglich ist. Außerdem muß eine hohe Dosiergenauigkeit garantiert werden.

Erfindungsgemäß läßt sich ein wirksames, stabiles injizierbares Präparat dadurch herstellen, daß Stickstofflostderivate mit Askorbinsäure gemischt werden. Dadurch wird erreicht, daß

1. mit Wasser für eine bestimmte Zeit eine stabile injizierbare Lösung hergestellt werden kann,
2. der erreichte pH-Wert der Lösung die Stabilität garantiert,
3. durch den Zusatz der Askorbinsäure die Dosiergenauigkeit beim Abfüllprozeß erhöht wird,
4. keine Inkompatibilitäten mit dem Wirkstoff auftreten,
5. die Mischung mit Askorbinsäure lagerfähig ist.

Ausführungsbeispiel:

γ-[1-Methyl-5-bis(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid	0,025 g
Askorbinsäure	0,175 g

Anwendung: Der Inhalt der Ampulle ist in 10 ml Wasser zur Injektion (DAB 7) zu lösen.

3

Patentanspruch:

Verfahren zur Herstellung eines stabilen injizierbaren Stickstofflostderiwat-Präparates, dadurch gekennzeichnet,

4

daß Stickstofflostderivate, insbesondere γ-[1-Methyl-5-bis-(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid, mit Askorbinsäure gemischt werden.

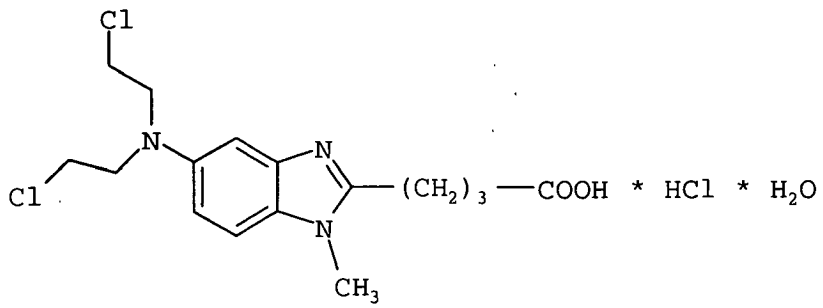
Vordruck-Leitverlag Berlin, Ag 300/71/DDR – (52) 9532

GDR Patent 80967

Method for preparing a stable injectable preparation of a nitrogen lost derivative

The invention relates to a method for obtaining a stable injectable preparation of a nitrogen lost derivative.

Nitrogen lost derivatives, in particular γ-[1-methyl-5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-butyric acid hydrochloride,



bearing the name IMET 3393 are used for a relapse and metastasis prophylaxis after operations of cancer as well as for myeloic leukaemia and lymphatic granulomatosis. These materials are locally extremely incompatible so that only the strict intravenous application is considered. The active agent has to be administered in very low dose.

It is already known that nitrogen lost compounds can be processed by lyophilization to injectable preparations.

The disadvantage of the method is that only nitrogen lost derivates being sufficiently stable can be processed. Moreover, a particular pH value range has to be guaranteed so

that the injection solutions remain undecomposed until application.

The invention purposes to permit the preparation of a stable injectable preparation of a nitrogen lost derivative.

The technical problem is to find an appropriate agent for nitrogen lost derivatives being not lyophilizable under the production conditions which guarantees a specific pH value range after addition of the solvent, shows no incompatibilities with the active agent and is physiological compatible. Moreover, a high accuracy of the dose has to be guaranteed.

According to the invention an effective stable injectable preparation can be prepared by mixing the nitrogen lost derivative with ascorbic acid. Thus, it can be achieved, that

1. for a particular time a stable injectable solution can be prepared using water,
2. the reached pH value of the solution guarantees the stability,
3. by addition of ascorbic acid the accuracy of the dose is increased during the filling process,
4. with the active agent no incompatibilities occur,
5. the mixture is storable with ascorbic acid.

Example:

γ-[1-methyl-5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-butyric acid hydrochloride	0.025 g
ascorbic acid	0.175 g

Application: the content of the ampoule has to be dissolved in 10 ml water for injection (DAB 7).

Claim:

1. Method for preparing a stable injectable preparation of a nitrogen lost derivative characterized in that the nitrogen lost derivative, in particular γ-[1-methyl-5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-butyric acid hydrochloride, is mixed with ascorbic acid.

(19) BUNDESREPUBLIK DEUTSCHLAND

PATENTSCHRIFT

(12) Ausschließungspatent

(11) DD 293 808 A5

Erteilt gemäß § 17 Absatz 1
Patentgesetz der DDR
vom 27.10.1983
in Übereinstimmung mit den entsprechenden
Festlegungen im Einigungsvertrag

5(51) C 07 D 235/16
A 61 K 31/415

DEUTSCHES PATENTAMT

In der vom Anmelder eingereichten Fassung veröffentlicht

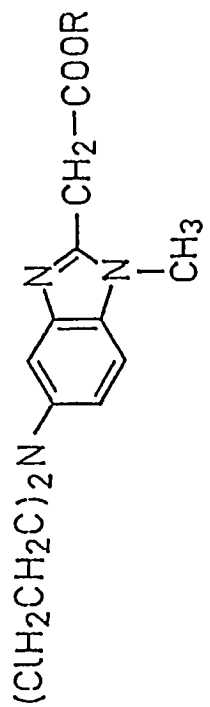
(21) DD C 07 D / 339 983 2 (22) 23.04.90 (44) 12.09.91

(71) Akademie der Wissenschaften, Patentabteilung, Otto-Nuschke-Straße 22/23, O - 1080 Berlin, DE
(72) Werner, Walter, Dr. sc. nat.; Letsch, Gerhard, DE
(73) Akademie der Wissenschaften, ZIMET, Beutenbergstraße 11, O - 6900 Jena, DE
(74) siehe (73)

(54) Verfahren zur Herstellung von (5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl(2))ethansäurealkylestern

(55)
(5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2))ethansäurealkylester; chemische Synthese; N^1-Methyl-4-nitro-o-phenylendiamin als Ausgangssubstanz; Kondensationsschritt; Solvolyse; Aufbau der Stickstofflostgruppe; potentielle Zytostatika; Bendamustin-Analoga

(57) Die Erfindung betrifft ein Verfahren zur Herstellung von (5-[Bis(2-chlorethyl)-amino]-1-methyl-benzimidazolyl-(2))ethansäurealkylestern der allgemeinen Formel, worin R CH_3, C_2H_5, C_3H_7, allgemein C_nH_{2n+1} bedeutet. Sie verfolgt das Ziel, die Verbindungen dieser Stoffgruppe in effektiver Weise herzustellen. Die dieser Zielstellung zugeordnete Aufgabe wird durch chemische Synthese in der Weise gelöst, daß N^1-Methyl-4-nitro-o-phenylendiamin mit Cyanoessigsäureethylester zu 2-Cyanomethyl-1-methyl-5-nitro-benzimidazol kondensiert sowie mit alkoholischer Salzsäure zum zugehörigen Alkylester solvolysiert und die Stickstofflostgruppe aus der Nitrogruppe durch Reduktion zur Aminogruppe, durch Addition von Ethylenoxid sowie durch Chlorierung mit Thionylchlorid aufgebaut wird. Die Zielverbindungen sind als Zytostatika (Bendamustin-Analoga) von potentiellem Interesse. Formel



ISSN 0433-6461

5 Seiten

Patentansprüche:

1. Verfahren zur Herstellung von {5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2)}ethansäurealkylestern der allgemeinen Formel.

A)

worin R, CH_3, C_2H_5, C_3H_7, allgemein C_nH_{2n+1} bedeutet, durch chemische Synthese, **gekennzeichnet dadurch,** daß man
- N^1-Methyl-4-nitro-o-phenylendiamin mit Cyanessigsäureethylester zu 2-Cyanomethyl-1-methyl-5-nitro-benzimidazol kondensiert sowie mit alkoholischer Salzsäure zum zugehörigen Alkylester solvolysiert und
- die Stickstofflostgruppe aus der Nitrogruppe durch Reduktion zur Aminogruppe, Addition von Ethylenoxid sowie Chlorierung mit Thionylchlorid aufbaut.

2. Verfahren gemäß Anspruch 1, **gekennzeichnet dadurch,** daß man aus den Zielverbindungen A) wasserlösliche Hydrochloride bzw. analoge Salze herstellt, indem man erstere entweder zunächst in Ether oder in Essigsäureethylester bzw. in einem niederen Alkohol löst und anschließend entweder Chlorwasserstoffgas einleitet oder eine organische bzw. anorganische Säure zusetzt.

Hierzu 2 Seiten Formel

Anwendungsgebiet der Erfindung

Die Erfindung betrifft ein Verfahren zur Herstellung von {5[Bis-(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2)}ethansäurealkylestern der allgemeinen Formel A (Abb. 1), in der R CH_3, C_2H_5, C_3H_7, allgemein C_nH_{2n+1} bedeutet.
Die Zielverbindungen sind durch Veresterung stabilisierte potentielle Metabolite des antineoplastisch wirkenden Arzneimittels 4-{5-[Bis-(2-chlorethyl)amino-1-methyl-1 H-benzimidazolyl-(2)}butansäure-hydrochlorid-hydrat (Bendamustin) und daher als Zytostatika von Interesse.
Das Anwendungsgebiet der Erfindung liegt somit in der pharmazeutisch-chemischen Forschung und Industrie.

Charakteristik des bekannten Standes der Technik

Verfahren zur Herstellung von Stickstofflostverbindungen sind in großer Zahl beschrieben sowie in die industrielle Praxis überführt. 4-{5-[Bis(2-chlorethyl)amino]-1-methyl-1H-benzimidazolyl(2)}-butansäure-hydrochlorid-hydrat (Bendamustin) ist dabei das den nachstehend beschriebenen Zielprodukten am nächsten verwandte Krebsarzneimittel. Im tierischen und menschlichen Organismus wird Bendamustin unter anderem zu dem entsprechenden 3-Hydroxybutansäure-Derivat biotransformiert. (R. Preiss, R. Sohr, M. Matthias, B. Brockmann, H. Müller, Pharmazie 40 [1985] 782; R. Preiss, R. Sohr, H. Matthias, H. Müller, W. Werner, W. Ihn, H. Wolff, im Druck). Der enzymatische Abbau zum zu erwartenden Ethansäure-Derivat wurden noch nicht nachgewiesen.
Die Darstellung von {5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl(2)}ethansäure wurde von W. Ozegowski u. Mitarb. ausgehend von einer Kondensation von N^1-Methyl-4-nitro-o-phenylendiamin mit Milchsäure und anschließender Oxidation mit CrO_3 in essigsaurer Lösung zu 2-Acetyl-1-methyl-5-nitro-benzimidazol versucht. Die Redox-Amidierung der vorstehenden Verbindung nach Willgerodt-Kindler zum Essigsäure-Derivat gelang jedoch nicht.
Ein weiterer in der Literatur beschriebener Versuch führte über die Kondensation von o-Phenylen-diamin mit Cyanessigsäureethylester zu 2-Cyano-methyl-benzimidazol. 1-Methylierung und saure Ethanolyse dieser Verbindung ergab (1-Methyl-benzimidazolyl-(2)ethansäureethylester, der in 5- und 6-Stellung nitriert wurde. Nach Isomeran-Trennung erfolgte die Reduktion der 5-Nitrogruppe zum 5-Amino-Derivat, an das Ethylenoxid addiert wurde. Zwar gelang die Chlorierung des Bis(2-hydroxyethyl)amino-Derivates mit $SOCl_2$; die saure Hydrolyse des Esters ergab jedoch nicht die substituierte Ethansäure, sondern nach Decarboxylierung lediglich 5-[Bis(2-chlorethyl)-amino]-1,2-dimethyl-benzimidazol (W. Ozegowski, D. Krebs; J. prakt. Chem. [4] 29 [1965] 18–25).
Bei diesen Methoden ist von Nachteil, daß die Synthese von Derivaten der oben genannten Stoffgruppe A von dem unsubstituierten o-Phenylendiamin ausgeht und das Benzimidazol-Derivat später noch N-methyliert und nitriert werden muß, wobei 5- und 6-Nitro-benzimidazol-Isomere entstehen, die anschließend noch zu trennen sind (nur das Isomere mit der Nitrogruppe in Position 5 des Benzimidazolringes ist von Interesse). Außerdem zerfiel die Zielverbindung schließlich durch spontane Decarboxylierung.

Ziel der Erfindung

Die Erfindung verfolgt das Ziel, (5[Bis(2-chlorethyl)-amino]-1-methylbenzimidazol-(2))ethansäurealkylester in effektiverer Weise herzustellen.

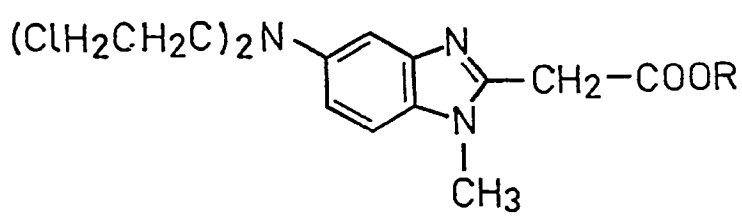
Darlegung des Wesens der Erfindung

Der Erfindung liegt die Aufgabe zugrunde, ein chemisch-synthetisches Verfahren anzugeben, mit dem (5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl(2))ethansäure in Form der stabilen Alkylester in effektiver Weise produziert werden kann.
Erfindungsgemäß wird diese Aufgabe dadurch gelöst, daß N^1-Methyl-4-nitro-o-phenylendiamin als Ausgangssubstanz zunächst mit Cyanessigsäureethylester zu 2-Cyanomethyl-1-methyl-5-nitro-benzimidazol (siehe 1 in Abb. 2) kondensiert und dieses Zwischenprodukt in bzw. mit alkoholischer Salzsäure zum jeweils zugehörigen Alkylester (vgl. etwa 2 in Abb. 2) solvolysiert wird. An diesen Estern wird anschließend in an sich bekannter Weise die Stickstoffflostgruppe aufgebaut, indem die Nitrogruppe durch hydrierende Reduktion zur Aminogruppe umgewandelt (vgl. etwa 3 in Abb. 2), danach an dieses Zwischenprodukt Ethylenoxid addiert (vgl. etwa 4 in Abb. 2) sowie im letzten Teilschritt durch Chlorierung mit Thionylchlorid der jeweilige (5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2))ethansäurealkylester, d.h. die jeweilige Zielverbindung A) (vgl. etwa 5 in Abb. 2), erhalten wird. Auf diese Weise werden stabile Verbindungen einer an sich labilen Carbonsäure erhalten.
Aus den Zielverbindungen A) lassen sich vorteilhaft noch wasserlösliche Hydrochloride bzw. analoge wasserlösliche Salze herstellen, indem man sie eingangs in Ether oder in Essigsäureethylester bzw. in einem niederen Alkohol löst sowie im 2. Teilschritt entweder Chlorwasserstoffgas einleitet oder eine organische bzw. anorganische Säure, vorzugsweise Eisessig oder Zitronensäure bzw. Schwefelsäure, zusetzt. Die somit gewonnenen Derivate von A) ließen sich auch intravenös applizieren.

Ausführungsbeispiel

- Methode und Geräte
  Die Schmelzpunkte wurden auf dem Mikroheiztisch nach Boetius (korr. Werte) bestimmt. Die massenspektrometrischen Aufnahmen erfolgten mit dem doppelfokussierenden Gerät der Firma JEOL, Typ JMS-D 100. Zur Dünnschichtchromatographie wurden Glasplatten mit Merck-Kieselgel GF254 (0,5 mm Schichtdicke) und als Laufmittel $n\text{-}C_4H_9OH/CH_3COOH/H_2O$ (4:1:2) verwendet.
- Darstellung von [1-Methyl-5-nitro-benzimidazolyl(2)]acetonitril (1)
  16,7 g (0,1 mol) N^1-Methyl-4-nitro-o-phenyldiamin und 45,2 g (0,4 mol) Cyanessigsäureethylester werden unter Rühren 50 Minuten auf 195°C bis 200°C unter Abdestillieren der flüchtigen Anteile erhitzt. Die nach Abkühlen erhaltene Masse wird mit 400 ml $CH_3COOC_2H_5$ ausgekocht und vom unlöslichen Rückstand abfiltriert. Nach Einengen im Vakuum liegen zunächst 12 g rotbraunes Rohprodukt von 1 (56% d. Th.) vor. Nach mehrmaliger Umkristallisation erhält man hellgelbe Nadeln vom Schmelzpunkt 183°C bis 184°C (C_2H_5OH); $C_{10}H_8N_4O_2$ (216,2).
- Darstellung von [1-Methyl-5-nitro-benzimidazolyl-(2)]-ethansäuremethylester (2)
  21,6 g (0,1 mol) von 1 werden in 350 ml methanolischer HCl (20 g HCl in 100 ml MeOH) 3,5 Stunden unter Rückfluß erhitzt. Nach Einengen im Vakuum wird in 350 ml H_2O gelöst, filtriert, mit $NaHCO_3$-Lösung neutralisiert und das ausgefallene Produkt abgesaugt. Die Rohausbeute an 2 betrug 22,1 g (89% d. Th.). Die Umkristallisation erfolgt unter Zusatz von Aktivkohle und Raney-Nickel. Man erhält hellgelbe Nadeln vom Schmelzpunkt 173°C bis 174°C (CH_3OH); R_F-Wert: 0,62; $C_{11}H_{11}N_3O_4$ (249,2).
- Darstellung von [5-Amino-1-methyl-benzimidazolyl-(2)]ethansäuremethylester (3)
  5 g (0,02 mol) von 2 werden bei 45°C in 900 ml CH_3OH gelöst und mit Raney-Nickel bei Normaldruck hydriert. Nach Aufnahme der erforderlichen H_2-Menge (Dauer etwa 2 Stunden) wird der Katalysator abgesaugt und das Filtrat im Vakuum eingeengt. Die Ausbeute an 3 betrug 2,5 g (57% d. Th.) hellgelbe Nadeln vom Schmelzpunkt 123°C bis 126°C (C_6H_6); R_F-Wert: 0,29; $C_{11}H_{13}N_3O_2$ (219,2).
- Darstellung von (5-[Bis(2-hydroxyethyl)amino]-1-methyl-benzimidazolyl-(2))ethansäuremethylester (4)
  0,7 g (3 mmol) von 3 werden in einem Gemisch aus 10 ml H_2O und 0,5 ml CH_3COOH gelöst und tropfenweise bei 0°C mit 3 ml (ca. 60 mmol) Ethylenoxid versetzt. Nach Stehen über Nacht bei 20°C wird mit $NaHCO_3$-Lösung neutralisiert und mit 75 ml $CH_3COOC_2H_5$ extrahiert. Nach dem Trocknen über Na_2SO_4 und Abdestillieren im Vakuum verbleiben 0,4 g von 4 (43% d. Th.) als gelblicher Sirup, der noch nicht kristallisierte.
  R_F-Wert von 4: 0,41 $C_{15}H_{21}N_3O_4$ (307,4).
- Darstellung von (5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2))-ethansäuremethylester (5)
  0,92 g (3 mmol) von 4 werden in 20 ml trockenem $CHCl_3$ gelöst, und 1,43 g (12 mmol) $SOCl_2$ werden bei −5°C bis 0°C zugetropft. Nach Stehen über Nacht wird für eine Stunde auf 40°C erwärmt, unter Eiskühlung mit 2 ml CH_3OH versetzt und im Vakuum eingeengt. Der ölige Rückstand wird in wenig Eiswasser gelöst, mit $NaHCO_3$-Lösung schwach alkalisiert, mit Ether extrahiert, über Na_2SO_4 getrocknet und erneut im Vakuum eingeengt. Es resultierte ein gelbliches Öl, das bisher noch nicht kristallisierte.
  Ausbeute 0,6 g an 5 (58% d. Th.),
  R_F: 0,44. $C_{15}H_{19}Cl_2N_3O_2$ (344,2),
  Massenspektrum: M^+ m/z
  ber.: 343,3186
  gef.: 343,3199
  – Herstellung des Hydrochlorides von 5:
  Die Verbindung 5 wird in Ether gelöst und etwa ein Äquivalent Chlorwasserstoffgas eingeleitet. Es scheidet sich ein öliges Hydrochlorid ab, das mit Essigsäureethylester zur Kristallisation gebracht wird.

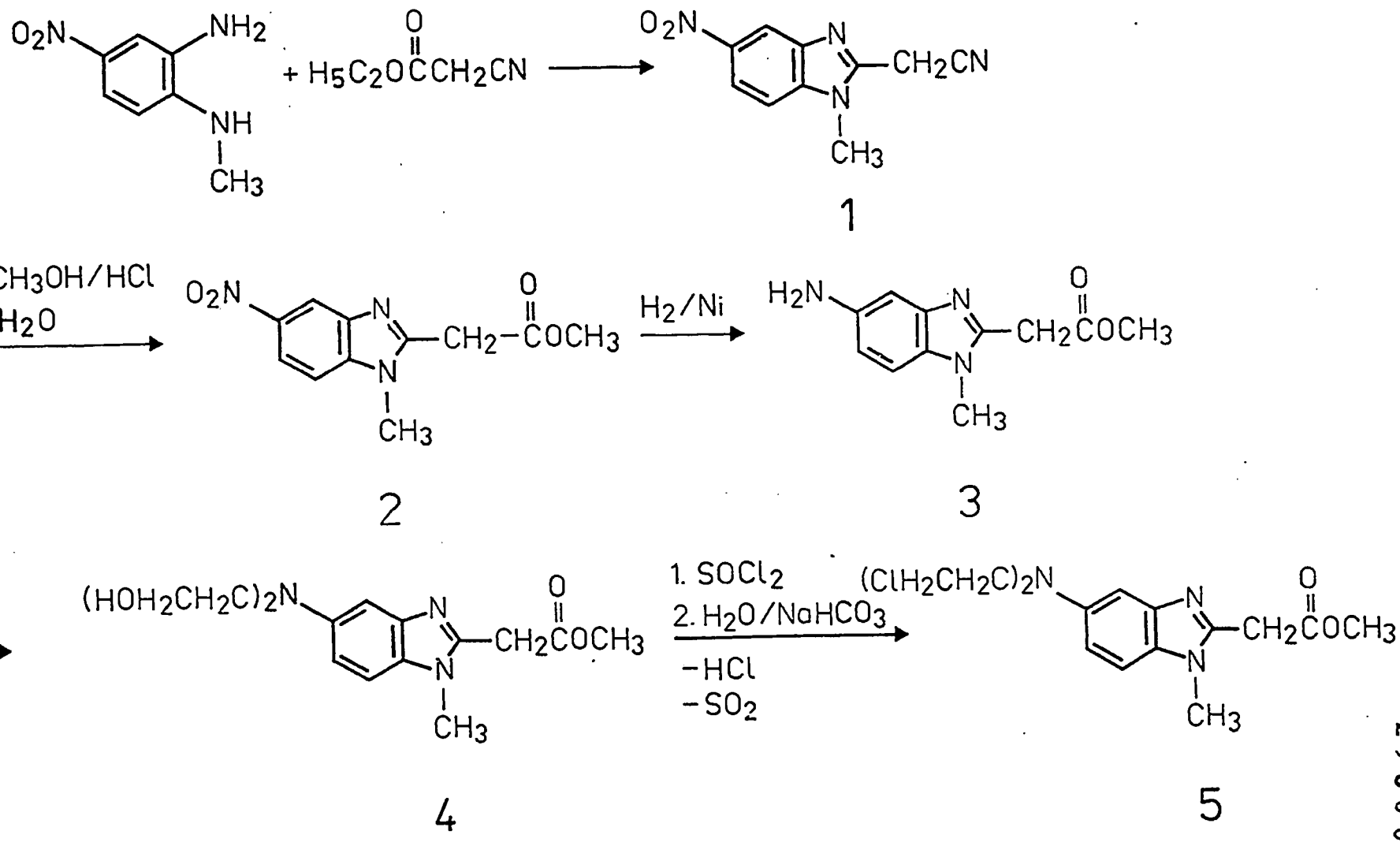
Abb. 1



$R = CH_3, C_2H_5, C_3H_7, C_nH_{2n+1}$

A

Abb. 2



(19) DEUTSCHE DEMOKRATISCHE REPUBLIK

PATENTSCHRIFT

Wirtschaftspatent

Erteilt gemäß § 5 Absatz 1 des Aenderungsgesetzes zum Patentgesetz

ISSN 0433-6461 (11) 1598 77

Int.Cl.³ 3(51) C 07 D235/04

AMT FUER ERFINDUNGS- UND PATENTWESEN

In der vom Anmelder eingereichten Fassung veroeffentlicht

(21) WP C 07 D/ 2307 454 (22) 12.06.81 (44) 13.04.83

(71) VEB JENAPHARM, JENA;DD;
(72) KRUEGER, WOLFGANG,DR. DIPL.-CHEM.;KRUEGER, GISELA;DD;
(73) siehe (72)
(74) VEB JENAPHARM, BFS, 3010 MAGDEBURG, V.-V.-UNRUH-STR. 4

(54) VERFAHREN ZUR HERSTELLUNG VON 4-[1-METHYL-5-BIS (2-CHLORAETHYL)AMINOBENZIMIDAZOLYL-2]-BUTTERSAEURE

(57) Die Erfindung betrifft ein verbessertes Verfahren zur Herstellung von 4[-1-Methyl-5-(bis-2-chloräthyl)-amino-benzimidazolyl-2]-buttersäure durch Reaktion von 4-[1-Methyl-5-bis-(2-hydroxyäthyl)-benzimidazolyl-2]-buttersäureestern mit Thionylchlorid und anschließender Verseifung des Esters. Die Verbindung ist bekannt als hochwirksames Chemotherapeutikum zur Bekämpfung des Wachstums von Tumoren.

Titel der Erfindung

Verfahren zur Herstellung von 4-/1-Methyl-5-bis (2-chloräthyl)amino-benzimidazolyl-2/-buttersäure

Anwendungsgebiet der Erfindung

Die Erfindung betrifft ein verbessertes Verfahren zur Herstellung von 4-/1-Methyl-5-(bis-2-chloräthyl)-amino-benzimidazolyl-2/-buttersäure. Die Verbindung ist bekannt als hochwirksames Chemotherapeutikum zur Bekämpfung des Wachstums von Tumoren.

Charakteristik der bekannten technischen Lösung

Die Herstellung der 4-/1-Methyl-5-bis-(2-chloräthyl)-amino-benzimidazolyl-2/-buttersäure erfolgt durch Reaktion von 4-/1-Methyl-5-bis-(2-hydroxyäthyl)-amino-benzimidazolyl-2/-buttersäureester mit anorganischen Säurechloriden, vorzugsweise Thionylchlorid (DDR-WP 34 727). Das Verfahren liefert sehr stark schwankende Ausbeuten, die zudem noch bei Vergrößerung der Ansätze stark abfallen. Dabei ist es zur Erzielung der erforderlichen Reinheit des Endproduktes notwendig, durch aufwendige Destillationsprozesse das überschüssige Thionylchlorid zu entfernen, weiterhin müssen bei der Reaktion entstandene Nebenprodukte durch eine kosten- und arbeitsintensive chromatographische Reinigung an Aluminiumoxid abgetrennt werden.

Ziel der Erfindung

Ziel der Erfindung ist die Herstellung der 4-/1-Methyl-5-bis-(2-chloräthyl)amino-benzimidazolyl-2/-buttersäure nach einem Verfahren mit unabhängig von der Ansatzgröße reproduzierbar guten Ausbeuten, bei dem einerseits die Entfernung des überschüssigen Chlorierungsmittels vereinfacht wird und bei dem insbesondere die sehr aufwendige chromatographische Reinigung entfallen kann.

Darlegung des Wesens der Erfindung

Es bestand die Aufgabe, einerseits die Ursachen der stark schwankenden Ausbeuten aufzudecken und zu beseitigen und außerdem die Aufarbeitung des Reaktionsgemisches so zu verändern, daß die Entfernung des übersohüssigen Thionylchlorides und der Nebenprodukte vereinfacht wurde um die kostenintensive ohromatographische Reinigung zu eliminieren. Durch eingehende Untersuchung des Verfahrens nach dem Stand der Technik wurde überraschenderweise gefunden, daß die Reaktion nach der angegebenen Reaktionszeit nur sehr unvollständig war und im Reaktionsgemisch zu diesem Zeitpunkt nur Spuren der Bis-chloräthylverbindung neben etwa gleichen Anteilen Mono-chloräthylverbindung und Ausgangsprodukt - durch Dünnsohichtchromatographie ermittelt - enthalten waren. Ein Fortschreiten der Reaktion erfolgte beim Abdestillieren des Lösungsmittels, wobei je nach den Destillationsbedingungen die Reaktion manchmal zum richtigen Endpunkt geführt wurde, häufig aber noch unvollständig war bzw. aber auch durch zu lange Reaktionszeit ein erhöhter Anteil an Nebenprodukten gebildet wurde. Nur durch Veränderung der Reaktionszeiten konnte eine Lösung des Problems nicht erreicht werden, da mit stöchiometrischen Mengen an Thionylchlorid die Reaktion keinen ausreichenden Umsatz ergab und ein Überschuß an Thionylchlorid beim Abdestillieren unkontrolliert die Bildung von Nebenprodukten verursachte. Überraschenderweise konnten alle geschilderten Probleme dadurch gelöst

werden, daß man zum Abbruch der Reaktion das Gemisch in wässrige Salzsäure einrührte. Dabei wurde einerseits in nicht exothermer Reaktion das überschüssige Thionylchlorid zersetzt und gleichzeitig das Reaktionsprodukt in der wässrigen Salzsäure gelöst. Da so ein genau definierter Abbruch der Reaktion erreicht wurde, konnte unter dünnschichtchromatographischer Kontrolle die Reaktion mit dem Thionylchlorid bis zum vollständigen Umsatz geführt werden. Wie sich zeigte ist nach definierten Zeiten der Hauptreaktion eine Mindestnachreaktionszeit von 10 Stunden erforderlich und 30 Stunden sollten nicht überschritten werden. Besonders vorteilhaft sind 16 Stunden. Bei der sich anschließenden Verseifung der Estergruppe werden gleichzeitig das Lösungsmittel der Chlorierungsreaktion abdestilliert und Verunreinigungen in unlöslicher Form abgeschieden. Die weitere Verarbeitung der salzsauren Lösung kann dann auf bekannte Weise erfolgen.

Das folgende Beispiel zeigt die bevorzugte Ausführungsform der Erfindung.

Ausführungsbeispiel

4,305 kg 4-/1-Methyl-5-bis-(2-hydroxyäthyl)-amino-benzimidazolyl-buttersäureäthylester (oder die entsprechenden Mengen Methyl-, Propyl oder Butylester) werden in 36 l Chloroform gelöst. Nach Abkühlen auf 0 °C werden 2,175 kg Thionylchlorid innerhalb 40 Min zugegeben, wobei man die Temperatur durch Kühlung bei 0....+5 °C hält. Man rührt 1 Stunde bei gleicher Temperatur nach, läßt innerhalb 2,5 bis 3 Stunden auf Raumtemperatur ansteigen und läßt anschließend 15 bis 16 Stunden bei Raumtemperatur stehen.

Die Lösung wird unter gutem Rühren in 37,5 l konz. Salzsäure dispergiert, wobei unter starker HCl- und SO_2-entwicklung das überschüssige Thionylchlorid zersetzt wird. Anschließend wird das Chloroform abdestilliert und anschließend noch 3 Stunden bei ca. 95 °C gerührt. Nach Zugabe von 0,78 kg Aktivkohle wird weitere 30 Min bei 95 °C gerührt,

die Lösung filtriert, im Reaktionsdünnschichtverdampfer i. V. eingeengt auf ca. 8 l und der Rückstand mit 24 l Wasser verdünnt und bis zur Kristallisation gerührt. Die weitere Reinigung erfolgt durch Umkristallisation aus Wasser Ausbeute 75 bis 80 % der Theorie.

Erfindungsanspruch

1. Verfahren zur Herstellung von 4-[1-Methyl-5-bis(2-chloräthyl)-benzimidazolyl-2]-buttersäure durch Reaktion von 4-[1-Methyl-5-bis-(2-hydroxyäthyl)-benzimidazolyl-2]-buttersäureestern mit Thionylchlorid und anschließende Verseifung des Esters dadurch gekennzeichnet, daß man die Reaktion bei Temperaturen von -5 bis 30 °C, bis zum vollständigen Umsatz der Hydroxyäthylgruppen führt, indem man an die bei 0 bis 5 °C durchgeführte Hauptreaktion eine 10 bis 30stündige Nachreaktion bei Temperaturen zwischen 20 bis 25 °C anschließt und daß man zum gezielten Abbruch der Reaktion das überschüssige Thionylchlorid durch Einrühren des Reaktionsgemisches in wässrige Chlorwasserstoffsäure hydrolysiert, vorzugsweise bei 15 bis 25 °C.

2. Verfahren nach Punkt 1 dadurch gekennzeichnet, daß die Nachreaktion vorzugsweise 15 bis 16 Stunden dauert.

GDR Patent 1598 77

Method for preparing 4-[1-methyl-5-bis(2-chloroethyl)amino-benzimidazolyl-2]-butyric acid

Title of the invention

Method for preparing 4-[1-methyl-5-bis(2-chloroethyl)amino-benzimidazolyl-2]-butyric acid.

Field of the invention

The invention relates to an improved method for preparing 4-[1-methyl-5-bis(2-chloroethyl)amino-benzimidazolyl-2]-butyric acid. The compound is known as a highly effective chemotherapeutic for treating the growth of tumors.

Characteristics of the known technical solution

The preparation of 4-[1-methyl-5-bis(2-chloroethyl)amino-benzimidazolyl-2]-butyric acid results from reaction of 4-[1-methyl-5-bis-(2-hydroxyethyl)-amino-benzimidazolyl-2]-butyric acid ester with inorganic acid chlorides, preferably thionyl chloride (DDR-WP 34 727). The method provides very unsteady yields decreasing in addition by increased starting amounts. At the same it is necessary to remove excess thionyl chloride by costly distillation processes to achieve the desired purity of the final product, wherein in addition by-products formed during the reaction have to be separated by a cost intensive and labour intensive chromatographic purification on aluminium oxide.

Object of the invention

Object of the invention is the preparation of 4-[1-methyl-5-bis-(2-chloroethyl)amino-benzimidazolyl-2]-butyric acid according to a method with reproducible good yields independent of the scheduled quantity wherein on the one hand

the removal of the excessive chlorinating agent is simplified and wherein in particular the extensive chromatographical purification can be omitted.

Demonstration of the inventive subject matter

On the one hand, there was the object to reveal and delete the reasons for the highly unsteady yields and furthermore to modify the working up of the reaction mixture so that the removal of the excessive thionyl chloride and of the by-products was simplified in order to eliminate the cost intensive chromatographic purification. By extensive examination of the method according to the state of the art it was surprisingly found that the reaction was very uncompleted after the indicated reaction time and at this time only traces of the bis-chloroethyl compound were contained in the reaction mixture besides equal amounts of mono-chloroethyl compound and starting compound. Progression of the reaction occurs during distillation of the solvent leading the reaction sometimes to the right final point depending on the distillation conditions, often being uncompleted and forming an increased amount of by-products due to reaction time that was too long, respectively. A solution of the problems could not be achieved by changing the reaction times only, as no adequate conversion of the reaction was achieved using stoichometric amounts of thionyl chloride and excessive thionyl chloride caused uncontrolled preparation of by-products during distillation. Surprisingly all described problems could be solved by introducing the mixture in aqueous hydrochloric acid for stopping of the reaction. Thereby, on the one hand, excessive thionyl chloride was decomposed in non-exothermic reaction and at the same time the reaction product was dissolved in aqueous hydrochloric acid. Achieving the exact definite stopping of the reaction, the reaction with the thionyl chloride could be

conducted to a complete conversion by thin film chromatographic control. It was found that after a defined period of time of the main reaction a minimum after-reaction of 10 hours was necessary but 30 hours should not be exceeded. Particular advantageous are 16 hours. At the same time the solvent of the chlorinating reaction is distilled and contaminations precipitate in an insoluble form following the esterification of the ester group. Further processing of the hydrochloric solution can be done in a known manner. The following example indicates the preferred embodiment of the invention.

Example

4.305 kg of 4-[1-methyl-5-bis-(2-hydroxyethyl)-amino-benzimidazolyl]butyric acid ester (or the corresponding amounts of methyl, propyl or butyl ester) are dissolved in 36 l chloroform. After cooling to 0°C 2.175 kg thionyl chloride are added within 40 min maintaining the temperature at 0-5°C by cooling. It is stirred for 1 hour at the same temperature, increased to room temperature within 2.5 to 3 hours and left for 15 to 16 hours at room temperature.

The solution is dispersed by strong agitation in 37.5 l concentrated sulphuric acid decomposing excessive thionyl chloride with heavy formation of HCl and SO_2. Subsequently the chloroform is distilled and afterwards it is stirred for 3 hours at about 95°C. After addition of 0.78 kg of activated carbon it is stirred for further 30 min at 95°C, the solution is filtered, concentrated to 8 l in the thin-film evaporator and the residue is diluted with 24 l water and is stirred until crystallisation starts. The further purification is achieved by re-crystallisation from water, yielding 75 to 80% of the theory.

Claims:

1. Method for preparing 4-[1-methyl-5-bis-(2-chloroethyl)-benzimidazolyl-2]-butyric acid by reaction of 4-[1-methyl-5-bis-(2-hydroxyethyl)-benzimidazolyl-2]-butyric acid ester with thionyl chloride and subsequent esterification of the ester characterized in that the reaction is drawn to complete conversion of the hydroxy ethyl groups at temperatures of -5 to 30°C by adding a 10 to 30 hours after-reaction at temperatures between 20 to 25°C subsequent to the main reaction conducted at 0 to 5°C and that the excessive thionyl chloride is hydrolysed by introducing the reaction mixture into aqueous hydrochloric acid preferably at 15 to 25°C for a specific stopping of the reaction.

2. Method according to claim 1, characterized in that the after-reaction lasts preferably 15 to 16 hours.

Abstract

The invention relates to an improved method for preparing of 4[-1-methyl-5-(bis-2-chloroethyl)-amino-benzimidazolyl-2]-butyric acid by reaction of 4-[1-methyl-5-bis-(2-hydroxyethyl)-benzimidazolyl-2]-butyric acid ester with thionyl chloride and subsequent esterification of the ester. The compound is known as highly effective chemotherapeutic for treating the growth of tumors.

(19) DEUTSCHE DEMOKRATISCHE REPUBLIK

PATENTSCHRIFT

Wirtschaftspatent

Erteilt gemaeß § 5 Absatz 1 des Aenderungsgesetzes zum Patentgesetz

ISSN 0433-6461 (11) 1592 89

Int.Cl.³ 3(51) A 61 K 31/41
A 61 K 9/08

AMT FUER ERFINDUNGS- UND PATENTWESEN

In der vom Anmelder eingereichten Fassung veroeffentlicht

(21) WP A 61 K/ 2304 291 (22) 01.06.81 (44) 02.03.83

(71) VEB JENAPHARM, JENA;DD;
(72) OLTHOFF, UWE,DR. DIPL.-CHEM.;REICHARDT, KARIN;JACOB, JOERG,DR.;DD;
(73) siehe (72)
(74) VEB JENAPHARM, BFS, 6900 JENA, OTTO-SCHOTT-STR. 13

(54) VERFAHREN ZUR HERSTELLUNG STABILER INJEKTIONSLOESUNGEN VON N-LOSTVERBINDUNGEN

(57) Die Erfindung beinhaltet ein Verfahren zur Herstellung von zur medizinischen Behandlung verwendbaren Injektionslösungen. Ziel ist die Herstellung einer stabilen und gebrauchsfertigen Injektionslösung von N-Lostverbindungen unter Umgehung der technischen Lösung als Trockenampulle. Die N-Lostderivate werden erfindungsgemäß in Konzentrationen von 25 mg/ml bis zu 100 mg/ml in einem ein- oder mehrwertigen Alkohol, insbesondere 1,2-Propylenglykol, gelöst wobei das Lösen, Abfüllen und Aufbewahren der Lösungu unter Inertgas erfolgt. Vor der Injektion werden die Lösungen mit einem wäßrigen Injektionsträger auf die gewünschte Konzentration verdünnt.

9 Seiten

31

bei der chronischen Lymphadenose, der Lymphoretikulose, der Lyphogranulomatose und bei Retikulosen eingeführt.

Bendamustinhydrochlorid ist eine relativ instabile Verbindung. In wäßrigen Lösungen erfolgt nach kurzer Zeit eine nahezu vollständige Hydrolyse der Lost-Halogengruppen. Deshalb ist die Herstellung der Injektionslösungen erst kurz vor der Injektion möglich. Die kinetischen Untersuchungen zur Chloridhydrolyse der N-Lostgruppe des Bendamustinhydrochlorids ergaben in sauren und neutralen Lösungen einen Reaktionsablauf, der sich nach pseudoerster Reaktionsordnung für eine Folgereaktion der symmetrischen Dihalogenidverbindung berechnen ließ (U. OLTHOFF, Abstr. Congr. Pharm. Hung. VI, Budapest 1974, S. 72). Die Chlorid- und Protonenabspaltung aus den ß-Chlorethylgruppen erfolgt unabhängig vom pH-Wert und von eingesetzten Puffersystemen vollständig und mit sehr großer Geschwindigkeit. Dabei übertrifft Bendamustinhydrochlorid sogar die besonders reaktionsfähigen Loste N-Methyl-Lost, Chlorambucil und Uracil-Lost. Diese Angaben begründen die besondere Schwierigkeiten, die sich der Herstellung stabiler medizinischer Zubereitungen des Bendamustinhydrochlorids entgegenstellen.

Nach der DDR-Patentschrift WP 80 967 muß das N-Lostderivat Bendamustinhydrochlorid steril, schwebstofffrei und in einer für die schnelle Auflösung geeigneten Kristallform hergestellt werden. Die Zubereitungsform ist eine Trockenampulle, die eine Mischung von 25 mg Bendamustinhydrochlorid und 175 mg Ascorbinsäure enthält. Vor der Anwendung ist der Ampulleninhalt in Wasser zur Injektion aufzulösen. Die Ascorbinsäure hat die Aufgaben, ein abfüllfähiges Pulvergemisch herzustellen, sowie die Haltbarkeit und den pH-Wert der Injektionslösung zu sichern. Außerdem soll auch die Lagerfähigkeit des trockenen Gemisches erreicht werden. Die Hydrolysegeschwindigkeit wird nach vorliegenden Versuchsergebnissen durch den Ascorbinsäurezusatz nicht beeinflußt.

Der pH-Wert einer Bendamustinhydrochlorid-Lösung, der im Bereich von 2,4 bis 3,0 liegt, wird durch den Ascorbinsäurezusatz nicht wesentlich verändert. Der Ascorbinsäurezusatz erfüllt demnach nicht die an einen Stabilisator zu stellenden Anforderungen. Bendamustinhydrochlorid zeigt als Festsubstanz nach einiger Lagerungszeit eine rosa bis braunrote Verfärbung, die von der Substanzoberfläche ausgeht und nach längerer Zeit die gesamte Substanz für die Herstellung von pharmazeutischen Zubereitungen ungeeignet macht. Auch die Mischung mit Ascorbinsäure zeigt diesen Effekt.

Weiterhin wurde vorgeschlagen, die wäßrige Lösung der Substanz Bendamustinhydrochlorid zu lyophilisieren und vor der Anwendung in Wasser oder Natriumchloridlösung aufzulösen (Herstellungsverfahren des ZIMET, Pharmazeutisches Gutachten IfAr/Nr. 180/80). Das erhaltene Lyophilisat (25 mg/Ampulle) weist erhebliche Nachteile für einen technologischen Herstellungsprozeß auf. Insbesondere die extreme Hygroskopizität und die Durchführung des Prozesses unter Inertgas erschweren die technologische Realisierbarkeit. Außerdem wurden während der Herstellung des Präparates deutliche Zersetzungserscheinungen im Bereich von 5 bis 10 % des Wirkstoffes nachgewiesen. Unbefriedigend ist auch die Feststellung großer Mengen von Mikropartikeln nach Auflösen des Lyophilisats, die auf eine weitere Instabilität des Systems hinweisen.

Der Nachteil der extremen Hygroskopizität des Lyophilisats kann durch Zusätze von bei Raumtemperatur festen Polyolen, insbesondere von Mannitol, behoben werden. Neben dem hohen technologischen Aufwand sind jedoch weiterhin die Nachteile einer erheblichen Zersetzung und des Auftretens von ungelösten Mikropartikeln gegeben.

Ziel der Erfindung

Ziel der Erfindung ist die Herstellung einer stabilen und gebrauchsfertigen Injektionslösung von N-Lostverbindungen unter Umgehung der technischen Lösung als Trockenampulle.

Darlegung des Wesens der Erfindung

Die bisher bekannten technischen Lösungen realisierten das Injektionspräparat entweder als Pulverabfüllung unter Zugabe eines Stabilisators bzw. andersartiger Hilfsstoffe, oder als Lyophilisat, ggf. unter Zusatz von Hilfsstoffen. Durch diese Maßnahmen gelingt es nicht bzw. nur mit erheblichen Nachteilen, ein geeignetes Injektionspräparat herzustellen. Die so erhaltenen Trockenabfüllungen sind durch mangelhafte chemische und physikalische Stabilität gekennzeichnet. Außerdem stellt die Lyophilisierung einen erheblichen technischen Mehraufwand dar, der sich als kapazitätsbegrenzend erweisen kann.

Überraschend wurde nunmehr gefunden, daß der Wirkstoff Bendamustinhydrochlorid in einwertigen Alkoholen, Glykolen und anderen mehrwertigen Alkoholen eine zur Herstellung von Injektionslösungen ausreichende Löslichkeit und vor allem eine auffällig hohe chemische Stabilität aufweist. Die Stabilität der erfindungsgemäß hergestellten Lösungen ist unerwartet, weil Verbindungen mit extremer Hydrolyseempfindlichkeit in der Regel auch empfindlich gegenüber anderen OH-Gruppen enthaltenden Lösungsmitteln sind. Im Falle von Alkoholen oder Polyolen sind derartige Reaktionen als Alkoholyse bekannt.

Es wurde nunmehr gefunden, daß N-Lostverbindungen vom Typ des Bendamustinhydrochlorids keine Alkoholysereaktion eingehen. Voraussetzung ist die Verwendung wasserfreier Lösungsmittel, um Zersetzungen durch die angegebene große Hydrolyseempfindlichkeit zu vermeiden. Unter diesen Voraussetzungen ist z. B. Bendamustinhydrochlorid über lange Zeiträume in der genannten Gruppe von Lösungsmitteln chemisch stabil und bildet nicht die aus wäßrigen Lösungen bekannten Mono- und Dihydroxy- bzw. -alkoxyderivate.

Zur Untersuchung der Stabilität wurde Bendamustinhydrochlorid in einer Konzentration von 25 mg/ml in Ethanol und 1,2-Propylenglykol gelöst und die Lösung bei Raumtemperatur, sowie erhöhten Temperaturen (50 °C, 75 °C, 130 °C) aufbe-

mittels eines spezifischen dünnschichtchromatographischen Verfahrens (Auftragmenge entspr. 0,025 mg Bendamustinhydrochlorid, Kieselgel G, Laufmittel: Butanol/Essigsäure/Wasser 4:1:5, Detektion UV 360 nm bzw. Dragendorff-Reagenz) auf die Bildung von Spaltprodukten hin untersucht, Befunde:

Bildung von Abbauprodukten in:

Zeit	Ethanol-Lösung		Propylenglykol-Lösung		
	25 °C	50 °C	25 °C	75 °C	130 °C
0,5 h	–	–	–	–	(Spuren)
1 h	–	–	–	ohne	–
1,5 h	–	–	–	–	(Spuren)
2 h	–	–	–	ohne	geringe Zersetzung
5 h	ohne	ohne	–	ohne	–
7 h	ohne	ohne	–	–	–
24 h	ohne	–	–	–	–
8 Wochen	ohne	–	ohne	–	–

Aus pharmakologischen und fertigungstechnischen Gründen sind einwertige Alkohole zur Herstellung von Injektionslösungen wenig geeignet. Häufiger verwendet wird 1,2-Propylenglykol. Die erzielbaren Löslichkeiten von Bendamustinhydrochlorid in einigen der benutzten Lösungsmittel betragen bei 25 °C etwa

Ethanol abs.	ca. 50 mg/ml
Propylenglykol	ca. 125 mg/ml und
Glycerol	ca. 50 mg/ml.

In den bisher bekannten Injektionsformen des Bendamustinhydrochlorids werden 25 mg Wirkstoff in 10 ml Lösungsmittel eingesetzt. Es wird nunmehr vorgeschlagen, zur Gewährleistung einer vereinfachten Herstellungstechnologie, einer verbesserten Wirkstoffstabilität bei der Herstellung der Lösungen und deren Aufbewahrung sowie einer vereinfachten Handhabung bei der Herstellung der injektionsfertigen Lösung den Wirkstoff in Polyolen, insbesondere in 1,2-Propylenglykol zu lösen und in Ampullen abzufüllen. Unmittelbar vor der vorgesehenen Injektion wird die Polyollösung durch

den Zusatz eines geeigneten wäßrigen Verdünnungsmittels (Natriumchloridlösung oder Wasser zur Injektion) so verdünnt, daß die zur Anwendung kommende Lösung nur noch ca. 10 % Polyol, ggf. auch weniger enthält. Die Polyollösungen sind mit den angegebenen Verdünnungsmitteln ohne Nachteil für den Wirkstoff beliebig verdünnbar. Durch die breite Variationsmöglichkeit des Polyolanteils und der Verdünnung ergeben sich weitere Vorteile für die Auswahl einer optimalen, besonders gut verträglichen Injektionszubereitung. Neben der Hydrolyseempfindlichkeit sind als weitere Stabilitätsfaktoren die Einwirkungen von Licht und Luftsauerstoff zu berücksichtigen. Trockene Zubereitungen und auch Lösungen verfärben sich unter Licht- und Lufteinwirkung langsam rosa oder bräunlich. Die Lösungen in Alkoholen und Polyolen, die in gut verschlossenen Ampullen unter Lichtausschluß aufbewahrt werden, zeigen keine Verfärbungserscheinungen. Es ist jedoch zu empfehlen, die Herstellung, Abfüllung und Aufbewahrung der Lösungen unter einem Inertgas, wie Argon oder Stickstoff vorzunehmen.

Ausführungsbeispiele

Beispiel 1:

Bendamustinhydrochlorid wird in einer Inertgasatmosphäre in 1,2-Propylenglykol in einer Konzentration von 2,5 g/100 ml unter Rühren gelöst. Die Lösung wird über eine geeignete Filtereinrichtung, ggf. nach Erwärmen auf 50 °C, schwebstofffrei und keimfrei filtriert. In 10-ml-Ampullen wird jeweils 1,0 ml der Lösung abgefüllt, die Ampullen mit Inertgas gefüllt und verschmolzen. Unmittelbar vor der Injektion sind in die geöffnete Ampulle 9,0 ml des vorgesehenen Verdünnungsmittels zu geben. Nach dem Umschütteln ist die Lösung injektionsfertig.

Beispiel 2:

5,0 g Bendamustinhydrochlorid werden in 100 ml Ethanol unter den bei Beispiel 1 genannten Bedingungen gelöst, filtriert

und in Ampullen abgefüllt. Die Aufbewahrung der Ampullen erfolgt bei Temperaturen von +15 °C bis +25 °C.

Erfindungsanspruch

1. Verfahren zur Herstellung stabiler Injektionslösungen von N-Lostverbindungen gekennzeichnet dadurch, daß N-Lostderivate in Konzentrationen von 25 mg/ml bis zu 100 mg/ml in einem wasserfreien ein- oder mehrwertigen Alkohol (Polyol) gelöst werden, wobei das Lösen, Abfüllen und Aufbewahren der Lösung unter Inertgas erfolgt, und die Lösung vor der medizinischen Anwendung im Verhältnis von 1:5 bis 1:20 mit einem wäßrigen Injektionsträger verdünnt wird.

2. Verfahren nach Punkt 1 gekennzeichnet dadurch, daß als Wirkstoff Benzimidazol-Loste, insbesondere Bendamustinhydrochlorid angewendet werden.

3. Verfahren nach Punkt 1 gekennzeichnet dadurch, daß als Polyol insbesondere 1,2-Propylenglykol verwendet wird.

Abstract: The invention relates to a method for producing stable injection solutions which can be used for medical treatment. It is the objective to produce a ready to use, stable injection solution of N-mustard compounds, avoiding the technical solution of a dry ampoule. According to the invention, the N-mustard compounds are used in concentrations of 25 mg/ ml to 100 mg/ ml, dissolved in an anhydrous monovalent or polyvalent alcohol (polyol), wherein the solution is dissolved, filled and stored under inert gas. Before the injection, the solutions are diluted by means of an aqueous injection medium in order to achieve the desired concentration.

Field of the Invention

The invention relates to a method for producing injection solutions which may be used for medical treatment.

Characteristic of the Background Art

N-mustard compounds have been used for some years as highly effective cytostatics. It is estimated that mustards can be used in the therapy of 70 % of all treated malign tumors. More recent synthesis research was aimed at, for example, synthesizing a multivalent antagonist type out of said compound group. In said experiments and in vast pharmacological and clinical trials, bendamustine hydrochloride (trial ID IMET 3393), the compound synthesized by OZEGOWSKI and KREBS in 1963, was for example selected (BRUNS, KNÖLL) from a larger number of compounds. Bendamustine hydrochloride is γ-[1-methyl-5-bis-(β-chloroethyl)-aminobenzimidazo-lyl-(2)]-butyric acid hydrochloride. In 1971, the compound was introduced as pharmaceutical preparation under the trade name of CYTOSTASAN® (0.025 g bendamustine hydrochloride per dry ampoule) for the therapy of hemoblastoses, particularly such as chronic lymphadenosis, lymphoreticulosis, lymphogranulomatosis and of reticuloses.

Bendamustine hydrochloride is a relatively instable compound. Its mustard-halogen groups are almost completely hydrolyzed in aqueous solutions after a short period of time. Therefore, the injection solutions can only be produced shortly before injection. The kinetic experiments regarding the chloride hydrolysis of the N-mustard group of the bendamustine hydrochloride showed a reaction course in acid and neutral solutions which could be calculated according to the pseudo first order of the reaction for a consecutive reaction of the symmetric di-halogen compound (U. OLTHOFF, Abstr. Congr. Pharm. Hung. VI; Budapest 1974, p. 72). Independently of the pH value and of the buffer system used, the separation of the chloride and protons from the β-chloroethyl groups is complete and occurs at very high velocity. As far as this aspect is concerned, bendamustine hydrochloride is even more reactive than the particularly reactive mustards N-methyl mustard, chlorambucil and uracil mustard.

These data explain particular difficulties encountered in the production of stable medical preparations out of bendamustine hydrochloride.

According to the patent from the former German Democratic Republic WP 80 967, the N-mustard derivative bendamustine hydrochloride has to be produced in a sterile crystal form which is free of aerosols and suited for quick dissolution. The preparation form is a dry ampoule containing a mixture of 25 mg of bendamustine hydrochloride and 175 mg of ascorbic acid. Before the application, the content of the ampoule has to be dissolved in water for the injection. The ascorbic acid is included for the objective to produce a powder mixture which can be filled and to ensure the durability and the pH value of the injection solution. Moreover, the shelf life of the dry mixture should be guaranteed. According to the present results of the experiments, the hydrolysis rate is not influenced by the addition of the ascorbic acid.

The pH value of a bendamustine hydrochloride solution, which is within the range of 2.4 to 3.0, is not significantly altered by the addition of the ascorbic acid. Thus, the addition of the ascorbic acid does not match the characteristics which are to be required from a stabilizer. After a certain storage period, solid bendamustine hydrochloride shows a pink to brown-red discoloration, which starts at the surface of the substance and which, after a longer period of time, renders the whole substance unsuitable for the production of pharmaceutical preparations. The mixture with ascorbic acid shows the same discoloration.

Furthermore, it was proposed to lyophilize the aqueous solution of the substance bendamustine hydrochloride and to dissolve it in water or sodium chloride solution before the application (production method of the ZIMET (= former Central Institute for microbiology and experimental therapy in the GDR), pharmaceutical expertise IfAr/ Nr. 180/ 80). The lyophilisate obtained (25 mg/ ampoule) has significant disadvantages for a technological production process. In particular, the technological realization is complicated even more by the extreme hygroscopicity and the fact that the process takes place under inert gas. Moreover, during the production of the preparation, clear signs for decomposition of 5 to 10 % of the active substance were observed. It is furthermore unsatisfying that high amounts of micro particles were

found after the dissolution of the lyophilisate, which indicate a further instability of the system.

The disadvantage of the extreme hygroscopicity of the lyophilisate can be eliminated by the addition of polyols which are solid at room temperature, such as in particular mannitol. In addition to the large technological efforts, also the disadvantages of significant decomposition and of the manifestation of undissolved micro particles have to be considered.

Objective of the Invention

It is the objective of the invention to produce a stable and ready-to-use injection solution out of N-mustard compounds, avoiding the technical solution of a dry ampoule.

Description of the Character of the Invention

In the technical solutions known so far, the injection preparation was realized either as a filled powder under addition of a stabilizer and other auxiliary substances respectively, or in form of a lyophilisate, if necessary, with auxiliary substances. By these measures, a suitable injection preparation can only be realized with significant disadvantages or can't be realized at all. The dry fillings thus obtained are characterized by insufficient chemical and physical stability. Besides, lyophilization means that significantly increased technical efforts have to be made, a fact which might be limiting as far as the capacities are concerned.

Surprisingly it has been found that the substance bendamustine hydrochloride has a sufficient solubility and particularly a extraordinarily high chemical stability for the production of injection solutions in monovalent alcohols, glycols and other polyvalent alcohols. The stability of the solutions produced according to the invention is unexpected, since compounds with extreme sensitivity to hydrolysis are generally also sensitive to other solvents which contain OH-groups. In the case of alcohols or polyols, such reactions are known as alcoholysis.

It has now been found that N-mustard compounds of the bendamustine hydrochloride type do not undergo an alcoholysis reaction. The use of anhydrous solvents is required in order to avoid decomposition caused by the mentioned sensitivity to hydrolysis. Under these conditions, bendamustine hydrochloride, for example, is chemically stable for long periods of time in the mentioned group of solvents and does not form the monohydroxy and dihydroxy or monoalkoxy or dialkoxy derivatives known from aqueous solutions.

In order to examine the stability, bendamustine hydrochloride in a concentration of 25 mg/ ml was dissolved in ethanol and 1,2-propylene glycol and the solution was stored at room temperature, as well as at increased temperatures (50° C, 75° C, 130° C)

[missing sentence]

examined for the formation of cleavage products by means of a specific, thin film chromatography procedure (quantity applied 0.025 mg bendamustine hydrochloride, silica gel G, mobile phase: butanol/ acetic acid/ water 4:1:5; detection UV 360 nm or respectively Dragendorff reagent); results:

Formation of decomposition products in:

time	ethanol solution		propylene glycol solution		
	25° C	50° C	25° C	75° C	130° C
0.5 h	-	-	-	-	(traces)
1 h	-	-	-	none	-
1.5 h	-	-	-	-	(traces)
2 h	-	-	-	none	minor decomposition
5 h	none	none	-	none	-
7 h	none	none	-	-	-
24 h	none	-	-	-	-
8 weeks	none	-	none	-	-

For pharmacological reasons and for reasons concerning the production, monovalent alcohols are of limited use for the production of injection solutions. 1,2-propylene glycol is used more frequently. The solubilites of bendamustine hydrochloride which can be achieved at 25° C in some of the solvents used are of about

ethanol abs.	ca.	50 mg/ ml
propylene glycol	ca.	125 mg/ ml and
glycerol	ca.	50 mg/ ml

In the injection forms of bendamustine hydrochloride known so far, 25 mg active substance are used in 10 ml solvent. It is now proposed to dissolve the active substance in polyols, particularly in 1,2-propylene glycol in order to ensure a more simple production technology, an improved stability of the active substance during the production and storing of the solutions as well as a simplified handling during the production of the ready to inject solution when the active substance is solved in polyols, particularly in 1,2-propylene glycol and filled into ampoules. Immediately before the injection, the polyol solution is diluted by means of the addition of an aqueous diluent (sodium chloride solution in water for injection) such that the solution which is to be applied contains only about 10% polyol, sometimes even less. The polyol solutions can be diluted as desired with the diluents described before, without causing any disadvantages for the active substance. Due to the numerous possible variations of the contents of polyol and the diluent, there are several other advantages for the choice of an optimized, particularly acceptable injection preparation. In addition to the sensitivity to hydrolysis, other stability factors which have to be considered are the influence of light and atmospheric oxygen. Dry preparations and solutions change color under the influence of light and air until they have a pink or brownish color. As far as the solutions in alcohols and polyols which are stored in closed ampoules and under exclusion of light are concerned, no discoloration was observed. It is, however, recommended that the solutions are produced, filled and stored under an inert gas, such as argon or nitrogen.

Exemplary embodiments:

Example 1:

Bendamustine hydrochloride is dissolved under stirring in an inert gas atmosphere in 1,2-propylene glycol in an concentration of 2.5 g/ 100 ml. The solution is filtered by means of a suitable filter device, if necessary after heating to 50° C, such that it is free of aerosols and germs. 1.0 ml of the solution is filled in 10 ml ampoules, the ampoules are filled with inert gas and sealed by heating. Immediately before the injection, 9.0 ml of the chosen diluent is to be added into the opened ampoule. After reshuffling, the solution is ready to inject.

Example 2:

5.0 g bendamustine hydrochloride are dissolved in 100 ml ethanol under the conditions described in example 1, filtered and filled into ampoules. The ampoules are stored at temperatures of +15° C to +25° C.

CLAIMS

1. Method for producing stable injection solutions of N-mustard compounds, characterized in that N-mustard derivatives in concentrations of 25 mg/ ml to 100 mg/ ml are dissolved in an anhydrous monovalent or polyvalent alcohol (polyol), wherein the solution is dissolved, filled and stored under inert gas and the solution is diluted before medical application in a ratio of 1:5 to 1:20 with an aqueous injection medium.

2. Method according to claim 1, characterized in that benzimidazole mustards are used as active substance, in particular bendamustine hydrochloride.

3. Method according to claim 1, characterized in that particularly 1,2-propylene glycol is used as polyol.

PTO/SB/08a (01-08)
Approved for use through 03/31/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	11/330,866
Filing Date	January 12, 2006
First Named Inventor	Brittain et al.
Art Unit	1616
Examiner Name	A. Soroush
Attorney Docket Number	CP391

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
		US-			
		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	C37	SCASNAR, V., et al.., Stability Studies of ^{14}C-Cytostasan® solutions and its extraction using dicarbolide of cobalt, *Pharmazie*, **1988**, pp. 176-179, Vol. 43.	

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] Applicant is to place a check mark here if English language Translation is attached.
This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

RECEIVED
CENTRAL FAX CENTER

OCT 0 7 2008

Attorney Docket No.: CP391

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	**Brittain et al.**	**Confirmation No.:**	**9998**
Serial No.:	**11/330,868**	**Group Art Unit.:**	**1616**
Filing Date:	**January 12, 2006**	**Examiner:**	**A. Soroush**
For:	**BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS**		

I hereby certify that this correspondence is being facsimile transmitted to the USPTO on the date shown below.

Lisa E. Obrecht

October 7, 2008
Date

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

Dear Sir:

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 C.F. R. §1.56 and in accordance with 37 C.F.R. §§1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as a representation that a search has been made or an admission that this information is material to the patentability as defined in 37 C.F.R. § 1.56(b).

This Information Disclosure Statement is being filed under the provisions of 37 C.F.R. §1.97(c), after the period set forth in 1.97(b) and before the mailing date of a Final Office action or before the mailing date of a Notice of Allowance along with authorization to charge the $180.00 fee specified in 37 C.F.R. §1.17(p) to the Deposit Account No. 03-1195.

A copy of each cited non-US patent reference is enclosed.

Please charge any deficiency or credit any overpayment to Deposit Account No. 03-1195.

Date: October 7, 2008

Robert T. Hrubiec
Attorney for Applicant
Registration No. 36,392

10/08/2008 CHOMP 00000020 031195 11330868
01 FC:1806 180.00 DA

CEPHALON, Inc.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Telephone: (610) 738-6356
Facsimile: (610) 727-7651

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

27573 7590 05/28/2009

Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

EXAMINER	
SOROUSH, ALI	

ART UNIT	PAPER NUMBER
1616	

MAIL DATE	DELIVERY MODE
05/28/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1616	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 January 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-78* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-78* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-24, 31-35, 46, 5156, 59, 60, 61, and 71-78, drawn to pharmaceutical composition comprising bendamustine, classified in class 514, subclass 395.

II. Claims 25-27, drawn to method of obtaining agency approval for bendamustine product, classified in class 434, subclass 283.

III. Claims 28-30, 36-45, 47-50, 52-55, 57, and 58, drawn to method of manufacturing lypholized bendamustine, classified in class 34, subclass 284.

IV. Claims 62-70, drawn to method of treating a medical condition, classified in class 514, subclasses 825, 883, 903, and 908.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make another and materially different product or (2) that the product as claimed can be made by another and materially different process (MPEP § 806.05(f)). In the instant case the process of lypholization as described in Invention II can be used to lyopholize a wide variety of active agents.

Inventions I and III are directed to an unrelated product and process. Product and process inventions are unrelated if it can be shown that the product cannot be used in, or made by, the process. See MPEP § 802.01 and § 806.06. In the instant case, the process of getting agency approval of Invention III does not result in the formation of a pharmaceutical composition comprising bendamustine.

Inventions I and IV are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the process of treating a medical condition of Invention IV can be practiced with a variety of different active compounds, such as humanized CD52 monoclonal antibody, depending of the condition to be treated. Furthermore, the composition of Invention I can be used to treat conditions not specifically disclosed by applicant such as for example in the treatment of sarcoma.

Inventions II and III are directed to related processes. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed, the method of manufacturing lyopholized bendamustine has a different design, mode of operation, function, and effect relative to the method of

obtaining agency approval of a composition comprising bendamustine. Furthermore, the inventions as claimed do not encompass overlapping subject matter and there is nothing of record to show them to be obvious variants.

Inventions II and IV are directed to related processes. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed , the method of manufacturing lyopholized bendamustine has a different design, mode of operation, function, and effect relative to the method of treating a medical condition comprising administering a bendamustine composition. Furthermore, the inventions as claimed do not encompass overlapping subject matter and there is nothing of record to show them to be obvious variants.

Inventions III and IV are directed to related processes. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed , the method of treating a medical condition comprising administering a bendamustine composition has a different design, mode of operation, function, and effect relative to the method of obtaining agency approval of a composition comprising bendamustine. Furthermore, the inventions as claimed do not encompass

overlapping subject matter and there is nothing of record to show them to be obvious variants.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Election of Species

This application contains claims directed to the following patentably distinct species chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin’s lymphoma, multiple myeloma, breast cancer, small cell lung cancer, rheumatoid arthritis, multiple scelrosis, lupus, and hperproliferative disorder. The species are independent or distinct because claims to the different species recite the mutually exclusive characteristics of

such species. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 62, 67 and 68 are generic.

There is an examination and search burden for these patentably distinct species due to their mutually exclusive characteristics. The species require a different field of search (e.g., searching different classes/subclasses or electronic resources, or employing different search queries); and/or the prior art applicable to one species would not likely be applicable to another species; and/or the species are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election of the species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the

requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species.

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

A telephone call was made to Scott Larsen on 05/13/2009 to request an oral election to the above restriction requirement, but did not result in an election being made.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder. All claims directed to a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder**. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ali Soroush whose telephone number is (571) 272-9925. The examiner can normally be reached on Monday through Thursday 8:30am to 5:00pm E.S.T.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor, Johann Richter can be reached on (571) 272-0646. 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.

Ali Soroush
Patent Examiner
Art Unit: 1616

/Johann R. Richter/

Supervisory Patent Examiner, Art Unit 1616

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009								
	1	÷								
	2	÷								
	3	÷								
	4	÷								
	5	÷								
	6	÷								
	7	÷								
	8	÷								
	9	÷								
	10	÷								
	11	÷								
	12	÷								
	13	÷								
	14	÷								
	15	÷								
	16	÷								
	17	÷								
	18	÷								
	19	÷								
	20	÷								
	21	÷								
	22	÷								
	23	÷								
	24	÷								
	25	÷								
	26	÷								
	27	÷								
	28	÷								
	29	÷								
	30	÷								
	31	÷								
	32	÷								
	33	÷								
	34	÷								
	35	÷								
	36	÷								

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009								
	37	÷								
	38	÷								
	39	÷								
	40	÷								
	41	÷								
	42	÷								
	43	÷								
	44	÷								
	45	÷								
	46	÷								
	47	÷								
	48	÷								
	49	÷								
	50	÷								
	51	÷								
	52	÷								
	53	÷								
	54	÷								
	55	÷								
	56	÷								
	57	÷								
	58	÷								
	59	÷								
	60	÷								
	61	÷								
	62	÷								
	63	÷								
	64	÷								
	65	÷								
	66	÷								
	67	÷								
	68	÷								
	69	÷								
	70	÷								
	71	÷								
	72	÷								

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009								
	73	÷								
	74	÷								
	75	÷								
	76	÷								
	77	÷								
	78	÷								

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	**Brittain et al.**	**Confirmation No.:**	**9998**
Application No.:	**11/330,868**	**Group Art Unit:**	**1616**
Filing Date:	**January 12, 2006**	**Examiner:**	**Ali Soroush**

For: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

DATE OF SUBMISSION: June 9, 2009

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

PRELIMINARY AMENDMENT AND
RESPONSE TO RESTRICTION REQUIREMENT

Sir:

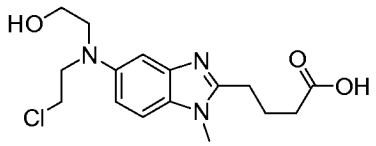
This response is to the Examiner's Office Action, having a Mailing Date of May 28, 2009 (hereinafter, "Office Action"), and a Shortened Statutory Period for Reply of One (1) Month. This response is timely filed.

The Commissioner is authorized to charge any required fees or credit any overpayments which may be required further to this submission under 37 C.F.R. §1.16 or §1.17 to Deposit Account No. 03-1195, upon which the undersigned is authorized to draw.

Preliminary to the examination of this application on the merits, please enter the following amendments, and consider the following remarks.

IN THE CLAIMS

1. (Currently amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II,



Formula II

wherein said HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

2. (Currently amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.5% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

3. (Currently amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.4% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

4. (Currently amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.3% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

5. (Currently amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride containing not more than about 0.9% (area percent of bendamustine) HP1 at release.

6. (Currently amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

7. (Original) The lyophilized preparation according to claim 5, wherein the preparation is packaged in a vial or other pharmaceutically acceptable container.

8. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about six months when stored at 5° C.

9. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 12 months when stored at 5° C.

10. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 24 months when stored at 5° C.

11. (Currently amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9% HP1, wherein said HP1 is the amount of HP1 present at release.

12. (Currently amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.5% HP1, wherein said HP1 is the amount of HP1 present at release.

13. (Currently amended) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 5 mg to about 500 mg of bendamustine or bendamustine hydrochloride.

14. (Currently amended) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 10 mg to about 300 mg of bendamustine or bendamustine hydrochloride.

15. (Currently amended) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 25 mg of bendamustine or bendamustine hydrochloride.

16. (Currently amended) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 100 mg of bendamustine or bendamustine hydrochloride.

17. (Original) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 200 mg of bendamustine or bendamustine hydrochloride.

18. (Original) A pharmaceutical dosage form comprising the lyophilized preparation of claim 5.

19. (Currently amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride comprising bendamustine or bendamustine hydrochloride containing not more than about 0.5% (area percent of bendamustine) HP1 and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release.

20. (Currently amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride according to claim 19 wherein the organic solvent is selected from the group consisting of one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane.

21. (Original) A pharmaceutical composition according to claim 20, wherein the organic solvent is selected from the group consisting of one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol.

22. (Original) A pharmaceutical composition according to claim 19, wherein the organic solvent is tertiary butanol.

23. (Original) A lyophilized preparation according to claim 5 further comprising a trace amount of an organic solvent.

24. (Original) A lyophilized preparation according to claim 23 wherein said organic solvent is tertiary butanol.

25. (Currently amended) In a method for obtaining agency approval for a bendamustine or bendamustine hydrochloride product, the improvement which comprises setting a release specification for bendamustine degradants at less than 4.0 % (area percent bendamustine) for a bendamustine or bendamustine hydrochloride product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

26. (Currently amended) In a method for obtaining agency approval for a bendamustine or bendamustine hydrochloride product, the improvement which comprises setting a release specification for bendamustine or bendamustine hydrochloride of HP1 at less than or equal to 1.5% for a bendamustine or bendamustine hydrochloride product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

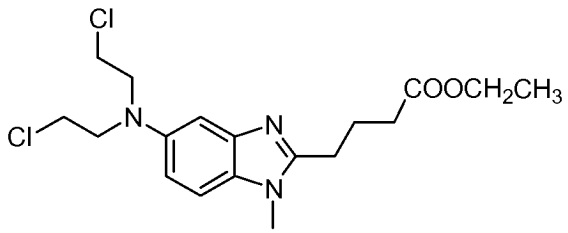
27. (Currently amended) In a method for obtaining agency approval for a bendamustine or bendamustine hydrochloride product, the improvement which comprises setting a shelf-life specification for bendamustine degradants at less than 7.0% (area percent bendamustine) for a bendamustine or bendamustine hydrochloride product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

28. (Currently amended) A process for manufacturing a lyophilized preparation of bendamustine or bendamustine hydrochloride which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of bendamustine degradants is less than 4.0 % (area percent of bendamustine) and the concentration of HP1 is less than 0.5% (area percent of bendamustine).

29. (Currently amended) A process for manufacturing a lyophilized preparation of bendamustine or bendamustine hydrochloride which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.9% (area percent of bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

30. (Currently amended) A process for manufacturing a lyophilized preparation of bendamustine or bendamustine hydrochloride which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.5% (area percent of bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

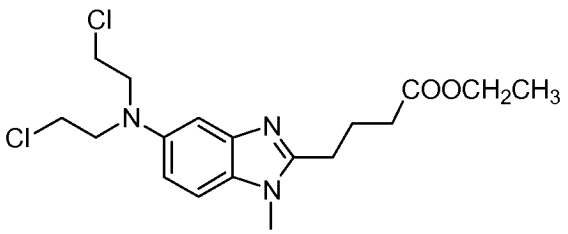
31. (Currently amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride wherein the concentration of bendamustine ethylester (as shown in Formula IV)



Formula IV

is no more than 0.2% greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

32. (Currently amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride according to claim 5 containing not more than about 0.5% bendamustine ethylester as shown in Formula IV



Formula IV.

33. (Currently amended) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.9% (area percent bendamustine).

34. (Currently amended) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.5% (area percent bendamustine).

35. (Original) The lyophilized powder produced from the pre-lyophilization solution or dispersion according to claim 33.

36. (Currently amended) A method of preparing a bendamustine or bendamustine hydrochloride lyophilized preparation comprising,

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine or bendamustine hydrochloride lyophilized preparation contains not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

37. (Currently amended) A method of preparing a bendamustine or bendamustine hydrochloride lyophilized preparation comprising,

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine or bendamustine hydrochloride lyophilized preparation contains not more than about 0.5% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

38. (Original) A method according to claim 36, wherein the alcohol concentration is between about 5% to about 99.9%.

39. (Original) A method according to claim 36, wherein said alcohol is selected from one or more of methanol, ethanol, propanol, iso-propanol, butanol, and tertiary-butanol.

40. (Original) A method according to claim 39, wherein said alcohol is tertiary-butanol.

41. (Original) A method according to claim 40, wherein said tertiary butanol is at a concentration of about 20% to 30%.

42. (Original) A method according to claim 40, wherein said tertiary butanol is at a concentration of about 30%.

43. (Original) A method according to claim 36, wherein an excipient is added before lyophilization.

44. (Original) A method according to claim 43, wherein the excipient is mannitol.

45. (Currently amended) A method according to claim 36, wherein the bendamustine or bendamustine hydrochloride concentration is about 2 to 50 mg/mL.

46. (Original) The lyophilized powder obtained from the method according to claim 36.

47. (Currently amended) A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to a temperature below about -40˚C to form a frozen solution;

ii) holding the frozen solution at or below -40˚C for at least 2 hours;

iii) ramping the frozen solution to a primary drying temperature between about -40˚C and about -10˚C to form a dried solution;

iv) holding for about 10 to about 70 hours;

v) ramping the dried solution to a secondary drying temperature between about 25˚C and about 40˚C; and

vii) holding for about 5 to about 40 hours to form a bendamustine or bendamustine hydrochloride lyophilized preparation.

48. (Original) A method according to claim 47, wherein said alcohol is tertiary-butanol.

49. (Original) A method according to claim 48, wherein said tertiary butanol is at a concentration of about 20% to 30%.

50. (Original) A method according to 49, wherein said tertiary butanol is at a concentration of about 30%.

51. (Original) The lyophilized powder obtained from the method according to claim 47.

52. (Original) A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution;

ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours;

iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution;

iv) holding at a primary drying temperature for about 10 to about 48 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours.

53. (Original) A method according to claim 52, wherein said alcohol is tertiary-butanol.

54. (Original) A method according to claim 53, wherein said tertiary butanol is at a concentration of about 20% to 30%.

55. (Original) A method according to 54, wherein said tertiary butanol is at a concentration of about 30%.

56. (Original) The lyophilized powder obtained from the method according to claim 53.

57. (Currently amended) A method according to claim 36 wherein step b) comprises: i) starting with a shelf temperature of about 5˚C for loading; ii) freezing to about -50˚C over about 8 hours;

iii) holding at -50˚C for about 4 hours; iv) ramping to about -20˚C over about 3 hours; v) holding at about -20˚C for 6 hours; vi) ramping to about -15°C over about 1 hour; ~~vi)~~ vii) holding at -15°C for about 20 hours; ~~vii) ramping to about -15˚C over about 1 hour; viii) holding at about -15˚C for about 20 hours;~~ ~~ix)~~ viii) ramping to about -12˚C over about 0.5 hours; ~~x)~~ ix) holding at about -12˚C for about 15.5 hours; ~~xi)~~ x) ramping to between about 25˚C and about 40˚C or higher for about 15 hours; ~~xii)~~ xi) holding between about 25˚C and about 40˚C for about 10 hours; ~~xiii)~~ xii) ramping to about 40˚C over about 1 hour; ~~andxiv)~~ xiii) holding at about 40˚C for about 5 hours; and xiv) unloading at about 5˚C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying steps iv, v, vi, vii, viii and ix and 50 microns throughout secondary drying steps x, xi, xii and xiii.

58. (Currently amended) A lyophilization cycle according to claim 57, wherein step ~~(xi)~~ (x) is ramped to about 30-35˚C for about 15 hours.

59. (Original) The lyophilized powder prepared from the lyophilization cycle of claim 57.

60. (Currently amended) A formulation for lyophilization comprising bendamustine or bendamustine hydrochloride at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water.

61. (Original) A lyophilized preparation made from the formulation according to claim 60.

62. (Original) A method of treating a medical condition in a patient comprising dissolving the preparation of claim 5 in a pharmaceutically acceptable solvent to produce a pharmaceutically acceptable solution and administering to said patient a therapeutically effective amount of said solution, wherein said condition is amenable to treatment with said preparation.

63. (Original) A method of treating according to claim 62, wherein said condition is selected from chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, breast cancer, small cell lung cancer, and an autoimmune disease.

64. (Original) A method of treating according to claim 63, wherein said condition is non-Hodgkin's lymphoma.

65. (Original) A method of treating according to claim 63, wherein said condition is chronic lymphocytic leukemia.

66. (Original) A method of treating according to claim 63, wherein said condition is multiple myeloma.

67. (Original) A method of treating according to claim 62 further comprising administering the dissolved preparation of claim 5 in combination with one or more anti-neoplastic agents wherein said antineoplastic agent is given prior, concurrently, or subsequent to the administration of the dissolved preparation of claim 5.

68. (Original) A method of treating according to claim 67 wherein the antineoplastic agent is an antibody specific for CD20, wherein said antibody is given prior, concurrently or subsequent to the administration of the dissolved preparation of claim 5.

69. (Original) A method of treating according to claim 62 wherein the autoimmune disease is rheumatoid arthritis, multiple sclerosis or lupus.

70. (Original) A method of treating according to claim 62, wherein the medical condition is a hyperproliferative disorder.

71. (Currently amended) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.9% HP1 (area percent of bendamustine) wherein

said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

72. (Currently amended) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.5% HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

73. (Currently amended) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 10 to about 500 mg/container.

74. (Currently amended) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 100 mg/container.

75. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 5 mg to about 2 g/container.

76. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 170 mg/container.

77. (Original) A pre-lyophilized pharmaceutical composition of bendamustine comprising about 15 mg/mL bendamustine HCl, about 25.5 mg/mL mannitol, about 30% (v/v) tertiary-butyl alcohol, and water.

78. (Original) The preparation of claim 5 which is a pharmaceutical composition.

REMARKS

Discussion of the Amendment

Claims 1 to 6, 11 to 17, 19, 20, 25 to 34, 36, 37, 45, 47, 60, and 71-74 have been amended to more particularly point out that bendamustine hydrochloride is contemplated in the claims, along with other bendamustine preparations and methods.

Support for the recitation of bendamustine hydrochloride is found throughout the specification, in particular for example at page 1, line 27, page 17, lines 18 and 19, and at pages 37 to 39.

Claim 57 has been amended to remove an inadvertent redundancy wherein the second portion of claim (v) as originally recited, and step (vi), as originally recited were redundant over previously recited steps (vii) and (viii). This redundancy has been removed by the present amendment, and the steps appropriately re-numbered.

Claim 57 has been amended also to remove any ambiguity as to what steps are encompassed by "primary drying" and "secondary drying". Support for this amendment to claim 57 is found in the specification, for example, at page 50, in the table of Example 7 showing the lyophilization cycle from which claim 57 is derived. This shows the particular steps, and pressures corresponding to the primary and secondary drying steps.

These amendments to the claims add no new matter.

Claims 1 to 78 are pending in this application.

Discussion of the Restriction Requirement

The Examiner has required restriction to one of the following inventions, as set forth in the Examiner's Groups I to IV.

I. Claims 1-24, 31-35, 46, 51, 56, 59, 60, 61 and 71-78, drawn to pharmaceutical composition comprising bendamustine…;

II. Claims 25 to 27, drawn to methods of obtaining agency approval for a bendamustine product…;

III. Claims 28 to 30, 36-45, 47-50, 52-55, 57 and 58, drawn to methods of manufacturing lyophilized bendamustine…; and

IV. Claims 62-70, drawn to methods of treatment of medical conditions…

(Office Action at 2).

The above Groups are as listed on page 2 of the Office Action, in the initial portion of the Restriction Requirement.

In the body of the Office Action, the Examiner appears to refer to Group II as though it were Group III (i.e., the methods of manufacture), and vice-versa. For purposes of this response, the Groups will be numbered as specified above (i.e., Group II will be treated as the methods for obtaining agency approval, and Group III will be treated as methods of manufacturing lyophilized bendamustine).

Although Applicants can appreciate restriction among the Examiner's Group II, and Groups I and III; and among Group IV, and Groups I and III, Applicants respectfully traverse the restriction between the Examiner's Groups I and III.

Even though the inventions of the Examiner's Groups I and III are patentably distinct, as the Examiner indicates, this is not the sole criterion for a proper restriction requirement. There must also be a serious burden on the Examiner to examine the inventions together.

The preparations and pharmaceutical compositions of the Examiner's Group I are specifically prepared by the methods of the Examiner's Group III. Thus, a search encompassing the invention of Group I would necessarily encompass the invention of the Examiner's Group III. Likewise the methods of the Examiner's Group III specifically result in the pharmaceutical compositions of the Examiner's Group I. Again, a search encompassing the invention of the Examiner's Group III would necessarily encompass the invention of the Examiner's Group I.

Accordingly, reconsideration and withdrawal of the restriction requirement between the Examiner's Groups I and III is respectfully requested.

Applicants note with appreciation the Examiner's discussion of rejoinder practice.

Discussion of the Election of Species Requirement

The Examiner indicates that, "This application contains claims directed to the following patentably distinct species, chronic lymphocytic leukemia, Hodgkin's disease…and hyperproliferative disorder." (Office Action at 6).

The election of species requirement appears to be directed only to the Examiner's Group IV. Because, as indicated below, it is Applicants' intention to elect the invention of the Examiner's Group I, the election of species requirement is not currently addressed.

Election

In order to comply with the requirements of 37 C.F.R. § 1.143, and in the event that the Examiner's Restriction Requirement is made final, Applicants elect, with traverse, the invention of the Examiner's Group I, i.e., that drawn to preparations and pharmaceutical compositions comprising bendamustine. The claims that read upon the elected invention are 1 to 24, 31 to 35, 46, 51, 56, 59 to 61, and 71 to 78.

Respectfully submitted,

/Paul R. Darkes/

Date: June 9, 2009

Paul R. Darkes
Registration No. 33,862
Attorney for Applicants

CEPHALON, Inc.
41 Moores Road
PO Box 4011
Frazer, PA 19355
Telephone: 610-738-6463
Telefax: 610-727-7651

Electronic Acknowledgement Receipt

EFS ID:	5478884
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Paul Randolph Darkes/Lisa Obrecht
Filer Authorized By:	Paul Randolph Darkes
Attorney Docket Number:	CP391
Receipt Date:	09-JUN-2009
Filing Date:	12-JAN-2006
Time Stamp:	16:24:46
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		CP391USPreAmRRR05282009.pdf	143430 36690b3fe2a41c1021c14158151ebf79da9a483c	yes	17

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response to Election / Restriction Filed	1	1
	Claims	2	14
	Applicant Arguments/Remarks Made in an Amendment	15	17
Warnings:			
Information:			
	Total Files Size (in bytes):	143430	

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.

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
11/330,868	01/12/2006	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
FOR	NUMBER FILED	NUMBER EXTRA					
☐ 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 06/09/2009

	(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))	* 78	Minus	** 78	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	* 21	Minus	***21	=	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	

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:
//TERRANCE LAWRENCE//

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

27573 7590 08/19/2009

Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

EXAMINER	
SOROUSH, ALI	
ART UNIT	PAPER NUMBER
1616	

MAIL DATE	DELIVERY MODE
08/19/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1616	

-- 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 June 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) *1-78* is/are pending in the application.

4a) Of the above claim(s) *25-30,36-45,47-50,52-55,57,58 and 62-70* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-24,31-35,46,51,56,59-61 and 71-78* 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 *01232008*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Acknowledgement of Receipt

Applicant's response filed on 06/09/2009 to the Office Action mailed on 05/28/2009 is acknowledged.

Status of the Claims

Claims 1-6, 12-20, 25-34, 36, 37, 45, 57, 58, 60, and 71-74 is currently amended. Claims 25-30, 36-45, 47-50, 52-55, 57, 58, and 62-70 are withdrawn as being directed to non-elected subject matter. Therefore, claims 1-78 are currently pending examination for patentability.

Election/Restrictions

Applicant's election of Invention of Group I (claims 1-24, 31-35, 46, 51, 56, 59, 60, 61 and 71-78) with traverse is acknowledged. Applicant argues the restriction between Groups I and III is not proper since it would not place an undue burden on the Examiner to search. Applicant's argument has been fully considered but found not to be persuasive. The search strategy needed to search Groups I and II would be different and therefore would result in an undue burden on the Examiner.

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 1-6, 12-20, 31-35, 51, 56, 59, 60, 61 and 71-74 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.

Claims 1-6, 31-34, and 72 are indefinite for including subject matter in parentheses. It is not clear if the subject matter in parentheses is intended to be limiting or not. It is suggested that the parenthetical subject matter be written without parentheses.

Claim Rejections - 35 USC § 102

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-12, 18, 19, 71, 72, and 78 are rejected under 35 U.S.C. 102(b) as being anticipated by Jacob et al. (DD 159289 A1, Published 03/02/1983).

Jacob et al. teach an injectable solution of bendamustine dissolved in ethanol or polyethylene glycol. (See abstract). The disclosed composition does not comprise any HP1 and therefore reads on the 0% HP1 which is less than 0.9% HP1. For the foregoing reasons the instant claims are anticipated.

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

1. Claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61 and 71-74 are rejected under 35 U.S.C. 103(a) as being unpatentable over Rubino (US Patent Application 2005/0020615 A1, Published 01/27/2005, Filed 07/19/2004) in view of Horres et al. (US Patent Application 2005/0060028 A1, Published 03/17/2005, Filed 10/15/2002).

Applicant Claims

Applicant claims a composition of bendamustine reconstituted from a lyophilized preparation of bendamustine, wherein the lyophilized preparation is a freeze-dried solution of bendamustine, mannitol, t-butyl alcohol, and water.

Determination of the Scope and Content of the Prior Art (MPEP §2141.01)

Rubino teaches a method of preparing lyophilized rapamycin derivative comprising the steps of, first preparing a solution of rapamycin derivative, mannitol, t-butyl alcohol, and water, and secondly freeze-drying the solution. (See claim 13). "The present invention provides pre-lyophilization formulations that provide freeze-dried CCI-779 of the invention with improved potency retention and stability under storage

conditions. More particularly, using the pre-lyophilization formulations if the invention, freeze-dried CCI-779 has been found to retain greater than 95% initial potency after one month storage ... The present invention also provides reconstituted CCI-779 formulations suitable for delivery parenterally or by other routes of delivery." (See paragraph 0008).

Ascertainment of the Difference Between Scope the Prior Art and the Claims (MPEP §2141.012)

Rubino lacks a teaching wherein the active compound is bendamustine. This deficiency is cured by the teachings of Horres et al.

Horres et al. teach that rapamycin and bendamustine are both antiproliferative agents for treating cancer such as leukemia. (See paragraph 0017).

Finding of Prima Facie Obviousness Rational and Motivation (MPEP §2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to combine the teachings of Robino with Horres et al. and produce the instant invention.

One would have been motivated to so because Robino teach that the lyophilization method taught would give a much more stable final active agent and Horres et al. teach that rapamycin and bendamusitne are suitable leukemia treatment

drugs. For the foregoing reasons the instant claimed kit would have been obvious to one of ordinary skill in the art at the time of the instant invention.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ali Soroush whose telephone number is (571) 272-9925. The examiner can normally be reached on Monday through Thursday 8:30am to 5:00pm E.S.T.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor, Johann Richter can be reached on (571) 272-0646. 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.

Ali Soroush
Patent Examiner
Art Unit: 1616

/Ernst V Arnold/

Primary Examiner, Art Unit 1616

Notice of References Cited	Application/Control No. 11/330,868	Applicant(s)/Patent Under Reexamination BRITTAIN ET AL.	
	Examiner ALI SOROUSH	Art Unit 1616	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2005/0020615 A1	01-2005	Rubino, Joseph T.	514/291
*	B	US-2005/0060028 A1	03-2005	Horres et al.	623/001.38
	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	159289 A	03-1983	DD	Jacob et al.	
	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.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009							
	1	÷	✓							
	2	÷	✓							
	3	÷	✓							
	4	÷	✓							
	5	÷	✓							
	6	÷	✓							
	7	÷	✓							
	8	÷	✓							
	9	÷	✓							
	10	÷	✓							
	11	÷	✓							
	12	÷	✓							
	13	÷	✓							
	14	÷	✓							
	15	÷	✓							
	16	÷	✓							
	17	÷	✓							
	18	÷	✓							
	19	÷	✓							
	20	÷	✓							
	21	÷	✓							
	22	÷	✓							
	23	÷	✓							
	24	÷	✓							
	25	÷	N							
	26	÷	N							
	27	÷	N							
	28	÷	N							
	29	÷	N							
	30	÷	N							
	31	÷	✓							
	32	÷	✓							
	33	÷	✓							
	34	÷	✓							
	35	÷	✓							
	36	÷	N							

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009							
	37	÷	N							
	38	÷	N							
	39	÷	N							
	40	÷	N							
	41	÷	N							
	42	÷	N							
	43	÷	N							
	44	÷	N							
	45	÷	N							
	46	÷	✓							
	47	÷	N							
	48	÷	N							
	49	÷	N							
	50	÷	N							
	51	÷	✓							
	52	÷	N							
	53	÷	N							
	54	÷	N							
	55	÷	N							
	56	÷	✓							
	57	÷	N							
	58	÷	N							
	59	÷	✓							
	60	÷	✓							
	61	÷	✓							
	62	÷	N							
	63	÷	N							
	64	÷	N							
	65	÷	N							
	66	÷	N							
	67	÷	N							
	68	÷	N							
	69	÷	N							
	70	÷	N							
	71	÷	✓							
	72	÷	✓							

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009							
	73	÷	✓							
	74	÷	✓							
	75	÷	✓							
	76	÷	✓							
	77	÷	✓							
	78	÷	✓							

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
EAST (See search history)	08/14/2009	AS
PALM Inventor search	08/14/2009	AS
Consulted Ernst Arnold	08/15/2009	AS

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L2	51578	"t-butanol" "tert-butanol" "t-butyl alcohol" "tert-butyl alcohol" "tertiary-butyl alcohol" "1,1-dimethylethanol" dimethylethanol "2-methyl-2-propanol"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 17:53
L3	6	L2 near mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 17:54
L4	4	"CCI-779" same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 18:02
L5	4	torisel same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 18:04
L6	7	temsirolimus same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 18:04
L7	12	rapamycin same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/14 18:05

S1	140851	("20030232874" | "20040053972" | "20040058956" | "20040072889" | "20040096436" | "20040152672" | "20040247600" | "20050060028" | "20050176678" | "20060051412" | "5204335" | "5227373" | "5750131" | "5770230" | "5776456" | "5955504" | "5972912" | "6034256" | "6077850" | "6090365" | "6271253" | "6380210" | "6492390" | "6545034" | "6569402" | "6573292" | "6613927")".PN"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:45
S2	51	("20030232874" | "20040053972" | "20040058956" | "20040072889" | "20040096436" | "20040152672" | "20040247600" | "20050060028" | "20050176678" | "20060051412" | "5204335" | "5227373" | "5750131" | "5770230" | "5776456" | "5955504" | "5972912" | "6034256" | "6077850" | "6090365" | "6271253" | "6380210" | "6492390" | "6545034" | "6569402" | "6573292" | "6613927").PN.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:45
S3	6	S2 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:46

S4	3	lyophilized same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:47
S5	0	lyophilize same bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:49
S6	5	(lyophilized lyophilization "freeze-dry" "freeze-drying" "freeze-dried" cryodessiccation)same (bendamustine ribomustine treanda "SDX-105")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:51
S7	139	reszka.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:52
S8	2	S7 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:53
S9	5	(lyophilize lyophilized lyophilization "freeze-dry" "freeze-drying" "freeze-dried" cryodessiccation)same (bendamustine ribomustine treanda "SDX-105")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 19:55
S10	2	"5770230".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 20:13
S11	256	(bendamustine ribomustine treanda "SDX-105")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2009/08/13 20:14

8/14/2009 7:03:50 PM
C:\Documents and Settings\asoroush\My Documents\EAST\Workspaces\11330868.wsp

FORM PTO-1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

List of Patent and Publications Cited by Applicant

Attorney Docket	CP391
Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Brittain
Group Art Unit	1616
Examiner Name	A. Soroush
Sheet	1 of 5

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number	Name	Date of Publication	Class	Subclass
/A.S./	A1	US-5204335	Sauerbier et al.	Apr. 20, 1993		
	A2	US-5227373	Alexander et al.	Jul. 13, 1993		
	A3	US-5750131	Wichert et al.	May 12, 1998		
	A4	US-5770230	Teagarden et al.	Jun. 23, 1998		
	A5	US-5776456	Anderson et al.	Jul. 7, 1998		
	A6	US-5955504	Wechter et al.	Sep. 21, 1999		
	A7	US-5972912	Marek et al.	Oct. 26, 1999		
	A8	US-6034256	Masferrer	Mar. 7, 2000		
	A9	US-6077850	Masferrer	Jun. 20, 2000		
	A10	US-6090365	Kaminski et al.	Jul. 18, 2000		
	A11	US-6271253 B1	Masferrer	Aug. 7, 2001		
	A12	US-6380210	Desimone et al.	Apr. 30, 2002		
	A13	US-6492390 B2	Masferrer	Dec. 12, 2002		
	A14	US-6545034 B1	Carson et al.	Apr. 8, 2003		
	A15	US-6569402	Cheesman et al.	May 27, 2003		
	A16	US-6573292 B1	Nardella	Jun. 3, 2003		
	A17	US-6613927 B1	Kwok	Sep. 2, 2003		
	A18	US-2003/0232874	Nardella	Dec 18, 2003		
	A19	US-2004/0053972	Nara	Mar. 18, 2004		
	A20	US-2004/0058956	Akiyama et al.	Mar. 25, 2004		
	A21	US-2004072889	Masjerrer	Apr. 15, 2004		
	A22	US-2004/0096436 A1	Carson et al.	May 20, 2004		
	A23	US-2004152672	Carson et al.	Aug. 5, 2004		
	A24	US-2004/0247600	Leoni	Dec. 9, 2004		
	A25	US-2005/0060028 A1	Horres et al.	Mar. 17, 2005		
	A26	US-2005/0176678	Horres et al.	Aug 11, 2005		
	A27	US-2006/0051412	Petereit et al.	Mar 9, 2006		

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	2 of 5

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Document No.	Date	Country	Translation YES	Translation NO
/A.S./	B1	DD 159289	Jun. 1, 1981	Germany	x	
	B2	DD 159877	Apr. 13, 1983	Germany	x	
	B3	DD 293808	Sep. 12, 1991	Germany		x
	B4	DE 80967	Jun. 1, 1970	Germany	x	
	B5	DE 10016077	Dec. 13, 2001	Germany		x
	B6	DE 10304403	Aug. 5, 2004	Germany	A27	
	B7	DE 10306724	Sep. 18, 2003	Germany		x
	B8	EP 1354952	Oct. 22, 2003	Germany		
	B9	EP 1444989	Aug. 11, 2004	Italy		
	B10	WO 96/28148	Mar. 13, 1998	Australia		
	B11	WO 03/066027 A1	Feb. 7, 2003	PCT		
	B12	WO 03/081238	Oct. 2, 2003	PCT		
	B13	WO 03/086470 A3	May 6, 2004	PCT		
	B14	WO 03/094990	Mar. 11, 2003	PCT	A26	

OTHER DOCUMENTS Non-Patent Literature Documents

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), Title of Article, Title of Journal (book, magazine, catalog, etc.) Date, Pertinent Pages, Volume-Issue Number, publisher, city and/or country where published.
/A.S./	C1	AIVADO, MANUEL et al., *Bendamustine in the treatment of chronic lymphocytic leukemia: Results and future perspectives*, Seminars in Oncology, **2002**, pp. 19-22, Vol. 29 No. 4, Suppl. 13.
/A.S./	C2	BARMAN BALFOUR, JULIA A. et al., *Bendamustine*, Drugs, **2001**, pp. 631-638, Vol. 61(5), Auckland, New Zealand
/A.S./	C3	BREMER, KARL, *High rates of long-lasting remissions after 5-day bendamustine chemotherapy cycles in pre-treated low-grade non-hodgkin's-lymphomas*, Journal of Cancer Research and Clinical Oncology, **2002**, pp.603-609, Vol. 128(11).

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	3 of 5

/A.S./	C4	CHOW, KAI et al., *Anti-CD20 antibody (IDEC-C2B8, rituximab) enchances efficacy of cytotoxic drugs on neoplastic lymphocytes in vitro: role of cytokines complement, and caspases*, Haematologica, **Jan. 2002**, pp. 33-43, Vol. 87(1)
	C5	CHOW, KAI U. et al., *In AML Cell Lines Ara-C Combined with Purine Analogues is Able to Exert Synergistic as Well as Antagonistic Effects on Proliferation, Apoptosis and Disruption of Mitochondrial Membrane Potential*, Leukemia & Lymphoma, **2003**, pp.165-173, Vol. 44(1).
	C6	CHOW, KAI U. et al., *In vitro induction of apoptosis of neoplastic cells in low-grade non-Hodkin's lymphomas by combinations of established cytotoxic drugs with bendamustine*, Haematologica, **2001**, pp. 485-493, Vol. 86(5).
	C7	CHOW, KAI UWE et al., *Synergistic effects of chemotherapeutic drugs in lymphoma cells are associated with down-regulation of inhibitor of apoptosis proteins (IAPs), prostate-apoptosis-response-gene 4(Par-4), death-associated protein (Dazz) and with enforced caspase activation*, Biochemical Pharmacology, **2003**, pp.711-724, Vol. 66(5).
	C8	DIEHL, VOLKER et al., *Bendamustine in the Treatment of Hematologic Malignancies*, Semin. Oncol., **2002**, Vol. 29(4) Suppl. 13, Saundes, Philadelphia, PA.
	C9	FICHTNER, I. et al., *Antineoplastic activity and toxicity of some alkylating cytostatics (cyclophosphamide, CCNU, cytostasan) encapsulated in liposomes in different murine tumor models*, Journal of Microencapsulation, **1986**, pp. 77-87, Vol. 3(2).
	C10	GHANDI, VARSHA, *Metabolism and mechanisms of action of bendamustine: Rationales for combination therapies*, Seminars in Oncology, **2002**, pp. 4-11, Vol. 29, No. 4, Suppl. 13.
	C11	GUST, R. et al., *Investigations on the Stability of Bendamustin, a Cytostatic Agent of the Nitrogen Mustard Type, I. Synthesis, Isolation, and Characterization of Reference Substances*, Monatshefte fur Chemie, **1997**, pp. 291-299, Vol. 128(3)
	C12	HEIDER, ANDREA et al., *Efficacy and toxicity of bendamustine in patients with relapsed low-grade non-Hodgkin's lymphomas*, Anti-Cancer Drugs, **2001**, pp. 725-729, Vol. 12(9).
	C13	KATH, R. et al., *Bendamustine monotherapy in advanced and refractory chronic lymphocytic leukemia*, Journal of Cancer Research and Clinical Oncology, **2001**, pp. 48-54, Vol. 127(1).

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	4 of 5

/A.S./	C14	KOENIGSMAN, MICHAEL et al., *Fludarabine and Bendamustine in Refractory and Relapsed Indolent Lymphoma – a Multicenter Phase I/II Trial of the East German Society of Hematology and Oncology (OSHO)*, Leukemia & Lymphoma. **2004**, pp.1821-1827, Vol.45(9)
	C15	KOESTER, WOLF et al., *Carboplatin in combination with bendamustine in previously untreated patients with extensive-stage small lung cancer (SCLC)*, Clinical Drug Investigation, **2004**, pp.611-618, Vol. 24(10).
	C16	KOLLMANNSBERGER, CHRISTIAN et al., *Phase II study of bendamustine in patients with relapsed or cisplatin-refractory germ cell cancer*, Anti-Cancer Drugs, **2000**, pp. 535-539, Vol. 11(7).
	C17	KONSTANTINOV, S. M. et al., *Cytotoxic efficacy of bendamustine in human leukemia and breast cancer cell lines*, Journal of Cancer Research and Clinical Oncology, **2002**, pp. 271-278, Vol. 128(5).
	C18	LEONI, LM et al., *Sdx-105 (Trenda), Active in Non-Hodgkins Lymphoma Cells, Induces the Mitotic Catastrophe Death Pathway*, Blood, **2004**, Vol. 104(11): Abs 4593.
	C19	MAAS, *Stabilitat von Benamustinhydrochlorid in Infusionslosungen* Pharmazie, vol. 49, no. 10, **1994**, pp. 775-777. (translation included)
	C20	NIEMEYER, CHRISTINA C. et al., *SDX-105 (bendamustine) is a clinically active chemotherapeutic agent with a distinct mechanism of action*, Proc Annu Meet Am Assoc Cancer Res, **2004**, Vol. 45:1st ed.
	C21	NOWAK, DANIEL et al., *Upon Drug-Induced Apoptosis in Lymphoma Cells X-linked Inhibitor of Apoptosis (XIAP) Translocates from the Cytosol to the Nucleus*, Leukemia & Lymphoma, **2004**, pp.1429-1436, Vol.45(7).
	C22	PONISCH, WOLFRAM et al., *Bendamustine in the treatment of Multiple Myeloma: Results and future perspectives*, Seminars in Oncology, **2002**, pp.23-26, Vol. 29 No. 4 Suppl. 13.
	C23	PREISS, R. et al., *Pharmacokinetics of bendamustin (Cytostasan) in patients*, Pharmazie, **1985**, pp. 782-784, Vol. 40(11). TRANSLATION
	C24	*Ribomustin: bendamustine Product Monograph*, **Jan. 2002**, pp. 3-54, Ribosepharm GMBH, Munchen, Germany
	C25	*Ribomustin: bendamustine Product Monograph*, **Mar. 2005**, pp. 3-73, Ribosepharm MBH, Munchen, Germany

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	5 of 5

/A.S./	C26	RUMMEL, MATHIAS J. et al., *Bendamustine in the treatment of non-Hodgkin's lymphoma: Results and future perspectives*, Seminars in Oncology, **2002**, pp.27-32, Vol. 29 No. 4 Suppl. 13.
	C27	RUMMEL, MATHIAS J. et al., *In Vitro Studies With Bendaustine: Enhanced Activity in Combination With Rituximab*, Seminars in Oncology, **Aug. 2002**, pp. 12-14, Vol. 29 No. 4 Suppl 13
	C28	SCASNAR et al., *Radiochemical Assay of Stability of ^{14}C-Cytostasan Solutions During Preparation and Storage*, vol. 121, no. 2m 1988 pgs 489-497.
	C29	SCHMIDT-HIEBER, MARTIN et al., *A phase II study of bendamustine chemotherapy as second-line treatment in metastatic uveal melanoma*, Melanoma Research, **2004**, pp.439-442, Vol.14(6)
	C30	SCHOFFSKI, P., *Repeated administration of short infusions of Bendamustine: a phase I study in patients with advanced progressive solid tumors*, Journal of Cancer Research and Clinical Oncology, **2000**, pp. 41-47, Vol. 126(1).
	C31	SCHRIJVERS, DIRK et al., *Phase I studies with bendamustine: An update*, Seminars in Oncology, **2002**, pp. 15-18, Vol. 29 No. 4 Suppl. 13.
	C32	STRUMBERG, DIRK et al., *Bendamustine hydrochloride activity against doxorubicin-resistant human breast carcinoma cell lines*, Anti-Cancer Drugs, **1996**, pp. 415-421, Vol. 7(4)
	C33	WEIDE, RUDOLF et al., *Bendamustine mitoxantrone and rituximab (BMR): a new effective regimen for refractory or relapsed indolent lymphomas*, Leukemia & Lymphoma, **2002**, pp.327-331, Vol. 43(2)
	C34	WEIDE, RUDOLF et al., *Bendamustine/Mitoxantrone/Rituximab (BMR): A Very Effective, Well Tolerated Outpatient Chemoimmunotherapy for Relapsed and Refractory CD20-positive Indolent Malignancies. Final Results of a Pilot Study*, Leukemia & Lymphoma, **2004**, pp. 2445-2449, Vol. 45(12)
	C35	WERNER, W. et al., *Hydrolyseprodukte des Cancerostaticums Cytostasan (Bendamustin)*, Pharmazie, **1987**, pp. 272-273, Vol. 42 (Translation included)
	C36	ZULKOWSKI, K. et al., *Regression of brain metastases from breast carcinoma after chemotherapy with bendamustine*, Journal of Cancer Research and Clinical Oncology, **2002**, pp. 111-113, Vol. 128(2).

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

PTO/SB/08a (01-08)
Approved for use through 03/31/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	11/330,866
Filing Date	January 12, 2006
First Named Inventor	Brittain et al.
Art Unit	1616
Examiner Name	A. Soroush
Attorney Docket Number	CP391

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-			
		US-			
		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/A.S./	C37	SCASNAR, V., et al.., Stability Studies of ^{14}C-Cytostasan® solutions and its extraction using dicarbolide of cobalt, *Pharmazie*, **1988**, pp. 176-179, Vol. 43.	

Examiner Signature	/Ali Soroush/	Date Considered	08/14/2009

*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] Applicant is to place a check mark here if English language Translation is attached.
This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jason Edward Brittain

Confirmation No.: **9998**

Application No.: **11/330,868**

Group Art Unit: **1616**

Filing Date: **January 12, 2006**

Examiner: **Ali Soroush**

For: **Bendamustine Pharmaceutical Compositions**

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

Sir:

REPLY PURSUANT TO 37 CFR § 1.111

In response to the Official Action dated **August 19, 2009**, reconsideration is respectfully requested in view of the amendments and/or remarks as indicated below:

☐ **Amendments to the Specification** begin on page of this paper.

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

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet.

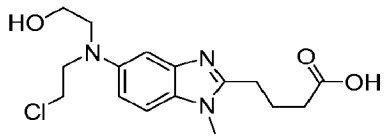
☒ **Remarks** begin on page 113 of this paper.

☐ **Request For Refund** submitted herewith.

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

Listing of Claims:

1. (Currently Amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9 area% ~~(area percent of bendamustine)~~ of HP1 ~~as shown in Formula II~~, relative to the amount of bendamustine



~~Formula II~~ HP1

wherein said HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

2. (Currently Amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.5 area% ~~(area percent of bendamustine)~~, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

3. (Currently Amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.4 area% ~~(area percent of bendamustine)~~, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

4. (Currently Amended) The composition according to claim 1, wherein the amount of HP1 is not more than 0.3 area% ~~(area percent of bendamustine)~~, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

5. (Canceled)

6. (Currently Amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% ~~(area percent of bendamustine)~~ of HP1, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride ~~release~~.

7. (Currently Amended) The lyophilized preparation according to claim 1 [[5]], wherein the preparation is packaged in a vial or other pharmaceutically acceptable container.

8. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about six months when stored at 5° C.

9. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 12 months when stored at 5° C.

10. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 24 months when stored at 5° C.

11. (Currently Amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9 area % of HP1, relative to the amount of bendamustine, wherein said HP1 is the amount of HP1 present at release.

12. (Currently Amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1, relative to the amount of bendamustine, wherein said HP1 is the amount of HP1 present at release.

13. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 5 mg to about 500 mg of bendamustine or bendamustine hydrochloride.

14. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 10 mg to about 300 mg of bendamustine or bendamustine hydrochloride.

15. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 25 mg of bendamustine or bendamustine hydrochloride.

16. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 100 mg of bendamustine or bendamustine hydrochloride.

17. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 200 mg of bendamustine or bendamustine hydrochloride.

18. (Currently Amended) A pharmaceutical dosage form comprising the lyophilized preparation of claim 1 [[5]].

19. (Currently Amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride comprising bendamustine or bendamustine hydrochloride containing not more than about 0.5 arca% ~~(area percent of bendamustine)~~ of HP1, rclativc to thc amount of bendamustine, and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release.

20. (Previously Presented) A pharmaceutical composition of bendamustine or bendamustine hydrochloride according to claim 19 wherein the organic solvent is selected from the group consisting of one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride,

dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane.

21. (Original) A pharmaceutical composition according to claim 20, wherein the organic solvent is selected from the group consisting of one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol.

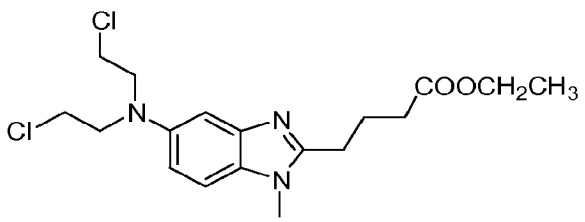
22. (Original) A pharmaceutical composition according to claim 19, wherein the organic solvent is tertiary butanol.

23. (Currently Amended) A lyophilized preparation according to claim 1 [[5]] further comprising a trace amount of an organic solvent.

24. (Original) A lyophilized preparation according to claim 23 wherein said organic solvent is tertiary butanol.

Claims 25-30 (Canceled)

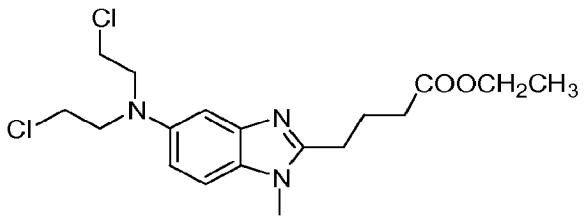
31. (Currently Amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride wherein the concentration of bendamustine ethylester ~~(as shown in Formula IV)~~



~~Formula IV~~bendamustine ethyl ester

is no more than 0.2% greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

32. (Currently Amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride according to claim 5 containing not more than about 0.5% bendamustine ethylester ~~as shown in Formula IV~~



~~Formula IV.~~bendamustine ethyl ester.

33. (Currently Amended) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.9area% ~~(area percent bendamustine)~~, relative to the amount of bendamustine.

34. (Currently Amended) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.5 area% ~~(area percent bendamustine)~~, relative to the amount of bendamustine.

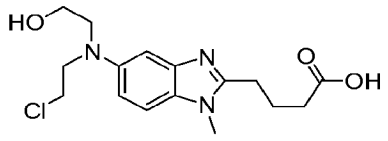
35. (Original) The lyophilized powder produced from the pre-lyophilization solution or dispersion according to claim 33.

Claims 36-45 (Canceled)

46. (Currently Amended) A ~~The~~ lyophilized powder obtained from by a ~~the~~ method comprising ~~according to claim 36~~a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said lyophilized powder contains not more than about 0.9 area% of HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release.

Claims 47-50 (Canceled)

51. (Currently Amended) A ~~The~~ lyophilized powder obtained from a ~~the~~ method comprising~~according to claim 47~~

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) freezing the pre-lyophilization solution to a temperature below about -40°C to form a frozen solution;

ii) holding the frozen solution at or below -40°C for at least 2 hours;

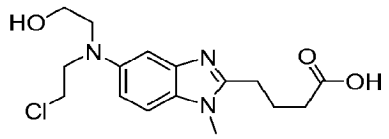
iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution;

iv) holding for about 10 to about 70 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vii) holding for about 5 to about 40 hours to form a bendamustine or bendamustine hydrochloride lyophilized preparation;

wherein said lyophilized powder contains not more than about 0.9 area% of HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release.

Claims 52-55 (Canceled)

56. (Currently Amended) A ~~The~~ lyophilized powder obtained from a ~~the~~ method comprising ~~according to claim 53~~

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of between about 5% to about 100% (v/v) tertiary-butanol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution;

ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours;

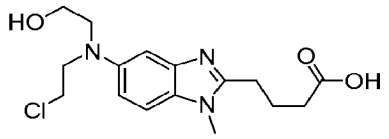
iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution;

iv) holding at a primary drying temperature for about 10 to about 48 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours;

wherein said lyophilized powder contains not more than about 0.9 area% HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release..

57. (Canceled)

58. (Canceled)

59. (Currently Amended) A ~~The~~ lyophilized powder prepared by a method comprising ~~from the lyophilization cycle of claim 57~~

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) starting with a shelf temperature of about 5°C for loading;

ii) freezing to about -50°C over about 8 hours;

iii) holding at -50°C for about 4 hours;

iv) ramping to about -20°C over about 3 hours;

v) holding at about -20°C for 6 hours;

vi) ramping to about -15°C over about 1 hour;

vii) holding at -15°C for about 20 hours;

viii) ramping to about -12°C over about 0.5 hours;

ix) holding at about -12°C for about 15.5 hours;

x) ramping to between about 25°C and about 40°C or higher for about 15 hours;

xi) holding between about 25°C and about 40°C for about 10 hours;

xii) ramping to about 40°C over about 1 hour;

xiii) holding at about 40°C for about 5 hours; and

xiv) unloading at about 5°C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns

throughout primary drying steps iv, v, vi, vii, viii and ix and 50 microns throughout secondary drying steps x, xi, xii and xiii.

60. (Previously Presented) A formulation for lyophilization comprising bendamustine or bendamustine hydrochloride at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water.

61. (Original) A lyophilized preparation made from the formulation according to claim 60.

62. (Withdrawn) A method of treating a medical condition in a patient comprising dissolving the preparation of claim 5 in a pharmaceutically acceptable solvent to produce a pharmaceutically acceptable solution and administering to said patient a therapeutically effective amount of said solution, wherein said condition is amenable to treatment with said preparation.

63. (Withdrawn) A method of treating according to claim 62, wherein said condition is selected from chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, breast cancer, small cell lung cancer, and an autoimmune disease.

64. (Withdrawn) A method of treating according to claim 63, wherein said condition is non-Hodgkin's lymphoma.

65. (Withdrawn) A method of treating according to claim 63, wherein said condition is chronic lymphocytic leukemia.

66. (Withdrawn) A method of treating according to claim 63, wherein said condition is multiple myeloma.

67. (Withdrawn) A method of treating according to claim 62 further comprising administering the dissolved preparation of claim 5 in combination with one or more anti-

neoplastic agents wherein said antineoplastic agent is given prior, concurrently, or subsequent to the administration of the dissolved preparation of claim 5.

68. (Withdrawn) A method of treating according to claim 67 wherein the antineoplastic agent is an antibody specific for CD20, wherein said antibody is given prior, concurrently or subsequent to the administration of the dissolved preparation of claim 5.

69. (Withdrawn) A method of treating according to claim 62 wherein the autoimmune disease is rheumatoid arthritis, multiple sclerosis or lupus.

70. (Withdrawn) A method of treating according to claim 62, wherein the medical condition is a hyperproliferative disorder.

71. (Currently Amended) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.9area% of HP1 ~~(area percent of bendamustine)~~, relative to the amount of bendamustine, wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

72. (Currently Amended) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1 ~~(area percent of bendamustine)~~, relative to the amount of bendamustine, wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

73. (Previously Presented) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 10 to about 500 mg/container.

74. (Previously Presented) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 100 mg/container.

75. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 5 mg to about 2 g/container.

76. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 170 mg/container.

77. (Original) A pre-lyophilized pharmaceutical composition of bendamustine comprising about 15 mg/mL bendamustine HCl, about 25.5 mg/mL mannitol, about 30% (v/v) tertiary-butyl alcohol, and water.

REMARKS

The finality of the restriction requirement is acknowledged. Claim 1-4, 6, 7, 19, 33, 34, 71, and 72 have been amended to delete the phrase "(area percent of bendamustine)." Claims 11 and 12 have been amended to language consistent with claims 1-4, 6, 7, 19, 33, 34, 71, and 72. Claims 18 and 23 have been amended to depend from claim 1. Claims 31 and 32 have been amended to replace "Formula IV" with "bendamustine ethyl ester." Claim 46 has been amended to recite the limitations of claim 36. Claim 51 has been amended to recite the limitations of claim 47. Claim 56 has been amended to recite the limitations of claim 53. Claim 59 has been amended to recite the limitations of claim 57. No new matter has been added.

Claims 5, 25-30, 36-45, 47-50, 52-55, 57, and 58 have been canceled. The Applicants reserve the right to file the canceled subject matter in one or more continuing or divisional applications.

Claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, and 61-77 are pending. Claims 62-70 have been withdrawn.

Rejection under 35 U.S.C. § 112

Claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61, and 71-74 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for including subject matter in parentheses, *i.e.*, "(area percent of bendamustine)." The parenthetical subject matter has been deleted, and the claims amended to recite that the amount of HP1 is the area% of HP1, relative to the amount of bendamustine. Support for the amendment can be found throughout the specification at, for example, page 17, lines 4-6 ("By 'area percent of bendamustine' is meant the amount of a specified degradant, e.g., HP1, relative to the amount of bendamustine as determined, e.g., by HPLC"). The rejection is moot.

Rejection under 35 U.S.C. § 102

Claims 1-12, 18, 19, 71, 72, and 78 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by DD159289 (Jacob). The Office alleges that the Jacob abstract does not indicated that the disclosed composition comprises any HP1 and that it therefore reads on

0% HP1. The Applicants disagree and request reconsideration and withdrawal of the rejection.

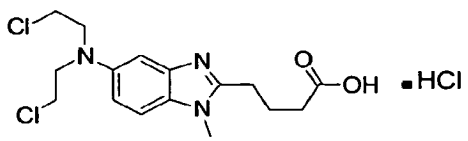
The Jacob abstract describes injectable bendamustine as a solution in alcohol or 1,2-propylene glycol. "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'" *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted); MPEP 2112.

The data set forth in the present specification demonstrates that variable amounts of HP1 form when bendamustine is exposed to alcohols. *See* Tables 4-9, pages 33-36. In view of this data, the Office cannot demonstrate that Jacobs always and inevitably produces bendamustine having less than 0.9% of HP1, as presently claimed. Withdrawal of the rejection is requested.

Rejection under 35 U.S.C § 103

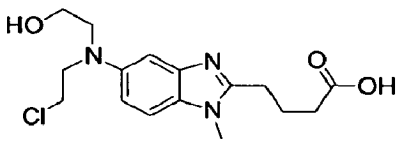
Claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61, and 71-74 stand rejected under 35 U.S.C. § 103 as allegedly obvious over U.S. Published Patent App. 2005/0020615 (Rubino) in view of U.S. Published Patent App. 2005/0060028 (Horres). The Applicants disagree and request withdrawal of the rejection.

The present invention is directed to pharmaceutical preparations of bendamustine or bendamustine hydrochloride



Bendamustine Hydrochloride

containing less than 0.9% of the impurity HP1.



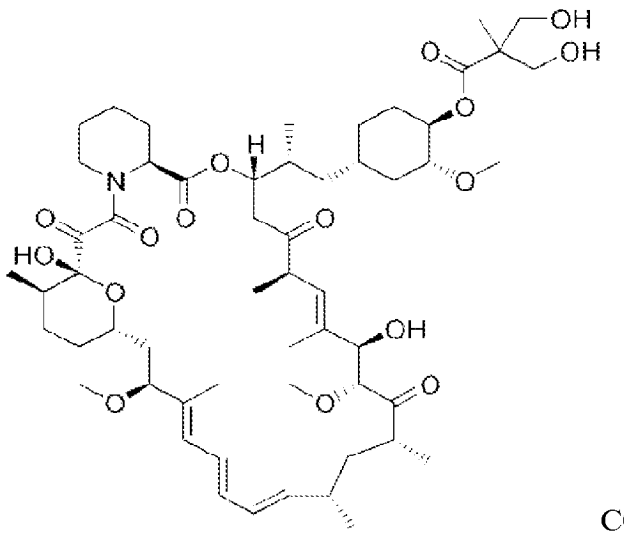
HP1

The desirability of keeping the amount of impurities low in a pharmaceutical composition is well known in the art.

Lyophilization is a known technique whereby a solution of drug substance is frozen and the solvent is removed via sublimation, generally at low pressures and temperatures. Countless variations in solvents, pressures, temperatures, and time are possible for any lyophilization process.

It has heretofore been unreported that the amount of HP1 in a bendamustine or bendamustine hydrochloride pharmaceutical preparation can be 0.9% or less. Moreover, it has been found that the amount of HP1 formed in the pharmaceutical preparations of the inventions can be reduced by using the lyophilization methods and solvents set forth in the present specification.

Rubino is directed to lyophilized preparations of CCI-779:



CCI-779 (Temsirolimus)

CCI-779 is structurally dissimilar from bendamustine and it cannot be disputed that HP1 is not an impurity found in any formulation of CCI-779. Moreover, Rubino simply describes a lyophilization method useful for preparing CCI-779; the applicability of that method to other compounds is not described or suggested in the reference or in the skill of one in the art.

Horres is directed to coating stents with antiproliferative and other agents. The Office has identified that Horres describes that CCI-779 and bendamustine are both antiproliferative active agents. It is undisputed that Horres does not teach or suggest lyophilization.

That CCI-779 and bendamustine are both antiproliferative agents is hardly sufficient to support a case of obviousness under *KSR v. Teleflex*. Importantly, the Office has failed to demonstrate the ***predictability*** of the alleged combination. CCI-779 and bendamustine are chemically dissimilar and the Office has not demonstrated that methods of stabilizing CCI-779 would predictably stabilize bendamustine or its hydrochloride salt to preclude formation of HP1. Merely identifying that both compounds are antiproliferative agents is insufficient evidence of obviousness. Withdrawal of the rejection is requested.

The Applicants assert that the foregoing constitutes a full and complete reply to the August 19, 2009 action and that claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, 61, and 71-77 are in condition for allowance. Rejoinder of claims 62-70 is also requested.

Date: November 17, 2009

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

Woodcock Washburn LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

Electronic Acknowledgement Receipt

EFS ID:	6469688
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	17-NOV-2009
Filing Date:	12-JAN-2006
Time Stamp:	15:24:18
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	Miscellaneous Incoming Letter	1410240_1.PDF	281600 d90abd8fa7ba7163732691832887648f5a37069b	no	2

Warnings:	
Information:	

2		1410313_1.PDF	197824 99b4544e2d6ed5b7db71870dac20e61cd3d973b8	yes	16

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	12
Applicant Arguments/Remarks Made in an Amendment	13	16

Warnings:	
Information:	
Total Files Size (in bytes):	479424

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.

Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Jason Edward Brittain
Art Unit	1616
Examiner Name	Ali Soroush
Attorney Docket Number	CP391/CEPH-4391

ENCLOSURES (*Check all that apply*)

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☑ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation
- ☐ Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please Identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Woodcock Washburn, LLP		
Signature	/Stephanie A. Barbosa/		
Printed name	Stephanie A. Barbosa		
Date	November 17, 2009	Reg. No.	51,430

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature			
Typed or printed name		Date	

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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
11/330,868	01/12/2006	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
FOR	NUMBER FILED	NUMBER EXTRA					
☐ 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 11/17/2009	(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))	* 49	Minus	** 78	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	* 15	Minus	***21	=	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	

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:
//TERRANCE LAWRENCE//

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

27573 7590 02/18/2010

Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

EXAMINER	
SOROUSH, ALI	

ART UNIT	PAPER NUMBER
1616	

MAIL DATE	DELIVERY MODE
02/18/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1616	

-- 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 *17 November 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) *1-4,6-24,31-35,46,51,56 and 59-77* is/are pending in the application.

4a) Of the above claim(s) *62-70* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,6-24,31-35,46,51,56,59-61 and 71-77* 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 _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Acknowledgement of Receipt

Applicant's response filed on 11/17/2009 to the Office Action mailed on 08/19/2009 is acknowledged.

Status of the Claims

Claims 6, 25-30, 36-45, 47-50, 52-55, 57, and 58 are cancelled, claims 62-70 are withdrawn, and claims 1-4, 6, 7, 11-13, 18, 19, 23, 31-34, 46, 51, 56, 59, 71, and 72 are currently amended. Therefore, claims 1-4, 6-24, 31-35, 46, 51, 56, 59-61, and 71-77 are currently pending examination for patentability.

Rejections and/or objections not reiterated from the previous Office Action are hereby withdrawn. The following rejections and/or objections are either reiterated or newly applied. They constitute the complete set of rejections and/or objections presently being applied to the instant application.

Claim Rejections - 35 USC § 102

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

The rejection of claims 1-12, 18, 19, 71, 72, and 77 under 35 U.S.C. 102(b) as being anticipated by Jacob et al. (DD 159289 A1, Published 03/02/1983) **is maintained**.

Jacob et al. teach an injectable solution of bendamustine dissolved in ethanol or polyethylene glycol. (See abstract). The disclosed composition does not comprise any HP1 and therefore reads on the 0% HP1 which is less than 0.9% HP1. For the foregoing reasons the instant claims are anticipated.

Response to Applicant's Arguments

Applicant argues that mere fact that a certain element HP1 is expressly disclosed does not necessarily mean it is not present. Applicant's argument has been fully considered but not found to be persuasive. Jacob et al. does not disclose that the composition comprises HP1. Absent data to the contrary, the Examiner holds that the composition does not comprise HP1 in any amount. For the foregoing reasons, the rejection of claims 1-12, 18, 19, 71, 72, and 77 under 35 U.S.C. 102(b) is maintained.

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

1. The rejection of claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61 and 71-74 under 35 U.S.C. 103(a) as being unpatentable over Rubino (US Patent Application

2005/0020615 A1, Published 01/27/2005, Filed 07/19/2004) in view of Horres et al. (US Patent Application 2005/0060028 A1, Published 03/17/2005, Filed 10/15/2002) **is maintained**.

Applicant Claims

Applicant claims a composition of bendamustine reconstituted from a lyophilized preparation of bendamustine, wherein the lyophilized preparation is a freeze-dried solution of bendamustine, mannitol, t-butyl alcohol, and water.

Determination of the Scope and Content of the Prior Art (MPEP §2141.01)

Rubino teaches a method of preparing lyophilized rapamycin derivative comprising the steps of, first preparing a solution of rapamycin derivative, mannitol, t-butyl alcohol, and water, and secondly freeze-drying the solution. (See claim 13). "The present invention provides pre-lyophilization formulations that provide freeze-dried CCI-779 of the invention with improved potency retention and stability under storage conditions. More particularly, using the pre-lyophilization formulations if the invention, freeze-dried CCI-779 has been found to retain greater than 95% initial potency after one month storage ... The present invention also provides reconstituted CCI-779 formulations suitable for delivery parenterally or by other routes of delivery." (See paragraph 0008).

Ascertainment of the Difference Between Scope the Prior Art and the Claims (MPEP §2141.012)

Rubino lacks a teaching wherein the active compound is bendamustine. This deficiency is cured by the teachings of Horres et al.

Horres et al. teach that rapamycin and bendamustine are both antiproliferative agents for treating cancer such as leukemia. (See paragraph 0017).

Finding of Prima Facie Obviousness Rational and Motivation (MPEP §2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to combine the teachings of Robino with Horres et al. and produce the instant invention.

One would have been motivated to so because Robino teach that the lyophilization method taught would give a much more stable final active agent and Horres et al. teach that rapamycin and bendamusitne are suitable leukemia treatment drugs. For the foregoing reasons, the instant claimed kit would have been obvious to one of ordinary skill in the art at the time of the instant invention.

Response to Applicant's Arguments

Applicant argues that one would not be motivated to substitute bendamustine for CCI-779 based on the teachings of Horres et al. Applicant's argument has been fully considered but not found to be persuasive. Horres et al. teach that bendamustine and

CCI-779 are both antiprolifertive agents and one would be motivated to use the method of Robino et al. in order to produce an active agent that will retain greater than 95% potency after one month of storage. For the foregoing reasons, the rejection of claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61 and 71-74 under 35 U.S.C. 103(a) is maintained.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ali Soroush whose telephone number is (571) 272-9925. The examiner can normally be reached on Monday through Thursday 8:30am to 5:00pm E.S.T.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor, Johann Richter can be reached on (571) 272-0646. 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.

Ali Soroush
Patent Examiner
Art Unit: 1616

/Johann R. Richter/

Supervisory Patent Examiner, Art Unit 1616

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010						
	1	÷	✓	✓						
	2	÷	✓	✓						
	3	÷	✓	✓						
	4	÷	✓	✓						
	5	÷	✓	-						
	6	÷	✓	✓						
	7	÷	✓	✓						
	8	÷	✓	✓						
	9	÷	✓	✓						
	10	÷	✓	✓						
	11	÷	✓	✓						
	12	÷	✓	✓						
	13	÷	✓	✓						
	14	÷	✓	✓						
	15	÷	✓	✓						
	16	÷	✓	✓						
	17	÷	✓	✓						
	18	÷	✓	✓						
	19	÷	✓	✓						
	20	÷	✓	✓						
	21	÷	✓	✓						
	22	÷	✓	✓						
	23	÷	✓	✓						
	24	÷	✓	✓						
	25	÷	N	-						
	26	÷	N	-						
	27	÷	N	-						
	28	÷	N	-						
	29	÷	N	-						
	30	÷	N	-						
	31	÷	✓	✓						
	32	÷	✓	✓						
	33	÷	✓	✓						
	34	÷	✓	✓						
	35	÷	✓	✓						
	36	÷	N	-						

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010						
	37	÷	N	-						
	38	÷	N	-						
	39	÷	N	-						
	40	÷	N	-						
	41	÷	N	-						
	42	÷	N	-						
	43	÷	N	-						
	44	÷	N	-						
	45	÷	N	-						
	46	÷	✓	✓						
	47	÷	N	-						
	48	÷	N	-						
	49	÷	N	-						
	50	÷	N	-						
	51	÷	✓	✓						
	52	÷	N	-						
	53	÷	N	-						
	54	÷	N	-						
	55	÷	N	-						
	56	÷	✓	✓						
	57	÷	N	-						
	58	÷	N	-						
	59	÷	✓	✓						
	60	÷	✓	✓						
	61	÷	✓	✓						
	62	÷	N	N						
	63	÷	N	N						
	64	÷	N	N						
	65	÷	N	N						
	66	÷	N	N						
	67	÷	N	N						
	68	÷	N	N						
	69	÷	N	N						
	70	÷	N	N						
	71	÷	✓	✓						
	72	÷	✓	✓						

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010						
	73	÷	✓	✓						
	74	÷	✓	✓						
	75	÷	✓	✓						
	76	÷	✓	✓						
	77	÷	✓	✓						
	78	÷	✓	✓						

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Jason Edward Brittain**	Confirmation No.: **9998**
Application No.: **11/330,868**	Group Art Unit: **1616**
Filing Date: **January 12, 2006**	Examiner: **Ali Soroush**

For: **Bendamustine Pharmaceutical Compositions**

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

Sir:

REPLY PURSUANT TO 37 CFR § 1.116

In response to the Official Action dated **February 18, 2010**, reconsideration is respectfully requested in view of the amendments and/or remarks as indicated below:

☐ **Amendments to the Specification** begin on page of this paper.

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

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet.

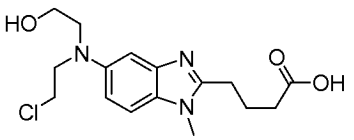
☒ **Remarks** begin on page 12 of this paper.

☐ **Request For Refund** submitted herewith.

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

Listing of Claims:

1. (Previously Presented) A pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9 area% of HP1 relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

2. (Previously Presented) The composition according to claim 1, wherein the amount of HP1 is not more than 0.5 area%, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

3. (Previously Presented) The composition according to claim 1, wherein the amount of HP1 is not more than 0.4 area%, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

4. (Previously Presented) The composition according to claim 1, wherein the amount of HP1 is not more than 0.3 area%, relative to the amount of bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

5. (Canceled)

6. (Previously Presented) A lyophilized preparation of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1, relative to the amount of

bendamustine, at time zero after reconstitution of a lyophilized preparation of bendamustine or bendamustine hydrochloride.

7. (Previously Presented) The lyophilized preparation according to claim 1, wherein the preparation is packaged in a vial or other pharmaceutically acceptable container.

8. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about six months when stored at 5° C.

9. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 12 months when stored at 5° C.

10. (Original) The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 24 months when stored at 5° C.

11. (Previously Amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.9 area % of HP1, relative to the amount of bendamustine, wherein said HP1 is the amount of HP1 present at release.

12. (Previously Amended) A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1, relative to the amount of bendamustine, wherein said HP1 is the amount of HP1 present at release.

13. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 5 mg to about 500 mg of bendamustine or bendamustine hydrochloride.

14. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 10 mg to about 300 mg of bendamustine or bendamustine hydrochloride.

15. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 25 mg of bendamustine or bendamustine hydrochloride.

16. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 100 mg of bendamustine or bendamustine hydrochloride.

17. (Previously Presented) A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 200 mg of bendamustine or bendamustine hydrochloride.

18. (Previously Amended) A pharmaceutical dosage form comprising the lyophilized preparation of claim 1.

19. (Previously Amended) A pharmaceutical composition of bendamustine or bendamustine hydrochloride comprising bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1, relative to the amount of bendamustine, and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release.

20. (Previously Presented) A pharmaceutical composition of bendamustine or bendamustine hydrochloride according to claim 19 wherein the organic solvent is selected from the group consisting of one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane.

21. (Original) A pharmaceutical composition according to claim 20, wherein the organic solvent is selected from the group consisting of one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol.

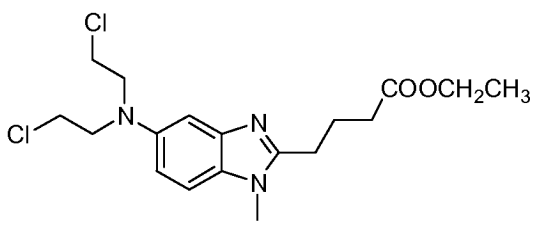
22. (Original) A pharmaceutical composition according to claim 19, wherein the organic solvent is tertiary butanol.

23. (Previously Amended) A lyophilized preparation according to claim 1 further comprising a trace amount of an organic solvent.

24. (Original) A lyophilized preparation according to claim 23 wherein said organic solvent is tertiary butanol.

Claims 25-30 (Canceled)

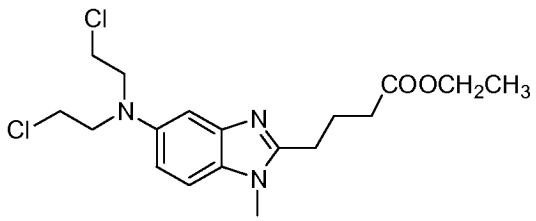
31. (Previously Amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride wherein the concentration of bendamustine ethylester



bendamustine ethyl ester

is no more than 0.2% greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

32. (Previously Amended) A lyophilized preparation of bendamustine or bendamustine hydrochloride according to claim 5 containing not more than about 0.5% bendamustine ethylester



bendamustine ethyl ester.

33. (Presently Amended) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.9area%, relative to the amount of bendamustine.

34. (Previously Presented) A bendamustine or bendamustine hydrochloride pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine or bendamustine hydrochloride so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.5 area%, relative to the amount of bendamustine.

35. (Original) The lyophilized powder produced from the pre-lyophilization solution or dispersion according to claim 33.

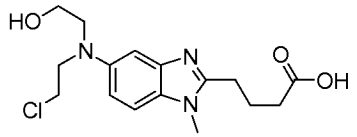
Claims 36-45 (Canceled)

46. (Previously Amended) A lyophilized powder obtained from by a method comprising

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said lyophilized powder contains not more than about 0.9 area% of HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release.

Claims 47-50 (Canceled)

51. (Previously Presented) A lyophilized powder obtained from a method comprising

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) freezing the pre-lyophilization solution to a temperature below about -40°C to form a frozen solution;

ii) holding the frozen solution at or below -40°C for at least 2 hours;

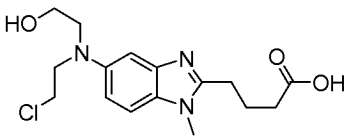
iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution;

iv) holding for about 10 to about 70 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vii) holding for about 5 to about 40 hours to form a bendamustine or bendamustine hydrochloride lyophilized preparation;

wherein said lyophilized powder contains not more than about 0.9 area% of HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release.

Claims 52-55 (Canceled)

56. (Previously Presented) A lyophilized powder obtained from a method comprising

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of between about 5% to about 100% (v/v) tertiary-butanol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution;

ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours;

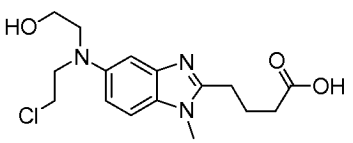
iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution;

iv) holding at a primary drying temperature for about 10 to about 48 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours;

wherein said lyophilized powder contains not more than about 0.9 area% HP1, relative to the amount of bendamustine



HP1

wherein said HP1 is the amount of HP1 present at release.

57. (Canceled)

58. (Canceled)

59. (Previously Presented) A lyophilized powder prepared by a method comprising

a) dissolving bendamustine or bendamustine hydrochloride in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution by

i) starting with a shelf temperature of about 5˚C for loading;

ii) freezing to about -50˚C over about 8 hours;

iii) holding at -50˚C for about 4 hours;

iv) ramping to about -20˚C over about 3 hours;

v) holding at about -20˚C for 6 hours;

vi) ramping to about -15°C over about 1 hour;

vii) holding at -15°C for about 20 hours;

viii) ramping to about -12˚C over about 0.5 hours;

ix) holding at about -12˚C for about 15.5 hours;

x) ramping to between about 25˚C and about 40˚C or higher for about 15 hours;

xi) holding between about 25˚C and about 40˚C for about 10 hours;

xii) ramping to about 40˚C over about 1 hour;

xiii) holding at about 40˚C for about 5 hours; and

xiv) unloading at about 5˚C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying steps iv, v, vi, vii, viii and ix and 50 microns throughout secondary drying steps x, xi, xii and xiii.

60. (Previously Presented) A formulation for lyophilization comprising bendamustine or bendamustine hydrochloride at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water.

61. (Original) A lyophilized preparation made from the formulation according to claim 60.

Claims 62-70 (Canceled)

71. (Previously Presented) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.9area% of HP1, relative to the amount of bendamustine, wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

72. (Previously Presented) A pharmaceutical dosage form of bendamustine or bendamustine hydrochloride containing not more than about 0.5 area% of HP1, relative to the amount of bendamustine, wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

73. (Previously Presented) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 10 to about 500 mg/container.

74. (Previously Presented) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine or bendamustine hydrochloride at a concentration of about 100 mg/container.

75. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 5 mg to about 2 g/container.

76. (Original) A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 170 mg/container.

77. (Original) A pre-lyophilized pharmaceutical composition of bendamustine comprising about 15 mg/mL bendamustine HCl, about 25.5 mg/mL mannitol, about 30% (v/v) tertiary-butyl alcohol, and water.

REMARKS

Claims 1-4, 6-24, 31-35, 46, 51, 56, and 59-77 are pending. Previously withdrawn claims 62-70 have been canceled. No other claim amendments have been made. Reconsideration and withdrawal of the pending final rejection is requested in view of the following remarks. An English translation of DD 159289 is submitted herewith in support of Applicants arguments *infra* that Jacob fails to describe a lyophilized formulation of bendamustine presently claimed.

The pending claims are directed to lyophilized bendamustine, as well as methods of producing a lyophilized form of bendamustine and methods of using lyophilized bendamustine in the treatment of cancer. The lyophilized bendamustine of the invention has fewer impurities than those lyophilized formulations previously described. Moreover, the claimed lyophilized formulation dissolves faster and more completely than those formulations previously described.

Rejection under 35 U.S.C. § 102

Claims 1-12, 18, 19, 71, 72, and 77 stand rejected under 35 U.S.C. 102 as allegedly anticipated by DD 159289 (Jacob). A copy of the English translation of Jacob accompanies this response. The claimed invention is directed to ***lyophilized*** bendamustine, as well as making and using lyophilized bendamustine. In contrast, Jacob is directed to liquid formulations wherein bendamustine is dissolved in an ***anhydrous*** solvent. Prior to injection, this solution is diluted with an aqueous medium.

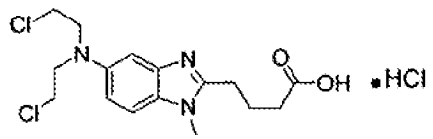
Jacob describes prior reports of lyophilized bendamustine, describing them as having "significant disadvantages" and producing a product that has "clear signs for decomposition of 5 to 10% of the active substance." Jacob Translation, page 3, ¶ 4. Moreover, those lyophilized formulation produce "unsatisfying [] high amounts of micro particles . . . which indicate a further instability of the system." Thus, not only is Jacob directed to different subject matter from the claimed lyophilized bendamustine, it actually *teaches away* from lyophilized preparations by pointing to disadvantages of prior art efforts at lyophilization of the drug.

As Jacob fails to describe the claimed invention, withdrawal of the rejection is requested.

Rejection under 35 U.S.C § 103

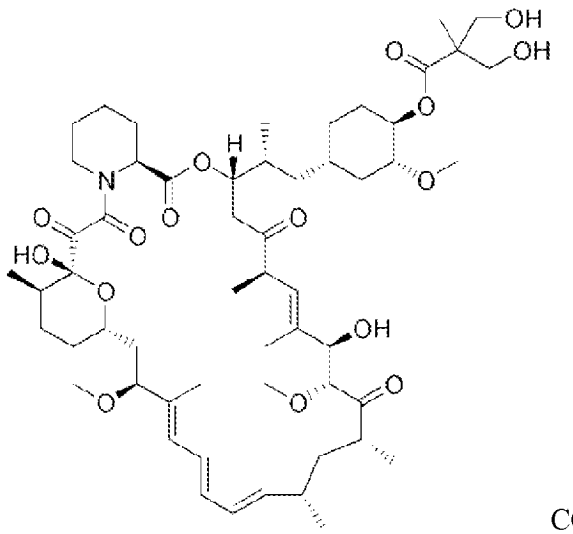
Claims 1-6, 12-20, 31-35, 51, 56, 59, 60, 61, and 71-74 stand rejected under 35 U.S.C. § 103 as allegedly obvious over U.S. Published Patent App. 2005/0020615 (Rubino) in view of U.S. Published Patent App. 2005/0060028 (Horres). The Applicants disagree and request withdrawal of the rejection.

The present invention is directed to pharmaceutical preparations of bendamustine or bendamustine hydrochloride:



Bendamustine Hydrochloride

Rubino is directed to lyophilized preparations of CCI-779:



CCI-779 (Temsirolimus)

Bendamustine and CCI-779 are significally distinct from each other, both structurally and physico-chemically. For example, bendamustine is a nitrogen mustard, *i.e.*, includes 2-chloroethylamine moieties that are not present in CCI-779. Nitrogen mustards, including bendamustine, are highly susceptible to hydrolysis. Bendamustine also includes a heteroaromatic ring and a carboxylic acid, both of which are absent in CCI-779. Applicants

note that Jacob instructs that lyophilization of bendamustine is undesirable because of its hygroscopicity and chemical instability.

CCI-779, in contrast to bendamustine, is a macrocyclic lactone that includes many structurally features not present in bendamustine, including conjugated double bonds, hydroxyketones, a pyran, a 1,2 bis-ketone, and an amide. Indeed, Rubino acknowledges that the physical-chemical properties of CCI-779, including its poor solubility in water and chemical instability, present challenges to its successful formulation. Rubino at [0005].

It is well known to those skilled in the art that the physical-chemical properties of a compound greatly impact the successful formulation of the compound. *See* Rubino at [0005]. Nevertheless, the Office has identified that Horres describes that CCI-779 and bendamustine are both antiproliferative active agents and alleges, therefore, that since CCI-779 and bendamustine are in the same drug class, that methods of lyophilizing CCI-779 could be extended to the lyophilization of bendamustine. This reasoning cannot support a finding of obviousness and withdrawal of the rejection is requested.

Importantly, the Office has failed to demonstrate that the suggested combination of references would ***predictably*** produce the claimed invention. That CCI-779 and bendamustine are in the same broad ***therapeutic*** class, *i.e.*, antiproliferative agents, is ***irrelevant*** to methods of formulating the compounds. Formulation, in particular, lyophilization, depends solely on the physical-chemical properties of the chemical compounds and has nothing whatever to do with the disease state to be treated with the compound. Indeed, Jacob teaches that lyophilization of bendamustine is undesirable. The Office has not demonstrated that methods of stabilizing CCI-779 would ***predictably*** stabilize the chemically dissimilar bendamustine or its hydrochloride salt. Merely identifying that both compounds are antiproliferative agents is insufficient evidence to establish *prima facie* obviousness. Withdrawal of the rejection is requested.

The Applicants assert that the foregoing constitutes a full and complete reply to the February 18, 2010 action and that claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, 61, and 71-77 are in condition for allowance. Rejoinder of claims 62-70 is also requested.

Date: April 1, 2010

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

Woodcock Washburn LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

Abstract: The invention relates to a method for producing stable injection solutions which can be used for medical treatment. It is the objective to produce a ready to use, stable injection solution of N-mustard compounds, avoiding the technical solution of a dry ampoule. According to the invention, the N-mustard compounds are used in concentrations of 25 mg/ ml to 100 mg/ ml, dissolved in an anhydrous monovalent or polyvalent alcohol (polyol), wherein the solution is dissolved, filled and stored under inert gas. Before the injection, the solutions are diluted by means of an aqueous injection medium in order to achieve the desired concentration.

Field of the Invention

The invention relates to a method for producing injection solutions which may be used for medical treatment.

Characteristic of the Background Art

N-mustard compounds have been used for some years as highly effective cytostatics. It is estimated that mustards can be used in the therapy of 70 % of all treated malign tumors. More recent synthesis research was aimed at, for example, synthesizing a multivalent antagonist type out of said compound group. In said experiments and in vast pharmacological and clinical trials, bendamustine hydrochloride (trial ID IMET 3393), the compound synthesized by OZEGOWSKI and KREBS in 1963, was for example selected (BRUNS, KNÖLL) from a larger number of compounds. Bendamustine hydrochloride is γ-[1-methyl-5-bis-(β-chloroethyl)-aminobenzimidazo-lyl-(2)]-butyric acid hydrochloride. In 1971, the compound was introduced as pharmaceutical preparation under the trade name of CYTOSTASAN® (0.025 g bendamustine hydrochloride per dry ampoule) for the therapy of hemoblastoses, particularly such as chronic lymphadenosis, lymphoreticulosis, lymphogranulomatosis and of reticuloses.

Bendamustine hydrochloride is a relatively instable compound. Its mustard-halogen groups are almost completely hydrolyzed in aqueous solutions after a short period of time. Therefore, the injection solutions can only be produced shortly before injection. The kinetic experiments regarding the chloride hydrolysis of the N-mustard group of the bendamustine hydrochloride showed a reaction course in acid and neutral solutions which could be calculated according to the pseudo first order of the reaction for a consecutive reaction of the symmetric di-halogen compound (U. OLTHOFF, Abstr. Congr. Pharm. Hung. VI; Budapest 1974, p. 72). Independently of the pH value and of the buffer system used, the separation of the chloride and protons from the β-chloroethyl groups is complete and occurs at very high velocity. As far as this aspect is concerned, bendamustine hydrochloride is even more reactive than the particularly reactive mustards N-methyl mustard, chlorambucil and uracil mustard.

These data explain particular difficulties encountered in the production of stable medical preparations out of bendamustine hydrochloride.

According to the patent from the former German Democratic Republic WP 80 967, the N-mustard derivative bendamustine hydrochloride has to be produced in a sterile crystal form which is free of aerosols and suited for quick dissolution. The preparation form is a dry ampoule containing a mixture of 25 mg of bendamustine hydrochloride and 175 mg of ascorbic acid. Before the application, the content of the ampoule has to be dissolved in water for the injection. The ascorbic acid is included for the objective to produce a powder mixture which can be filled and to ensure the durability and the pH value of the injection solution. Moreover, the shelf life of the dry mixture should be guaranteed. According to the present results of the experiments, the hydrolysis rate is not influenced by the addition of the ascorbic acid.

The pH value of a bendamustine hydrochloride solution, which is within the range of 2.4 to 3.0, is not significantly altered by the addition of the ascorbic acid. Thus, the addition of the ascorbic acid does not match the characteristics which are to be required from a stabilizer. After a certain storage period, solid bendamustine hydrochloride shows a pink to brown-red discoloration, which starts at the surface of the substance and which, after a longer period of time, renders the whole substance unsuitable for the production of pharmaceutical preparations. The mixture with ascorbic acid shows the same discoloration.

Furthermore, it was proposed to lyophilize the aqueous solution of the substance bendamustine hydrochloride and to dissolve it in water or sodium chloride solution before the application (production method of the ZIMET (= former Central Institute for microbiology and experimental therapy in the GDR), pharmaceutical expertise IfAr/ Nr. 180/ 80). The lyophilisate obtained (25 mg/ ampoule) has significant disadvantages for a technological production process. In particular, the technological realization is complicated even more by the extreme hygroscopicity and the fact that the process takes place under inert gas. Moreover, during the production of the preparation, clear signs for decomposition of 5 to 10 % of the active substance were observed. It is furthermore unsatisfying that high amounts of micro particles were

found after the dissolution of the lyophilisate, which indicate a further instability of the system.

The disadvantage of the extreme hygroscopicity of the lyophilisate can be eliminated by the addition of polyols which are solid at room temperature, such as in particular mannitol. In addition to the large technological efforts, also the disadvantages of significant decomposition and of the manifestation of undissolved micro particles have to be considered.

Objective of the Invention

It is the objective of the invention to produce a stable and ready-to-use injection solution out of N-mustard compounds, avoiding the technical solution of a dry ampoule.

Description of the Character of the Invention

In the technical solutions known so far, the injection preparation was realized either as a filled powder under addition of a stabilizer and other auxiliary substances respectively, or in form of a lyophilisate, if necessary, with auxiliary substances. By these measures, a suitable injection preparation can only be realized with significant disadvantages or can't be realized at all. The dry fillings thus obtained are characterized by insufficient chemical and physical stability. Besides, lyophilization means that significantly increased technical efforts have to be made, a fact which might be limiting as far as the capacities are concerned.

Surprisingly it has been found that the substance bendamustine hydrochloride has a sufficient solubility and particularly a extraordinarily high chemical stability for the production of injection solutions in monovalent alcohols, glycols and other polyvalent alcohols. The stability of the solutions produced according to the invention is unexpected, since compounds with extreme sensitivity to hydrolysis are generally also sensitive to other solvents which contain OH-groups. In the case of alcohols or polyols, such reactions are known as alcoholysis.

It has now been found that N-mustard compounds of the bendamustine hydrochloride type do not undergo an alcoholysis reaction. The use of anhydrous solvents is required in order to avoid decomposition caused by the mentioned sensitivity to hydrolysis. Under these conditions, bendamustine hydrochloride, for example, is chemically stable for long periods of time in the mentioned group of solvents and does not form the monohydroxy and dihydroxy or monoalkoxy or dialkoxy derivatives known from aqueous solutions.

In order to examine the stability, bendamustine hydrochloride in a concentration of 25 mg/ ml was dissolved in ethanol and 1,2-propylene glycol and the solution was stored at room temperature, as well as at increased temperatures (50° C, 75° C, 130° C)
[missing sentence]
examined for the formation of cleavage products by means of a specific, thin film chromatography procedure (quantity applied 0.025 mg bendamustine hydrochloride, silica gel G, mobile phase: butanol/ acetic acid/ water 4:1:5; detection UV 360 nm or respectively Dragendorff reagent); results:

Formation of decomposition products in:

time	ethanol solution		propylene glycol solution		
	25° C	50° C	25° C	75° C	130° C
0.5 h	-	-	-	-	(traces)
1 h	-	-	-	none	-
1.5 h	-	-	-	-	(traces)
2 h	-	-	-	none	minor decomposition
5 h	none	none	-	none	-
7 h	none	none	-	-	-
24 h	none	-	-	-	-
8 weeks	none	-	none	-	-

For pharmacological reasons and for reasons concerning the production, monovalent alcohols are of limited use for the production of injection solutions. 1,2-propylene glycol is used more frequently. The solubilites of bendamustine hydrochloride which can be achieved at 25° C in some of the solvents used are of about

ethanol abs.	ca.	50 mg/ ml
propylene glycol	ca.	125 mg/ ml and
glycerol	ca.	50 mg/ ml

In the injection forms of bendamustine hydrochloride known so far, 25 mg active substance are used in 10 ml solvent. It is now proposed to dissolve the active substance in polyols, particularly in 1,2-propylene glycol in order to ensure a more simple production technology, an improved stability of the active substance during the production and storing of the solutions as well as a simplified handling during the production of the ready to inject solution when the active substance is solved in polyols, particularly in 1,2-propylene glycol and filled into ampoules. Immediately before the injection, the polyol solution is diluted by means of the addition of an aqueous diluent (sodium chloride solution in water for injection) such that the solution which is to be applied contains only about 10% polyol, sometimes even less. The polyol solutions can be diluted as desired with the diluents described before, without causing any disadvantages for the active substance. Due to the numerous possible variations of the contents of polyol and the diluent, there are several other advantages for the choice of an optimized, particularly acceptable injection preparation. In addition to the sensitivity to hydrolysis, other stability factors which have to be considered are the influence of light and atmospheric oxygen. Dry preparations and solutions change color under the influence of light and air until they have a pink or brownish color. As far as the solutions in alcohols and polyols which are stored in closed ampoules and under exclusion of light are concerned, no discoloration was observed. It is, however, recommended that the solutions are produced, filled and stored under an inert gas, such as argon or nitrogen.

Exemplary embodiments:

Example 1:

Bendamustine hydrochloride is dissolved under stirring in an inert gas atmosphere in 1,2-propylene glycol in an concentration of 2.5 g/ 100 ml. The solution is filtered by means of a suitable filter device, if necessary after heating to 50° C, such that it is free of aerosols and germs. 1.0 ml of the solution is filled in 10 ml ampoules, the ampoules are filled with inert gas and sealed by heating. Immediately before the injection, 9.0 ml of the chosen diluent is to be added into the opened ampoule. After reshuffling, the solution is ready to inject.

Example 2:

5.0 g bendamustine hydrochloride are dissolved in 100 ml ethanol under the conditions described in example 1, filtered and filled into ampoules. The ampoules are stored at temperatures of +15° C to +25° C.

CLAIMS

1. Method for producing stable injection solutions of N-mustard compounds, characterized in that N-mustard derivatives in concentrations of 25 mg/ ml to 100 mg/ ml are dissolved in an anhydrous monovalent or polyvalent alcohol (polyol), wherein the solution is dissolved, filled and stored under inert gas and the solution is diluted before medical application in a ratio of 1:5 to 1:20 with an aqueous injection medium.

2. Method according to claim 1, characterized in that benzimidazole mustards are used as active substance, in particular bendamustine hydrochloride.

3. Method according to claim 1, characterized in that particularly 1,2-propylene glycol is used as polyol.

Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Jason Edward Brittain
Art Unit	1616
Examiner Name	Ali Soroush
Attorney Docket Number	CP391/CEPH-4391

ENCLOSURES (*Check all that apply*)

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☑ Amendment/Reply
  - ☑ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation
- ☐ Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please Identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Woodcock Washburn, LLP		
Signature	/Stephanie A. Barbosa/		
Printed name	Stephanie A. Barbosa		
Date	April 1, 2010	Reg. No.	51,430

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature			
Typed or printed name		Date	

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	7338082
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	01-APR-2010
Filing Date:	12-JAN-2006
Time Stamp:	17:31:28
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		CEPH-4391-reply-to-02-18-10.PDF	254181 723dc761ba921c8b3922ca8722c3169164f5246a	yes	23

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment After Final	1	1
	Claims	2	11
	Applicant Arguments/Remarks Made in an Amendment	12	23

Warnings:					
Information:					
2	Transmittal Letter	CEPH-4391CP391-Transmittal-reply-to-02-18-10.PDF	276472 a567e5ae9fd9c06856f7658dceb72bad6b830746	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	530653		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

PTO/SB/31 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

NOTICE OF APPEAL FROM THE EXAMINER TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

Docket Number (Optional): CEPH-4391/CP391

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to "Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" [37 CFR 1.8(a)]
on _____

Signature _____

Typed or printed name _____

In re Application of: Jason Edward Brittain

Application Number	Filed
11/330,868	January 12, 2006

For: Bendamustine Pharmaceutical Compositions

Art Unit	Examiner
1616	Ali Soroush

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 540.00

☐ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ _____

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 233050 .

☑ A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest.
See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed.
(Form PTO/SB/96)

☑ attorney or agent of record.
Registration number 51,430.

☐ attorney or agent acting under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34. _____

/Stephanie A. Barbosa/
Signature

Stephanie A. Barbosa
Typed or printed name

215-568-3100
Telephone number

August 18, 2010
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required.
Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 2 forms are submitted.

This collection of information is required by 37 CFR 41.31. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11, 1.14 and 41.6. 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/22 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2009** (*Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).*)	Docket Number (Optional) CEPH-4391/CP391
Application Number 11/330,868	Filed January 12, 2006
For Bendamustine Pharmaceutical Compositions	
Art Unit 1616	Examiner Ali Soroush

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
☑	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 1,110.00
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 233050.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 51,430

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Stephanie A. Barbosa/ — Signature

August 18, 2010 — Date

Stephanie A. Barbosa — Typed or printed name

215-568-3100 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 2 forms are submitted.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	11330868
Filing Date:	12-Jan-2006
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Filer:	Stephanie A. Barbosa/Patricia Salazar
Attorney Docket Number:	CP391

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:				
Notice of appeal	1401	1	540	540
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1110	1110
Miscellaneous:				
			Total in USD ($)	**1650**

Electronic Acknowledgement Receipt

EFS ID:	8246964
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Stephanie A. Barbosa/Patricia Salazar
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	18-AUG-2010
Filing Date:	12-JAN-2006
Time Stamp:	17:52:49
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1650
RAM confirmation Number	4228
Deposit Account	233050
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 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance 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	Notice of Appeal Filed	1697256_1.PDF	249726 442e520d37a0756180bb6198251fa7b9af357e67	no	2
Warnings:					
Information:					
2	Extension of Time	1697258_1.PDF	327631 c526072757389f282fdfcf6afe09333dc4de8bb0	no	2
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	31891 3f4649a2a8c3fde34050fc37544bc5b3dd8d8141	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	609248		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

27573 7590 08/19/2010

Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

EXAMINER	
SOROUSH, ALI	
ART UNIT	PAPER NUMBER
1617	

NOTIFICATION DATE	DELIVERY MODE
08/19/2010	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):

uspatentcounsel@cephalon.com

Office Action Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1616	

-- 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 *01 April 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-4,6-24,31-35,46,51,56,59-61 and 71-77* 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) *1-4,6-24,31-35,46,51,56,59-61 and 71-77* 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 _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Acknowledgement of Receipt

Applicant's response filed on 04/01/2010 to the Office Action mailed on 02/18/2010 is acknowledged.

Status of the Claims

Claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, 61 and 71-77 are currently pending examination for patentability.

Rejections and/or objections not reiterated from the previous Office Action are hereby withdrawn. The following rejections and/or objections are either reiterated or newly applied. They constitute the complete set of rejections and/or objections presently being applied to the instant application.

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

1. Claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, 61 and 71-77 are rejected under 35 U.S.C. 103(a) as being unpatentable over Horres et al. (US Patent Application 2005/0060028 A1, Published 03/17/2005, Filed 10/15/2002) in view of Ku et al. (EP Patent Application 0656211 A1, Published 06/07/1995).

Applicant Claims

Applicant claims a composition of bendamustine reconstituted from a lyophilized preparation of bendamustine, wherein the lyophilized preparation is a freeze-dried solution of bendamustine, mannitol, t-butyl alcohol, and water.

Determination of the Scope and Content of the Prior Art (MPEP §2141.01)

Horres et al. teach that rapamycin and bendamustine are both antiproliferative agents for treating cancer such as leukemia. (See paragraph 0017).

Ascertainment of the Difference Between Scope the Prior Art and the Claims (MPEP §2141.012)

Horres lacks a teaching wherein the bendamustine is lyophilized. This deficiency is cured by the teachings of Ku et al.

Ku et al. teach a stable lyophilized composition of an antitumor alkylating agent. (See abstract). It is known in the art that lyophilization of a product which is relatively unstable in aqueous solution can result in a product that is stabilized and therefore has a longer shelf life than an aqueous solution. (See page 2, Lines 43-51).

Finding of Prima Facie Obviousness Rational and Motivation (MPEP §2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to combine the teachings of Ku et al. with Horres et al. and produce the instant invention.

One would have been motivated to so because Ku et al. teach that the lyophilization method taught would give a much more stable final active agent. For the foregoing reasons, the instant claimed composition would have been obvious to one of ordinary skill in the art at the time of the instant invention.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ali Soroush whose telephone number is (571) 272-9925. The examiner can normally be reached on Monday through Thursday 8:30am to 5:00pm E.S.T.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor, Johann Richter can be reached on (571) 272-0646. 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.

Ali Soroush
Patent Examiner
Art Unit: 1616

/Johann R. Richter/

Supervisory Patent Examiner, Art Unit 1616

Notice of References Cited	Application/Control No. 11/330,868	Applicant(s)/Patent Under Reexamination BRITTAIN ET AL.	
	Examiner ALI SOROUSH	Art Unit 1616	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2005/0060028 A1	03-2005	Horres et al.	623/001.38
	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	EP-0656211-A1	06-1995	EP	Ku et al.	
	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.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010	08/14/2010					
	1	÷	✓	✓	✓					
	2	÷	✓	✓	✓					
	3	÷	✓	✓	✓					
	4	÷	✓	✓	✓					
	5	÷	✓	-	-					
	6	÷	✓	✓	✓					
	7	÷	✓	✓	✓					
	8	÷	✓	✓	✓					
	9	÷	✓	✓	✓					
	10	÷	✓	✓	✓					
	11	÷	✓	✓	✓					
	12	÷	✓	✓	✓					
	13	÷	✓	✓	✓					
	14	÷	✓	✓	✓					
	15	÷	✓	✓	✓					
	16	÷	✓	✓	✓					
	17	÷	✓	✓	✓					
	18	÷	✓	✓	✓					
	19	÷	✓	✓	✓					
	20	÷	✓	✓	✓					
	21	÷	✓	✓	✓					
	22	÷	✓	✓	✓					
	23	÷	✓	✓	✓					
	24	÷	✓	✓	✓					
	25	÷	N	-	-					
	26	÷	N	-	-					
	27	÷	N	-	-					
	28	÷	N	-	-					
	29	÷	N	-	-					
	30	÷	N	-	-					
	31	÷	✓	✓	✓					
	32	÷	✓	✓	✓					
	33	÷	✓	✓	✓					
	34	÷	✓	✓	✓					
	35	÷	✓	✓	✓					
	36	÷	N	-	-					

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010	08/14/2010					
	37	÷	N	-	-					
	38	÷	N	-	-					
	39	÷	N	-	-					
	40	÷	N	-	-					
	41	÷	N	-	-					
	42	÷	N	-	-					
	43	÷	N	-	-					
	44	÷	N	-	-					
	45	÷	N	-	-					
	46	÷	✓	✓	✓					
	47	÷	N	-	-					
	48	÷	N	-	-					
	49	÷	N	-	-					
	50	÷	N	-	-					
	51	÷	✓	✓	✓					
	52	÷	N	-	-					
	53	÷	N	-	-					
	54	÷	N	-	-					
	55	÷	N	-	-					
	56	÷	✓	✓	✓					
	57	÷	N	-	-					
	58	÷	N	-	-					
	59	÷	✓	✓	✓					
	60	÷	✓	✓	✓					
	61	÷	✓	✓	✓					
	62	÷	N	N	-					
	63	÷	N	N	-					
	64	÷	N	N	-					
	65	÷	N	N	-					
	66	÷	N	N	-					
	67	÷	N	N	-					
	68	÷	N	N	-					
	69	÷	N	N	-					
	70	÷	N	N	-					
	71	÷	✓	✓	✓					
	72	÷	✓	✓	✓					

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

✓	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	05/13/2009	08/14/2009	02/13/2010	08/14/2010					
	73	÷	✓	✓	✓					
	74	÷	✓	✓	✓					
	75	÷	✓	✓	✓					
	76	÷	✓	✓	✓					
	77	÷	✓	✓	✓					
	78	÷	✓	✓						

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
EAST (See search history)	08/14/2010	AS
PALM Inventor search	08/14/2010	AS

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2	treanda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
L2	0	bendamustine same (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
L3	10	bendamustine and (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
L4	46	bendamustine and (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
L5	3	bendamustine same (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
L6	88851	lyophilize lyophilization freeze $dry freeze$dried free$1drying	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:56
L7	22	L6 same (alkylating adj agent)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:57

L8	2	bendamustine same (aqueous adj solution) same unstable	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:03
L9	0	"cephalon.in"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:04
L10	563	cephalon.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
L11	11	L10 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
L12	4	bendamustine same (aqueous adj solution)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
L13	458	bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
L14	30	bendamustine adj hydrochloride	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
L15	58	bendamustine same injection	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:07

L16	18	bendamustine same solid	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:12
L17	2	bendamustine same unstable	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:13

8/ 14/ 2010 8:28:11 PM
C:\ Documents and Settings\ asoroush\ My Documents\ EAST\ Workspaces\ 11330868.wsp

DOCKET NO.: CP391/CEPH-4391 **PATENT**
Application No.: 11/330,868
Office Action Dated: August 19, 2010

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jason Edward Brittain

Confirmation No.: **9998**

Application No.: **11/330,868**

Group Art Unit: **1616**

Filing Date: **January 12, 2006**

Examiner: **Ali Soroush**

For: **Bendamustine Pharmaceutical Compositions**

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

Sir:

REPLY PURSUANT TO 37 CFR § 1.111

In response to the Official Action dated **August 19, 2010**, reconsideration is respectfully requested in view of the amendments and/or remarks as indicated below:

☐ **Amendments to the Specification** begin on page of this paper.

☒ **A Listing of the Claims** is reflected in the listing of the claims which begins on page 2 of this paper.

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet.

☒ **Remarks** begin on page 4 of this paper.

☐ **Request For Refund** submitted herewith.

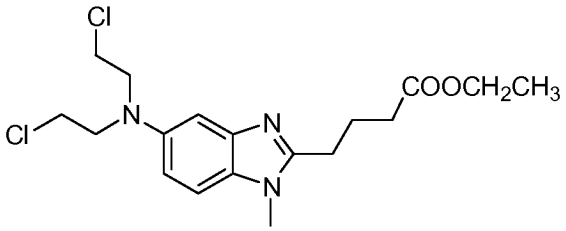
☒ The Commissioner is hereby authorized to charge any fee deficiency, charge any additional fees, or credit any overpayment of fees, associated with this application in connection with this filing, or any future filing, submitted to the U.S. Patent and Trademark Office during the pendency of this application, to Deposit Account No. 23-3050.

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

Listing of Claims:

Claims 1- 30 (Canceled)

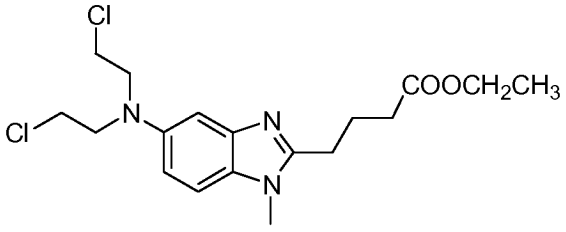
31. (Currently Amended) A lyophilized pharmaceutical composition ~~lyophilized preparation~~ of bendamustine or bendamustine hydrochloride prepared from a pre-lyophilized pharmaceutical composition wherein the concentration of bendamustine ethylester



bendamustine ethyl ester

in said lyophilized preparation is no more than 0.2% greater than the concentration of bendamustine ethylester ~~as found~~ present in the pre-lyophilized pharmaceutical composition ~~in the drug substance used to make the lyophilized preparation~~.

32. (Currently Amended) A lyophilized pharmaceutical composition ~~lyophilized preparation~~ of bendamustine or bendamustine hydrochloride ~~according to claim 5~~ containing not more than about 0.5% bendamustine ethylester



bendamustine ethyl ester.

Claims 33 – 77 (Canceled)

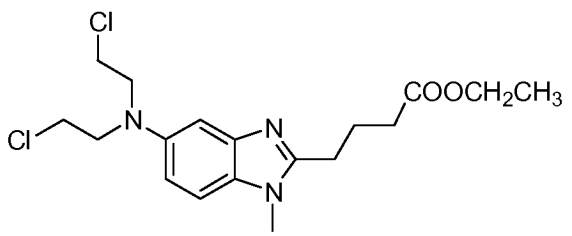
78. (New) A pre-lyophilized pharmaceutical composition comprising bendamustine or bendamustine hydrochloride, mannitol, tertiary-butyl alcohol, and water.

79. (New) The pre-lyophilized pharmaceutical composition according to claim 78, wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 12 to 17 mg/mL, said mannitol is present at a concentration of about 20-30 mg/mL, and said tertiary-butyl alcohol is present at a concentration of about 10-50% (v/v).

80. (New) The pre-lyophilized pharmaceutical composition of claim 79 wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 15 mg/mL, said mannitol is present at a concentration of about 25.5. mg/mL, and said tertiary-butyl alcohol is present at a concentration of about 30% (v/v).

81. (New) A lyophilized pharmaceutical composition made from the pre-lyophilized pharmaceutical composition according to any one of claims 78 to 80.

82. (New) The lyophilized pharmaceutical composition according to claim 81, wherein the concentration of bendamustine ethylester



bendamustine ethyl ester

in said lyophilized preparation is no more than 0.2% greater than the concentration of bendamustine ethylester present in said pre-lyophilized pharmaceutical composition.

REMARKS

A response to the final rejection dated February 18, 2010 was filed April 1, 2010 and should have provoked an Advisory Action prior to the six month response date, August 18, 2010. Despite several calls by the undersigned to the Office inquiring about the status of the Advisory Action, no Advisory Action was issued prior to the six month response date. As a result, the Applicants filed a Notice of Appeal on August 18, 2010. On August 19, 2010, the Applicants were notified of the pending non-final office action. This reply is responsive to the August 19, 2010 office action and no appeal brief will be filed at this time.

After entry of the present amendment, previously presented claims 31 and 32, along with new claims 78-82, will be pending. Support for the new claims can be found throughout the specification, for example, at page 5, lines 1-11, page 25, lines 2-3, 14-16, page 26, line 2, and page 39, lines 1-5. Cancellation of subject matter is done without prejudice to the presentation of that subject matter in one or more continuing applications.

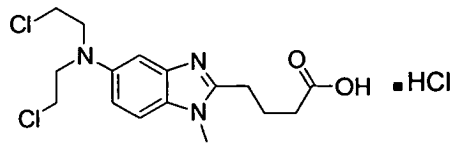
The present claims are directed to, among other things, pre-lyophilized pharmaceutical compositions for the lyophilization of bendamustine or bendamustine hydrochloride, as well as lyophilized pharmaceutical compositions of bendamustine or bendamustine hydrochloride. Prior to the invention, bendamustine was historically lyophilized from a solution of ethanol, water, mannitol, and bendamustine. As described in the specification, bendamustine ethyl ester is a degradant formed when bendamustine reacts with ethyl alcohol. Specification at page 32, lines 8-10. Prior to the invention, it was not appreciated that the ethanol-containing lyophilization procedure resulted in an increase in the amount of bendamustine ethyl ester present in the lyophilized product over that present in the pre-lyophilized pharmaceutical composition. *See* Specification at page 5, lines 1-11. The present inventors discovered that the amount of bendamustine ethyl ester in the lyophilized preparation could be minimized by creating an entirely new pre-lyophilization procedure. Moreover, as discussed in the specification (*see* page 48, lines 21 to 26), while this formulation avoided formation of bendamustine ethyl ester, the

inventors also discovered that the new formulation did not lead to formation of a bendamustine tert-butyl ester.

Rejection under 35 U.S.C. § 103

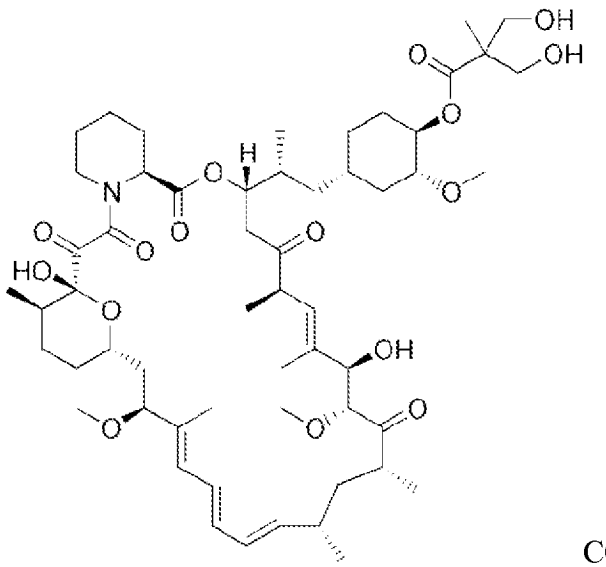
Claims 1-4, 6-24, 31-35, 46, 51, 56, 59, 60, 61, and 71-77 stand rejected under 35 U.S.C. § 103 as allegedly obvious over U.S. Published Application No. 2005/0060028 (Horres) in view of EP 0656211 (Ku). The Applicants disagree and request withdrawal of the rejection and allowance of the claims.

The present invention is directed to, among other things, pre-lyophilized pharmaceutical compositions for the lyophilization of bendamustine or bendamustine hydrochloride, as well as lyophilized pharmaceutical compositions of bendamustine or bendamustine hydrochloride:



Bendamustine Hydrochloride

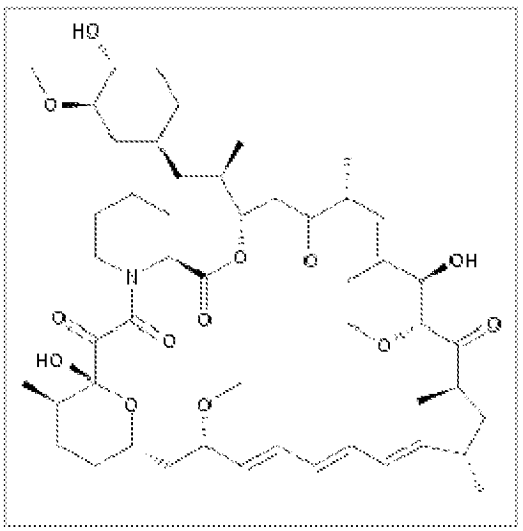
In the February 18, 2010 Office Action, the claims were rejected over U.S. Published Patent App. 2005/0020615 (Rubino) in view of Horres. As argued in the Applicants April 1, 2010 response, Rubino is directed to lyophilized preparations of CCI-779 (temsirolimus), a rapamycin derivative:



CCI-779 (Temsirolimus)

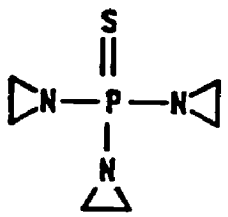
Bendamustine and CCI-779 are significally distinct from each other, both structurally and physico-chemically. Nevertheless, the Office identified that Rubino describes that CCI-779 and bendamustine are both antiproliferative active agents. The Office alleged, therefore, that since CCI-779 and bendamustine are in the same drug class, that methods of lyophilizing CCI-779 could be extended to the lyophilization of bendamustine. The Office failed to demonstrate that the suggested modification of Rubino would ***predictably*** produce the claimed invention. That CCI-779 and bendamustine are in the same broad ***therapeutic*** class, *i.e.*, antiproliferative agents, is ***irrelevant*** to methods of formulating the compounds. Based on the Applicants' arguments, the rejection over Rubino was withdrawn.

In the present action, the Office states that Horres teaches that rapamycin:



(rapamycin)

which is similar in structure to CCI-779, and bendamustine are both antiproliferative agents for treating cancer such as leukemia. The Office acknowledges that Horres lacks a teaching wherein bendamustine is lyophilized and asserts Ku against the pending claims. Ku describes the lyophilization of thiotepa:



Thiotepa

a compound that is structurally and physico-chemically different from bendamustine. For example, thiotepa includes three aziridine rings and a P=S moiety that are not present in bendamustine. Moreover, thiotepa, is described as being useful for treating breast, ovarian, and bladder cancer. Ku at paragraph [0002]. Bendamustine is not approved for the treatment of breast, ovarian, or bladder cancer.

Not only does Ku describe the formulation of a compound that is structually and physico-chemically different from bendamustine, the lyophilization procedure described in Ku is unrelated to the present application. Ku fails to use an alcohol in the aqueous pre-lyophilization solution. Moreover, Ku describes that it is imperative that the pH of the pre-lyophilization be controlled by the addition of base. Ku at [0015]. The present invention does not require the addition of base for the lyophilization to be successful.

The combination of cited art fails, again, to establish a *prima facie* case of obviousness. As previously argued, that a rapamycin and bendamustine are both antiproliferative active agents is completely ***irrelevant*** to the inquiry of whether bendamustine can be successful formulated as a lyophilized composition, as claimed. It is well established that it is the physical-chemical properties of a compound that impact the successful formulation of the compound, ***not*** the mechanism of action or therapeutic indication of the compound. As such, the disclosure of Horres is completely irrelevant to whether bendamustine can be successfully lyophilized as claimed.

Ku describes the lyophilization of thiotepa, instructing that thiotepa must be co-lyophilized with a base such that the composition has a pH of 7-9 upon reconstitution. Ku at paragraph [0012]. The claimed lyophilization methods do not include the addition of a base. For at least this reason, the skilled person, reading the disclosure of Ku, would not have been led to the claimed lyophilization methods that do not include a base.

Thiotepa is structurally unrelated to bendamustine, as stated above. Importantly, while bendamustine hydrolyzes upon exposure to water, thiotepa is theorized to polymerize. Ku at paragraph [0004]. As a result, one skilled in the art, reading the disclosure of Ku, could not have predicted the effects the lyophilization methods described in Ku would have on bendamustine.

The obviousness inquiry requires that the cited art ***predictably*** produce the claimed invention. MPEP 2141. Based on the numerous and significant physical-chemical differences between bendamustine and thiotepa, the skilled person would not have been able to predict that lyophilization of bendamustine would predictably be successful in producing the claimed invention.

Simply because Ku describes lyophilization of an antitumor alkylating agent is insufficient to support a finding of obviousness. The present invention does not seek to claim lyophilization, generally. Rather, the claims encompass pre-lyophilized pharmaceutical compositions and lyophilized pharmaceutical compositions of specific compounds – bendamustine and bendamustine hydrochloride. Nothing in the cited art suggests that bendamustine could successfully and predictably be lyophilized, as presently claimed. Indeed, as previously argued, Jacob, previously cited by the Office teaches that lyophilization of bendamustine is undesirable.

Moreover, the cited references fail to teach or suggest the advantages provided by the present invention. By using tertiary butyl alcohol, the formation of bendamustine ethyl ester is eliminated. Moreover, the tertiary butyl alcohol does not contribute to the formation of the teriary butyl ester of bendamustine. Specification at 48, lines 25-26. In addition, the new lyophilization formulation produces a lyophilized cake that is well-formed, stable, easily reconstituted, more easily manufactured, and resistant to breakage during handling than the cakes produced using ethanol in the pre-lyophilization solution. Specification at page 46, lines 18-21; page 47, lines 12-25, lines 26-29. The formulation also enables the use of smaller lyophilization vials for a 100 mg bendamustine presentation, which leads to savings of cost and resources. *See* specification at page 47, lines 6-11 and 26 to 31.

None of the cited art suggests that bendamustine or bendamustine hydrochloride can be successfully and predictably lyophilized from a solution comprising mannitol, tertiary-butyl alcohol, and water. Accordingly, Applicants submit that the pending claims are patentable over the cited art. An early Notice to this effect is, therefore, earnestly solicited.

The Applicants assert that the application is now in condition for allowance. If the examiner is of a contrary opinion, the Applicants hereby request a telephonic interview prior to the issuance of a further office action.

Date: November 19, 2010

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

Woodcock Washburn LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

Electronic Acknowledgement Receipt

EFS ID:	8873034
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	19-NOV-2010
Filing Date:	12-JAN-2006
Time Stamp:	10:21:03
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	Transmittal Letter	CEPH-4391-Transmittal-reply-to-08-19-10.PDF	283239 a38a79938a90d135117d43d2862603e99b50f88b	no	2

Warnings:	
Information:	

2		CEPH-4391-Reply-to-08-19-10.PDF	183897 50ef17b88061bdb3433bd3363e7399c43a5f8e16	yes	9

	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	9

Warnings:	
Information:	
Total Files Size (in bytes):	467136

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.

Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Jason Edward Brittain
Art Unit	1616
Examiner Name	Ali Soroush
Attorney Docket Number	CP391/CEPH-4391

ENCLOSURES (*Check all that apply*)

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☑ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation
- ☐ Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please Identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Woodcock Washburn, LLP		
Signature	/Stephanie A. Barbosa/		
Printed name	Stephanie A. Barbosa		
Date	November 19, 2010	Reg. No.	51430

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature			
Typed or printed name		Date	

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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
11/330,868	01/12/2006	

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))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT 11/17/2009

	(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))	* 49	Minus	** 78	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 15	Minus	***21	= 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 11/19/2010

	(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))	* 7	Minus	** 78	= 0	X $ =		OR	X $52 =	0
Independent (37 CFR 1.16(h))	* 4	Minus	*** 21	= 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

* 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:
/GLENN BURNS JR/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

27573 7590 05/13/2011

Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

EXAMINER	
SOROUSH, ALI	
ART UNIT	PAPER NUMBER
1617	

NOTIFICATION DATE	DELIVERY MODE
05/13/2011	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):

uspatentcounsel@cephalon.com

Interview Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1617	

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

(1) *ALI SOROUSH*. (3)_____.

(2) *Stephanie Barbosa*. (4)_____.

Date of Interview: *04/26 & 05/05/2011*.

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: _____.

Claim(s) discussed: *31, 32, and 78-82*.

Identification of prior art discussed: *NA*.

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: *See Continuation Sheet*.

(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.

/ALI SOROUSH/ Examiner, Art Unit 1617	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: The Examiner contacted Applicant's attorney on 04/26/2011 to propose amendments to the claims which the Examiner and Examiner's SPE believe put the claims in condition for allowance. The proposed claim amendments are as such:

1-31. Cancelled.

32. The Lyophilized pharmaceutical compositionaccording to claim 81 containing not more than about 0.5% bendamustine ethylester

33.-77 Cancelled.

78. A pharmaceutical composition comprising bendamustine or bendamustine hydrochloride, mannitol, tertiary-butyl alcohol, and water.

79. The pharmaceutical composition according to claim 78, wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 12 to 17 mg/mL, said mannitol is present at a concentration of about 20-30 mg/mL, and said tertiary-butyl alcohol is present at a concentration of about 10-50% (v/v).

80. The pharmaceutical composition according to claim 79, wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 15 mg/mL, said mannitol is present at a concentration of about 25.5. mg/mL, and said tertiary-butyl alcohol is present at a concentration of about 30% (v/v).

81. A lyophilized pharmaceutical composition made from the pharmaceutical composition according to any one of claims 78 to 80.

82. Cancelled.

Ms. Barbosa indicated that she would discuss the proposed amendments with Applicant and contact the Examiner at a later date. The Examiner spoke with Ms. Barbosa on 05/05/2011 at which time she indicated that Applicant's did not agree to the claim amendments.

.

Office Action Summary	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1617	

-- 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 *19 November 2010*.

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) *31,32 and 78-82* 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) *31,32 and 78-82* 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 _____.
4) ☒ Interview Summary (PTO-413) Paper No(s)/Mail Date. *05052011* .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Acknowledgement of Receipt

Applicant's response filed on 11/19/2010 to the Office Action mailed on 08/19/2010 is acknowledged.

Claim Status

Claims 31, 32, and 78-82 are pending.

Claims 1-30 and 33-77 are cancelled.

Claims 31 and 32 are currently amended.

Claims 78-82 are newly added.

Claims 31, 32, and 78-82 have been examined.

Claims 31, 32, and 78-82 are rejected.

Rejections and/or objections not reiterated from the previous Office Action are hereby withdrawn. The following rejections and/or objections are either reiterated or newly applied. They constitute the complete set of rejections and/or objections presently being applied to the instant application.

Priority

Priority to application 60/644,354 filed on 01/14/2005 is acknowledged.

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 31, 32 and 78-82 are rejected under 35 U.S.C. 103(a) as being unpatentable over Battelli et al. (US Patent 4670262, Published 06/02/1987) in view of Klaveness et al. (US Patent 2002/0102215 A1, Published 08/01/2002).

Claim 32 is directed to a lyophilized composition comprising less than 0.5% bendamustine ethylester. Claims 78-80 are directed to a pre-lyophilized composition comprising bendamustine, mannitol, tertiary-butyl alcohol, and water. The claims are further directed to the composition comprising about 15mg/ml of bendamustine, 25.5mg/ml mannitol, and 30% tertiary-butyl alcohol. Claims 31 and 81-82 are directed to the lyophilized composition of the pre-lyophilized composition.

Battelli et al. teach a process for producing a cisplatin (cis-platinumm (II) diaminedichloride) by forming a lyophilization (freeze-dry) composition comprising 10 to 50mg cisplatin, 45 to 450mg sodium chloride, 100 to 1500mg mannitol, 0.2 to 5% tertiary-butyl alcohol and water; and lyophilizing the composition (column 8, lines 34-47). Cisplatin is used in chemotherapy of cancer in man (column 1, lines 8-15). The lyophilized composition provide a more advantageous manner in antitumor chemotherapy (abstract).

Battelli et al. lacks a teaching wherein the composition comprises bendamustine.

Klaveness et al. teach preparation therapeutically active agents involves lyophilization which advantageously include cryoprotectant/lycoprotectant or bulking agents such as mannitol and tert-butanol (paragraph 0042). Representative active agents useful in accordance with the invention include cisplatin and bendamustine (paragraph 0146).

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to substitute bendamustine for cisplatin. One would have been motivated to do so in order to provide a bendamustine composition that is easily

reconstituted for use. One would have expected success since Klaveness et al. teach that cisplatin and bendamustine are functional alternatives as chemotherapy agents and further teach that bendamustine can be lyophilized with mannitol and tert-butanol, absent an unexpected result. With regard to the amount of bendamustine ester, since the instant method and the method made obvious by the prior art are the same it would be expected that the amount of ester formed would necessarily also be the same.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALI SOROUSH whose telephone number is (571)272-9925. The examiner can normally be reached on M-F (9am-6pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Fereydoun G. Sajjadi can be reached on (571)272-3311. 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.

/A. S./
Examiner, Art Unit 1617

/KARLHEINZ R SKOWRONEK/
Primary Examiner, Art Unit 1631

April 22, 2011

Notice of References Cited	Application/Control No. 11/330,868	Applicant(s)/Patent Under Reexamination BRITTAIN ET AL.	
	Examiner ALI SOROUSH	Art Unit 1617	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-4,670,262	06-1987	Battelli et al.	424/649
*	B	US-2002/0102215 A1	08-2002	Klaveness et al.	424/9.52
	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.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
see search history printouts	04/22/2011	AS
Inventor search (Jason Edward Brittain, Joe Craig Franklin)	04/22/2011	AS

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

/A.S./ Examiner.Art Unit 1617	

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	benadmustine with mannitol with alcohol	EPO	OR	ON	2011/04/22 20:07
L2	0	benadmustine	EPO	OR	ON	2011/04/22 20:07
L3	11	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl) amino]-1-methylbenzimidazol-2-yl] butanoic acid" "16506-27-7"	EPO	OR	ON	2011/04/22 20:20
L4	775	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl) amino]-1-methylbenzimidazol-2-yl] butanoic acid" "16506-27-7"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:20
L5	10	L4 with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
L6	13	L4 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
L7	13	L4 with alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
L8	22	L4 same alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:22

L9	23	L4 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:24
L10	345	L4 and mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:36
L11	52	L10 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:38
L12	108	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) with (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:44
L13	31	L12 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:44
L14	2	"5362718".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:52
L15	1	L4 same (freeze$1dry freez $1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55

L16	15	L4 and (freeze$1dry freez $1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55
L17	18	L4 with rapamycin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:56
L18	23	L4 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
L19	6	L4 same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
L20	132	L4 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
L21	299	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02

L22	7	L21 and L4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02
L23	65	cyclophosphamide with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07
L24	17	L23 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07
L25	0	L24 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:12
L26	17166	(nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
L27	113050	L26 sme (lyophilization lyophilize freeze$1dry freeze $1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
L28	6	L26 same (lyophilization lyophilize freeze$1dry freeze $1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
L29	2335	L26 and (lyophilization lyophilize freeze$1dry freeze $1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14

L30	4	L9 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:15
L31	3	L4 same tablet	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:18
L32	60242	(t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
L33	81388	lyophilization lyophilize freeze $1dry freeze$1drying	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
L34	477	L32 same L33	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
L35	52	L34 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:23
L36	7	chlorambucil same lyophilization	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:41

L37	49972	freeze$1dry freez$1drying lyophilisation lyophilization cryodesiccation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
L38	82	L37 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
L39	6	l12 and L38	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:46
L40	13	L4 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:48
L41	10	fishman.in. and K4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50
L42	0	fishman.in. and L4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50
L43	2	"20020102215"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:53
L44	986	brittain.in. franklin.in. and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 22:53
L45	2	(brittain.in. franklin.in.) and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 22:54

4/22/2011 10:54:25 PM
C:\Documents and Settings\asoroush\My Documents\EAST\Workspaces\11330868.wsp

PTO/SB/81 (01-09)
Approved for use through 11/30/2011. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

POWER OF ATTORNEY OR REVOCATION OF POWER OF ATTORNEY WITH A NEW POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS

Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Jason Edward Brittain
Title	Bendamustine Pharmaceutical Compositions
Art Unit	1617
Examiner Name	Ali Soroush
Attorney Docket Number	CP391

I hereby revoke all previous powers of attorney given in the above-identified application.

☐ A Power of Attorney is submitted herewith.

OR

☒ I hereby appoint Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith: **46347**

OR

☐ I hereby appoint Practitioner(s) named below as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith:

Practitioner(s) Name	Registration Number

Please recognize or change the correspondence address for the above-identified application to:

☒ The address associated with the above-mentioned Customer Number.

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the:

☐ Applicant/Inventor.

OR

☒ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) (Form PTO/SB/96) submitted herewith or filed on _____.

SIGNATURE of Applicant or Assignee of Record

Signature		Date	
Name	Ross J. Oehler	Telephone	610-344-0200
Title and Company	Vice President, Cephalon, Inc.		

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☒ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/96 (04-09)
Approved for use through 05/31/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Jason Edward Brittain, Joe Craig Franklin

Application No./Patent No.: 11/330,868 Filed/Issue Date: January 12, 2006

Titled: Bendamustine Pharmaceutical Compositions

Cephalon, Inc. , a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in (The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made) the patent application/patent identified above, by virtue of either:

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 017302, Frame 0847, or for which a copy therefore is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____

The document was recorded in the United States 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(s).

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

_____ 5/18/11

Signature Date

Ross J. Oehler Vice President

Printed or Typed Name Title

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	10123023
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	27573
Filer:	Stephanie A. Barbosa/Ann Trevisani
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	18-MAY-2011
Filing Date:	12-JAN-2006
Time Stamp:	20:13:46
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	Power of Attorney	Power_of_Attorney_CP391.pdf	160265 44b3ddab9bd620e70bc8f578d000288cdaf6db8e	no	2

Warnings:	
Information:	

2	Assignee showing of ownership per 37 CFR 3.73(b).	Statement_373_CP391.pdf	150056 80e0aab673a1022757df77f38e4a87671de1ebbd	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	310321

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.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

POA ACCEPTANCE LETTER

46347
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

OC00000047867147

Date Mailed: 05/27/2011

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 05/18/2011.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/erimando/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/330,868	01/12/2006	Jason Edward Brittain	CP391

CONFIRMATION NO. 9998

POWER OF ATTORNEY NOTICE

27573
Ross J. Oehler
CEPHALON, Inc.
41 MOORES ROAD
PO BOX 4011
FRAZER, PA 19355

OC000000047866957

Date Mailed: 05/27/2011

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 05/18/2011.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

/erimando/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PTO/SB/31 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

NOTICE OF APPEAL FROM THE EXAMINER TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

Docket Number (Optional): CEPH-4391/CP391

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to "Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" [37 CFR 1.8(a)]
on _____

Signature _____

Typed or printed name _____

In re Application of: Jason Edward Brittain

Application Number	Filed
11/330,868	January 12, 2006

For: Bendamustine Pharmaceutical Compositions

Art Unit	Examiner
1616	Ali Soroush

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 620

☐ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ _____

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 233050 .

☑ A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest.
See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed.
(Form PTO/SB/96)

☑ attorney or agent of record.
Registration number 51,430.

☐ attorney or agent acting under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34. _____

/Stephanie A. Barbosa/
Signature

Stephanie A. Barbosa
Typed or printed name

215-568-3100
Telephone number

October 31, 2011
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required.
Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 41.31. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11, 1.14 and 41.6. 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.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) CEPH-4391/CP391
Application Number 11/330,868	Filed January 12, 2006
For Bendamustine Pharmaceutical Compositions	
Art Unit 1616	Examiner Ali Soroush

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$150	$75	$
☐	Two months (37 CFR 1.17(a)(2))	$560	$280	$
☑	Three months (37 CFR 1.17(a)(3))	$1270	$635	$ 1270
☐	Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☐	Five months (37 CFR 1.17(a)(5))	$2690	$1345	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 233050.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 51,430

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Stephanie A. Barbosa/ — Signature

October 31, 2011 — Date

Stephanie A. Barbosa — Typed or printed name

215-568-3100 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	11330868
Filing Date:	12-Jan-2006
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Filer:	Stephanie A. Barbosa/D. McCarty
Attorney Docket Number:	CP391

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:				
Notice of appeal	1401	1	620	620
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1270	1270
Miscellaneous:				
			Total in USD ($)	**1890**

Electronic Acknowledgement Receipt

EFS ID:	11299011
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	31-OCT-2011
Filing Date:	12-JAN-2006
Time Stamp:	13:14:32
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1890
RAM confirmation Number	10135
Deposit Account	233050
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 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance 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	Notice of Appeal Filed	CEPH-4391-Notice-of-Appeal.PDF	243603 dd87a34d36e3d2b008e70d13869c3cca5841ff3f	no	2
Warnings:					
Information:					
2	Extension of Time	CEPH-4391-Extension-of-time.PDF	287704 5b589fe1277c9e6ba0052cb1cf5081b24791e354	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32196 57014dc1504b2cd3b490f4fbbd100c941700a72c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	563503		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	11/330,868	Filing Date	2006-01-12	Docket Number (if applicable)	CEPH-4391/CP391	Art Unit	1617
First Named Inventor	Jason Edward Brittain			Examiner Name	Ali Soroush		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☒ Other Petition for Extension of Time

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 233050

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

☐ Applicant Signature

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Signature of Registered U.S. Patent Practitioner			
Signature	/Stephanie A. Barbosa/	Date (YYYY-MM-DD)	2012-05-30
Name	Stephanie A. Barbosa	Registration Number	51430

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) CEPH-4391/CP391
Application Number 11/330,868	Filed January 12, 2006
For Bendamustine Pharmaceutical Compositions	
Art Unit 1617	Examiner Ali Soroush

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$150	$75	$
☐ Two months (37 CFR 1.17(a)(2))	$560	$280	$
☐ Three months (37 CFR 1.17(a)(3))	$1270	$635	$
☐ Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☑ Five months (37 CFR 1.17(a)(5))	$2690	$1345	$ 2,690.00

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 233050.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 51,430

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Stephanie A. Barbosa/ — Signature

May 30, 2012 — Date

Stephanie A. Barbosa — Typed or printed name

215-568-3100 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

DOCKET NO.: CP391/CEPH-4391 **PATENT**
Application No.: 11/330,868
Office Action Dated: May 13, 2011

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Jason Edward Brittain**	**Confirmation No.: 9998**
Application No.: 11/330,868	**Group Art Unit: 1617**
Filing Date: January 12, 2006	**Examiner: Ali Soroush**

For: Bendamustine Pharmaceutical Compositions

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

Sir:

SUBMISSION PURSUANT TO 37 CFR § 1.114

In response to the Official Action dated **May 13, 2011**, reconsideration is respectfully requested in view of the amendments and/or remarks as indicated below:

☐ **Amendments to the Specification** begin on page of this paper.

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

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet.

☒ **Remarks** begin on page 4 of this paper.

☒ The Commissioner is hereby authorized to charge any fee deficiency, charge any additional fees, or credit any overpayment of fees, associated with this application in connection with this filing, or any future filing, submitted to the U.S. Patent and Trademark Office during the pendency of this application, to Deposit Account No. 23-3050.

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

Listing of Claims:

Claims 1- 82 (Canceled)

83. (New) A pharmaceutical composition comprising bendamustine or bendamustine hydrochloride, mannitol, tertiary-butyl alcohol and water.

84. (New) The pharmaceutical composition according to claim 83, wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 12 to 17 mg/ml, said mannitol is present at a concentration of about 20-30 mg/ml, and said tertiary-butyl alcohol is present at a concentration of about 10-50% (v/v).

85. (New) The pharmaceutical composition according to claim 84, wherein said bendamustine or bendamustine hydrochloride is present at a concentration of about 15 mg/ml, said mannitol is present at a concentration of about 25.5. mg/ml, and said tertiary-butyl alcohol is present at a concentration of about 30% (v/v).

86. (New) A lyophilized pharmaceutical composition made from the pharmaceutical composition according to claim 83.

87. (New) The lyophilized pharmaceutical composition according to claim 86, wherein said bendamustine or bendamustine hydrochloride is present in said pharmaceutical composition at a concentration of about 12 to 17 mg/ml, said mannitol is present in said pharmaceutical composition at a concentration of about 20-30 mg/ml, and said tertiary-butyl alcohol is present in said pharmaceutical composition at a concentration of about 10-50% (v/v).

88. (New) The lyophilized pharmaceutical composition according to claim 87, wherein said bendamustine or bendamustine hydrochloride is present in said pharmaceutical composition at a concentration of about 15 mg/ml, said mannitol is present in said pharmaceutical composition at a concentration of about 25.5 mg/ml, and said tertiary-butyl alcohol is present in said pharmaceutical composition at a concentration of about 30% (v/v).

89. (New) The lyophilized pharmaceutical composition according to claim 86 containing not more than about 0.5% bendamustine ethylester.

90. (New) The lyophilized pharmaceutical composition according to claim 87 containing not more than about 0.5% bendamustine ethylester.

91. (New) The lyophilized pharmaceutical composition according to claim 88 containing not more than about 0.5% bendamustine ethylester.

REMARKS

This submission accompanies a request for continued examination.

Claims 1-82 have been canceled, and claims 83-91 have been added.

The pending claims correspond to claims to those that the Examiner and the Examiner's SPE indicated were allowable in the Interview Summary mailed on May 13, 2011, modified to remove multiple dependencies.

Rejection under 35 U.S.C. § 103

Prior claims 31, 32, and 78-82 stood rejected under 35 U.S.C. § 103 as allegedly obvious over U.S. 4,670,262 ("Battelli") in view of U.S. Published Application No. 2002/0102215 ("Klaveness"). However, the cited references fail to teach or suggest compositions of bendamustine as claimed. Accordingly, Applicants assert that the combination of cited art fails to support *a prima facie* case of obviousness of the pending claims, as evidenced, *inter alia,* by the Office's indication in the Interview Summary that such claims are allowable over the art. To the extent that the pending rejections would be applied to the amended claims, reconsideration and withdrawal of the rejection is requested.

Claims 83-91 are allowable over the art. An early notice to that effect is, therefore, earnestly solicited.

Date: May 30, 2012

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

Woodcock Washburn LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

Electronic Patent Application Fee Transmittal

Application Number:	11330868
Filing Date:	12-Jan-2006
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Filer:	Stephanie A. Barbosa/D. McCarty
Attorney Docket Number:	CP391

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:				
Extension - 5 months with $0 paid	1255	1	2690	2690

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	930	930
		Total in USD ($)		**3620**

Electronic Acknowledgement Receipt

EFS ID:	12895115
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	30-MAY-2012
Filing Date:	12-JAN-2006
Time Stamp:	16:51:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$3620
RAM confirmation Number	4066
Deposit Account	233050
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 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance 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	Request for Continued Examination (RCE)	CEPH-4391-Request-for-Continued-Examination.PDF	697829 db8f2964c486b93396d1830601014260debe5085	no	3
Warnings:					
Information:					
2	Extension of Time	CEPH-4391-Petition-for-Extension-of-Time.PDF	286650 cca3c7eaa287e97844645373de0971cef3b142e8	no	2
Warnings:					
Information:					
3		CEPH-4391-submission-RCE.PDF	100694 8e848f257672d8bc805bb783cb7d3386499be8be	yes	4

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	3
Applicant Arguments/Remarks Made in an Amendment	4	4

Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	32065 011f55fd82efbff2de9922040bf595b2aea70df3	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	1117238	

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.

Substitute for 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
(use as many sheets as necessary)				**Examiner Name**	Ali Soroush
Sheet	1	of	2	**Attorney Docket Number**	CEPH-4391 / CP391

U. S. PUBLICATION AND PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number Number – Kind Code (if known)	Publication or Grant Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document
	79	2004/0072889	04-15-2004	Masferrer
	80	2006/0128777	06-15-2006	Bendall et al.
	81	2009/0264488	10-22-2009	Cooper et al.
	82	2012/0071532	03-22-2012	Cooper et al.

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
	83	DD 34727	12-28-1964	Krebs Dietrich	X
	84	WO 2006/076620	07-20-2006	Cephalon, Inc.	
	85	WO 2009/120386	10-01-2009	Cephalon, Inc.	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author, 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
	86	Berge et al., "Pharmaceutical Salts", Journal of pharmaceutical sciences, January 1977, 66(1), 1-19	
	87	Byrn et al., "Pharmaceutical Solids: A Strategic Approach to Regulatory Consideration", Pharmaceutical Research, July 1995, 12(7), 945-954	
	88	EC Safety Data Sheet: Ribomustin® in http://www.docstoc.com/docs/22323231/EC-Safety-Data-Sheet-Bendamustin (published: July 3, 1998; updated March 1, 2007), 8 pages	
	89	Goodman et al., The Pharmacological Basis of Therapeutics, 1985, 7th edition, Macmillan publishing company, New York	
	90	Ni et al., "Use of pure t-butanol as a solvent for freeze-drying: a case study", International Journal of Pharmaceutics, September 2001, 226(1-2), 39-46	

Examiner Signature		**Date Considered**	

Substitute for 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
(use as many sheets as necessary)				**Examiner Name**	Ali Soroush
Sheet	2	of	2	**Attorney Docket Number**	CEPH-4391 / CP391

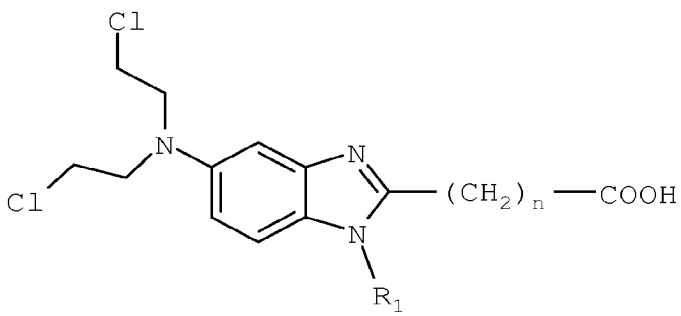
NON PATENT LITERATURE DOCUMENTS			
	91	Ozegowski et al., "IMET 3393, gamma-(1-methyl-5-bis-(ß-chloräthyl)-amino-benzimidazolyl(2)-buttersäure-hydrochlorid, ein neues Zytostatikum aus der Reihe der Benzimidazol-Loste", Zbl Pharm., 1971;110, Heft 10, 1013–1019 (Translation Included)	X
	92	Remington: Pharmaceutical Sciences, 1990, Mack Publishing company, Easton, Pennsylvania	
	93	Schwanen et al., "In Vitro Evaluation of Bendamustine Induced Apoptosis in B-Chronic Lymphocytic Leukemia", Leukemia, October 2002, 16(10), 2096-2105	
	94	Weidmann et al., "Bendamustine is Effective in Relapsed or Refractory Aggressive non-Hodgkin's Lymphoma", Annals of Oncology, August 2002, 13(8), 1285-1289	

Examiner Signature		**Date Considered**	

GDR Patent 34727

Method of preparing ω-[5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-alkane carboxylic acids substituted in 1-position

The invention relates to a method of preparing ω-[5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-alkane carboxylic acids substituted in 1-position of the general formula



wherein R_1 is an alkyl residue or aryl residue and n = 2, 3 or 4.

The compounds prepared according to the method are useful as chemotherapeutics for treatment of growth of tumors.

The use of compounds exhibiting the benzimidazole ring in connection with the bis-(β-chloroethyl)amino group is already known as cytostatic agent (E. HIRSCHBERG, A. GELLHORN and W. GUMP, Cancer Research, Bd. 17 (1957), p. 904 to 910; W. KNOBLOCH, Berichte der Deutschen Chemischen Gesellschaft, Bd. 91 (1958), p. 2557 to 2561). In the 2-[bis-(β-chloroethyl)aminomethyl]-benzimidazole (I) the bis-(β-chloroethyl)amino group is bound to the heteroaromatic system via a methylene bridge. In this compound the basicity of the nitrogen atom of the bis-(β-chloroethyl)amino group is not

influenced by the heteroaromatic system. As the basicity of the nitrogen atom is correlated with the cytostatic activity, an "a priori" alkylating effect of this compound has to be expected, on the other hand a high toxicity of compound I is foreseen from the bis-(β-chloroethyl)amino group linked with the aliphatic methylene group. As a further factor the presence of the benzimidazole ring has to be regarded as antagonist of the purine resulting in an enhancement of the cancer inhibiting effect.

It has to be expected from the linkage of the bis-(β-chloroethyl)amino group with the 5-position of the benzimidazole ring that the toxicity of these compounds is lower as the toxicity from I, as the basicity of the amino nitrogen atom of such compounds is decreased due to the electron attraction of the aromatic ring. Such compounds having a reduced toxicity involve a benefit if the alkylating effect is not reduced substantially vis-à-vis compound I and if it matches with I in its anti-cancer activity, respectively.

It is known that highly proliferated tissue has a high demand of amino acids. Therefore, it has to be assumed that an increased rate of incorporation of amino acids exists in cancer tissue. Amino acid antagonists are suitable for treatment of tumors due to their ability of negatively influencing the growth of cancer cells. Also the benzimidazolyl alkane carboxylic acids can be regarded as amino acid antagonists and purine antagonists and they can be expected to have an inhibiting influence on the growth of cancer cells. The anti-cancer activity of the basic compound should be enforced substantially by linking with a nitrogen lost group in 5-position of the benzimidazole ring holding the toxicity of the overall compound at a low level.

The object of the invention is to introduce the benzimidazole alkane carboxylic acid residue for the synthesis of the anti-cancer substances and to substantially intensify the existing antagonism by incorporating the nitrogen lost group in 5-position of the benzimidazole ring without substantially increasing the toxicity of the overall compound. The fatty acid residue linked with the 2-position of the benzimidazole ring causes an enforcement of the water solubility of the substances and an adaption of the substances produced according to the invention to the organism. The substances prepared according to the invention depict an enrichment of the field of pharmaceuticals in the field of treating tumors.

It has been found that ω-[5-bis-(β-chloroethyl)-aminobenzimidazolyl-(2)]-alkane carboxylic acids substituted in 1-position have a high cytostatic activity at the Ehrlich Ascites-Carcinom of the white mouse, although merely a minor alkylating effect has been expected due to the minor basicity of the nitrogen atom bound directly to the aromatic ring of the bis-(β-chloroethyl)-amino group of this compound.

In animal assays (mouse) it has been observed that the compounds prepared according to the method having increased cytostatic activity show an considerable lower toxicity than the known 2-[bis-(β-chloroethyl)-amino-methyl]-benzimidazole, which is effective as cytostatic.

The N^1-substituted ω-[5-nitro-benzimidazolyl-(2)]-alkane carboxylic acid esters used as starting material are obtained starting from the known N^1-substituted 4-nitro-phenylenediamines-(1.2), e.g. N^1-methyl-4-nitro-phenylene-diamin-(1.2) having as substituents alkyl- or aryl residues and reacting these in a known manner with anhydrides of α, ω-

alkane dicarboxylic acids such as succhinic acid, glutaric acid or adipinic acid. The resulting N^1-substituted α, ω-alkane dicarboxylic acid-2-amino-5-nitro-monoanilides are cyclized to the corresponding 1-substituted ω-[5-nitro-benzimidazolyl-(2)]-alkane carboxylic acids in diluted hydrochloric acid and reacted with alcohols such as ethyl alcohol in acidic solution to 1-substituted ω-[5-nitro-benzimidazolyl-(2)]-alkane carboxylic acid esters. The preparation of the foregoing alkane carboxylic acid esters serving as starting material is not part of the invention.

The advantages provided by the substances prepared according to the invention with respect to the inhibition of cancer growth are indicated by means of 2 examples:

The substance β-[5-bis-(β-chloroethyl)-amino-1-methyl-benzimidazolyl-(2)]-propionic acid hydrochloride has been tested for its cancer inhibiting effect on the Ehrlich-Ascites-carzinom. It shows an at least equipollent inhibiting effect over β-[5-bis-(β-chloroethyl)-amino-1-methyl-benzimidazolyl-(2)]-propion acid hydrochloride. However, it is less toxic vis-à-vis the comparison substance already applied in human medicine and having its benefit vis-à-vis the comparison substance. If the optimal dose (single dose) of β-[5-bis-(β-chloroethyl)-amino-1-methyl-benzimidazolyl-(2)]-propionic acid hydrochloride is 0.05 to 0.35 mg/20g mouse daily, that is 3.76 to 26.31% of LD_{50}, the value of the comparison substance is between 0.1 to 0.2 mg/20g mouse daily (= 15 to 30% of LD_{50}). Cf. table 1.

The substance γ-[5-bis-(β-chloroethyl)-amino-1-methyl-benzimidazolyl-(2)]-butyric acid hydrochloride has been tested for its cancer inhibiting effect on the Ehrlich-Ascites-carcinom. It shows at least an equipollent inhibiting

effect vis-à-vis [bis-(β-chloroethyl)-aminomethyl]-benzimidazole hydrochloride. However, it is less toxic vis-à-vis the comparison substance already applied in human medicine and having therein its benefit vis-à-vis the comparison substance. If the optimal dose (single dose) of γ-[5-bis-(β-chloroethyl)-amino-1-methyl-benzimidazolyl-(2)]-butyric acid hydrochloride is 0.05 to 0.5 mg/20g mouse, that is 3.5 to 35% of LD_{50}, the value of the comparison substance is between 0.1 to 0.2 mg/20g mouse (= 15 to 30% of LD_{50}). Cf. table 2.

Remarks regarding the performance of the experiments

Mice have been used from AB/Jena (colony culture) strain weighing from 20 to 25 g and separated in 3 groups in every experiment: 1. Ascites-control group, 2. Ascites + substance group, 3. substance control group. The groups 1 and 2 were injected with about 20 mill. Ascites cells intraperitoneal. After 48 hours the substance injections began for the groups 2 and 3 also interperitonial. The treatment was made with respect to the health condition of the animals (but no more than 1 injection per day).

In the tables, Q_T stands for the ratio of middle survival time of the treated animals to middle survival time of the Ascites-control animals, that is the extension of the middle survival time of the treated animals.

In the table it means:

tox. = toxic
\- = no inhibition
(+) = marginal inhibition
++ = reasonable inhibition
+++ = strong inhibition

Example 1:

a) β-[5-Nitro-1-methyl-benzimidazolyl-(2)]-propionic acid ethyl ester (II)

37.1 g β-[5-Nitro-1-methyl-benzimidazolyl-(2)]-propionic acid (0.15 M) (Fp. 271 to 273.5°C) are refluxed in 190 ml abs. alcohol and 10 ml concentrated sulphuric acid for 3 hours. The solution is poured into 400 ml water after cooling and neutralized with sodium carbonate. The precipitated ester is sucked off and dried.

Yield: 39.8 g (72% of the theory) of colourless crystals of 50% alcohol, F. 105 to 106°C

$C_{13}H_{15}N_3O_4$ (277.3)

calculated: C 56.30; H 5.45; N 15.14

found: C 56.12; H 5.87; N 14.87

b) β-[5-amino-1-methyl-benzimidazolyl-(2)]-propionic acid ethyl ester (III)

33.3 g β-[5-nitro-1-methyl-benzimidazolyl-(2)]-propionic acid ethyl ester (0.12 M) are dissolved in 800 ml acetic acid-methanol (1:1) and hydrogenated at normal pressure by addition of Raney-nickel. The catalyst is filtered when the calculated amount of hydrogen has been absorbed and the filtrate is concentrated in vacuum. The resulting syrup purely crystallizes. The crystals melt at 50 to 55°C.

Yield: 24 g (81% of the theory)

For identification the picrate is made. Fp. 197.5 to 199°C. Yellow crystals from alcohol.

$C_{13}H_{17}N_3O_2 \cdot C_6H_3N_3O_7$ (476.4)

calculated: C 47.90; H 4.23; N 17.64

found: C 47.70; H 4.12; N 17.65

c) β-[5-bis(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-propionic acid ethyl ester (IV)

β-[5-amino-1-methyl- benzimidazolyl-(2)]-propionic acid ethyl ester (0.04 M) are dissolved in 80 ml pure acetic acid and 80 ml water and at 0°C 20 ml ethylene oxide (~0.4 M) are added. After stirring for 48 hours at room temperature the solution is poured into 320 ml water and neutralized with sodium hydrogen carbonate. The neutral solution is shaked out with a total of 1 l acetic ether. After drying of the extracts over sodium sulfate the acetic ether is distilled in vaccum.
Yield: 12.1 g of a brown syrup (90.5% of the theory)
For identification the picrate is made. Yellow needles from alcohol, Fp. 152 to 153°C.
$C_{17}H_{25}N_3O_4 \cdot C_6H_3N_3O_7$ (564.5)
calculated: C 48.94; H 5.00; N 14.89
found: C 48.51; H 5.07; N 14.64

d) β-[5-bis(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-propionic acid hydrochloride (V)

β-[5-bis(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-propionic acid ethyl ester (0.01 mol) are dissolved in 100 ml chloroform and 10 g thionyl chloride (0.08 m) are added dropwise at -5°C. It is stirred for one hour in ice water and for 3 hours at room temperature. The light brown solution is concentrated in vacuum. The resulting syrup is dissolved in 100 ml concentrated hydrochloric acid and is refluxed for 3 hours for esterification. When concentrated the hydrochloride precipitates.
Yield: 1.5 g (38.5% of the theory), colourless needles in water, Fp. 181 to 185°C. The substance crystallizes with ½ M crystal water.
$C_{15}H_{19}Cl_2N_3O_2 \cdot HCl \cdot ½ H_2O$ (389.7)

calculated: C 46.23; H 5.43; N 10.78; H_2O 2.31
found: C 46.23; H 5.48; N 10.74; H_2O 2.47

Example 2:

a) γ-[5-nitro-1-methyl-benzimidazolyl-(2)]-butyric-acid-ethyl ester is prepared analogue II.

Yield: 68% of the theory, light yellow crystals from diluted alcohol, Fp. 109 to 110°C
$C_{14}H_{17}Cl_2N_3O_4$ (291.3)
calculated: C 57.72; H 5.88; N 14.43
found: C 57.59; H 5.93; N 14.44

b) γ-[5-amino-1-methyl-benzimidazolyl-(2)]-butyric-acid-ethyl ester is prepared analogue III.

Yield: 97.3% of the theory, almost colourless crystal powder from acetic ether, Fp. 130.5 to 132°C.
$C_{14}H_{19}N_3O_2$ (261.3)
calculated: C 64.35; H 7.33; N 16.08
found: C 64.15; H 7.57; N 16.16

c) γ-[5-bis-(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-butyric-acid-ethyl ester

is prepared analogue IV.
Yield: 91% of the theory, Fp. 108 to 109°C, almost colourless needles from benzene.
$C_{18}H_{27}N_3O_4$ (349.4)
calculated: C 61.87; H 7.79; N 12.02
found: C 61.88; H 7.79; N 12.07

d) γ-[5-bis-(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-butyric-acid-hydrochloride

is prepared analogue V.

Yield: 85% of the theory, colourless crystals from alcohol, Fp. 148 to 151°C.

The substance crystallizes with 1 M crystal water.

$C_{16}H_{21}Cl_2N_3O_2 \cdot HCl \cdot H_2O$ (412.8)

calculated: C 46.55; H 5.86; N 10.18; H_2O 4.35

found: C 47.18; H 6.00; N 10.28; H_2O 5.02

Example 3:

a) δ-[5-nitro-1-methyl-benzimidazolyl-(2)]-valeric acid ethyl ester is prepared analogue II.

Yield: 66.5% of the theory, almost colourless crystals from alcohol, Fp. 92.5 to 93.5°C.

$C_{15}H_{19}N_3O_4$ (305.3)

calculated: C 59.01; H 6.27; N 13.77

found: C 59.06; H 6.17; N 13.72

b) δ-[5-amino-1-methyl-benzimidazolyl-(2)]-valeric acid ethyl ester

is prepared analogue III.

Yield: theoretic brown syrup which not crystallizes.

For identification the picrate is made, Fp. 146.5 to 147°C, yellow needles from alcohol.

$C_{15}H_{21}N_3O_2 \cdot C_6H_3N_3O_7$ (504.5)

calculated: C 49.99; H 4.80; N 16.67

found: C 50.03; H 4.97; N 16.84

c) δ-[5-bis-(β-hydroxyethyl)-amino-1-methyl-benzimidazolyl-(2)]-valeric acid ethyl ester

is prepared analogue IV.

Yield: 63.5% of the theory, colourless crystals from benzene, Fp. 93 to 95°C.

$C_{19}H_{29}N_3O_4$ (363.5)

calculated: C 62.78; H 8.04; N 11.57

found: C 62.58; H 8.06; N 11.56

d) δ-[5-bis-(β-chlorethyl)-amino-1-methyl-benzimidazolyl-(2)]-valeric acid hydrochloride

is prepared analogue V.

Yield: 66% of the theory, colourless crystals from water, Fp. 173 to 176°C. The substance crystallizes with 1 M crystal water.

$C_{17}H_{23}Cl_2N_3O_2 \cdot HCl \cdot 1\ H_2O$ (426.8)

calculated: C 47.84; H 6.14; N 9.85; H_2O 4.22

found: C 47.70; H 6.01; N 10.22; H_2O 4.82

Example 4:

a) β-[5-nitro-1-phenyl-benzimidazolyl-(2)]-propionic acid ethyl ester

is made analogue II.

Yield: 58% of the theory, colourless crystals from alcohol, Fp. 116 to 117°C.

$C_{18}H_{17}N_3O_4$ (339.4)

calculated: C 63.71; H 5.04; N 12.38

found: C 63.33; H 5.03; N 12.48

b) β-[5-amino-1-phenyl-benzimidazolyl-(2)]-propionic acid ethyl ester

is prepared analogue III.

Yield: almost quantitative, colourless needles from acetic ester, Fp. 124 to 126°C.

$C_{18}H_{19}N_3O_2$ (309.4)
calculated: C 69.87; H 6.20; N 13.59
found: C 69.98; H 6.26; N 13.60

c) β-[5-bis-(β-hydroxyethyl)-amino-1-phenyl-benzimidazolyl-(2)]-propionic acid ethyl ester

is prepared analogue IV.
Yield: almost quantitative, brown syrup which not crystallizes.
For identification the picrate is made. Fp. 163 to 164°C, yellow needles from alcohol.
$C_{22}H_{27}N_3O_4 \cdot C_6H_3N_3O_7$ (626.6)
calculated: C 63.67; H 4.82; N 13.38
found: C 63.66; H 4.88; N 13.46

d) β-[5-bis-(β-chloroethyl)-amino-1-phenyl-benzimidazolyl-(2)]-propionic acid hydrochloride

is prepared analogue V.
Yield: 59% of the theory, colourless needles from alcohol, Fp. 200 to 203°C.
$C_{20}H_{21}Cl_2N_3O_2 \cdot HCl$ (442.8)
calculated: C 54.24; H 5.01; N 9.50
found: C 54.28; H 5.14; N 9.36

Example 5:
a) γ-[5-nitro-1-phenyl-benzimidazolyl-(2)]-butyric acid ethyl ester

is prepared analogue II.
Yield: 60% of the theory, light yellow crystals from alcohol, Fp. 74.5 to 75.5°C.
$C_{19}H_{19}N_3O_4$ (353,4)

calculated: C 64.56; H 5.42; N 11.89
found: C 64.69; H 5.44; N 12.17

b) γ-[5-amino-1-phenyl-benzimidazolyl-(2)]-butyric acid ethyl ester

is prepared analogue III.
Yield: 82% of the theory, crystals from acetic ether, Fp. 56 to 61°C.
For identification the picrate is made. Fp. 147 to 152°C, yellow needles from alcohol.
$C_{19}H_{21}N_3O_2 \cdot C_6H_3N_3O_7$ (552.5)
calculated: C 54.35; H 4.38; N 15.21
found: C 54.45; H 4.46; N 15.12

c) γ-[5-bis-(β-hydroxyethyl)-amino-1-phenyl-benzimidazolyl-(2)]-butyric acid ethyl ester

is prepared analogue IV.
Yield: almost quantitative, brown syrup, which not crystallizes.
For identification the picrate is made, Fp. 113 to 114°C, yellow crystals from alcohol.
$C_{23}H_{29}N_3O_4 \cdot C_6H_3N_3O_7$ (640.6)
calculated: C 54.37; H 5.03; N 13.06
found: C 54.50; H 4.87; N 13.22

d) γ-[5-bis-(β-chloroyethyl)-amino-1-phenyl-benzimidazolyl-(2)]-butyric acid

is prepared analogue V.
Yield: 62% of the theory, brown syrup, which not crystallizes.

For identification the picrate is made, which crystallizes with 1 M crystal alcohol. Yellow flakes from alcohol, Fp. 132 to 135°C.

$C_{21}H_{23}Cl_2N_3O_2 \cdot C_6H_3N_3O_7 \cdot C_2H_5OH$ (659.5)

calculated: C 50.08; H 4.64; N 12.08

found: C 50.01; H 4.82; N 11.79

Example 6:

a) δ-[5-nitro-1-phenyl-benzimidazolyl-(2)]-valeric acid ethyl ester

is prepared analogue II.

Yield: 62% of the theory, yellowish crystals from diluted alcohol, Fp. 63 to 65°C.

$C_{20}H_{21}N_3O_4$ (367.4)

calculated: C 65.38; H 5.76; N 11.44

found: C 65.45; H 5.74; N 11.43

b) δ-[5-amino-1-phenyl-benzimidazolyl-(2)]-valeric acid ethyl ester

is prepared analogue III.

Yield: 82% of the theory, brown syrup, which not crystallizes.

For identification the picrate is made, which crystallizes wit 1 M crystal alcohol. Fp. 134 to 135°C, fine yellow needles from diluted alcohol.

$C_{20}H_{23}N_3O_2 \cdot C_6H_3N_3O_7$ (566.5)

calculated: C 55.12; H 4.63; N 14.83

found: C 55.28; H 4.70; N 14.81

c) δ-[5-bis-(β-hydroxyethyl)-amino-1-phenyl-benzimidazolyl-(2)]-valeric acid ethyl ester

is prepared analogue IV.

Yield: theoretic brown syrup, which not crystallizes.

For identification the picrate is made, Fp. 68 to 72°C, fine yellow crystals from alcohol.

$C_{24}H_{31}N_3O_4 \cdot C_6H_3N_3O_7$ (654.7)

calculated: C 55.02; H 5.23; N 12.86

found: C 54.69; H 5.30; N 12.67

d) δ-[5-bis-(β-chloroethyl)-amino-1-phenyl-benzimidazolyl-(2)]-valeric acid

is prepared analogue V.

Yield: 90.5% of the theory, brown syrup, which not crystallizes.

For identification the picrate is made, which crystallizes with ½ M crystal alcohol; yellow needles from alcohol, Fp. 91 to 92°C.

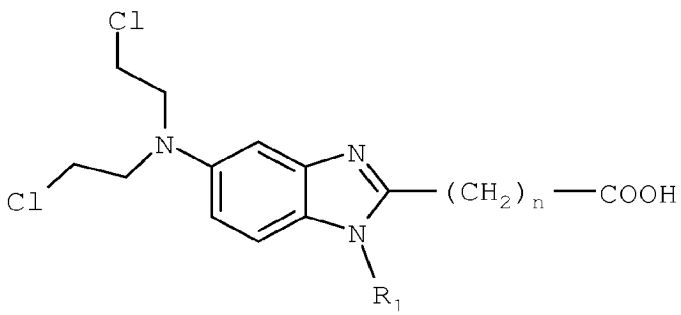
$C_{22}H_{25}N_3O_2Cl_2 \cdot C_6H_3N_3O_7 \cdot ½\ C_2H_5OH$ (678.5)

calculated: C 51.35; H 4.60; N 12.39

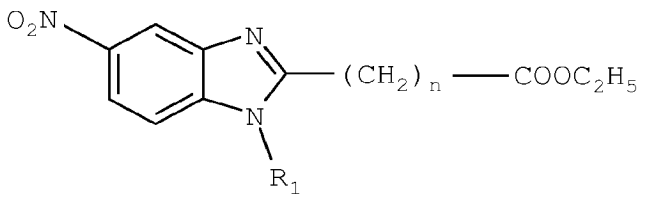
found: C 52.04; H 4.74; N 12.47

Claim:

1. Method of preparing ω-[5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-alkane carboxylic acids substituted in 1-position of the general formula



wherein R_1 is an alkyl residue or an aryl residue and n = 2, 3 or 4, characterized in that ω-[5-nitro-benzimidazolyl-(2)]-alkane carboxylic acid substituted in 1-position of the general formula



wherein R_1 is an alkyl residue or an aryl residue, is treated with hydrogen in the presence of catalysts in a known manner, the resulting ω-[5-amino-benzimidazolyl-(2)]-alkane carboxylic acid ester substituted in 1-position is reacted with ethylene oxide, the obtained ω-[5-bis-(β-hydroxyethyl)amino-benzimidazolyl-(2)]-alkane carboxylic acid ester substituted in 1-position at R_1 is treated with inorganic acid chlorides and subsequently with concentrated hydrochloric acid.

Results :

Table 1

single dose (mg)	number of injections	total dose (mg)	number of tested animals			Q_T	evaluation of experiments
			Asc. - conc.	Asc. + conc.	subst. - conc.		
0,5	6	3	3	10	3	1,13	++ (tox.)
0,35	23	8,05	3	3	3	2,1	+++/++++
0,25	33	8,25	3	3	3	3,5	+++
0,25	19	4,75	3	10	3	2,01	++/+++
0,1	23	2,3	3	3	3	2,11	++/+++
0,1	33	2,3	3	10	3	1,4	++/+++
0,05	31	1,55	3	3	3	1,3	++
0,05	20	1,0	3	10	3	1,86	++/+++
0,025	14	0,35	3	3	3	1,61	+/++
0,01	8	0,08	3	10	3	0,78	-

LD_{50} i.p. : 1.33 mg/20 g mouse; 65.5 mg/kg mouse
most effective single dose: 0.05 to 0.25 mg = 3.76 to 26.31% of LD_{50}

Results:

Table 2

single dose (mg)	number of injections	total dose (mg)	number of tested animals			Q_T	evaluation of experiments
			Asc. - conc.	Asc. + conc.	subst. - conc.		
1	3	3	3	3	3	0,26	tox.
0,5	9	4,5	3	3	3	2,29	+++/++++
0,25	33	8,25	3	3	3	4,54	+++
0,1	30	3	3	3	3	2,04	+++
0,1	29	2,9	3	10	3	2,02	+++
0,05	32	1,6	3	3	3	2,58	+++
0,05	22	1,1	3	3	3	1,45	++
0,05	29	1,45	3	10	3	2,88	+++
0,025	15	0,375	3	3	3	1,06	(+)
0,025	13	0,325	3	10	3	0,71	-

LD_{50} i.p. : 1.42 mg/20 g mouse; 71 mg/kg mouse
most effective single dose: 0.05 to 0.5 mg (3.5 to 35% of LD_{50})

Table:

results

single dose [mg]	number of injections	total dose [mg]	number of tested animals			Qc	evaluation of experiments
			Asc. - conc.	Asc. + conc.	subst. - conc.		
1	3	3	3	3	3	0,26	tox.
0,3	9	4,5	3	3	3	2,29	++/+++
0,25	33	8,25	3	3	3	4,54	+++
0,1	30	3	3	3	3	2,04	+++
0,1	29	2,9	3	10	3	2,08	+++
0,05	32	1,6	3	3	3	2,26	+++
0,05	22	1,1	3	3	3	1,45	++
0,05	29	1,45	3	10	3	2,88	+++
0,025	15	0,375	3	3	3	1,86	(*)
0,025	13	0,325	3	10	3	0,71	-

LD_{50} i.p. : 1.42 mg/20 g mouse; 71 mg/kg mouse
most effective single dose: 0.05 to 0.5 mg (3.5 to 35% of LD_{50})

Deutsche Demokratische Republik

Amt für Erfindungs- und Patentwesen

PATENTSCHRIFT 34727

Wirtschaftspatent

Erteilt gemäß § 5 Absatz 1 des Änderungsgesetzes zum Patentgesetz

Zusatzpatent zum Patent: —

Anmeldetag: 21. XII. 1963 (WP 12 p / 102 091)

Priorität: —

Ausgabetag: 28. XII. 1964

Kl.: 12 p, 9

IPK.: C 07 d 2

DK.:

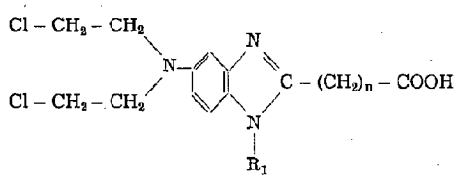
Erfinder zugleich Inhaber:

Dietrich Krebs, Jena
Dr. Werner Ozegowski, Jena
Dipl.-Chem. Walter Gutsche, Jena
Dr. Horst Heinecke, Jena
Dr. Walter Jungstand, Jena
Prof. Dr. Hans Knöll, Jena
Dipl.-Chem. Heinrich Wiankge, Jena

Verfahren zur Herstellung von in 1-Stellung substituierten ω-[5-Bis-(β-chloräthyl)-amino-benzimidazolyl-(2)]-alkancarbonsäuren

1

Die Erfindung betrifft ein Verfahren zur Herstellung von in 1-Stellung substituierten ω-[5-Bis-(β-chloräthyl)-amino-benzimidazolyl-(2)]-alkancarbonsäuren der allgemeinen Formel



in der R_1 einen Alkyl- oder Arylrest und n = 2, 3 oder 4 bedeuten.

Die verfahrensgemäß hergestellten Verbindungen sind als Chemotherapeutika zur Bekämpfung des Wachstums von Tumoren geeignet.

Die Verwendung von Verbindungen, die den Benzimidazolring in Verbindung mit der Bis-(β-chloräthyl)-amino-Gruppe aufweisen, ist als cytostatisches Mittel bereits bekannt (E. HIRSCHBERG, A. GELLHORN und W. GUMP, Cancer Research, Bd. 17 (1957), S. 904 bis 910; W. KNOBLOCH, Berichte der Deutschen Chemischen Gesellschaft, Bd. 91 (1958), S. 2557 bis 2561). Bei dem 2-[Bis-(β-chloräthyl)-amino-methyl]-benzimidazol (I) ist die Bis-(β-chloräthyl)-aminogruppe über eine Methylenbrücke mit dem heteroaromatischen System verknüpft. In dieser Verbindung wird die Basizität des

2

Stickstoffatoms der Bis-(β-chloräthyl)-aminogruppe durch das heteroaromatische System nicht beeinflußt. Da aber die Basizität des Stickstoffatoms mit der cytostatischen Wirksamkeit in Zusammenhang steht, so ist zwar a priori eine alkylierende Wirkung dieser Verbindung zu erwarten, andererseits ist aber auch von der Bis-(β-chloräthyl)-aminogruppe, die mit der aliphatischen Methylengruppe verknüpft ist, eine hohe Toxizität der Verbindung I vorauszusehen. Als ein weiterer Faktor, der zu einer Verstärkung der krebshemmenden Wirkung führt, ist das Vorhandensein des Benzimidazolringes als Antagonist des Purins anzusehen.

Es ist durch Verknüpfung der Bis-(β-chloräthyl)-aminogruppe mit der 5-Stellung des Benzimidazolringes zu erwarten, daß die Toxizität dieser Verbindungen geringer ist als die von I, da die Basizität des Aminostickstoffatoms solcher Verbindungen infolge des Elektronensogs durch den aromatischen Ring erniedrigt wird. Solche Verbindungen mit einer herabgesetzten Toxizität bedeuten aber einen Vorzug, wenn ihre alkylierende Wirkung nicht wesentlich gegenüber der Verbindung I gemindert ist bzw. wenn sie in ihrer Antikrebswirksamkeit mit der von I übereinstimmen.

Es ist bekannt, daß stark proliferierendes Gewebe einen hohen Bedarf an Aminosäuren besitzt. Deswegen ist anzunehmen, daß im Krebsgewebe eine erhöhte Einbaurate an Aminosäuren besteht. Aminosäureantagonisten sind infolge ihrer Fähigkeit, das

Wachstum von Krebszellen negativ zu beeinflussen, zur Behandlung von Tumoren geeignet. Auch die Benzimidazolyl-alkancarbonsäuren können als Aminosäureantagonisten und als Purinantagonisten angesehen werden, und es ist von ihnen ein hemmender Einfluß auf das Wachstum von Krebszellen zu erwarten. Die Antikrebswirksamkeit des Grundkörpers soll durch die Verknüpfung mit der Stickstofflostgruppe in der 5-Stellung des Benzimidazolringes noch wesentlich verstärkt werden, wobei aber die Toxizität der Gesamtverbindung niedrig bleiben soll.

Der Erfindung liegt die Aufgabe zugrunde, den Benzimidazolylalkancarbonsäurerest für die Synthese von Antikrebssubstanzen einzusetzen und den bereits bestehenden Antagonismus durch den Einbau der Stickstofflostgruppe in 5-Stellung des Benzimidazolringes noch wesentlich zu verstärken, ohne hierbei die Toxizität der Gesamtverbindung wesentlich zu steigern. Der Fettsäurerest, der mit der 2-Stellung des Benzimidazolringes verknüpft ist, bewirkt eine Verstärkung der Wasserlöslichkeit der Substanzen und eine Anpassung der erfindungsgemäß hergestellten Substanzen an den Organismus. Die nach der Erfindung hergestellten Substanzen stellen eine Bereicherung des Arzneischatzes auf dem Gebiet zur Behandlung von Tumoren dar.

Es wurde gefunden, daß in 1-Stellung substituierte ω-[5-Bis-(β-chloräthyl)-amino-benzimidazolyl-(2)]-alkancarbonsäuren eine hohe cytostatische Wirksamkeit am Ehrlichschen Ascites-Carcinom der weißen Maus besitzen, obwohl auf Grund der geringen Basizität des direkt am aromatischen Ring befindlichen Stickstoffatoms der Bis-(β-chloräthyl)-aminogruppe dieser Verbindung lediglich eine geringe alkylierende Wirkung zu erwarten war.

Bei Tierversuchen (Maus) wurde festgestellt, daß die verfahrensgemäß hergestellten Verbindungen bei etwas verstärkter cytostatischer Wirksamkeit eine erheblich geringere Toxizität als das bekannte cytostatisch wirksame 2-[Bis-(β-chloräthyl)-aminomethyl]-benzimidazol aufweisen.

Die als Ausgangsmaterial verwendeten N^1-substituierten ω-[5-Nitro-benzimidazolyl-(2)]alkancarbonsäureester werden erhalten, indem man von den bereits bekannten N^1-substituierten 4-Nitro-phenylendiaminen-(1.2), z. B. N^1-Methyl-4-nitro-phenylendiamin-(1.2) ausgeht, die als Substituenten Alkyl- oder Arylreste aufweisen, und diese in an sich bekannter Weise mit den Anhydriden von α, ω-Alkandicarbonsäuren, wie z. B. Bernsteinsäure, Glutarsäure oder Adipinsäure, umsetzt. Die erhaltenen N^2-substituierten α, ω-Alkandicarbonsäure-2-amino-5-nitro-monoanilide werden zu den entsprechenden 1-substituierten ω-[5-Nitro-benzimidazolyl-(2)]-alkancarbonsäuren in verdünnter Salzsäure cyclisiert und mit Alkohol z. B. Äthylalkohol in saurer Lösung zu den 1-substituierten ω-[5-Nitro-benzimidazolyl-(2)]-alkancarbonsäureestern umgesetzt. Die Herstellung der vorstehend beschriebenen als Ausgangsmaterial dienenden Alkancarbonsäureester ist nicht Gegenstand der Erfindung.

Die Vorteile, die die erfindungsgemäß hergestellten Substanzen in bezug auf Hemmung des Krebswachstums bieten, werden an Hand von 2 Beispielen

belegt:

Die Substanz β-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-propionsäure-hydrochlorid wurde am Ehrlich-Ascites-Carcinom auf ihre krebshemmende Wirkung geprüft. Sie zeigte gegenüber 2-[Bis-(β-chloräthyl)-aminomethyl]-benzimidazol-hydrochlorid eine mindestens gleichstarke Hemmwirkung. Sie ist jedoch gegenüber der in der Humanmedizin bereits angewandten Vergleichssubstanz weniger toxisch, und darin liegt ihr Vorzug gegenüber der Vergleichssubstanz. Beträgt die optimale Dosis (Einzeldosis) bei β-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-propionsäure-hydrochlorid 0,05 bis 0,35 mg/20 g Maus täglich, das sind gleich 3,76 bis 26,31% der LD_{50}, so liegt der Wert bei der Vergleichssubstanz zwischen 0,1 bis 0,2 mg/20 g Maus täglich (= 15 bis 30% der LD_{50}). Siehe Tabelle 1.

Die Substanz γ-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-buttersäure-hydrochlorid wurde am Ehrlich-Ascites-Carcinom auf ihre krebshemmende Wirkung geprüft. Sie zeigte gegenüber 2-[Bis-(β-chloräthyl)-aminomethyl]-benzimidazol-hydrochlorid eine mindestens gleichstarke Hemmwirkung. Sie ist jedoch gegenüber der in der Humanmedizin bereits angewandten Vergleichssubstanz weniger toxisch, und darin liegt ihr Vorzug gegenüber der Vergleichssubstanz. Beträgt die optimale Dosis (Einzeldosis bei γ-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-buttersäure-hydrochlorid 0,05 bis 0,5 mg/20 g Maus, das sind gleich 3,5 bis 35% der LD_{50}, so liegt der Wert bei der Vergleichssubstanz zwischen 0,1 bis 0,2 mg/20 g Maus (= 15 bis 30% der LD_{50}). Siehe Tabelle 2.

Bemerkungen zur Durchführung der Versuche

Es wurden Mäuse vom Stamm AB/Jena (Kolonienzucht) mit einem Gewicht von 20 bis 25 g verwendet und bei jedem Versuch in 3 Gruppen getrennt: 1. Ascites-Kontrollgruppe, 2. Ascites + Substanz-Gruppe, 3. Substanz-Kontrollgruppe. Die Gruppen 1. und 2. bekamen eine Injektion von etwa 20 Mill. Asciteszellen intraperitoneal. 48 Stunden danach begannen für die Gruppen 2. und 3. die Substanz-Injektionen, ebenfalls intraperitoneal. Die Behandlung erfolgte mit Rücksicht auf den Gesundheitszustand der Tiere (jedoch nicht mehr als 1 Injektion je Tag).

In den Tabellen bedeutet Q_T das Verhältnis von mittlerer Überlebenszeit der behandelten Tiere zur mittleren Überlebenszeit der Ascites-Kontrolltiere, also die Verlängerung der mittleren Überlebenszeit der behandelten Tiere.

Es bedeutet in der Tabelle:

tox. = toxisch
– = keine Hemmung
(+) = unbedeutende Hemmung
+ – = mäßige Hemmung
+ – + = starke Hemmung

Beispiel 1:

a) β-[5-Nitro-1-methyl-benzimidazolyl-(2)]-propionsäureäthylester (II)

37,1 g β-[5-Nitro-1-methyl-benzimidazolyl-(2)]-propionsäure (0,15 Mol) (Fp. 271 bis 273,5 °C) werden mit 190 ml abs. Alkohol und 10 ml konz. Schwefel-

säure drei Stunden unter Rückfluß erhitzt. Die Lösung gießt man nach dem Erkalten in 400 ml Wasser und neutralisiert mit Natriumcarbonat. Der ausgefallene Ester wird abgesaugt und getrocknet.

Ausbeute: 39,8 g (72% der Th.) farblose Kristalle aus 50proz. Alkohol, F. 105 bis 106 °C.

$C_{13}H_{15}N_3O_4$ (277,3)

berechnet: C 56,30; H 5,45; N 15,14

gefunden: C 56,12; H 5,87; N 14,87

b) 3-[5-Amino-1-methyl-benzimidazolyl-(2)]-propionsäureäthylester (III)

33,3 g β-[5-Nitro-1-methyl-benzimidazolyl-(2)]-propionsäure-äthylester (0,12 Mol) werden in 600 ml Essigester-Methanol (1:1) gelöst und unter Zusatz von Raney-Nickel bei Normaldruck hydriert. Der Katalysator wird nach Aufnahme der berechneten Menge Wasserstoff abfiltriert und das Filtrat im Vakuum eingeengt. Der zurückbleibende Sirup kristallisiert schwer. Die Kristalle schmelzen bei 50 bis 55 °C.

Ausbeute: 24 g (81% der Th.).

Zur Identifizierung wird das Pikrat hergestellt. Fp. 197,5 bis 199 °C. Gelbe Kristalle aus Alkohol.

$C_{13}H_{17}N_3O_2 \cdot C_6H_3N_3O_7$ (476,4)

berechnet: C 47,90; H 4,23; N 17,64

gefunden: C 47,70; H 4,12; N 17,65

c) β-[5-Bis-(β-hydroxyäthyl)-amino-1-methyl-benzimidazolyl-(2)]-propionsäureäthylester (IV)

9,9 g β-[5-Amino-1-methyl-benzimidazolyl-(2)]-propionsäure-äthylester (0,04 Mol) werden in 80 ml Eisessig und 80 ml Wasser gelöst und bei 0 °C mit 20 ml Äthylenoxyd (~ 0,4 Mol) versetzt. Nach 48stündigem Rühren bei Zimmertemperatur gießt man die Lösung in 320 ml Wasser und neutralisiert mit Natriumhydrogencarbonat. Die neutrale Lösung wird mit insgesamt 1 l Essigester ausgeschüttelt. Nach Trocknen des Extraktes über Natriumsulfat wird der Essigester im Vakuum abdestilliert.

Ausbeute: 12,1 g eines braunen Sirups (90,5% der Th.).

Zur Identifizierung wird das Pikrat hergestellt. Gelbe Nadeln aus Alkohol, Fp. 152 bis 153 °C

$C_{17}H_{25}N_3O_4 \cdot C_6H_3N_3O_7$ (564,5)

berechnet: C 48,94; H 5,00; N 14,89

gefunden: C 48,51; H 5,07; N 14,64

d) β-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-propionsäurehydrochlorid (V)

3,35 g β-[5-Bis-(β-hydroxyäthyl)-amino-1-methyl-benzimidazolyl-(2)]-propionsäureäthylester (0,01 Mol) werden in 100 ml Chloroform gelöst und bei −5 °C mit 10 g Thionylchlorid (0,08 Mol) tropfenweise versetzt. Es wird 1 Stunde in Eiswasser und 3 Stunden bei Zimmertemperatur nachgerührt. Die hellbraune Lösung engt man im Vakuum ein. Der zurückbleibende Sirup wird in 100 ml konzentrierter Salzsäure gelöst und drei Stunden zur Verseifung unter Rückfluß erhitzt. Beim Einengen fällt das Hydrochlorid aus.

Ausbeute: 1,5 g (38,5% der Th.), farblose Nadeln aus Wasser, Fp. 181 bis 185 °C. Die Substanz kristallisiert mit einem halben Mol Kristallwasser.

$C_{15}H_{19}Cl_2N_3O_2 \cdot HCl \cdot {}^1/_2 H_2O$ (389,7)

berechnet: C 46,23; H 5,43; N 10,78; H_2O 2,31

gefunden: C 46,23; H 5,48; N 10,74; H_2O 2,47

Beispiel 2:

a) γ-[5-Nitro-1-methyl-benzimidazolyl-(2)]-buttersäureäthylester wird analog II hergestellt.

Ausbeute: 68% der Th., schwach gelbe Kristalle aus verdünntem Alkohol, Fp. 109 bis 110 °C.

$C_{14}H_{17}N_3O_4$ (291,3)

berechnet: C 57,72; H 5,88; N 14,43

gefunden: C 57,59; H 5,93; N 14,44

b) γ-[5-Amino-1-methyl-benzimidazolyl-(2)]-buttersäureäthylester wird analog III hergestellt.

Ausbeute: 97,3% der Th., fast farbloses Kristallpulver aus Essigester, Fp. 130,5 bis 132 °C.

$C_{14}H_{19}N_3O_2$ (261,3)

berechnet: C 64,35; H 7,33; N 16,08

gefunden: C 64,15; H 7,57; N 16,16

c) γ-[5-Bis-(β-hydroxyäthyl)-amino-1-methyl-benzimidazolyl-(2)]-buttersäureäthylester wird analog IV hergestellt.

Ausbeute: 91% der Th., Fp. 108 bis 109 °C, fast farblose Nadeln aus Benzol.

$C_{18}H_{27}N_3O_4$ (349,4)

berechnet: C 61,87; H 7,79; N 12,02

gefunden: C 61,88; H 7,79; N 12,07

d) γ-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimidazolyl-(2)]-buttersäurehydrochlorid wird analog V hergestellt.

Ausbeute: 85% der Th., farblose Kristalle aus Wasser, Fp. 148 bis 151 °C.

Die Substanz kristallisiert mit einem Mol Kristallwasser.

$C_{16}H_{21}Cl_2N_3O_2 \cdot HCl \cdot H_2O$ (412,3)

berechnet: C 46,55; H 5,86; N 10,18; H_2O 4,35

gefunden: C 47,18; H 6,00; N 10,28; H_2O 5,02

Beispiel 3:

a) δ-[5-Nitro-1-methyl-benzimidazolyl-(2)]-valeriansäureäthylester wird analog II hergestellt.

Ausbeute: 66,5% der Th., fast farblose Kristalle aus Alkohol, Fp. 92,5 bis 93,5 °C.

$C_{15}H_{19}N_3O_4$ (305,3)

berechnet: C 59,01; H 6,27; N 13,77

gefunden: C 59,06; H 6,17; N 13,72

b) δ-[5-Amino-1-methyl-benzimidazolyl-(2)]-valeriansäureäthylester wird analog III hergestellt.

Ausbeute: theor., brauner Sirup, der nicht kristallisiert.

Zur Identifizierung wird das Pikrat hergestellt, Fp. 146,5 bis 147 °C, gelbe Nadeln aus Alkohol.

$C_{15}H_{21}N_3O_2 \cdot C_6H_3N_3O_7$ (504,5)

berechnet: C 49,99; H 4,80; N 16,67

gefunden: C 50,03; H 4,97; N 16,84

c) δ-[5-Bis-(β-hydroxyäthyl)-amino-1-methyl-benzimidazolyl-(2)]-valeriansäureäthylester wird analog IV hergestellt.

Ausbeute: 63,5% der Th., farblose Kristalle aus Benzol, Fp. 93 bis 95 °C.

$C_{19}H_{29}N_3O_4$ (363,5)

berechnet: C 62,78; H 8,04; N 11,57

gefunden: C 62,58; H 8,06; N 11,56

d) δ-[5-Bis-(β-chloräthyl)-amino-1-methyl-benzimid-

azolyl-(2)]-valeriansäure-hydrochlorid
wird analog V hergestellt.
Ausbeute: 66% der Th., farblose Kristalle aus Wasser, Fp. 173 bis 176 °C. Die Substanz kristallisiert mit einem Mol Kristallwasser aus.
$C_{17}H_{23}Cl_2N_3O_2 \cdot HCl \cdot 1\,H_2O$ (426,8)
berechnet: C 47,84; H 6,14; N 9,85; H_2O 4,22
gefunden: C 47,70; H 6,01; N 10,22; H_2O 4,82

Beispiel 4:
a) β-[5-Nitro-1-phenyl-benzimidazolyl-(2)]-propionsäureäthylester
wird analog II hergestellt.
Ausbeute: 58% der Th., farblose Kristalle aus Alkohol, Fp. 116 bis 117 °C.
$C_{19}H_{17}N_3O_4$ (339,4)
berechnet: C 63,71; H 5,04; N 12,38
gefunden: C 63,33; H 5,03; N 12,48
b) β-[5-Amino-1-phenyl-benzimidazolyl-(2)]-propionsäureäthylester
wird analog III hergestellt.
Ausbeute: fast quantitativ, farblose Nadeln aus Essigester, Fp. 124 bis 126 °C.
$C_{18}H_{19}N_3O_2$ (309,4)
berechnet: C 69,87; H 6,20; N 13,59
gefunden: C 69,98; H 6,26; N 13,60
c) β-[5-Bis-(β-hydroxyäthyl)-amino-1-phenyl-benzimidazolyl-(2)]-propionsäureäthylester
wird analog IV hergestellt.
Ausbeute: fast quantitativ, brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt. Fp. 163 bis 164 °C, gelbe Nadeln aus Alkohol.
$C_{22}H_{27}N_3O_4 \cdot C_6H_3N_3O_7$ (626,6)
berechnet: C 63,67; H 4,82; N 13,38
gefunden: C 63,66; H 4,88; N 13,46
d) β-[5-Bis-(β-chloräthyl)-amino-1-phenyl-benzimidazolyl-(2)]-propionsäurehydrochlorid
wird analog V hergestellt.
Ausbeute: 59% der Th., farblose Nadeln aus Alkohol, Fp. 200 bis 203 °C.
$C_{20}H_{21}Cl_2N_3O_2 \cdot HCl$ (442,8)
berechnet: C 54,24; H 5,01; N 9,50
gefunden: C 54,28; H 5,14; N 9,36

Beispiel 5:
a) γ-[5-Nitro-1-phenyl-benzimidazolyl-(2)]-buttersäureäthylester
wird analog II hergestellt.
Ausbeute: 60% der Th., hellgelbe Kristalle aus Alkohol, Fp. 74,5 bis 75,5 °C.
$C_{19}H_{19}N_3O_4$ (353,4)
berechnet: C 64,56; H 5,42; N 11,89
gefunden: C 64,69; H 5,44; N 12,17
b) γ-[5-Amino-1-phenyl-benzimidazolyl-(2)]-buttersäureäthylester
wird analog III hergestellt.
Ausbeute: 82% der Th., Kristalle aus Essigester, Fp. 56 bis 61 °C.
Zur Identifizierung wird das Pikrat hergestellt. Fp. 147 bis 152 °C, gelbe Nadeln aus Alkohol.
$C_{19}H_{21}N_3O_2 \cdot C_6H_3N_3O_7$ (552,5)
berechnet: C 54,35; H 4,38; N 15,21
gefunden: C 54,45; H 4,46; N 15,12
c) γ-[5-Bis-(β-hydroxyäthyl)-amino-1-phenyl-benz-

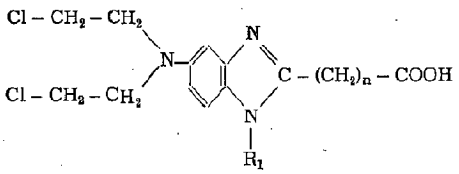
imidazolyl-(2)]-buttersäureäthylester
wird analog IV hergestellt.
Ausbeute: fast quantitativ; brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt. Fp. 113 bis 114 °C, gelbe Kristalle aus Alkohol.
$C_{23}H_{29}N_3O_4 \cdot C_6H_3N_3O_7$ (640,6)
berechnet: C 54,37; H 5,03; N 13,06
gefunden: C 54,50; H 4,87; N 13,22
d) γ-[5-Bis-(β-chloräthyl)-amino-1-phenyl-benzimidazolyl-(2)]-buttersäure
wird analog V hergestellt.
Ausbeute: 62% der Th., brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt, das mit einem Mol Kristallalkohol auskristallisiert. Gelbe Blättchen aus Alkohol, Fp. 132 bis 135 °C.
$C_{21}H_{23}Cl_2N_3O_2 \cdot C_6H_3N_3O_7 \cdot C_2H_5OH$ (659,5)
berechnet: C 50,08; H 4,64; N 12,08
gefunden: C 50,01; H 4,82; N 11,79

Beispiel 6:
a) δ-[5-Nitro-1-phenyl-benzimidazolyl-(2)]-valeriansäureäthylester
wird analog II hergestellt.
Ausbeute: 62% der Th., gelbliche Kristalle aus verdünntem Alkohol, Fp. 63 bis 65 °C.
$C_{20}H_{21}N_3O_4$ (367,4)
berechnet: C 65,38; H 5,76; N 11,44
gefunden: C 65,45; H 5,74; N 11,43
b) δ-[5-Amino-1-phenyl-benzimidazolyl-(2)]-valeriansäureäthylester
wird analog III hergestellt.
Ausbeute: 82% der Th., brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt, Fp. 134 bis 135 °C, feine, gelbe Nadeln aus verdünntem Alkohol.
$C_{20}H_{23}N_3O_2 \cdot C_6H_3N_3O_7$ (565,5)
berechnet: C 55,12; H 4,63; N 14,83
gefunden: C 55,28; H 4,70; N 14,31
c) δ-[5-Bis-(β-hydroxyäthyl)-amino-1-phenyl-benzimidazolyl-(2)]-valeriansäureäthylester
wird analog IV hergestellt.
Ausbeute: theoret.; brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt, Fp. 68 bis 72 °C, gelbe Kristalle aus Alkohol.
$C_{24}H_{31}N_3O_4 \cdot C_6H_3N_3O_7$ (654,7)
berechnet: C 55,02; H 5,23; N 12,86
gefunden: C 54,69; H 5,30; N 12,67
d) δ-[5-Bis-(β-chloräthyl)-amino-1-phenyl-benzimidazolyl-(2)]-valeriansäure
wird analog V hergestellt.
Ausbeute: 90,5% der Th., brauner Sirup, der nicht kristallisiert.
Zur Identifizierung wird das Pikrat hergestellt, das mit einem halben Mol Kristallalkohol kristallisiert; gelbe Nadeln aus Alkohol, Fp. 91 bis 92 °C.
$C_{22}H_{25}N_3O_2Cl_2 \cdot C_6H_3N_3O_7 \cdot$
$\frac{1}{2}\,C_2H_5OH$ (678,5)
berechnet: C 51,35; H 4,60; N 12,39
gefunden: C 52,04; H 4,74; N 12,47

9

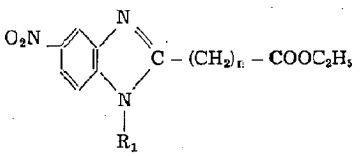
Patentanspruch:

Verfahren zur Herstellung von in 1-Stellung substituierten ω-[5-Bis-(β-chloräthyl)-amino-benzimidazolyl-(2)]-alkancarbonsäuren der allgemeinen Formel



in der R_1 einen Alkyl- oder Arylrest und n = 2, 3 oder 4 bedeuten, dadurch **gekennzeichnet**, daß man in an sich bekannter Weise in der 1-Stellung substituierte ω-[5-Nitro-benzimidazolyl-(2)]-alkancarbonsäureester der allgemeinen Formel

10



in der R_1 einen Alkyl- oder Arylrest bedeutet, mit Wasserstoff in Gegenwart von Katalysatoren behandelt, die erhaltenen in 1-Stellung substituierten ω-[5-Amino-benzimidazolyl-(2)]-alkancarbonsäureester mit Äthylenoxyd umsetzt, die daraus gewonnenen in 1-Stellung R_1-substituierten ω-[5-Bis-(β-hydroxyäthyl)-amino-benzimidazolyl-(2)]-alkancarbonsäureester mit anorganischen Säurechloriden und anschließend mit konzentrierter Chlorwasserstoffsäure behandelt.

Vordruck-Leitverlag Berlin, Ag 300/64/DDR – (52) 9138

Testergebnisse:

Tabelle 1

Einzeldosis mg	Anzahl d. Injektionen	Gesamtdosis mg	Anzahl der Versuchstiere			Q_T	Versuchsbewertung
			Asc. – Kontr.	Asc. + Subst.	Subst. – Kontr.		
0,5	6	3	3	10	3	1,13	++ (tox.)
0,35	23	8,05	3	3	3	2,1	++/+++
0,25	33	8,25	3	3	3	2,6	+++
0,25	19	4,75	3	10	3	2,01	++/+++
0,1	23	2,3	3	3	3	2,11	++/+–+
0,1	22	2,2	3	10	3	1,4	++/+++
0,05	31	1,55	3	3	3	1,3	++
0,05	20	1,0	3	10	3	1,93	++/+++
0,025	14	0,35	3	3	3	1,61	+/++
0,01	8	0,08	3	10	3	0,78	–

LD_{50} i. p.: 1,33 mg/20 g Maus; 65,5 mg/kg Maus
Wirksamste Einzeldosis: 0,05 bis 0,35 mg = 3,76 bis 26,31% der LD_{50}.

Testergebnisse:

Tabelle 2

Einzeldosis mg	Anzahl d. Injektionen	Gesamtdosis mg	Anzahl der Versuchstiere			Q_T	Versuchsbewertung
			Asc. – Kontr.	Asc. + Subst.	Subst. – Kontr.		
1	3	3	3	3	3	0,26	tox.
0,5	9	4,5	3	3	3	2,29	++/+++
0,25	33	8,25	3	3	3	4,54	+++
0,1	30	3	3	3	3	2,04	+++
0,1	29	2,9	3	10	3	2,08	+++
0,05	32	1,6	3	3	3	2,56	+++
0,05	22	1,1	3	3	3	1,45	++
0,05	29	1,45	3	10	3	2,88	+++
0,025	15	0,375	3	3	3	1,06	(+)
0,025	13	0,325	3	10	3	0,71	–

LD_{50} i. p.: 1,42 mg/20 g Maus; 71 mg/kg Maus
Wirksamste Einzeldosis: 0,05 bis 0,5 mg (3,5 bis 35% der LD_{50})

Tabelle:

Testergebnisse

Einzeldosis (mg)	Anzahl der Injektionen	Gesamtdosis (mg)	Anzahl der Versuchstiere			Q_T	Versuchsbewertung
			Asc.-Kontr.	Asc. + Subst.	Subst.-Kontr.		
1	3	3	3	3	3	0,26	tox.
0,5	9	4,5	3	3	3	2,29	++/+++
0,25	33	8,25	3	3	3	4,54	+++
0,1	30	3	3	3	3	2,04	+++
0,1	29	2,9	3	10	3	2,08	+++
0,05	32	1,6	3	3	3	2,56	+++
0,05	22	1,1	3	3	3	1,45	++
0,05	29	1,45	3	10	3	2,88	+++
0,025	15	0,375	3	3	3	1,06	(+)
0,025	13	0,325	3	10	3	0,71	–

LD_{50} i. p.: 1,42 mg/20 g Maus; 71 mg/kg Maus
Wirksamste Einzeldosis: 0,05 bis 0,5 mg (3,5 bis 35% der LD_{50})

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 July 2006 (20.07.2006)

PCT

(10) International Publication Number
WO 2006/076620 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2006/001308

(22) International Filing Date: 13 January 2006 (13.01.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/644,354	14 January 2005 (14.01.2005)	US
11/330,868	12 January 2006 (12.01.2006)	US

(71) Applicant *(for all designated States except US)*: **CEPHALON, INC.** [US/US]; 41 Moores Road, P.O. Box 4011, Frazer, Pennsylvania 19355 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BRITTAIN, Jason, Edward** [US/US]; 1580 Chiswick Court, El Cajon, California 92020 (US). **FRANKLIN, Joe, Craig** [US/US]; 3708 45th Street, Tulsa, Oklahoma 74135 (US).

(74) Agent: **MILLER, Suzanne, E.**; Woodcock Washburn LLP, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 (US).

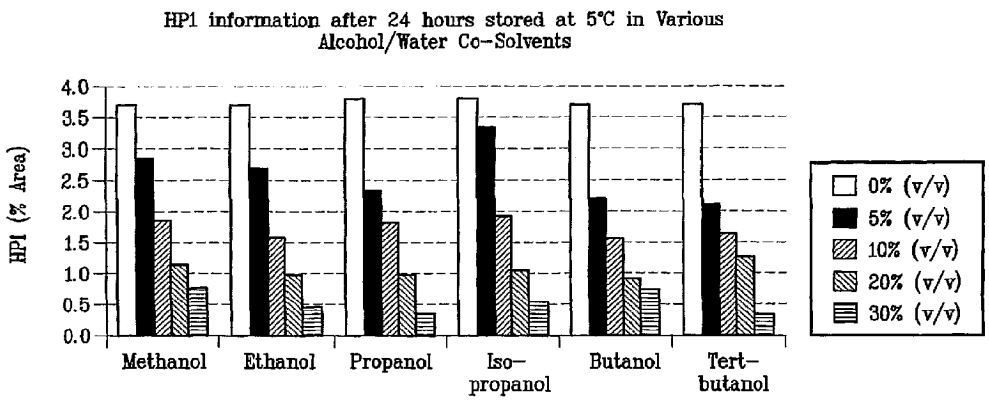
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, 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.

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

Published:
— *without international search report and to be republished upon receipt of that report*

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

(54) Title: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS



(57) Abstract: The present invention provides pharmaceutical formulations of lyophilized bendamustine suitable for pharmaceutical use. The present invention further provides methods of producing lyophilized bendamustine. The pharmaceutical formulations can be used for any disease that is sensitive to treatment with bendamustine, such as neoplastic diseases.

WO 2006/076620 A2

BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

FIELD OF THE INVENTION

The present invention pertains to the field of pharmaceutical compositions for the treatment of various disease states, especially neoplastic diseases and autoimmune diseases. Particularly, it relates to pharmaceutical formulations comprising nitrogen mustards, particularly the nitrogen mustard bendamustine, e.g., bendamustine HCl.

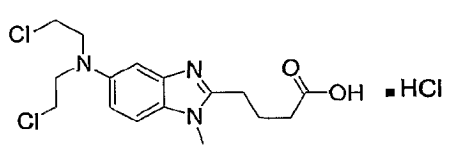
BACKGROUND OF THE INVENTION

The present invention claims the benefit of and priority to US Serial No. 60/644,354, filed January 14, 2005, entitled, "Bendamustine Pharmaceutical Compositions," which is incorporated herein by reference in its entirety, including figures and claims.

The following description includes information that may be useful in understanding the present invention. It is not an admission that any such information is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

Because of their high reactivity in aqueous solutions, nitrogen mustards are difficult to formulate as pharmaceuticals and are often supplied for administration in a lyophilized form that requires reconstitution, usually in water, by skilled hospital personal prior to administration. Once in aqueous solution, nitrogen mustards are subject to degradation by hydrolysis, thus, the reconstituted product should be administered to a patient as soon as possible after its reconstitution.

Bendamustine, (4-{5-[Bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl} butyric acid, is an atypical structure with a benzimidazole ring, whose structure includes an active nitrogen mustard (see Formula I, which shows bendamustine hydrochloride).



Formula I

Bendamustine was initially synthesized in 1963 in the German Democratic Republic (GDR) and was available from 1971 to 1992 in that location under the name Cytostasan®. Since that time, it has been marketed in Germany under the tradename Ribomustin®. It has been widely used in Germany to treat chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, and breast cancer.

Due to its degradation in aqueous solutions (like other nitrogen mustards), bendamustine is supplied as a lyophilized product. The current lyophilized formulation of bendamustine (Ribomustin®) contains bendamustine hydrochloride and mannitol in a sterile lyophilized form as a white powder for intravenous use following reconstitution. The finished lyophilisate is unstable when exposed to light. Therefore, the product is stored in brown or amber-colored glass bottles. The current lyophilized formulation of bendamustine contains degradation products that may occur during manufacturing of the drug substance and/or during the lyophilization process to make the finished drug product.

Currently bendamustine is formulated as a lyophilized powder for injection with 100 mg of drug per 50 mL vial or 25 mg of drug per 20 mL vial. The vials are opened and reconstituted as close to the time of patient administration as possible. The product is reconstituted with 40 mL (for the 100 mg presentation) or 10 mL (for the 25 mg presentation) of Sterile Water for Injection. The reconstituted product is further diluted into 500 mL, q.s., 0.9% Sodium Chloride for Injection. The route of administration is by intravenous infusion over 30 to 60 minutes.

Following reconstitution with 40 mL Sterile Water for Injection, vials of bendamustine are stable for a period of 7 hours under room temperature storage or for 6 days upon storage at 2-8°C. The 500 mL admixture solution must be administered to the patient within 7 hours of vial reconstitution (assuming room temperature storage of the admixture).

The reconstitution of the present bendamustine lyophilized powder is difficult. Reports from the clinic indicate that reconstitution can require at least fifteen minutes and may require as long as thirty minutes. Besides being burdensome and time-consuming for the healthcare professional responsible for reconstituting the product, the lengthy exposure of bendamustine to water during the reconstitution process

increases the potential for loss of potency and impurity formation due to the hydrolysis of the product by water.

Thus, a need exists for lyophilized formulations of bendamustine that are easier to reconstitute and which have a better impurity profile than the current lyophilate (lyophilized powder) formulations of bendamustine.

German (GDR) Patent No. 34727 discloses a method of preparing ω-[5-bis-(β-chloroethyl)-amino-benzimidazolyl-(2)]-alkane carboxylic acids substituted in the 1-position.

German (GDR) Patent No. 80967 discloses an injectable preparation of γ-[1-methyl-5-bis-(β-chloroethyl)-amino-benzimaidazolyl-(2)]-butric acid hydrochloride.

German (GDR) Patent No. 159877 discloses a method for preparing 4-[1-methyl-5-bis (2-chloroethyl) amino-benzimidazolyl-2)-butyric acid.

German (GDR) Patent No. 159289 discloses an injectable solution of bendamustine.

Ribomustin® bendamustine Product monograph (updated 1/2002) http://www.ribosepharm.de/pdf/ribomustin_bendamustin/productmonograph.pdf provides information about Ribomustin® including product description.

Ni et al. report that the nitrosourea SarCNU was more stable in pure tertiary butanol than in pure acetic acid, dimethyl sulfoxide, methylhydroxy, water or in TBA/water mixtures (Ni et al. (2001) *Intl. J. Phamaceutics* 226:39-46).

Lyophilized cyclophoshamide is known in the art see e.g., US Patent Nos. 5,418,223; 5,413,995; 5,268,368; 5,227,374; 5,130,305; 4,659,699; 4,537,883; and 5,066,647.

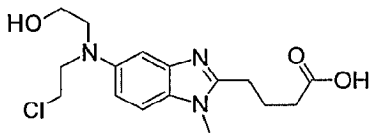
The lyophilized nitrogen mustard Ifosfamide is disclosed in International Publication No. WO 2003/066027; US Pat. Nos. 6,613,927; 5,750,131; 5,972,912; 5,227,373; and 5,204,335.

Teagarden et al. disclose lyophilized formulations of prostaglandin E-1 made by dissolving PGE-1 in a solution of lactose and tertiary butyl alcohol (US Pat. No. 5,770,230).

SUMMARY OF THE INVENTION

The present invention is directed to stable pharmaceutical compositions of nitrogen mustards, in particular lyophilized bendamustine and its use in treatment of various disease states, especially neoplastic diseases and autoimmune diseases.

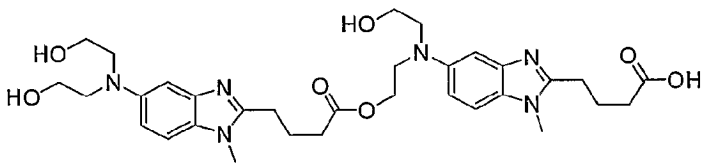
An embodiment of the invention is a pharmaceutical composition of bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine) HP1, as shown in Formula II,



Formula II

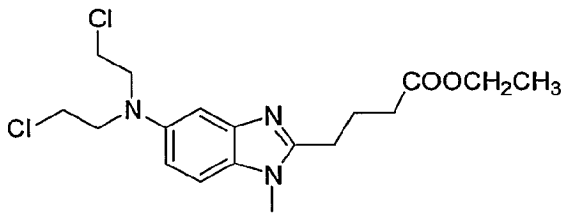
at the time of release or where the HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized pharmaceutical composition of bendamustine as described herein. In a preferred embodiment is a pharmaceutical composition of bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%.

Another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.1 % to about 0.3 % bendamustine dimer as shown in Formula III at release or at time zero after reconstitution



Formula III.

Yet another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.5%, preferably 0.15% to about 0.5%, bendamustine ethylester, as shown in Formula IV at release or at time zero after reconstitution



Formula IV.

Yet another embodiment of the invention is a lyophilized preparation of bendamustine wherein the concentration of bendamustine ethylester (Formula IV) is no more than 0.2%, preferably 0.1%, greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

In another embodiment of the invention is a lyophilized preparation of bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine) HP1 at the time of drug product release. In a preferred embodiment is a lyophilized preparation of bendamustine containing not more than about 0.50% (area percent of bendamustine) HP1, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%. An aspect of this embodiment is lyophilized preparations of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, (area percent of bendamustine) HP1 at the time of release of drug product where the lyophilized preparation is packaged in a vial or other pharmaceutically acceptable container.

In yet another aspect of the invention, the lyophilized preparations of bendamustine are stable with respect to the amount of HP1 for at least about 6 months, preferably 12 months, preferably 24 months, to about 36 months or greater when stored at about 2° to about 30°. Preferred temperatures for storage are about 5° C and about room temperature.

Another embodiment of the invention is a pharmaceutical dosage form that includes a pharmaceutical composition of bendamustine containing not more than about 0.5% to about 0.9% HP1, preferably not more than about 0.50%, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%, where the HP1 is the amount of HP1 present at release or at time zero after reconstitution of a lyophilized preparation of bendamustine of the present invention. In preferred aspects

of the invention, the dosage form can be about 5 to about 500 mg of bendamustine, about 10 to about 300 mg of bendamustine, about 25 mg of bendamustine, about 100 mg of bendamustine, and about 200 mg of bendamustine.

Yet another embodiment of the invention is a pharmaceutical dosage form that includes a lyophilized preparation of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, HP1. Preferred dosage forms can be about 5 to about 500 mg of bendamustine, about 10 to about 300 mg of bendamustine, about 25 mg of bendamustine, about 100 mg of bendamustine, and about 200 mg of bendamustine.

In still another embodiment, the invention includes a pharmaceutical composition of bendamustine including bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine), preferably not more than about 0.50%, preferably not more than about 0.45%, more preferably not more than about 0.40%, more preferably not more than about 0.35%, even more preferably not more than 0.30%, and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release or time zero after reconstitution of a lyophilized pharmaceutical composition of bendamustine as disclosed herein. In different aspects of this embodiment, the organic solvent is selected from one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane. Preferred organic solvents include one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol. A more preferred organic solvent is tertiary butanol, also known as TBA, t-butanol, tert-butyl alcohol or tertiary butyl alcohol.

The present invention involves a method for obtaining agency approval for a bendamustine product, the improvement which includes setting a release specification for bendamustine degradants at less than about 4.0%, preferably about 2.0 % to about 4.0 %, (area percent bendamustine) or otherwise to achieve the pharmaceutical compositions described herein. An aspect of this embodiment is a method for obtaining agency approval for a bendamustine product which includes setting a release specification for HP1 to be less than or equal to 1.5% (area percent Bendamustine).

The bendamustine product herein contains not more than about 0.5% (area percent of bendamustine) HP1 at release.

Another embodiment is a method for obtaining agency approval for a bendamustine product, the improvement which includes setting a shelf-life specification for bendamustine degradants at less than about 7.0%, preferably about 5.0% to about 7.0%, (area percent bendamustine) where the product is stored at about 2°C to about 30°C. Preferred temperatures for storage are about 5°C and about room temperature. The bendamustine product herein contains not more than about 0.5% (area percent of bendamustine) HP1 at release.

Another embodiment of the invention is a process for manufacturing a lyophilized preparation of bendamustine which includes controlling for the concentration of bendamustine degradants in the final product, such that the concentration of bendamustine degradants is less than about 4.0%, preferably no more than about 2.0 % to about 4.0 %, (area percent of bendamustine) at release or otherwise to achieve the pharmaceutical compositions described herein. The bendamustine product herein contains not more than about 0.5% to about 0.9%, preferably about 0.5%, (area percent of bendamustine) HP1 at release.

The present invention discloses a process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of HP1 is less than 0.9%, preferably 0.5%, (area percent of bendamustine) and, at the time of product expiration, the concentration of bendamustine degradants is less than about 7.0%, preferably no more than about 5.0% to about 7.0%; wherein said product is stored at about 2°C to about 30°C.

Another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization from about 0 to 24 hours does not exceed about 0.5% to about 0.9% (area percent of bendamustine) preferably 0.50%, preferably 0.45%, more preferably 0.40%, more preferably 0.35%, even more preferably 0.30%. An aspect of this embodiment is the lyophilized powder produced from the pre-lyophilization solution or dispersion.

Still another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of bendamustine ethylester produced during lyophilization from about 0 to 24 hours does not exceed about 0.5% (area percent bendamustine). An aspect of this embodiment is the lyophilized powder produced from the pre-lyophilization solution or dispersion.

Still another embodiment of the invention is a bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents where the solution or dispersions include at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of bendamustine ethylester (as shown in Formula IV) produced during lyophilization from about 0 to 24 hours is no more than 0.2%, preferably 0.1%, greater than the concentration of bendamustine ethylester as found in the drug substance used to make the pre-lyophilization solution. A preferred organic solvent is tertiary butanol.

The invention also discloses methods for preparing a bendamustine lyophilized preparation that includes dissolving bendamustine in a stabilizing concentration of an alcohol solvent of between about 5% to about 100% (v/v alcohol to form a pre-lyophilization solution; and lyophilizing the pre-lyophilization solution; wherein the bendamustine lyophilized preparation made from such methods contains not more than about 0.5% to about 0.9%, preferably 0.5%, (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release or at time zero after reconstitution of the lyophilized pharmaceutical composition of bendamustine. Other alcohol concentrations include about 5% to about 99.9%, about 5% to about 70%, about 5% to about 60%, about 5% to about 50%, about 5% to about 40%, about 20% to about 35%. Preferred concentrations of alcohol are from about 20% to about 30%. Preferred alcohols include one or more of methanol, ethanol, propanol, iso-propanol, butanol, and tertiary-butanol. A more preferred alcohol is tertiary-butanol. A preferred concentration of tertiary-butanol is about 20% to about 30%, preferably about 30%. An aspect of this embodiment is the addition of an excipient before lyophilization. A preferred excipient is mannitol. Preferred pre-lyophilized concentrations of bendamustine are from about 2 mg/mL to about 50 mg/mL.

In a preferred method for preparing a bendamustine lyophilized preparation, lyophilizing the pre-lyophilization solution comprises i) freezing the pre-lyophilization solution to a temperature below about -40°C, preferably -50°C, to form a frozen solution; ii) holding the frozen solution at or below -40°C, preferably -50°C, for at least 2 hours; iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution; iv) holding for about 10 to about 70 hours; v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and vii) holding for about 5 to about 40 hours to form a bendamustine lyophilized preparation. In a more preferred method lyophilizing the pre-lyophilization solution comprises i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution; ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours; iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution; iv) holding at a primary drying temperature for about 10 to about 48 hours; v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours. A preferred alcohol is tertiary-butanol. A preferred concentration of tertiary-butanol is about 20% to about 30%, preferably about 30%. An aspect of this embodiment is the addition of an excipient before lyophilization. A preferred excipient is mannitol. Preferred pre-lyophilized concentrations of bendamustine are from about 2 mg/mL to about 50 mg/mL.

Another embodiment of the invention is the lyophilized powder or preparation obtained from the methods of preparing a bendamustine lyophilized preparation disclosed herein.

The invention also involves bendamustine formulations for lyophilization that include an excipient and a stabilizing concentration of an organic solvent. A preferred formulation includes bendamustine at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water. Included in this embodiment of the invention are the lyophilized preparations made from such bendamustine formulations.

Included in the inventions are methods of treating a medical condition in a patient that involve administering a therapeutically effective amount of a pharmaceutical composition of the invention where the condition is amenable to

treatment with said pharmaceutical composition. Some conditions amenable to treatment with the compositions of the invention include chronic lymphocytic leukemia (CLL), Hodgkin's disease, non-Hodgkin's lymphoma (NHL), multiple myeloma (MM), breast cancer, small cell lung cancer, hyperproliferative disorders, and an autoimmune disease. Preferred conditions include NHL, CLL, breast cancer, and MM. Preferred autoimmune diseases include rheumatoid arthritis, multiple sclerosis or lupus.

Included in the inventions are the use of the pharmaceutical compositions or pharmaceutical preparations of the invention in the manufacture of a medicament for the treatment of a medical condition, as defined herein, in a patient that involve administering a therapeutically effective amount of a pharmaceutical composition of the invention where the condition is amenable to treatment with said pharmaceutical composition.

Also included in the invention are methods of treating in which the pharmaceutical compositions of the invention are in combination with one or more anti-neoplastic agents where the antineoplastic agent is given prior, concurrently, or subsequent to the administration of the pharmaceutical composition of the invention. Preferred antineoplastic agents are antibodies specific for CD20.

Another embodiment of the invention is a lyophilization cycle for producing lyophilized bendamustine preparations of the invention. A preferred lyophilization cycle includes a) freezing to about -50°C over about 8 hours; b) holding at -50°C for about 4 hours; c) ramping to -25°C over about 3 hours; d) holding at -10°C for 30 hours; e) ramping to between about 25°C and about 40°C or higher for about 3 hours; f) holding between about 25°C and about 40°C for about 25 hours; g) ramping to about 20°C in 1 hour; h) unloading at about 20°C, at a pressure of 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying. An aspect of this cycle involves step (e) which is ramped to about 30-35°C for 3 hours and then ramped to 40°C for 5 hours. Another aspect of this embodiment is the lyophilized powered prepared from such lyophilization cycles. A more preferred lyophilization cycle includes i) starting with a shelf temperature of about 5°C for loading; ii) freezing to about -50°C over about 8 hours; iii) holding at -50°C for about 4 hours; iv) ramping to about -20°C over about 3 hours; v) holding at about -20°C for 6

hours; ramping to about -15°C over about 1 hour; vi) holding at -15°C for about 20 hours; vii) ramping to about -15°C over about 1 hour; viii) holding at about -15°C for about 20 hours; ix) ramping to about -12°C over about 0.5 hours; x) holding at about -12°C for about 15.5 hours; xi) ramping to between about 25°C and about 40°C or higher for about 15 hours; xii) holding between about 25°C and about 40°C for about 10 hours; xiii) ramping to about 40°C over about 1 hour; and xiv) holding at about 40°C for about 5 hours; unloading at about 5°C, at a pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying. In a preferred embodiment step (xi) is ramped to about 30-35°C for about 15 hours.

The invention also encompasses a pharmaceutical dosage form of bendamustine containing not more than about 0.5% to about 0.9%, preferably 0.5%, HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form. Preferred concentrations of bendamustine include about 10 to about 500 mg/container, about 100 mg/container, about 5 mg to about 2 g/container and about 170 mg/container.

The present invention also includes pre-lyophilized pharmaceutical compositions of bendamustine. A preferred pre-lyophilized composition includes bendamustine HCl about 15 mg/mL, mannitol about 25.5 mg/mL, about 30% (v/v) tertiary-butyl alcohol, and water.

These and other embodiments of the invention are described hereinbelow or are evident to persons of ordinary skill in the art based on the following disclosures.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the solubility of bendamustine at various temperatures for two different solutions of bendamustine in tertiary butanol.

Fig. 2 shows the purity results of an HPLC analysis after incubating bendamustine in various alcohols for 24 hours at 5°C. Results are presented as the area percent of the bendamustine peak.

Fig. 3 shows HP1 (Formula II) formation after 24 hours in various alcohol/water co-solvents at 5°C

Fig 4 shows dimer (Formula III) formation after 24 hours in various alcohol/water co-solvents at 5°C

Fig. 5- shows a lyophilization cycle for bendamustine using a TBA/water co-solvent.

Fig. 6 shows a chromatogram for Ribomustin® using HPLC method No. 1.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms "formulate" refers to the preparation of a drug, e.g., bendamustine, in a form suitable for administration to a mammalian patient, preferably a human. Thus, "formulation" can include the addition of pharmaceutically acceptable excipients, diluents, or carriers.

As used herein, the term "lyophilized powder" or "lyophilized preparation" refers to any solid material obtained by lyophilization, i.e., freeze-drying of an aqueous solution. The aqueous solution may contain a non-aqueous solvent, i.e. a solution composed of aqueous and one or more non-aqueous solvent(s). Preferably, a lyophilized preparation is one in which the solid material is obtained by freeze-drying a solution composed of aqueous and one or more non-aqueous solvents, more preferably the non-aqueous solvent is an alcohol.

By "stable pharmaceutical composition" is meant any pharmaceutical composition having sufficient stability to have utility as a pharmaceutical product. Preferably, a stable pharmaceutical composition has sufficient stability to allow storage at a convenient temperature, preferably between -20°C and 40°C, more preferably about 2°C to about 30°C, for a reasonable period of time, e.g., the shelf-life of the product which can be as short as one month but is typically six months or longer, more preferably one year or longer even more preferably twenty-four months or longer, and even more preferably thirty-six months or longer. The shelf-life or expiration can be that amount of time where the active ingredient degrades to a point below 90% purity. For purposes of the present invention stable pharmaceutical composition includes reference to pharmaceutical compositions with specific ranges of impurities as described herein. Preferably, a stable pharmaceutical composition is one which has minimal degradation of the active ingredient, e.g., it retains at least about 85

% of un-degraded active, preferably at least about 90 %, and more preferably at least about 95%, after storage at 2-30°C for a 2-3 year period of time.

By "stable lyophilized preparation" is meant any lyophilized preparation having sufficient stability, such characteristics as similarly defined herein for a stable pharmaceutical composition, to have utility as a pharmaceutical product

By "degraded" is meant that the active has undergone a change in chemical structure.

The term "therapeutically effective amount" as used herein refers to that amount of the compound being administered that will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to the treatment of neoplasms, a therapeutically effective amount refers to that amount which has the effect of (1) reducing the size of the tumor, (2) inhibiting (that is, slowing to some extent, preferably stopping) tumor metastasis, (3) inhibiting to some extent (that is, slowing to some extent, preferably stopping) tumor growth, and/or, (4) relieving to some extent (or, preferably, eliminating) one or more symptoms associated with the cancer. Therapeutically effective amount can also mean preventing the disease from occurring in an animal that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment). Further, therapeutically effective amount can be that amount that increases the life expectancy of a patient afflicted with a terminal disorder. Typical therapeutically effective doses for bendamustine for the treatment of non-Hodgkin's lymphoma can be from about 60-120 mg/m^2 given as a single dose on two consecutive days. The cycle can be repeated about every three to four weeks. For the treatment of chronic lymphocytic leukemia (CLL) bendamustine can be given at about 80-100 mg/m^2 on days 1 and 2. The cycle can be repeated after about 4 weeks. For the treatment of Hodgkin's disease (stages II-IV), bendamustine can be given in the "DBVBe regimen" with daunorubicin 25 mg/m^2 on days 1 and 15, bleomycin 10 mg/m^2 on days 1 and 15, vincristine 1.4 mg/m^2 on days 1 and 15, and bendamustine 50 mg/m^2 on days 1-5 with repetition of the cycle about every 4 weeks. For breast cancer, bendamustine (120 mg/m^2) on days 1 and 8 can be given in combination with methotrexate 40 mg/m^2 on days 1 and 8, and 5-fluorouracil 600 mg/m^2 on days 1 and 8 with repetition of the cycle about every 4 weeks. As a second-line of therapy for breast cancer, bendamustine can be given at

about 100-150 mg/m^2 on days 1 and 2 with repetition of the cycle about every 4 weeks.

As used herein "neoplastic" refers to a neoplasm, which is an abnormal growth, such growth occurring because of a proliferation of cells not subject to the usual limitations of growth. As used herein, "anti-neoplastic agent" is any compound, composition, admixture, co-mixture, or blend which inhibits, eliminates, retards, or reverses the neoplastic phenotype of a cell.

As used herein "hyperproliferation" is the overproduction of cells in response to a particular growth factor. "Hyperproliferative disorders" are diseases in which the cells overproduce in response to a particular growth factor. Examples of such "hyperproliferative disorders" include diabetic retinopathy, psoriasis, endometriosis, cancer, macular degenerative disorders and benign growth disorders such as prostate enlargement.

As used herein, the term "vial" refers to any walled container, whether rigid or flexible.

"Controlling" as used herein means putting process controls in place to facilitate achievement of the thing being controlled. For example, in a given case, "controlling" can mean testing samples of each lot or a number of lots regularly or randomly; setting the concentration of degradants as a release specification; selecting process conditions, e.g., use of alcohols and/or other organic solvents in the pre-lyophilization solution or dispersion, so as to assure that the concentration of degradants of the active ingredient is not unacceptably high; etc. Controlling for degradants by setting release specifications for the amount of degradants can be used to facilitate regulatory approval of a pharmaceutical product by a regulatory agency, such as the U.S. Food and Drug Administration and similar agencies in other countries or regions ("agency").

The term "pharmaceutically acceptable" as used herein means that the thing that is pharmaceutically acceptable, e.g., components, including containers, of a pharmaceutical composition, does not cause unacceptable loss of pharmacological activity or unacccptable adverse side effects. Examples of pharmaceutically acceptable components are provided in The United States Pharmacopeia (USP), The National Formulary (NF), adopted at the United States Pharmacopeial Convention, held in Rockville, Md. in 1990 and FDA Inactive Ingredient Guide 1990, 1996 issued

by the U.S. Food and Drug Administration (both are hereby incorporated by reference herein, including any drawings). Other grades of solutions or components that meet necessary limits and/or specifications that are outside of the USP/NF may also be used.

The term "pharmaceutical composition" as used herein shall mean a composition that is made under conditions such that it is suitable for administration to humans, e.g., it is made under GMP conditions and contains pharmaceutically acceptable excipients, e.g., without limitation, stabilizers, bulking agents, buffers, carriers, diluents, vehicles, solubilizers, and binders. As used herein pharmaceutical composition includes but is not limited to a pre-lyophilization solution or dispersion as well as a liquid form ready for injection or infusion after reconstitution of a lyophilized preparation.

A "pharmaceutical dosage form" as used herein means the pharmaceutical compositions disclosed herein being in a container and in an amount suitable for reconstitution and administration of one or more doses, typically about 1-2, 1-3, 1-4, 1-5, 1-6, 1-10, or about 1-20 doses. Preferably, a "pharmaceutical dosage form" as used herein means a lyophilized pharmaceutical composition disclosed herein in a container and in an amount suitable for reconstitution and delivery of one or more doses, typically about 1-2, 1-3, 1-4, 1-5, 1-6, 1-10, or about 1-20 doses. The pharmaceutical dosage form can comprise a vial or syringe or other suitable pharmaceutically acceptable container. The pharmaceutical dosage form suitable for injection or infusion use can include sterile aqueous solutions or dispersions or sterile powders comprising an active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The prevention of the growth of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

As used herein, the term "excipient" means the substances used to formulate active pharmaceutical ingredients (API) into pharmaceutical formulations; in a preferred embodiment, an excipient does not lower or interfere with the primary

therapeutic effect of the API. Preferably, an excipient is therapeutically inert. The term "excipient" encompasses carriers, diluents, vehicles, solubilizers, stabilizers, bulking agents, and binders. Excipients can also be those substances present in a pharmaceutical formulation as an indirect or unintended result of the manufacturing process. Preferably, excipients are approved for or considered to be safe for human and animal administration, i.e., GRAS substances (generally regarded as safe). GRAS substances are listed by the Food and Drug administration in the Code of Federal Regulations (CFR) at 21 CFR § 182 and 21 CFR § 184, incorporated herein by reference. Preferred excipients include, but are not limited to, hexitols, including mannitol and the like.

As used herein "a stabilizing concentration of an organic solvent" or "a stabilizing concentration of an alcohol" means that amount of an organic solvent or alcohol that reduces the level of degradation of bendamustine to achieve a specified level of degradants in the final drug product. For example, with respect to the degradant HP1, a stabilizing concentration of an organic solvent is that amount which results in an HP1 concentration (area percent of bendamustine) of less than about 0.5%, preferably less than 0.45 %, preferably less than 0.40 %, more preferably less than 0.35%, more preferably less than 0.30%, and even more preferably less than 0.25%. With respect to the overall or total degradant concentration of the final drug product, a stabilizing concentration of an organic solvent is that amount that results in a total degradant concentration (at the time of drug product release) of less than about 7% (area percent bendamustine), preferably less than about 6%, more preferably less than about 5%, and even more preferably less than about 4.0%. By "area percent of bendamustine" is meant the amount of a specified degradant, e.g., HP1, relative to the amount of bendamustine as determined, e.g., by HPLC.

The term "organic solvent" means an organic material, usually a liquid, capable of dissolving other substances.

As used herein, "trace amount of an organic solvent" means an amount of solvent that is equal to or below recommended levels for pharmaceutical products, for example, as recommended by ICH guidelines (International Conferences on Harmonization, Impurities-- Guidelines for Residual Solvents. Q3C. Federal Register. 1997;62(247):67377). The lower limit is the lowest amount that can be detected.

The term "release" or "at release" means the drug product has met the release specifications and can be used for its intended pharmaceutical purpose.

A. General

The invention provides stable, pharmaceutically acceptable compositions prepared from bendamustine. In particular, the invention provides formulations for the lyophilization of bendamustine HCl. The lyophilized powder obtained from such formulations is more easily reconstituted than the presently available lyophilized powder of bendamustine. Further, the lyophilized products of the present invention have a better impurity profile than Ribomustin® with respect to certain impurities, in particular HP1, bendamustine dimer, and bendamustine ethylester, prior to reconstitution, upon storage of the lyophilate, or following reconstitution and admixture.

The present invention further provides formulations of bendamustine useful for treating neoplastic diseases. The formulations described herein can be administered alone or in combination with at least one additional anti-neoplastic agent and/or radioactive therapy.

An aspect of the invention is conditions and means for enhancing the stability of bendamustine prior to and during the lyophilization process, upon shelf storage or upon reconstitution.

Anti-neoplastic agents which may be utilized in combination with the formulations of the invention include those provided in the Merck Index 11, pp 16-17, Merck & Co., Inc. (1989) and The Chemotherapy Source Book (1997). Both books are widely recognized and readily available to the skilled artisan.

There are large numbers of antineoplastic agents available in commercial use, in clinical evaluation and in pre-clinical development, which could be selected for treatment of neoplasia by combination drug chemotherapy. Such antineoplastic agents fall into several major categories, namely, antibiotic-type agents, covalent DNA-binding drugs, antimetabolite agents, hormonal agents, including glucocorticoids such as prednisone and dexamethasone, immunological agents, interferon-type agents, differentiating agents such as the retinoids, pro-apoptotic agents, and a category of miscellaneous agents, including compounds such as antisense, small interfering RNA, and the like. Alternatively, other anti-neoplastic agents, such as metallomatrix proteases (MMP) inhibitors, SOD mimics or $alpha_v$ $beta_3$ inhibitors may be used.

One family of antineoplastic agents which may be used in combination with the compounds of the inventions consists of antimetabolite-type antineoplastic agents. Suitable antimetabolite antineoplastic agents may be selected from the group consisting of alanosine, AG2037 (Pfizer), 5-FU-fibrinogen, acanthifolic acid, aminothiadiazole, brequinar sodium, carmofur, Ciba-Geigy CGP-30694, cyclopentyl cytosine, cytarabine phosphate stearate, cytarabine conjugates, Lilly DATHF, Merrel Dow DDFC, dezaguanine, dideoxycytidine, dideoxyguanosine, didox, Yoshitomi DMDC, doxifluridine, Wellcome EHNA, Merck & Co. EX-015, fazarabine, floxuridine, fludarabine phosphate, 5-fluorouracil, N-(2'-furanidyl)-5-fluorouracil, Daiichi Seiyaku FO-152, isopropyl pyrrolizine, Lilly LY-188011, Lilly LY-264618, methobenzaprim, methotrexate, Wellcome MZPES, norspermidine, NCI NSC-127716, NCI NSC-264880, NCI NSC-39661, NCI NSC-612567, Warner-Lambert PALA, pentostatin, piritrexim, plicamycin, Asahi Chemical PL-AC, Takeda TAC-788, thioguanine, tiazofurin, Erbamont TIF, trimetrexate, tyrosine kinase inhibitors, tyrosine protein kinase inhibitors, Taiho UFT and uricytin.

A second family of antineoplastic agents which may be used in combination with the compounds of the invention consists of covalent DNA-binding agents . Suitable alkylating-type antineoplastic agents may be selected from the group consisting of Shionogi 254-S, aldo-phosphamide analogues, altretamine, anaxirone, Boehringer Mannheim BBR-2207, bestrabucil, budotitane, Wakunaga CA-102, carboplatin, carmustine, Chinoin-139, Chinoin-153, chlorambucil, cisplatin, cyclophosphamide, American Cyanamid CL-286558, Sanofi CY-233, cyplatate, Degussa D-19-384, Sumimoto DACHP(Myr)2, diphenylspiromustine, diplatinum cytostatic, Erba distamycin derivatives, Chugai DWA-2114R, ITI E09, elmustine, Erbamont FCE-24517, estramustine phosphate sodium, fotemustine, Unimed G-6-M, Chinoin GYKI-17230, hepsul-fam, ifosfamide, iproplatin, lomustine, mafosfamide, melphalan, mitolactol, Nippon Kayaku NK-121, NCI NSC-264395, NCI NSC-342215, oxaliplatin, Upjohn PCNU, prednimustine, Proter PTT-119, ranimustine, semustine, SmithKline SK&F-101772, Yakult Honsha SN-22, spiromustine, Tanabe Seiyaku TA-077, tauromustine, temozolomide, teroxirone, tetraplatin and trimelamol.

Another family of antineoplastic agents which may be used in combination with the compounds disclosed herein consists of antibiotic-type antineoplastic agents. Suitable antibiotic-type antineoplastic agents may be selected from the group

consisting of Taiho 4181-A, aclarubicin, actinomycin D, actinoplanone, alanosine, Erbamont ADR-456, aeroplysinin derivative, Ajinomoto AN-201-II, Ajinomoto AN-3, Nippon Soda anisomycins, anthracycline, azino-mycin-A, bisucaberin, Bristol-Myers BL-6859, Bristol-Myers BMY-25067, Bristol-Myers BMY-25551, Bristol-Myers BMY-26605, Bristol-Myers BMY-27557, Bristol-Myers BMY-28438, bleomycin sulfate, bryostatin-1, Taiho C-1027, calichemycin, chromoximycin, dactinomycin, daunorubicin, Kyowa Hakko DC-102, Kyowa Hakko DC-79, Kyowa Hakko DC-88A, Kyowa Hakko DC89-Al, Kyowa Hakko DC92-B, ditrisarubicin B, Shionogi DOB-41, doxorubicin, doxorubicin-fibrinogen, elsamicin-A, epirubicin, erbstatin, esorubicin, esperamicin-Al, esperamicin-Alb, Erbamont FCE-21954, Fujisawa FK-973, fostriecin, Fujisawa FR-900482, glidobactin, gregatin-A, grincamycin, herbimycin, idarubicin, illudins, kazusamycin, kesarirhodins, Kyowa Hakko KM-5539, Kirin Brewery KRN-8602, Kyowa Hakko KT-5432, Kyowa Hakko KT-5594, Kyowa Hakko KT-6149, American Cyanamid LL-D49194, Meiji Seika ME 2303, menogaril, mitomycin, mitoxantrone, SmithKline M-TAG, neoenactin, Nippon Kayaku NK-313, Nippon Kayaku NKT-01, SRI International NSC-357704, oxalysine, oxaunomycin, peplomycin, pilatin, pirarubicin, porothramycin, pyrindamycin A, Tobishi RA-I, rapamycin, rhizoxin, rodorubicin, sibanomicin, siwenmycin, Sumitomo SM-5887, Snow Brand SN-706, Snow Brand SN-07, sorangicin-A, sparsomycin, SS Pharmaceutical SS-21020, SS Pharmaceutical SS-7313B, SS Pharmaceutical SS-9816B, steffimycin B, Taiho 4181-2, talisomycin, Takeda TAN-868A, terpentecin, thrazine, tricrozarin A, Upjohn U-73975, Kyowa Hakko UCN-10028A, Fujisawa WF-3405, Yoshitomi Y-25024 and zorubicin.

A fourth family of antineoplastic agents which may be used in combination with the compounds of the invention include a miscellaneous family of antineoplastic agents selected from the group consisting of alpha-carotene, alpha-difluoromethyl-arginine, acitretin, arsenic trioxide, Avastin® (bevacizumab), Biotec AD-5, Kyorin AHC-52, alstonine, amonafide, amphethinile, amsacrine, Angiostat, ankinomycin, anti-neoplaston A10, antineoplaston A2, antineoplaston A3, antineoplaston A5, antineoplaston AS2-1, Henkel APD, aphidicolin glycinate, asparaginase, Avarol, baccharin, batracylin, benfluron, benzotript, Ipsen-Beaufour BIM-23015, bisantrene, Bristo-Myers BMY-40481, Vestar boron-10, bromofosfamide, Wellcome BW-502, Wellcome BW-773, caracemide, carmethizole hydrochloride, Ajinomoto CDAF,

chlorsulfaquinoxalone, Chemes CHX-2053, Chemex CHX-100, Warner-Lambert CI-921, Warner-Lambert CI-937, Warner-Lambert CI-941, Warner-Lambert CI-958, clanfenur, claviridenone, ICN compound 1259, ICN compound 4711, Contracan, Yakult Honsha CPT-ll, crisnatol, curaderm, cytochalasin B, cytarabine, cytocytin, Merz D-609, DABIS maleate, dacarbazine, datelliptinium, didemnin- B, dihaematoporphyrin ether, dihydrolenperone, dinaline, distamycin, Toyo Pharmar DM-341, Toyo Pharmar DM-75, Daiichi Seiyaku DN-9693, elliprabin, elliptinium acetate, epothionesTsumura EPMTC, erbitux, ergotamine, erlotnib, etoposide, etretinate, fenretinide, Fujisawa FR-57704, gallium nitrate, genkwadaphnin, Gleevec® (imatnib), Chugai GLA-43, Glaxo GR-63178, gefitinib, grifolan NMF-5N, hexadecylphosphocholine, Green Cross HO-221, homoharringtonine, hydroxyurea, BTG ICRF-187, indanocine, ilmofosine, isoglutamine, isotretinoin, Otsuka JI-36, Ramot K-477, Otsuak K-76COONa, Kureha Chemical K-AM, MECT Corp KI-8110, American Cyanamid L-623, leukoregulin, lonidamine, Lundbeck LU-23-112, Lilly LY-186641, NCI (US) MAP, marycin, mefloquine, Merrel Dow MDL-27048, Medco MEDR-340, merbarone, merocyanine derivatives, methylanilinoacridine, Molecular Genetics MGI-136, minactivin, mitonafide, mitoquidone, mopidamol, motretinide, Zenyaku Kogyo MST-16, N-(retinoyl)amino acids, Nisshin Flour Milling N-021, N-acylated-dehydroalanines, nafazatrom, Taisho NCU-190, nocodazole derivative, Normosang, NCI NSC-145813, NCI NSC-361456, NCI NSC-604782, NCI NSC-95580, octreotide, Ono ONO-112, oquizanocine, Akzo Org-10172, paclitaxel, pancratistatin, pazelliptine, Warner-Lambert PD-111707, Warner-Lambert PD-115934, Warner-Lambert PD-131141, Pierre Fabre PE-1001, ICRT peptide D, piroxantrone, polyhaematoporphyrin, polypreic acid, Efamol porphyrin, probimane, procarbazine, proglumide, Invitron protease nexin I, Tobishi RA-700, razoxane, Sapporo Breweries RBS, restrictin-P, retelliptine, retinoic acid, Rhone-Poulenc RP-49532, Rhone-Poulenc RP-56976, Rituxan® (and other anti CD20 antibodies, e.g. Bexxar®, Zevalin®), SmithKline SK&F-104864, statins (Lipitor® etc.), Sumitomo SM-108, Kuraray SMANCS, SeaPharm SP-10094, spatol, spirocyclopropane derivatives, spirogermanium, Unimed, SS Pharmaceutical SS-554, strypoldinone, Stypoldione, Suntory SUN 0237, Suntory SUN 2071, superoxide dismutase, Thalidomide, Thalidomide analogs, Toyama T-506, Toyama T-680, taxol, Teijin TEI-0303, teniposide, thaliblastine, Eastman Kodak TJB-29, tocotrienol, Topostin, Teijin

TT-82, Kyowa Hakko UCN-01, Kyowa Hakko UCN-1028, ukrain, Eastman Kodak USB-006, vinblastine sulfate, vincristine, vindesine, vinestramide, vinorelbine, vintriptol, vinzolidine, withanolides and Yamanouchi YM-534, Zometa®.

Examples of radioprotective agents which may be used in the combination chemotherapy of this invention are AD-5, adchnon, amifostine analogues, detox, dimesna, 1-102, MM-159, N-acylated-dehydroalanines, TGF-Genentech, tiprotimod, amifostine, WR-151327, FUT-187, ketoprofen transdermal, nabumetone, superoxide dismutase (Chiron and Enzon).

Methods for preparation of the antineoplastic agents described above may be found in the literature. Methods for preparation of doxorubicin, for example, are described in U.S. Pat. Nos. 3,590,028 and 4,012,448. Methods for preparing metallomatrix protease inhibitors are described in EP 780386. Methods for preparing .alpha$_v$ .beta$_3$ inhibitors are described in WO 97/08174.

Preferred anti-neoplastic agents include, without limitation, one or more of daunorubicin, bleomycin, vincristine, doxorubicin, dacarbazine, prednisolone, mitoxantrone, prednisone, methotrexate, 5-flurouracil, dexamethasone, thalidomide, thalidomide derivatives, 2ME2, Neovastat, R 11 5777, arsenic trioxide, bortezomib, tamoxifen, G3139 (antisense), and SU5416, mitomycin, anti-CD20 antibodies, such as Rituxan® and R-etodolac.

Preferred drug regimens for which the present formulation may be used in conjunction with or as a replacement for one or more of the components includes, without limitation, ABVD (doxorubicin, bleomycin, vincristine, dacarbazine), DBV (daunorubicin, belomycin, vincristine), CVPP (cyclophosphamide, vinblastine, procarbazine, prednisolone), COP (cyclophosphamide, vincristine, prednisolone), CHOP (cyclophosphamide, doxorubicin,

vincristine and prednisone) and CMF (cyclophosphamide, methotrexate, 5-fluorouracil). Additional regimens are given in Table A below.

Table A- Cancer Therapeutic Regimens

Abbreviation	Drugs Used	Disease
AC	Doxorubicin & Cyclophosphamide	Breast cancer
CFM (CF, FNC)	Cyclophosphamide,	Breast cancer

	Fluorouracil, Mitaxantrone	
CMF	Cyclophosphamide, Methotrexate, Fluorouracil	Breast cancer
NFL	Mitoxantrone, Fluorouracil, Leucovorin	Breast cancer
Sequential Dox-CMF	Doxorubicin	Breast cancer
VATH	Vinblastine, Doxorubicin, Thiotepa, Fluoxymesterone	Breast cancer
EMA-86	Etoposide, Mitoxantrone, Ctyarabine	AML (induction)
7 + 3	Cytarabine WITH Daunorubicin OR Idarobicin OR Mitoxantrone	AML (induction)
5 + 2	Cytarabine WITH Daunorubicin OR Mitoxantrone	AML (induction)
HiDAC	Cytarabine	AML (post-remission)
ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine	Hodgkin's
ChlVPP	Chlorambucil, Vinblastine, Procarbazine, Prednisone	Hodgkin's
EVA	Etoposide, Vinblastine, Doxorubicin	Hodgkin's
MOPP	Mechlorethamine, Vincristine, Procarbazine, Prednisone	Hodgkin's
MOPP/ABV Hybrid	Mechlorethamine, Vincristine, Procarbazine, Prednisone, Doxorubicin, Bleomycin, Vinblastine	Hodgkin's
MOPP/ABVD	Mechlorethamine,	Hodgkin's

	Doxorubicin, Vinblastine, Bleomycin, Etoposide, Prednisone	
CNOP	Cyclophosphamide, Mitoxantrone, Vincristine, Prednisone	Non-Hodgkin's
COMLA	Cyclophosphamide, Vincristine, Methotrexate, Leucovorin, Cytarabine	Non-Hodgkin's
DHAP	Dexamethasone, Cisplatin, Cytarabine	Non-Hodgkin's
ESHAP	Etoposide, Methylprednisilone, Cisplatin, Cytarabine	Non-Hodgkin's
MACOP-B	Methotrexate, Leucovorin, Doxorubicin, Cyclophosphamide, Vincristine, Prednisone, Bleomycin, Septra, Ketoconazole	Non-Hodgkin's
m-BACOD	Methotrexate, Leucovorin, Bleomycin, Doxorubicin, Cyclophosphamide, Vincristine, Dexamethasone	Non-Hodgkin's
MINE-ESHAP	Mesna, Ifosfamide, Mitoxantrone, Etoposide	Non-Hodgkin's
NOVP	Mitoxantrone, Vinblastine, Prednisone, Vincristine	Non-Hodgkin's
ProMACE/cytaBOM	Prednisone, Doxorubicin,	Non-Hodgkin's

	Cyclophosphamide, Etoposide, Cytarabine, Bleomycin, Vincristine, Methotrexate, Leucovorin, Septra	
M2	Vincristine, Carmustine, Cyclophosphamide, Melphalan, Prednisone	Multiple Myeloma
MP	Melphalan, Prednisone	Multiple Myeloma
VAD	Vincristine, Doxorubicin, Dexamethasone	Multiple Myeloma
VBMCP	Vincristine, Carmustine, Melphalan, Cyclophosphamide, Prednisone	Multiple Myeloma

As described herein, a lyophilized formulation of bendamustine is achieved following removal of an organic solvent in water. The most typical example of the solvent used to prepare this formulation is tertiary butanol (TBA). Other organic solvents can be used including ethanol, n-propanol, n-butanol, isoproponal, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, acetone, 1-pentanol, methyl acetate, methanol, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, cyclohexane. These preceding solvents may be used individually or in combination. Useful solvents must form stable solutions with bendamustine and must not appreciably degrade or deactivate the API. The solubility of bendamustine in the selected solvent must be high enough to form commercially useful concentrations of the drug in solvent. Additionally, the solvent should be capable of being removed easily from an aqueous dispersion or solution of the drug product, e.g., through lyophilization or vacuum drying. Preferably, a solution having a concentration of

about 2-80 mg/mL, preferably about 5 to 40 mg/mL, more preferably 5-20 mg/mL and even more preferably 12 to 17 mg/mL bendamustine is used.

A pharmaceutically acceptable lyophilization excipient can be dissolved in the aqueous phase. Examples of excipients useful for the present invention include, without limitation, sodium or potassium phosphate, citric acid, tartaric acid, gelatin, glycine, and carbohydrates such as lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose and hetastarch. Mannitol is a preferred excipient. Other excipients that may be used if desired include antioxidants, such as, without limitation, ascorbic acid, acetylcysteine, cysteine, sodium hydrogen sulfite, butyl-hydroxyanisole, butyl-hydroxytoluene or alpha-tocopherol acetate, or chelators.

A typical formulation and lyophilization cycle useful in accordance with the present invention is provided below. Lyophilization can be carried out using standard equipment as used for lyophilization or vacuum drying. The cycle may be varied depending upon the equipment and facilities used for the fill/finish.

In accordance with a typical embodiment of the present invention, an aqueous pre-lyophilization solution or dispersion is first formulated in a pharmaceutically acceptable compounding vessel. The solution is aseptically filtered into a sterile container, filled into an appropriate sized vial, partially stoppered and loaded into the lyophilizer. Using lyophilization techniques described herein the solution is lyophilized until a moisture content in the range of about 0.1 to about 8.0 percent is achieved. The resulting lyophilization powder is stable as a lyophilized powder for about six months to greater than about 2 years, preferably greater than about 3 years at about 5°C to about 25° C and can be readily reconstituted with Sterile Water for Injection, or other suitable carrier, to provide liquid formulations of bendamustine, suitable for internal administration e.g., by parenteral injection. For intravenous administration, the reconstituted liquid formulation, i.e., the pharmaceutical composition, is preferably a solution.

The pre-lyophilization solution or dispersion normally is first formulated in a pharmaceutically acceptable container by: 1) adding an excipient, such as mannitol (about 0 to about 50 mg/mL) with mixing to water (about 65% of the total volume) at ambient temperature, 2) adding an organic solvent (0.5- 99.9% v/v), such as TBA to the aqueous solution with mixing at about 20°-35°C, 4) adding bendamustine HCl to the desired concentration with mixing, 5) adding water to achieve the final volume,

and 6) cooling the solution to about 1°C to about 30°C, preferably about 5°C. Although the preceding steps are shown in a certain order, it is understood that one skilled in the art can change the order of the steps and quantities as needed. Quantities can be prepared on a weight basis also.

The pre-lyophilization solution or dispersion can be sterilized prior to lyophilization, sterilization is generally performed by aseptic filtration, e.g., through a 0.22 micron or less filter. Multiple sterilization filters can be used. Sterilization of the solution or dispersion can be achieved by other methods known in the art, e.g., radiation.

In this case, after sterilization, the solution or dispersion is ready for lyophilization. Generally, the filtered solution will be introduced into a sterile receiving vessel, and then transferred to any suitable container or containers in which the formulation may be effectively lyophilized. Usually the formulation is effectively and efficiently lyophilized in the containers in which the product is to be marketed, such as, without limitation, a vial, as described herein and as known in the art.

A typical procedure for use in lyophilizing the pre-lyophilization solutions or dispersions is set forth below. However, a person skilled in the art would understand that modifications to the procedure or process may be made depending on such things as, but not limited to, the pre-lyophilization solution or dispersion and lyophilization equipment.

Initially, the product is placed in a lyophilization chamber under a range of temperatures and then subjected to temperatures well below the product's freezing point, generally for several hours. Preferably, the temperature will be at or below about -40°C for at least 2 hours. After freezing is complete, the chamber and the condenser are evacuated through vacuum pumps, the condenser surface having been previously chilled by circulating refrigerant. Preferably, the condenser will have been chilled below the freezing point of the solution preferably to about -40°, more preferably to about -50°C or lower, even more preferably to about -60°C or lower. Additionally, evacuation of the chamber should continue until a pressure of about 10 to about 600 microns, preferably about 50 to about 150 microns is obtained.

The product composition is then warmed under vacuum in the chamber and condenser. This usually will be carried out by warming the shelves within the lyophilizer on which the product rests during the lyophilization process at a pressure

ranging from about 10 to about 600 microns. The warming process will optimally take place very gradually, over the course of several hours. For example, the product temperature should initially be increased from about -30°C to about -10°C and maintained for about 10-70 hours. Additionally, the product temperature can be increased from the freezing temperature to about 25°C-40°C over a period of 30-192 hours. To prevent powder ejection of the lyophilate from vials, complete removal of the organic solvent and water should be done during the initial drying phase. Complete drying can be confirmed by stabilization of vacuum, condenser temperature and product shelf temperature. After the initial drying, the product temperature should be increased to about 25°C-40°C and maintained for about 5-40 hours.

Once the drying cycle is completed, the pressure in the chamber can be slowly released to atmospheric pressure (or slightly below) with sterile, dry-nitrogen gas (or equivalent gas). If the product composition has been lyophilized in containers such as vials, the vials can be stoppered, removed and sealed. Several representative samples can be removed for purposes of performing various physical, chemical, and microbiological tests to analyze the quality of the product.

The lyophilized bendamustine formulation is typically marketed in pharmaceutical dosage form. The pharmaceutical dosage form of the present invention, although typically in the form of a vial, may be any suitable container, such as ampoules, syringes, co-vials, which are capable of maintaining a sterile environment. Such containers can be glass or plastic, provided that the material does not interact with the bendamustine formulation. The closure is typically a stopper, most typically a sterile rubber stopper, preferably a bromobutyl rubber stopper, which affords a hermetic seal.

After lyophilization, the bendamustine lyophilization powder may be filled into containers, such as vials, or alternatively the pre-lyophilization solution can be filled into such vials and lyophilized therein, resulting in vials which directly contain the lyophilized bendamustine formulation. Such vials are, after filling or lyophilization of the solution therein, sealed, as with a stopper, to provide a sealed, sterile, pharmaceutical dosage form. Typically, a vial will contain a lyophilized powder including about 10-500 mg/vial, preferably about 100 mg/vial, bendamustine and about 5mg-2g/vial, preferably about 170 mg/vial, mannitol.

The lyophilized formulations of the present invention may be reconstituted with water, preferably Sterile Water for Injection, or other sterile fluid such as co-solvents, to provide an appropriate solution of bendamustine for administration, as through parenteral injection following further dilution into an appropriate intravenous admixture container, for example, normal saline.

B. Solubility

The solubility of bendamustine HCl (bendamustine) in water (alone) and with varying amounts of alcohols commonly used in lyophilization, e.g., methanol, ethanol, propanol, isopropanol, butanol and tertiary-butyl alcohol (TBA) was determined by visual inspection. Amounts of bendamustine at 15 mg/mL, combined with mannitol at 25.5 mg/mL were prepared in 10 mL of the indicated alcohol solutions at room temperature (see Table 1). Samples were then refrigerated at 5°C and inspected after 0, 3, 6 and 24 hours for particulates and/or precipitates.

The results shown in Table 1 indicate that bendamustine solubility is dependant on temperature and the amount of alcohol in aqueous solutions. For the alcohols tested, the solubility of bendamustine increased as the concentration of alcohol increased. The formation of a precipitant was also dependent on the temperature and time. Bendamustine did not precipitate immediately with any alcohol, but crystallized after storage at 5°C. Alcohols varied in their effect on solubility. Without wishing to be bound to any particular theory, smaller alcohols such as methanol and ethanol have less of an effect on solubility as compared with larger alcohols (tertiary-butanol and n-butanol). However, the shape of the alcohol is also important. For example n-propanol was found to be better than iso-propanol in preventing precipitation in this system. The two alcohols with the greatest effect on solubility were n-propanol and tertiary-butanol.

Table 1. Bendamustine solubility over a 24 hour period in various alcohols when stored at 5°C.

	Zero Time	3 Hours	6 Hours	24 Hours
Methanol (v/v)				
0% (Water Only)	CCS	CCS	Precipitate	Precipitate
5%	CCS	CCS	Precipitate	Precipitate
10%	CCS	CCS	CCS	Precipitate
20%	CCS	CCS	CCS	Precipitate
30%	CCS	CCS	CCS	CCS
Ethanol (v/v)				
1.9%	CCS	CCS	Precipitate	Precipitate
5%	CCS	CCS	Precipitate	Precipitate
10%	CCS	CCS	CCS	Precipitate

20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
n-Propanol (v/v/)				
5%	CCS	CCS	CCS	Precipitate
10%	CCS	CCS	CCS	CCS
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
Iso-propanol (v/v)				
5%	CCS	Precipitate	Precipitate	Precipitate
10%	CCS	CCS	CCS	CCS
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS
n-Butanol (v/v)				
5%	CCS	CCS	CCS	CCS
10%	CCS	CCS	CCS	CCS
20%	2 layers	2 layers	2 layers	2 layers
30%	2 layers	2 layers	2 layers	2 layers
Tert-Butanol (v/v)				
5%	CCS	CCS	CCS	Precipitate
10%	CCS	CCS	CCS	Precipitate
20%	CCS	CCS	CCS	CCS
30%	CCS	CCS	CCS	CCS

CCS stands for clear colorless solution

Experiments to quantitatively determine the solubility of bendamustine at various temperatures for three different solutions are summarized in Figure 1 and Table 2. The amount of TBA, 20% (v/v) and 30% (v/v), used in the experiment was based on stability studies (results described below). For both solutions tested, the solubility of bendamustine decreased linearly with temperatures from 25°C to 0°C. This experiment confirmed the data shown in Table 1 and highlights the difference in bendamustine solubility for 20% and 30% TBA solutions.

Table 2. Solubility of bendamustine in TBA

	-8°C	0°C	5°C	25°C
20% (v/v) TBA 25.5 mg/mL mannitol Water, q.s. to desired volume	14 mg/mL	11 mg/mL	17 mg/mL	47 mg/mL
30% (v/v) TBA 25.5 mg/mL mannitol Water, q.s. to desired volume	20 mg/mL	18 mg/mL	27 mg/mL	65 mg/mL

C. Stability

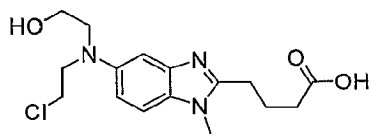
Because of its instability in aqueous solutions due to hydrolysis with water, bendamustine requires lyophilization in order to make a product suitable for

pharmaceutical use. However, during the manufacturing of lyophilized drug products, aqueous solutions are commonly needed for filling, prior to lyophilization. Thus, the use of aqueous solutions during the compounding and fill processes for bendamustine and other nitrogen mustards can result in degradation of the drug product.

Consequently, the effect of various alcohols on the degradation of bendamustine was evaluated to determine if formulations could be found that would allow longer fill-finish times, provide lyophilate powders that could be reconstituted more quickly than the current Ribomustin® formulation, and/or provide lyophilized preparations of bendamustine with a better impurity profile with respect to certain impurities, e.g., HP1, and BM1 dimer than Ribomustin®.

Preferably, a lyophilized preparation of the invention is stable with respect to HP1, i.e., the amount of HP1 does not increase appreciably (does not exceed the shelf-life specifications), for 6 months, more preferably 12 months, and even more preferably greater than 24 months, e.g., 36 months, when stored at about 2°C to about 30°C, preferably 5°C.

Table 3 shows the stability results of bendamustine in water with no addition of alcohol over a 24 hour period at 5°C. Bendamustine degrades rapidly in water alone and forms predominantly the hydrolysis product, HP1 (monohydroxy bendamustine).



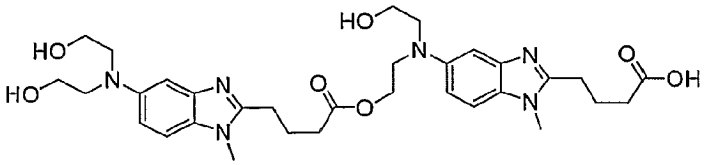
Monohydroxy bendamustine (HP1)

Formula II

Table 3. Stability of bendamustine in water

	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
0% Alcohol, i.e., Water Alone	0 hours	99.11	0.60	0.11
	3 hours	98.83	0.86	0.13
	6 hours	98.44	1.22	0.17
	24 hours	95.67	3.81	0.29

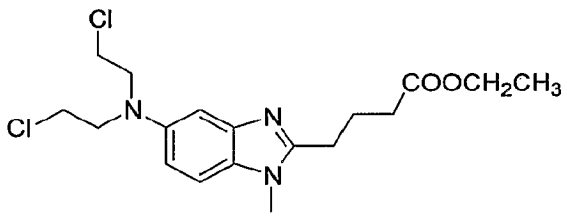
The other major degradant observed during this study and other long term stability studies was the dimer of bendamustine.



Bendamustine Dimer (BM1 Dimer)

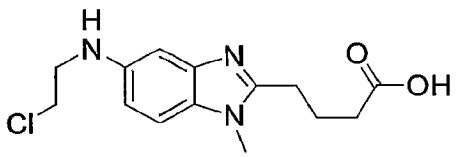
Formula III

Other degradants contained in the Ribomustin lyophilized product are bendamustine ethylester (BM1EE) (Formula IV) and BM1DCE (Formula V). BM1EE is formed when bendamustine reacts with ethyl alcohol.



Bendamustine ethylester (BM1EE)

Formula IV



BM1DCE

Formula V

Figure 2 summarizes the purity results of an HPLC analysis after incubating bendamustine in various alcohols for 24 hours at 5°C. Results are presented as the area percent of the total peak area. The numerical values for Figure 2 are provided in Tables 3-9. The purity was highest in solutions containing higher concentration of alcohols, regardless of the alcohol. Of the alcohols evaluated, bendamustine degraded the least in a solution containing about 30% (v/v) TBA. In about 10% and about 20 % alcohol solutions, n-butanol was superior in preventing degradation of bendamustine. At 20% and 30% (v/v), n-butanol in water resulted in a biphasic system due to the insolubility of n-butanol in water at these concentrations.

Figures 3 and 4 show the amount of degradation of bendamustine as measured by HP1 and dimer formation quantified by HPLC (as described herein). HP1 and dimer formation increased as the amount of alcohol concentration decreased regardless of the alcohol. This increase in impurities occurred with an anticipated time dependence (see Tables 3-9). Tert-butanol and n-butanol appeared superior to other alcohols in preventing degradation of the product. As seen in Table 10, mannitol had no effect on the stabilization of bendamustine with TBA.

Table 4. HPLC stability results for the stability of bendamustine in various ethyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

V/V alcohol	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
1.9% Ethanol	0 hours	99.11	0.64	0.12
	3 hours	98.83	0.90	0.14
	6 hours	98.60	1.12	0.15
	24 hours	96.16	3.41	0.27
5% Ethanol	0 hours	99.31	0.44	0.12
	3 hours	99.10	0.64	0.13
	6 hours	98.87	0.86	0.14
	24 hours	96.89	2.68	0.25
10% Ethanol	0 hours	99.44	0.33	0.11
	3 hours	99.28	0.48	0.12
	6 hours	99.10	0.65	0.12
	24 hours	98.03	1.57	0.18
20% Ethanol	0 hours	99.54	0.22	0.10
	3 hours	99.45	0.30	0.11
	6 hours	99.36	0.39	0.11
	24 hours	98.61	0.96	0.15
30% Ethanol	0 hours	99.62	0.15	0.10
	3 hours	99.56	0.21	0.11
	6 hours	99.52	0.24	0.12
	24 hours	99.21	0.45	0.12

Table 5. HPLC stability results for bendamustine in various Tert-butanol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Tert-butanol	0 hours	99.34	0.41	0.12
	3 hours	99.10	0.64	0.14
	6 hours	98.85	0.88	0.13
	24 hours	97.58	2.09	0.20
10% Tert-butanol	0 hours	99.46	0.30	0.11
	3 hours	99.26	0.48	0.12
	6 hours	99.05	0.69	0.13
	24 hours	98.04	1.64	0.19
20% Tert-butanol	0 hours	99.59	0.17	0.11
	3 hours	99.48	0.29	0.11
	6 hours	99.35	0.40	0.12
	24 hours	98.35	1.27	0.20
30% Tert-butanol	0 hours	99.63	0.13	0.10
	3 hours	99.60	0.16	0.10
	6 hours	99.58	0.18	0.11
	24 hours	99.42	0.34	0.12

Table 6. HPLC stability results for various n-propyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% n-Propanol	0 hours	99.25	0.43	0.13
	3 hours	99.00	0.66	0.15
	6 hours	98.72	0.94	0.16
	24 hours	97.24	2.33	0.26
10% n-Propanol	0 hours	99.34	0.33	0.15
	3 hours	99.17	0.48	0.14
	6 hours	98.92	0.70	0.16
	24 hours	97.67	1.83	0.28
20% n-Propanol	0 hours	99.45	0.33	0.13
	3 hours	99.42	0.26	0.13
	6 hours	99.29	0.39	0.14
	24 hours	98.60	0.97	0.24
30% n-Propanol	0 hours	99.53	0.15	0.13
	3 hours	99.51	0.15	0.15
	6 hours	99.44	0.20	0.11
	24 hours	99.27	0.36	0.17

Table 7. HPLC stability results for bendamustine in various iso-propyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Iso-propanol	0 hours	99.21	0.48	0.13
	3 hours	98.65	0.72	0.14
	6 hours	98.56	1.02	0.14
	24 hours	96.14	3.35	0.26
10% Iso-propanol	0 hours	99.32	0.37	0.12
	3 hours	99.11	0.55	0.14
	6 hours	98.85	0.75	0.16
	24 hours	97.68	1.92	0.21
20% Iso-propanol	0 hours	99.49	0.21	0.11
	3 hours	99.39	0.31	0.12
	6 hours	99.22	0.42	0.13
	24 hours	98.61	1.04	0.17
30% Iso-propanol	0 hours	99.56	0.15	0.10
	3 hours	99.47	0.20	0.12
	6 hours	99.40	0.24	0.11
	24 hours	99.15	0.52	0.14

Table 8. HPLC stability results for bendamustine in various methyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Methanol	0 hours	99.35	0.40	0.12
	3 hours	98.97	0.70	0.14
	6 hours	98.66	0.95	0.14
	24 hours	96.65	2.83	0.23
10% Methanol	0 hours	99.42	0.34	0.11
	3 hours	99.01	0.59	0.12
	6 hours	98.86	0.80	0.12

	24 hours	97.65	1.85	0.18
20% Methanol	0 hours	99.56	0.22	0.11
	3 hours	99.31	0.38	0.11
	6 hours	98.99	0.50	0.12
	24 hours	98.31	1.15	0.16
30% Methanol	0 hours	99.59	0.18	0.10
	3 hours	99.43	0.27	0.11
	6 hours	99.25	0.34	0.11
	24 hours	98.65	0.76	0.13

Table 9. HPLC stability results for bendamustine in various n-butyl alcohol concentrations over a 24 hour period. HP1 and Dimer were impurities that increased in this study.

Concentration alcohol (v/v)	Hold Time	Purity (%Area)	HP1 (%)	Dimer (%)
5% Butanol	0 hours	99.25	0.49	0.13
	3 hours	98.94	0.73	0.14
	6 hours	98.76	0.91	0.14
	24 hours	97.46	2.20	0.21
10% Butanol	0 hours	99.44	0.30	0.11
	3 hours	99.18	0.49	0.12
	6 hours	99.03	0.64	0.12
	24 hours	98.13	1.55	0.17
20% Butanol[a]	0 hours	99.54	0.23	0.10
	3 hours	99.45	0.31	0.11
	6 hours	99.30	0.40	0.11
	24 hours	98.81	0.91	0.14
30% Butanol[a]	0 hours	99.55	0.24	0.10
	3 hours	99.40	0.29	0.10
	6 hours	99.40	0.37	0.11
	24 hours	99.00	0.74	0.12

a – Both solutions had 2 layers/phases of liquids in the vial. Solutions were vortexed prior to sample preparation.

The results in Tables 1-9 indicate that the stability of bendamustine HCl with respect to HP1 and dimer improves with increasing alcohol concentration.

Table 10. HPLC stability results for bendamustine in TBA with and without mannitol over a 24 hour period.

Sample	Purity (%Area)	HP1 (%)
TBA 20% (v/v) with Mannitol		
0 hours	99.59	0.17
24 hours @ 5°C	99.35	1.27
TBA 20% (v/v) without Mannitol		
0 hours	100.0	0.00
24 hours @ 5°C	98.80	1.21

NOTE: The samples analyzed without mannitol were analyzed by HPLC using a normal phase method while the samples analyzed with mannitol used a reverse phase HPLC method. Slight variability may be seen in other samples analyzed between the two methods.

D. Lyophilization Cycle Development

Different pre-lyophilization formulations were prepared at various concentrations of bendamustine, mannitol, and alcohols in water. The cycle

development was changed and optimized at each step for freezing (fast vs. slow), primary drying (both temperature and pressure), and secondary drying as described herein.

Based upon all of the information detailed above on solubility, stability, and ease of lyophilization, preferred formulations include the following:

Ingredients	Concentration
Bendamustine	about 2-40 mg/mL
Mannitol	about 0-50 mg/mL
Alcohol	about 0.5%-40% (v/v)
Water, q.s. to	desired volume

wherein the alcohol is selected from methanol, n-propanol, or isopropanol

Ingredients	Concentration
Bendamustine	about 5-20 mg/mL
Mannitol	10-30 mg/mL
Alcohol	1-20% (v/v)
Water, q.s. to	desired volume

wherein the alcohol is selected from methanol, n-propanol, or isopropanol

Ingredients	Concentration
Bendamustine	about 5-20 mg/mL
Mannitol	10-30 mg/mL
Alcohol	5-40% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Alcohol	about 5-15% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Alcohol	about 10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-40 mg/mL
Mannitol	about 0-50 mg/mL
Butanol	about 0.5-20% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 5-20 mg/mL
Mannitol	about 10-30 mg/mL
Butanol	about 1-10 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Butanol	about 1-10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Butanol	about 10% (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-100 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-99.9 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration

Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 0.5-99 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 2-50 mg/mL
Mannitol	about 0-50 mg/mL
Tertiary butanol	about 90-99 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 5-20 mg/mL
Mannitol	about 10-30 mg/mL
Tertiary butanol	about 5-80 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12-17 mg/mL
Mannitol	about 20-30 mg/mL
Tertiary butanol	about 10-50 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 12.5-15 mg/mL
Mannitol	about 0-30 mg/mL
Ethanol	about 20-30 % (v/v)
Water, q.s. to	desired volume

Ingredients	Concentration
Bendamustine HCl	about 15 mg/mL
Mannitol	about 25.5 mg/mL
Tertiary butanol	about 30 % (v/v)
Water, q.s. to	desired volume

EXAMPLES

The following Examples are provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. These

Examples are in no way to be considered to limit the scope of the invention in any manner.

Materials:

Bendamustine HCl, (Degussa, Lot #s 0206005 and 0206007)
Mannitol, NF or equivalent (Mallinckrodt)
Ethyl Alcohol Dehydrated (200 proof), USP or equivalent (Spectrum)
Tertiary-butyl alcohol, ACS (EM Science)
Methanol (Spectrum and EMD)
Propanol (Spectrum)
Iso-propanol (Spectrum)
Butanol (Spectrum)
Water, HPLC grade or equivalent (EMD)
Acetonitrile, HPLC grade or equivalent (EMD)
Trifluoroacetic Acid, J.T. Baker
Methanol, HPLC grade or equivalent (EM Science, Cat # MX0488P-1)
Trifluoroacetic Acid, HPLC grade or equivalent (JT Baker, Cat# JT9470-01)

Equipment:

Waters 2695 Alliance HPLC system with photodiode array detector
Waters 2795 Alliance HPLC system with dual wavelength detector
Analytical Balance (Mettler AG285, ID #1028) and (Mettler XS205)
VirTis Lyophilizer AdVantage
Agilent Zorbax SB-C18 5 µm 80Å 4.6 × 250 mm column, Cat# 880975-902

Example 1- HPLC Procedures

Method 1

Mobile Phase A:	0.1% TFA; H_2O
Mobile Phase B:	0.1% TFA; 50% ACN:50% H_2O
UV: 230 nm	
Flow rate:	1.0 mL/min
Column temp.:	30 °C
Column:	Zorbax SB-C18 5 µm 80 Å 4.6 × 250 mm
Sample temp.:	5 °C
Injection Volume:	10 µL
Sample Concentration:	0.25 mg/mL in MeOH
Gradient:	20%B for 1 min
	20 – 90%B in 23 min
	90%B for 6 min

back to 20%B in 1 min
hold at 20%B for 4 min

Run time: 30 min
Post run time: 5 min

Method 2

Mobile Phase A: 0.1% TFA; H_2O:ACN (9:1)
Mobile Phase B: 0.1% TFA; H_2O:ACN (5:5)
UV: 230 nm
Flow rate: 1.0 mL/min
Column: Zorbax SB-C18 5 μm 80 Å 4.6 × 250 mm
Column temp.: 30 °C
Sample temp.: 5 °C
Injection Volume: 10 μL
Sample Concentration:0.25 mg/mL in MeOH
Gradient: 0%B for 3 min
0 – 50%B in 13 min
50 – 70%B in 17 min
70 – 90%B in 2 min
90%B for 5 min
back to 0%B in 1 min
hold at 0%B for 4 min
Run time: 40 min
Post run time: 5 min

Method 3

Phase A: HPLC grade water with 0.1 % TFA(v/v)
Phase B: HPLC grade ACN / water(1:1v/v) with 0.1%TFA(v/v)
UV: 254 nm
Flow rate: 1.0 mL/min
Column: Zorbax SB-C18 5 μm 80 Å 4.6 × 250 mm
Column temp.: 30 °C
Sample temp.: 5 °C
Injection Volume: 5 μL
Acquisition time: 30 min
Post time: 9 min
Diluent: methanol

Gradient:

Time (min.)	% Phase A	% Phase B
0.0	82	18
7.0	60	40
11.0	60	40
15.0	20	80
30.0	20	80
31.0	82	18

Sample preparation- dissolve the drug product with 200 mL MeOH. Sonicate 6 minutes. The solution can be injected directly into the HPLC (ca. 0.5 mg/mL)

Method 4

Phase A: HPLC grade water with 0.1 % TFA(v/v)
Phase B: HPLC grade ACN with 0.1%TFA(v/v)
UV: 254 nm
Flow rate: 1.0 mL/min
Column: Zorbax Bonus RP-C14 5 μm 4.6 × 150 mm
Column temp.: 30°C
Sample temp.: 5°C
Injection Volume: 2 μL
Acquisition time:31 min
Post time: 5 min
Diluent: NMP/0.1% TFA in water (50:50 v/v)

Gradient:

Time (min.)	% Phase A	% Phase B
0.0	93	7
5	93	7
13	73	27
16	73	27
25	10	90
31	10	90

Sample preparation for method 4- dissolve the drug product with a known amount of diluent to prepare a concentration of 4.2 mg/mL for injection directly into the HPLC. It may be necessary to perform a second dilution (the 100 mg/vial dosage form) to obtain a 4.2 mg/mL sample concentration.

Results

The retention times for some Bendamustine impurities using HPLC Method 1 described above are shown in Table 11. An HPLC chromatograph for Ribomustin® using the HPLC procedure described herein is shown in Fig. 6.

Table 11: Retention Time for Bendamustine and some of its Impurities using HPLC Method 1

Sample Name	Retention Time (min)
HP1	14.110
Bendamustine	22.182
BM1 Dimer	24.824
BM1EE	26.968

Although HPLC Method 1 was capable of resolving impurities found in bendamustine it was not capable of separating a potential impurity formed during analysis, the methyl ester of bendamustine (BM1ME). The retention time difference between BM1ME and BM1 Dimer was only 0.3 minutes. In order to resolve BM1 Dimer, another HPLC method (# 2) was developed. HPLC method #2 was capable of separating all the impurities but required a longer run time of 45 minutes (Table 12).

Table 12: Retention Time for bendamustine and impurities using HPLC Method 2.

Sample Name	Retention Time (min)
HP1	15.694
BM1	25.420
BM1ME	31.065
BM1 Dimer	32.467
BM1EE	36.038

The impurity profile of various lots of Ribomustin using HPLC Method 3 are shown in Table 13.

Table13- Ribomustine Impuirty Profile using HPLC Method 3

% Area					
Batch	**Bendamustine(HCl)**	**HP1**	**BM1EE**	**BM1 Dimer**	**BM1DCE**
03H08	98.14	1.07	0.21	0.34	0.03
03H07	97.67	1.5	0.2	0.33	0.04
02K27	96.93	0.93	0.29	1.18	0.08

03C08	97.61	1.24	0.19	0.46	0.02

Example 2- Solubility

The solubility of bendamustine HCl (bendamustine) in water (alone) and with varying amounts of methanol, ethanol, propanol, isopropanol, butanol and tertiary-butyl alcohol (TBA) was determined by visual inspection. Amounts of bendamustine at 15 mg/mL, mannitol at 25.5 mg/mL were prepared in 10 mL of the indicated alcohol solutions (Table 1) at room temperature. Samples were then refrigerated at 5°C and inspected after 0, 3, 6 and 24 hours for particulates and/or precipitates.

Results summarized in Table 1 indicate that bendamustine solubility is dependant on temperature and the amount of alcohol in aqueous solutions. For all alcohols the solubility of bendamustine increased as the concentration of alcohol increased. The formation of a precipitant was also dependent on the temperature and time.

The solubility of bendamustine was also determined in 20% (v/v) TBA containing 25.5 mg/mL mannitol in water, and 30% (v/v) TBA containing 25.5 mg/mL mannitol in water (Fig 1). Bendamustine was added to 4 mL of each solution while mixing until it would no longer dissolve. The saturated solutions were allowed to mix for 1 hour at -8°C, 0°C, 5°C, or 25°C. The samples were centrifuged and placed back at the original temperature for a minimum of 30 minutes. The -8°C sample was placed into an ice bath containing sodium chloride, which lowers the temperature of the ice bath, and the temperature was measured when the sample was pulled for analysis. An aliquot of each sample was taken and prepared for HPLC analysis.

The results of these experiments are shown in Figure 1 and Table 2. The amount of TBA, 20% (v/v) and 30% (v/v), used in the experiment (Fig. 1) was based on stability studies described herein.

As indicated in Fig. 1, the solubility of bendamustine decreased linearly with temperature (25°C to 0°C). The solubility of bendamustine was temperature dependant whether it was dissolved in water alone or with an alcohol. The 20% (v/v) TBA may likely be the lower limit required for efficient and robust pharmaceutical manufacturing due to the stability and solubility of bendamustine. A filling solution of 15 mg/mL bendamustine is close to the saturation limit of 17.2 mg/mL bendamustine at 5°C but higher than the limit at 0°C. The 30% (v/v) TBA is the recommended

concentration of TBA for the final formulation and is well within the solubility limit regardless of temperature.

Example 3-Stability

A. Stability in Water

Solutions of bendamustine (15 mg/mL), and mannitol (25.5 mg/mL) were prepared in water at room temperature and immediately placed in an ice bath (to lower the temperature quickly to about 5°C) for 10 minutes and then refrigerated at 5°C. A sample of each formulation was analyzed by HPLC using the methods described herein after 0, 3, 6 and 24 hours when stored at 5°C.

B. Stability in Alcohols

Solutions containing 15 mg/mL bendamustine, 25.5 mg/mL mannitol, and 1.9%, 5%, 10%, 20% or 30% (v/v) ethyl alcohol in water or 5%, 10%, 20% or 30% (v/v) TBA, methanol, propanol, iso-propanol, or butanol in water were prepared at room temperature, placed into an ice bath for 10 minutes and then refrigerated at 5°C. A sample of each formulation was analyzed by HPLC after 0, 3, 6 and 24 hours when stored at 5°C.

C. Stability Results

Table 3 shows the stability results of bendamustine in water with no addition of alcohol over a 24 hour period at 5°C. Bendamustine degrades quickly in water but the stability of bendamustine increases with increasing alcohol concentrations (Figs. 2, 3 and 4). Although alcohols are frequently used in lyophilization to aid in solubility problems, the effect of alcohols on bendamustine stability is unique, unexpected and useful in manufacturing bendamustine with fewer impurities since an aqueous solution can be used while maintaining the stability of bendamustine. TBA was found to be the best stabilizer of the six alcohols tested (Figs. 2, 3, and 4). All alcohols at 30% (v/v) reduced the formation of impurities HP1 and Dimer at 5°C for up to 24 hours. With respect to TBA, HP1 reaches only about 0.4% when stored at 5°C for up to 24 hours. Lower concentrations of alcohol may not be efficient, when formulated at 15 mg/mL bendamustine and stored at 5°C due to bendamustine precipitation and impurity formation.

Example 4- Formulation Optimization

After the solubility and stability of bendamustine were determined, the formulation was optimized for lyophilization. Since the concentration of

bendamustine is higher in a 30% TBA/water saturated solution as compared with other alcohol solutions, it is anticipated that the vial size required to fill 100 mg of bendamustine can be decreased from the current Ribomustin® presentation. Although a saturated solution of bendamustine contains 18 mg/mL at 0°C, a concentration of 15 mg/mL was selected for the formulation to compensate for slight differences in API solubility due to differences in bulk API purity as a result of batch differences. A concentration of 15 mg/mL bendamustine requires 6.67 mL to fill 100 mg of bendamustine HCl per vial.

The surface (sublimation) area to volume ratio is critical to producing a lyophilized product with good appearance that freeze dries quickly. Generally, lyophilized products occupy between 30% to 50% of the vial volume. A 20 mL vial with 6.67 mL contains about 30% of its capacity and has a surface area ratio of 0.796 cm^2/mL.

Mannitol was selected as the bulking agent in order to maintain a formulation similar to Ribomustin®. Studies were performed to evaluate the effect of mannitol on bendamustine solubility and appearance of the product. Mannitol decreases the solubility of bendamustine (at 15 mg/mL) in both ethanol and TBA aqueous solutions. For example, solutions containing 5% and 10% ethanol and TBA without mannitol did not precipitate over 24 hours. However, for samples with mannitol (Table 1) precipitate was observed within 24 hours. There was no precipitate with aqueous solutions containing 30% (v/v) TBA, 15 mg/mL bendamustine, and 25.5 mg/mL mannitol. In order to maintain a well formed cake resistant to breakage during handling, a minimum of 134 mg/vial of mannitol was required with no difference observed in vials up to 200 mg/vial of mannitol.

All alcohols tested increased the stability and solubility of bendamustine. However, a significant mole fraction was required to affect the stability of the filling solution and the ease of manufacturing. Smaller alcohols have the undesirable effect of lowering the freezing point of the bulk solution and thus requiring long lyophilization cycles at lower temperatures. Higher concentrations of methanol and ethanol produced unattractive cakes that were difficult to reconstitute. 10% ethanol, 20% ethanol, 10% iso-propanol, 20% iso-propanol, or 30% TBA aqueous solutions containing bendamustine (15 mg/mL), mannitol (25.5 mg/mL) were prepared and lyophilized. The lyophilized vials filled from solutions of 10% ethanol, 20% ethanol,

10% iso-propanol, 20% iso-propanol produced either a collapsed cake or a film residue. The only solvent system producing an acceptable cake was 30% TBA. Additionally, reconstitution of 10% ethanol, 20% ethanol, 10% iso-propanol, 20% iso-propanol lyophilized vials were difficult and did not fully dissolve until >45 minutes.

The ability to utilize a smaller vial is constrained by the concentration or solubility of bendamustine in the aqueous/organic solution. At lower concentrations of ethanol, methanol, isopropanol and n-propanol, which produced acceptable cake appearance, a more dilute solution of bendamustine is required due to solubility limitations. To maintain a presentation with 100 mg of bendamustine per vial, a vial larger than 50 mL would be required. Also, stability studies herein indicated that at the lower alcohol concentration, the chemical stability was not sufficient to allow for acceptable filling times.

One of the factors affecting the ease of reconstitution is the porosity of the lyophilate. In general, amorphously precipitated solids with little surface area are more difficult to solubilize. Most lyophilates containing mannitol will reconstitute within 3-5 minutes as long as there is no precipitate formed during lyophilization, frequently caused by evaporation of a liquid (melt back). Based on our experience with several lyophilization solvent systems and not wishing to be bound to any particular theory, the problems associated with Ribomustin® reconstitution may be associated with precipitation caused by melt back during lyophilization. Most organic solvents do not lyophilize efficiently and cause melt back because of their low melting point. TBA (tertiary butyl alcohol) has a high melting point and a similar vapor pressure as compared to water. TBA is removed by sublimation, not evaporation, at about the same rate as water. Lyophilates produced with 30% (v/v) TBA according to the invention reconstitute within 3-10 minutes as compare to commercially available Ribomustin which may take 30-45 minutes.

Based upon the solubility, stability, ease of reconstitution and manufacturing considerations, the following is a preferred pre-lyophilization formulation of the present invention: bendamustine HCl about 15 mg/mL, mannitol about 25.5 mg/mL, about 30% (v/v) tertiary-butyl alcohol, and q.s. using water for Injection. The formulation is then filled at 5°C using 6.67 mL in an amber 20 mL, 20 mm vial and partially stoppered with a bromobutyl stopper and loaded into a pre-chilled lyophilizer.

Example 5- Impurity assessment

Major impurities introduced during Ribomustin® manufacturing, compounding, fill, and lyophilization procedure, as determined by HPLC analysis (Fig. 6), are the hydrolysis product HP1, the Dimer, and the ethyl ester of bendamustine, BM1EE. BM1EE can be formed during drug substance manufacturing, e.g., during recrystalization and/or purification processes. BM1EE is known to be a more potent cytotoxic drug than bendamustine. Experiments were undertaken to determine if the use of a 30% TBA aqueous filling solution would lead to the formation of bendamustine t-butyl ester.

Experiments were performed using traditional Fisher esterfication reaction conditions required for the formation of t-butyl ester of bendamustine. Bendamustine was heated in 60°C TBA with HCl for 20 hours. No reaction was observed. This result indicated that it would be very difficult to form the tert-butyl ester of bendamustine during the fill/finish process. No new impurities in drug product manufactured from TBA have been observed in stability studies to date.

To aid in the testing of the drug product, synthetic routes using more reactive sources of the t-butyl moiety were developed. Another attempt to make tert-butyl ester was carried out by formation of the acyl chloride of bendamustine. A suspension of bendamustine in methylene chloride was treated with oxalyl chloride and N,N-dimethylformamide. After acyl chloride was formed, the solvent was concentrated. The residue was added to methylene chloride, tert-butanol, triethylamine, and 4-dimethylaminopyridine and the mixture was stirred at room temperature overnight. After adding all solvents and purification, an unknown compound was given. The LC-MS did not match the molecular weight of bendamustine tert-butyl ester and the proton NMR did not showed the peak for tert-butyl. Therefore, this attempt also failed to produce the bendamustine tert-butyl ester. Thus, using TBA as the co-solvent has an additional benefit of not forming the ester from the alcohol.

Example 6- Lyophilization Cycle Development

Numerous lyophilization cycles were performed to evaluate the critical stages of lyophilization and achieve the most efficient drying cycle. Experiments were performed to evaluate the effect of the freezing rate, primary drying temperature, time, and pressure on the product.

A. Freezing Rate

The literature reports that TBA adopts different crystal forms depending on the freeze rate. In some TBA solutions, the slower the product froze, the quicker it dried. Larger crystals formed during slow freezing producing bigger pores allowing more efficient sublimation. However, during studies with bendamustine, the freezing rate was not found to be a critical processing parameter when evaluated at 2 and 8 hours.

B. Primary and Secondary Drying

During the first attempts to lyophilize from 30% TBA solutions, the lyophilized cake fractured and powder was ejected from the vial. These cakes appeared to contain amorphous particles within the lyophilate, an indication of melt back. This phenomenon was reproducible and occurred when the product reached about -10°C (refer to Fig. 5) independent of the warming rate. Several variables were tested to determine the cause and solution to the problem of the powder ejection. The pressure was raised from 50 μm to 150 μm during primary drying, but powder ejection was still observed but to a lesser extent. This experiment was then repeated except the freezing rate was extended to 8 hours from 2 hours. This change had no effect.

The length of primary drying was next evaluated. For example, the following very slow drying cycle was evaluated: freezing from +25°C to -50°C in eight hours; holding at -50°C for 5 hours, warming and drying from-50°C to -25°C in seven hours; holding for twenty hours at -25°C, warming and drying from -25°C to -15°C in two hours and holding for twenty hours at -15°C, warming and drying from -15°C to 40°C in six hours and holding for twenty hours at 40°C while maintaining a chamber pressure of 150 μm throughout drying. No powder ejection (Fig 5) was observed. This cycle resulted in a well-formed cake without fracture that reconstituted readily. Without wishing to be bound to a particular theory, the problems with powder ejection and difficulty with reconstitution may be the result of drying the lyophilate too quickly, thus resulting in strong vapor flow out of the cake as well as melt back. With the use of a less aggressive drying cycle an aesthetic, stable, and easy to reconstitute cake was reproducibly formed. Thus, removing all unbound water and tertiary-butyl alcohol prior to secondary drying may prevent melt back as well as powder ejection. The lyophilization cycle was further optimized under these gentle conditions (Fig. 5). There were no immediate degradation products as a result of drying at 40°C for up to 20 hours.

Example 7- Lyophilization cycle

Step	Description	Time (Hour)	Temperature (°C)	Pressure (Microns)
1	Hold	0.25	5°C	-
2	Ramp	8	-50°C	-
3	Hold	4	-50°C	-
4	Ramp	3	-20°C	150
5	Hold	6	-20°C	150
6	Ramp	1	-15°C	150
7	Hold	20	-15°C	150
8	Ramp	0.5	-12°C	150
9	Hold	15.5	-12C	150
10	Ramp	15	35C	50
11	Hold	10	35°C	50
12	Ramp	1	40C	50
	Hold	5	40C	50
Total		89.25	-	-

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the spirit and scope of the invention. More specifically, it will be apparent that certain solvents which are both chemically and physiologically related to the solvents disclosed herein may be substituted for the solvents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit and scope of the invention as defined by the appended claims.

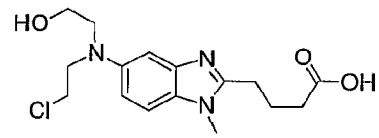
All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in

each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

What is claimed is:

1. A pharmaceutical composition of bendamustine containing not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II,



Formula II

wherein said HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized preparation of bendamustine.

2. The composition according to claim 1, wherein the amount of HP1 is not more than 0.5% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

3. The composition according to claim 1, wherein the amount of HP1 is not more than 0.4% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

4. The composition according to claim 1, wherein the amount of HP1 is not more than 0.3% (area percent of bendamustine) at time zero after reconstitution of a lyophilized preparation of bendamustine.

5. A lyophilized preparation of bendamustine containing not more than about 0.9% (area percent of bendamustine) HP1 at release.

6. A lyophilized preparation of bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

7. The lyophilized preparation according to claim 5, wherein the preparation is packaged in a vial or other pharmaceutically acceptable container.

8. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about six months when stored at 5° C.

9. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 12 months when stored at 5° C.

10. The lyophilized preparation according to claim 6, wherein said preparation is stable with respect to the amount of HP1 for at least about 24 months when stored at 5° C.

11. A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine containing not more than about 0.9% HP1, wherein said HP1 is the amount of HP1 present at release.

12. A pharmaceutical dosage form comprising a pharmaceutical composition of bendamustine containing not more than about 0.5% HP1, wherein said HP1 is the amount of HP1 present at release.

13. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 5 mg to about 500 mg of bendamustine.

14. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 10 mg to about 300 mg of bendamustine.

15. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 25 mg of bendamustine.

16. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 100 mg of bendamustine.

17. A pharmaceutical dosage form of claim 11, wherein the pharmaceutical dosage form comprises about 200 mg of bendamustine.

18. A pharmaceutical dosage form comprising the lyophilized preparation of claim 5.

19. A pharmaceutical composition of bendamustine comprising bendamustine containing not more than about 0.5% (area percent of bendamustine) HP1 and a trace amount of one or more organic solvents, wherein said HP1 is the amount of HP1 present at release.

20. A pharmaceutical composition of bendamustine according to claim 19 wherein the organic solvent is selected from the group consisting of one or more of tertiary butanol, n-propanol, n-butanol, isopropanol, ethanol, methanol, acetone, ethyl acetate, dimethyl carbonate, acetonitrile, dichloromethane, methyl ethyl ketone, methyl isobutyl ketone, 1-pentanol, methyl acetate, carbon tetrachloride, dimethyl sulfoxide, hexafluoroacetone, chlorobutanol, dimethyl sulfone, acetic acid, and cyclohexane.

21. A pharmaceutical composition according to claim 20, wherein the organic solvent is selected from the group consisting of one or more of ethanol, methanol, propanol, butanol, isopropanol, and tertiary butanol.

22. A pharmaceutical composition according to claim 19, wherein the organic solvent is tertiary butanol.

23. A lyophilized preparation according to claim 5 further comprising a trace amount of an organic solvent.

24. A lyophilized preparation according to claim 23 wherein said organic solvent is tertiary butanol.

25. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a release specification for bendamustine degradants at less than 4.0 % (area percent bendamustine) for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

26. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a release specification for bendamustine of HP1 at less than or equal to 1.5% for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

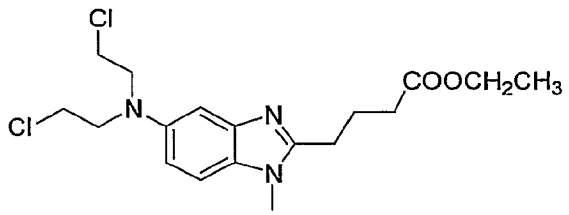
27. In a method for obtaining agency approval for a bendamustine product, the improvement which comprises setting a shelf-life specification for bendamustine degradants at less than 7.0% (area percent bendamustine) for a bendamustine product containing not more than about 0.5% (area percent of bendamustine) HP1 at release.

28. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of bendamustine degradants is less than 4.0 % (area percent of bendamustine) and the concentration of HP1 is less than 0.5% (area percent of bendamustine).

29. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.9% (area percent of bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

30. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that the concentration of HP1 is less than 0.5% (area percent of bendamustine) at release and the concentration of bendamustine degradants is less than 7.0% at the time of product expiration; wherein said product is stored at 5°C.

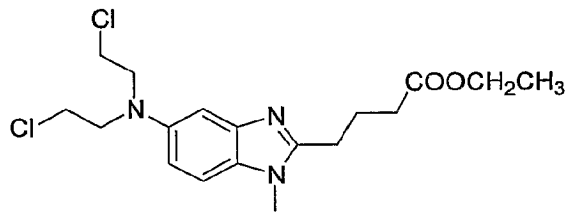
31. A lyophilized preparation of bendamustine wherein the concentration of bendamustine ethylester (as shown in Formula IV)



Formula IV

is no more than 0.2% greater than the concentration of bendamustine ethylester as found in the drug substance used to make the lyophilized preparation.

32. A lyophilized preparation of bendamustine according to claim 5 containing not more than about 0.5% bendamustine ethylester as shown in Formula IV



Formula IV.

33. A bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.9% (area percent bendamustine).

34. A bendamustine pre-lyophilization solution or dispersion comprising one or more organic solvents, wherein said solution or dispersion comprises at least one stabilizing concentration of an organic solvent which reduces the level of degradation of bendamustine so that the amount of HP1 produced during lyophilization, from about 0 to 24 hours, does not exceed 0.5% (area percent bendamustine).

35. The lyophilized powder produced from the pre-lyophilization solution or dispersion according to claim 33.

36. A method of preparing a bendamustine lyophilized preparation comprising,

a) dissolving bendamustine in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine lyophilized preparation contains not more than about 0.9% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

37. A method of preparing a bendamustine lyophilized preparation comprising,

a) dissolving bendamustine in a stabilizing concentration of an alcohol solvent comprising between about 5% to about 100% (v/v) alcohol to form a pre-lyophilization solution; and

b) lyophilizing the pre-lyophilization solution;

wherein said bendamustine lyophilized preparation contains not more than about 0.5% (area percent of bendamustine) HP1 as shown in Formula II, wherein said HP1 is the amount of HP1 present at release.

38. A method according to claim 36, wherein the alcohol concentration is between about 5% to about 99.9%.

39. A method according to claim 36, wherein said alcohol is selected from one or more of methanol, ethanol, propanol, iso-propanol, butanol, and tertiary-butanol.

40. A method according to claim 39, wherein said alcohol is tertiary-butanol.

41. A method according to claim 40, wherein said tertiary butanol is at a concentration of about 20% to 30%.

42. A method according to claim 40, wherein said tertiary butanol is at a concentration of about 30%.

43. A method according to claim 36, wherein an excipient is added before lyophilization.

44. A method according to claim 43, wherein the excipient is mannitol.

45. A method according to claim 36, wherein the bendamustine concentration is about 2 to 50 mg/mL.

46. The lyophilized powder obtained from the method according to one of claims 36 to 45.

47. A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to a temperature below about -40°C to form a frozen solution;

ii) holding the frozen solution at or below -40°C for at least 2 hours;

iii) ramping the frozen solution to a primary drying temperature between about -40°C and about -10°C to form a dried solution;

iv) holding for about 10 to about 70 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vii) holding for about 5 to about 40 hours to form a bendamustine lyophilized preparation.

48. A method according to claim 47, wherein said alcohol is tertiary-butanol.

49. A method according to claim 48, wherein said tertiary butanol is at a concentration of about 20% to 30%.

50. A method according to 49, wherein said tertiary butanol is at a concentration of about 30%.

51. The lyophilized powder obtained from the method according to one of claims 47 to 50.

52. A method according to claim 36 wherein step b) comprises:

i) freezing the pre-lyophilization solution to about -50°C to form a frozen solution;

ii) holding the frozen solution at about -50°C for at least 2 hours to about 4 hours;

iii) ramping to a primary drying temperature between about -20°C and about -12°C to form a dried solution;

iv) holding at a primary drying temperature for about 10 to about 48 hours;

v) ramping the dried solution to a secondary drying temperature between about 25°C and about 40°C; and

vi) holding at a secondary drying temperature for at least 5 hours up to about 20 hours.

53. A method according to claim 52, wherein said alcohol is tertiary-butanol.

54. A method according to claim 53, wherein said tertiary butanol is at a concentration of about 20% to 30%.

55. A method according to 54, wherein said tertiary butanol is at a concentration of about 30%.

56. The lyophilized powder obtained from the method according to one of claims 52 to 55.

57. A method according to claim 36 wherein step b) comprises: i) starting with a shelf temperature of about 5°C for loading; ii) freezing to about -50°C over about 8 hours; iii) holding at -50°C for about 4 hours; iv) ramping to about -20°C over about 3 hours; v) holding at about -20°C for 6 hours; ramping to about -15°C over about 1 hour; vi) holding at -15°C for about 20 hours; vii) ramping to about -15°C over about 1 hour; viii) holding at about -15°C for about 20 hours; ix) ramping to about -12°C over about 0.5 hours; x) holding at about -12°C for about 15.5 hours; xi) ramping to between about 25°C and about 40°C or higher for about 15 hours; xii) holding between about 25°C and about 40°C for about 10 hours; xiii) ramping to about 40°C over about 1 hour; andxiv) holding at about 40°C for about 5 hours; unloading at about 5°C, at a

pressure of about 13.5 psi in a pharmaceutically acceptable container that is hermetically sealed; wherein the pressure is about 150 microns throughout primary drying and 50 microns throughout secondary drying.

58. A lyophilization cycle according to claim 57, wherein step (xi) is ramped to about 30-35°C for about 15 hours.

59. The lyophilized powder prepared from the lyophilization cycle of claim 57 or 58.

60. A formulation for lyophilization comprising bendamustine at a concentration of about 15 mg/mL, mannitol at a concentration of about 25.5 mg/mL, tertiary-butyl alcohol at a concentration of about 30% (v/v) and water.

61. A lyophilized preparation made from the formulation according to claim 60.

62. A method of treating a medical condition in a patient comprising dissolving the preparation of claim 5 in a pharmaceutically acceptable solvent to produce a pharmaceutically acceptable solution and administering to said patient a therapeutically effective amount of said solution, wherein said condition is amenable to treatment with said preparation.

63. A method of treating according to claim 62, wherein said condition is selected from chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, breast cancer, small cell lung cancer, and an autoimmune disease.

64. A method of treating according to claim 63, wherein said condition is non-Hodgkin's lymphoma.

65. A method of treating according to claim 63, wherein said condition is chronic lymphocytic leukemia.

66. A method of treating according to claim 63, wherein said condition is multiple myeloma.

67. A method of treating according to claim 62 further comprising administering the dissolved preparation of claim 5 in combination with one or more anti-neoplastic agents wherein said antineoplastic agent is given prior, concurrently, or subsequent to the administration of the dissolved preparation of claim 5.

68. A method of treating according to claim 67 wherein the antineoplastic agent is an antibody specific for CD20, wherein said antibody is given prior, concurrently or subsequent to the administration of the dissolved preparation of claim 5.

69. A method of treating according to claim 62 wherein the autoimmune disease is rheumatoid arthritis, multiple sclerosis or lupus.

70. A method of treating according to claim 62, wherein the medical condition is a hyperproliferative disorder.

71. A pharmaceutical dosage form of bendamustine containing not more than about 0.9% HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

72. A pharmaceutical dosage form of bendamustine containing not more than about 0.5% HP1 (area percent of bendamustine) wherein said dosage form comprises a vial or other pharmaceutically acceptable container, wherein said HP1 is the amount of HP1 present pre-reconstitution or at time zero after reconstitution of said dosage form.

73. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine at a concentration of about 10 to about 500 mg/container.

74. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container contains bendamustine at a concentration of about 100 mg/container.

75. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 5 mg to about 2 g/container.

76. A pharmaceutical dosage form according to claim 72, wherein the vial or other pharmaceutically acceptable container further comprises mannitol at a concentration of about 170 mg/container.

77. A pre-lyophilized pharmaceutical composition of bendamustine comprising about 15 mg/mL bendamustine HCl, about 25.5 mg/mL mannitol, about 30% (v/v) tertiary-butyl alcohol, and water.

78. The preparation of claim 5 which is a pharmaceutical composition.

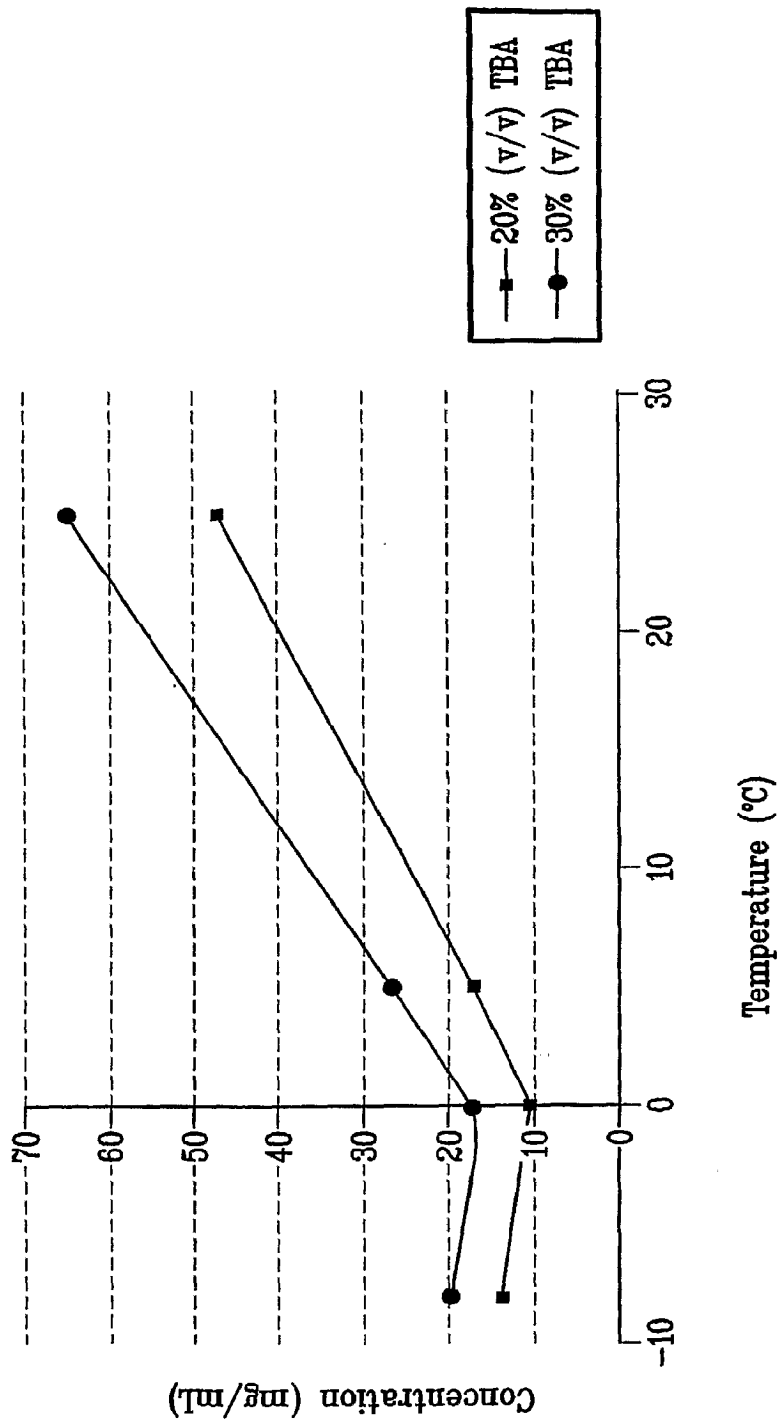


FIG. 1

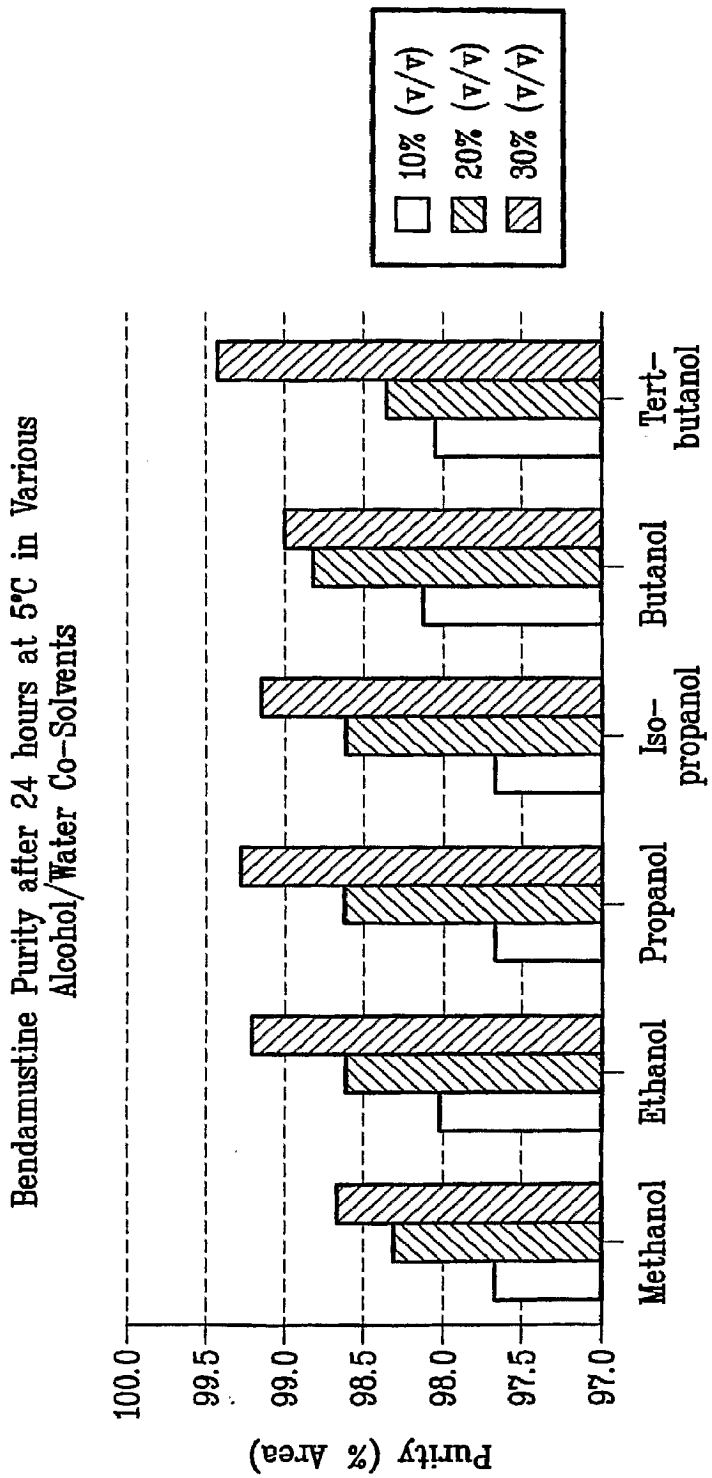


FIG. 2

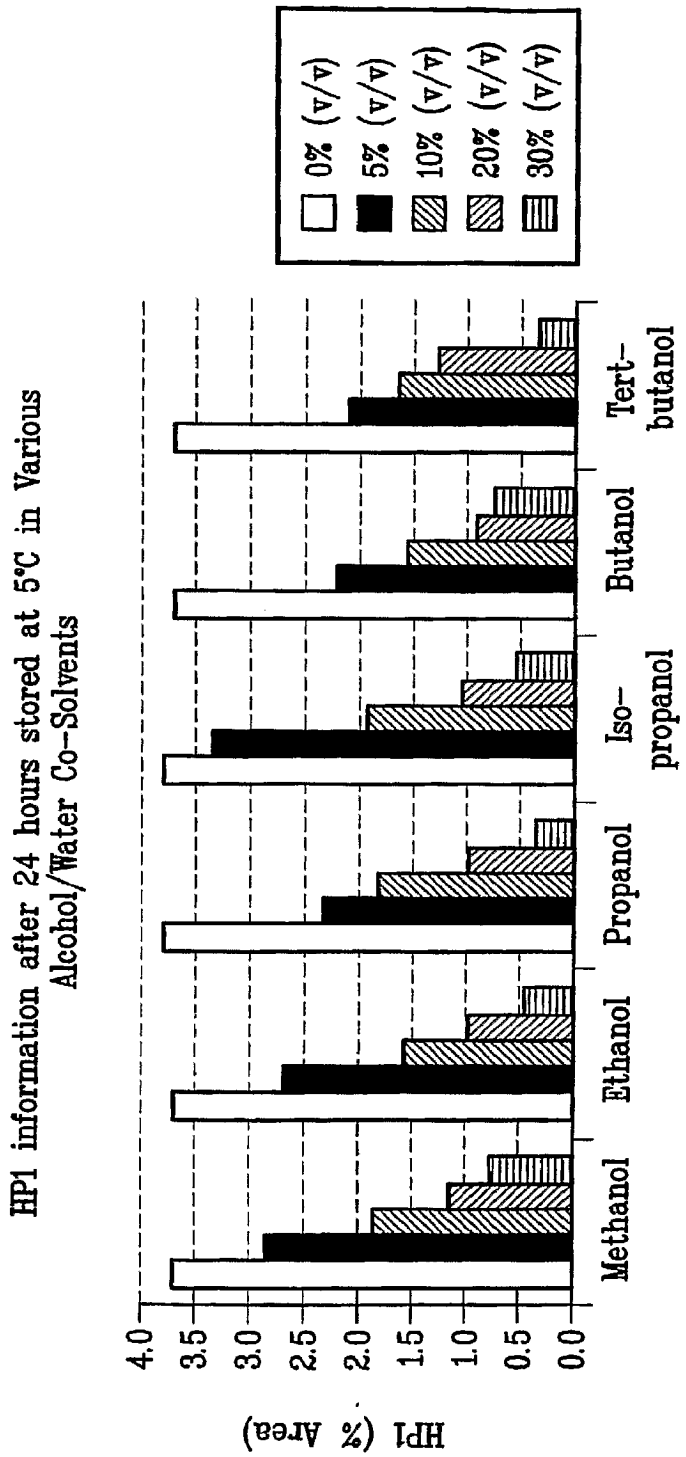


FIG. 3

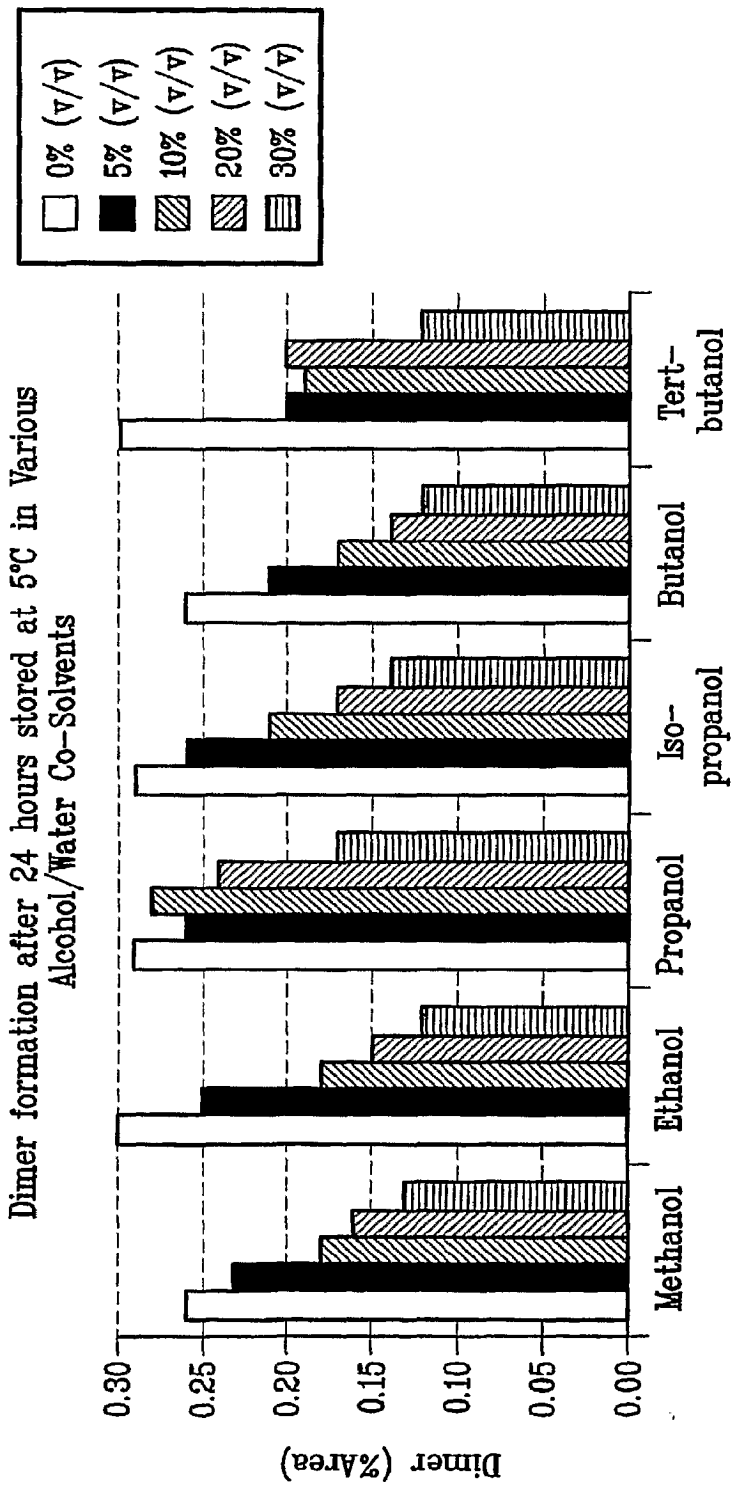


FIG. 4

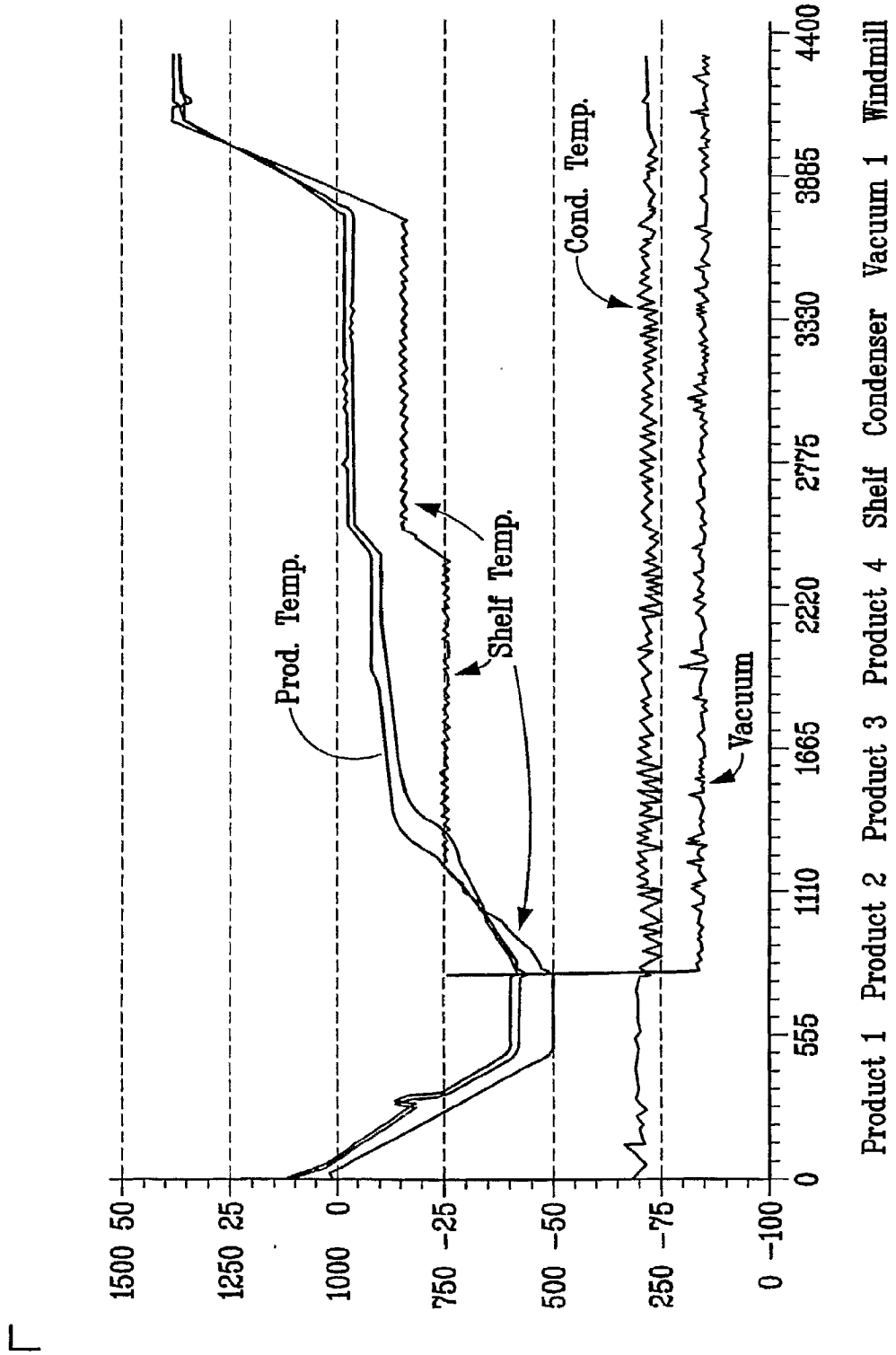


FIG. 5

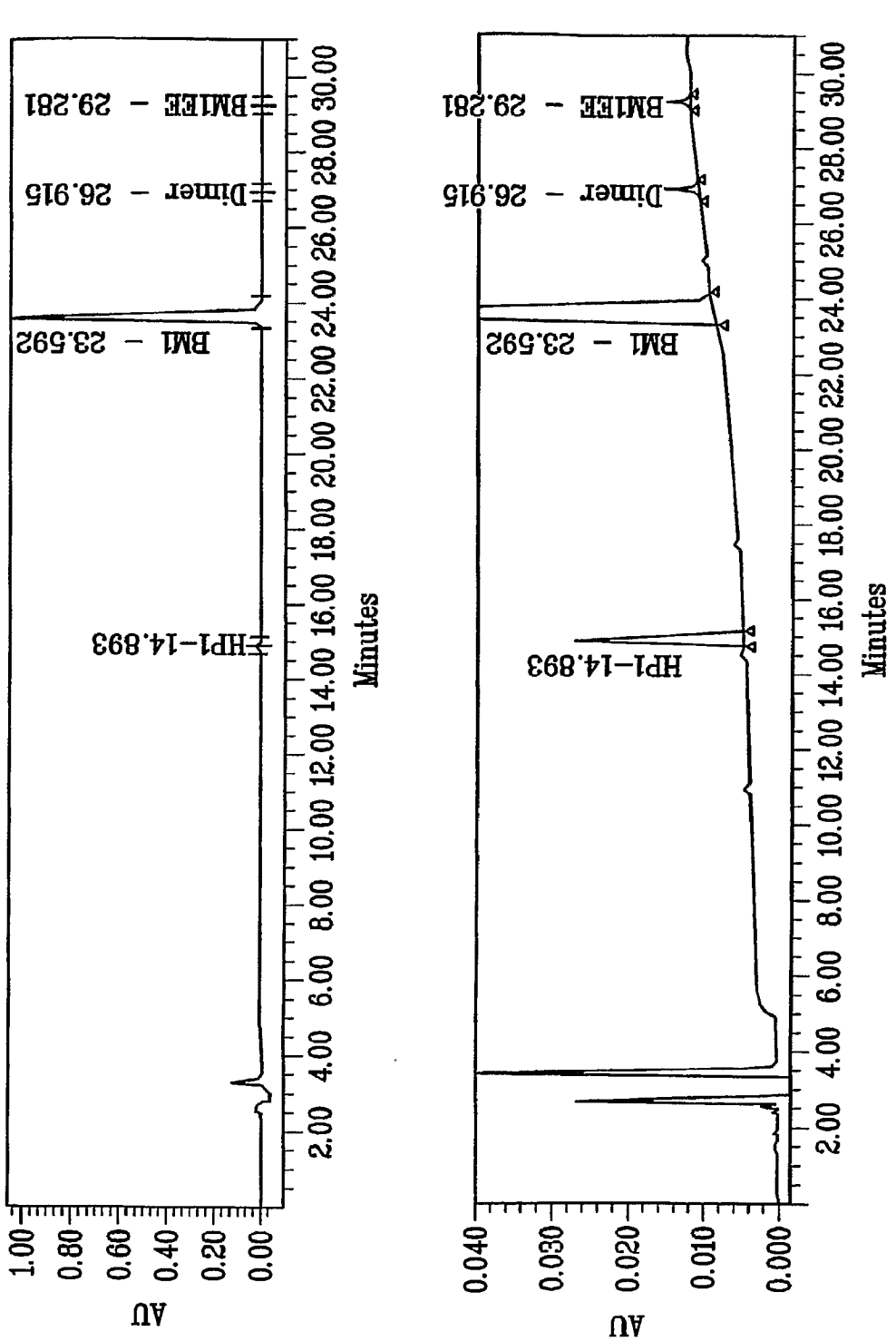


FIG. 6

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 October 2009 (01.10.2009)

PCT

(10) International Publication Number
WO 2009/120386 A2

(51) International Patent Classification:
A61K 9/19 (2006.01)

(21) International Application Number:
PCT/US2009/001956

(22) International Filing Date:
26 March 2009 (26.03.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/039,752 26 March 2008 (26.03.2008) US

(71) Applicant *(for all designated States except US)*: **CEPHALON, INC.** [US/US]; 41 Moores Road, P.o. Box 4011, Frazer, PA 19355 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **COOPER, Martin, Ian** [GB/GB]; 29 West Hill Road, Foxton, Cambridge CB22 6SZ (GB). **COURVOISIER, Laurent, D.** [FR/US]; 212 Turnberry Drive, Thorndale, PA 19372 (US). **EDDLESTON, Mark** [GB/GB]; 17 Harlech Rise, Chilwell, Nottingham NG9 5PD (GB). **MCKEAN, Robert, E.** [US/US]; 16 Houndstooth Lane, Chester Springs, PA 19425 (US).

(74) Agent: **HRUBIEC, Robert, T.**; Cephalon Inc., 41 Moores Road, P.O. Box 4011, Frazer, PA 19355 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: 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.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title**: NOVEL SOLID FORMS OF BENDAMUSTINE HYDROCHLORIDE

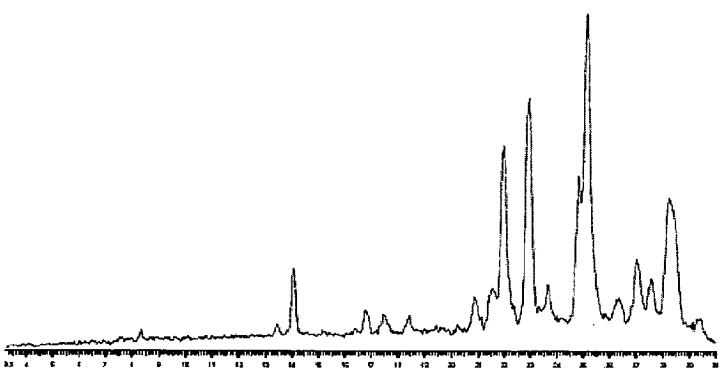


FIG. 2

(57) **Abstract**: Novel solid forms of bendamustine hydrochloride are described, as well as methods of their preparation and use.

WO 2009/120386 A2

NOVEL SOLID FORMS OF BENDAMUSTINE HYDROCHLORIDE

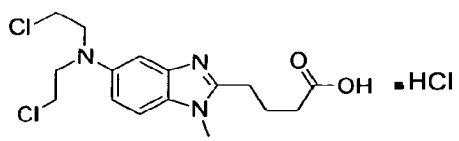
FIELD OF THE INVENTION

This invention pertains to bendamustine-containing compositions, pharmaceutical compositions comprising bendamustine, processes to reproducibly make them, and methods of treating patients using them.

BACKGROUND OF THE INVENTION

Active pharmaceutical ingredients (APIs) can be prepared in a variety of different forms, for example, chemical derivatives, solvates, hydrates, co-crystals, or salts. APIs may also be prepared in different solid forms, in that they may be amorphous, may exist as different crystalline polymorphs, and/or in different solvation or hydration states. By varying the form of an API, it is possible to vary the physical properties thereof. For instance, solid forms of an API typically have different solubilities such that a more thermodynamically stable solid form is less soluble than a less thermodynamically stable solid form. Solid forms can also differ in properties such as shelf-life, bioavailability, morphology, vapor pressure, density, color, and compressibility. Accordingly, variation of the solid state of an API is one of many ways in which to modulate the physical and pharmacological properties thereof.

Bendamustine, 4-{5-[Bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl} butyric acid:



Bendamustine Hydrochloride

was initially synthesized in 1963 in the German Democratic Republic (GDR) and was available from 1971 to 1992 there under the tradename Cytostasan®. *See, e.g.,* W.

Ozegowski and D. Krebs, IMET 3393 γ-[1-methyl-5-bis-(β-chloroethyl)-aminobenzimidazolo-(2)]-butyryl chloride, a new cytostatic agent of the group of benzimidazole nitrogen mustards. Zbl. Pharm. *110*, (1971) Heft 10, 1013-1019, describing the synthesis of bendamustine hydrochloride monohydrate. Since that time, it has been marketed in Germany under the tradename Ribomustin®. Bendamustine is an alkylating agent that has been shown to have therapeutic utility in treating diseases such as chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, and breast cancer.

While bendamustine has been demonstrated as efficacious, it is known to be unstable, especially in aqueous solutions, leading to technical difficulties in its preparation and administration. Researchers, therefore, have investigated methods of improving the preparation and stability of bendamustine and its formulations. For example, German (GDR) Patent No. 159877 discloses a method for preparing bendamustine free base by reaction of the bis-hydroxyl precursor with thionyl chloride followed by recrystallization from water.

German (GDR) Patent No. 34727 discloses a method of preparing derivatives of bendamustine. The described derivatives differ from bendamustine in the substitution at the 1-position.

German (GDR) Patent No. 80967 discloses an injectable preparation of bendamustine hydrochloride monohydrate, ascorbic acid, and water. GDR 80967 describes that lyophilization of compounds such as bendamustine is only possible if the compound is of sufficient stability that it can withstand the processing conditions. The preparation described in GDR 80967 is not lyophilized.

German (GDR) Patent No. 159289 discloses a ready-to use, injectable solution of bendamustine hydrochloride that avoids lyophilization. GDR 159289 describes an anhydrous solution of bendamustine hydrochloride in 1,2-propylene glycol or ethanol.

U.S. Application No. 11/330,868, filed January 12, 2006, assigned to Cephalon, Inc., Frazer, PA, discloses methods of preparing lyophilized pharmaceutical compositions comprising bendamustine hydrochloride.

In light of the potential benefits of different solid forms of APIs and in light of the efficacy of bendamustine, a need exists to identify and prepare novel solid forms of bendamustine hydrochloride.

SUMMARY OF THE INVENTION

Solid forms of bendamustine hydrochloride are described, as well as methods of their preparation. For example, in some embodiments, the invention is directed to a solid form of bendamustine hydrochloride that comprises at least one of bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, amorphous bendamustine hydrochloride, or a mixture thereof. This solid form of bendamustine hydrochloride may be one that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 25.1, 24.9, 22.9, 22.0, and/or 14.1 ± 0.2 degrees 2θ, or that produces an X-ray powder diffraction pattern further comprising one or more of the following reflections: 16.8, 17.5, 18.5, 24.9, and/or 28.3 ± 0.2 degrees 2θ. Alternatively, the solid form of bendamustine hydrochloride may produce an X-ray powder diffraction pattern comprising one or more of the following reflections: 26.1, 27.9, and/or 28.1 ± 0.2 degrees 2θ, or that further produces an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.6, 15.6, and/or 19.8 ± 0.2 degrees 2θ. Other embodiments may produce an X-ray powder diffraction pattern comprising one or more of the following reflections: 10.8, 15.5, 20.5, and/or 23.6 ± 0.2 degrees 2θ, or that produce an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.3, 19.6, 20.7, 21.2, 25.8 and/or 27.6 ± 0.2 degrees 2θ.

Another embodiment of the invention is directed to compositions comprising a solid form of bendamustine hydrochloride, such as described above. In certain embodiments, the composition is a pharmaceutical composition that further comprises at least one pharmaceutically acceptable excipient. In other embodiments, the composition is a lyophilized composition. In certain embodiments the composition comprises a single solid form of bendamustine hydrochloride and is substantially free of other solid forms. Alternatively, the composition may contain a mixture of solid forms, such as a mixture of a crystalline form of bendamustine hydrochloride and amorphous bendamustine. Thus, the composition may, for example, be a lyophilized composition that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 7.98, 10.58, 15.43, 19.64, and/or 19.89 ± 0.2 degrees 2θ.

Methods of preparing the compositions, and methods of using same for use in treating chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma or breast cancer are also described.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a ^{1}H NMR spectrum of bendamustine hydrochloride

FIG. 2 is an X-ray Powder Diffractogram (XRPD) of bendamustine hydrochloride Form 1

FIG. 3 is a Differential Scanning Calorimetry (DSC) Thermogram of bendamustine hydrochloride Form 1

FIG. 4 is a Thermo-Gravimetric Analysis (TGA) Thermogram of bendamustine hydrochloride Form 1

FIG. 5 is a Gravimetric Vapor Sorption (GVS) trace of bendamustine hydrochloride Form 1

FIG. 6 is an X-ray Powder Diffractogram of bendamustine hydrochloride Form 2

FIG. 7A is a DSC Thermogram of bendamustine hydrochloride Form 2

FIG. 7B is a DSC Thermogram of bendamustine hydrochloride Form 2 using a 2 °C per minute heating rate.

FIG. 8 is a TGA Thermogram of bendamustine hydrochloride Form 2

FIG. 9 is a GVS trace of bendamustine hydrochloride Form 2

FIG. 10 is an X-ray Powder Diffractogram of bendamustine hydrochloride Form 3

FIG. 11 is an X-ray Powder Diffractogram of bendamustine hydrochloride Form 4

FIG. 12 is a DSC Thermogram of bendamustine hydrochloride Form 4

FIG. 13 is an X-ray Powder Diffractogram of amorphous bendamustine hydrochloride

FIG. 14 is an X-ray Powder Diffractogram of one embodiment of the present invention comprising amorphous bendamustine hydrochloride, bendamustine hydrochloride Form 4, and mannitol (Lot#426804).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Four polymorphs of crystalline bendamustine hydrochloride are disclosed herein (referred to herein as Form 1, Form 2, Form 3, and Form 4). Also described is amorphous (*i.e.*, non-crystalline) bendamustine hydrochloride. Spectral data relating to these solid forms of bendamustine hydrochloride is depicted in FIGS. 1-14, and methods of preparing each of these forms is presented

In preferred embodiments are solid forms of bendamustine hydrochloride that comprise Form 1, Form 2, Form 3, Form 4, or mixtures thereof. More preferred embodiments are solid forms of bendamustine hydrochloride that are Form 1, Form 3,

Form 4, amorphous bendamustine hydrochloride, or mixtures thereof. In other embodiments, solid forms of the invention may further comprise bendamustine hydrochloride Form 2. These polymorphic solid forms may be identified, for example, by X-ray powder diffraction and characterized by one, two, three, four, five, or more reflection peaks that are characteristic of each polymorphic form. The four crystalline polymorphs (Form 1, Form 2, Form 3, Form 4) and amorphous bendamustine ydrochloride can also be identified by reference to their DSC thermograms, TGA thermograms, and/or GVS traces, which are set forth in FIGS. 1-14. Methods of making solid forms of bendamustine, including each of the described polymorphs, or a mixture of polymorphs, and amorphous bendamustine hydrochloride can be preformed using the techniques described herein.

Any of the solid forms of bendamustine hydrochloide described herein can be a component of a composition comprising bendmustine hydrochloride. In some embodiments, these compositions comprising at least one of the solid forms of bendamustine hydrochloride described herein are substantially free of other solid forms of bendamustine hydrochloride.

Certain of the preferred embodiments of the invention may be characterized, at least in part, by X-ray Powder Diffraction. As is known in the art, crystalline solids produce a distinctive diffraction pattern of peaks, represented in what is referred to as a diffractogram. The peak assignments for a given crystalline material, for example, degree 2θ values, may vary slightly, depending on the instrumentation used to obtain the diffractogram and certain other factors, for example, sample preparation. Nevertheless, these variations should not be more than +/- 0.2 degrees 2θ and the relative spacing between the peaks in the diffractogram will always be the same, regardless of the instrumentation used or the method of sample preparation, and the like.

In preferred embodiments, compositions of the invention are pharmaceutical compositions that further comprise at least one pharmaceutically acceptable excipient. Preferred excipients include, for example, sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof. A more preferred pharmaceutical excipient is mannitol.

In another embodiment of the invention are pharmaceutical compositions comprising Form 1, Form 2, Form 3, Form 4, or mixtures thereof, of bendamustine

hydrochloride. In more preferred embodiments are compositions, preferably pharmaceutical compositions, that comprise Form 1, Form 3, Form 4, amorphous, or mixtures thereof, of bendamustine hydrochloride. In other embodiments, the pharmaceutical compositions further comprise Form 2 or bendamustine hydrochloride. More preferred embodiments of the invention are pharmaceutical compositions comprising one or more of Form 1, Form 2, Form 3, and Form 4 with amorphous bendamustine hydrochloride.

In another embodiment of the invention are lyophilized compositions comprising at least one solid form of bendamustine hydrochloride as described herein. Preferred lyophilized compositions of the invention include those that comprise a mixture of amorphous bendamustine hydrochloride and at least one crystalline form of bendamustine hydrochloride. More preferred lyophilized compositions of the invention include those that comprise a mixture of amorphous bendamustine hydrochloride and bendamustine hydrochloride Form 4.

Lyophilized compositions of the invention can further include at least one pharmaceutically acceptable excipient. Preferred excipients include, for example, sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof. A more preferred pharmaceutical excipient is mannitol. A preferred lyophilzed composition of the invention comprises a mixture of amorphous bendamustine hydrochloride, bendamustine hydrochloride Form 4, and at least one pharmaceutically acceptable excipient that is preferably mannitol. More preferred are lyophilized compositions consisting essentially of amorphous bendamustine hydrochloride, bendamustine hydrochloride Form 4, and mannitol. (*See, e.g.*, FIG. 14)

Form 1 was characterized as a white powder consisting of lath shaped particles. Form 1 was crystalline by X-ray Powder Diffraction (XRPD), the ^{1}H NMR spectrum was consistent with the structure of the molecule, and the purity was 97.2%. Thermal analysis showed an endotherm with onset 167°C (ΔH 103J/g) corresponding to a melting event. (Peak = 170 °C). Degradation occurred above this temperature. The sample became amorphous by XRPD (FIG. 13) on heating to 180°C (melt) and remained amorphous on cooling to ambient temperature. Form 1 was found to have low hygroscopicity, showing a 0.7% weight increase between 0 and 90% relative humidity (RH). This did not lead to a significant change in XRPD pattern upon reanalysis under ambient conditions. There were

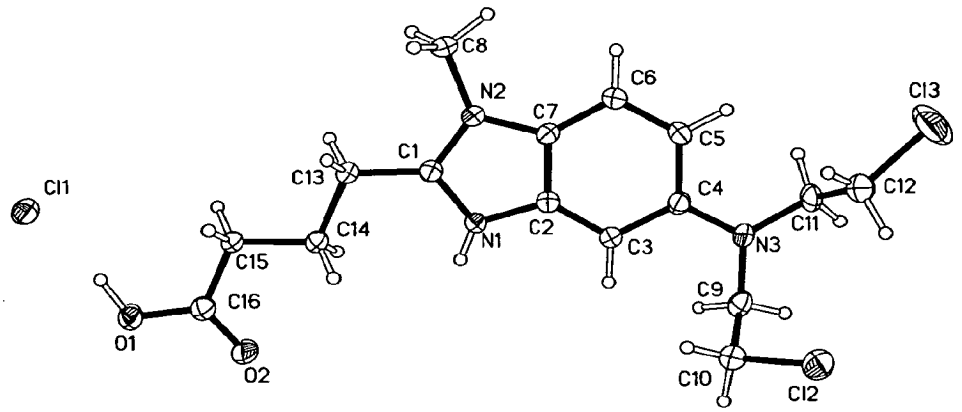
no significant changes during 1 week of storage at 40°C / 75%RH or 3 weeks of storage at 40°C / 11%RH. The data from the XRPD is shown below.

XRPD Data for Bendamustine HCl Form 1

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
8.349	10.59033	110	6.8
13.503	6.55757	129	8
14.049	6.30377	394	24.5
16.824	5.26978	190	11.8
17.51	5.06473	172	10.7
18.452	4.80825	167	10.4
20.239	4.38767	130	8.1
20.904	4.24957	257	16
21.544	4.12484	295	18.3
21.972	4.04537	980	60.9
22.354	3.97705	210	13.1
22.922	3.87977	1213	75.4
23.305	3.81696	215	13.4
23.672	3.7586	317	19.7
24.851	3.58278	833	51.8
25.122	3.54475	1608	100
25.858	3.44558	173	10.7
26.35	3.38229	254	15.8
27.082	3.29256	437	27.2
27.591	3.23295	343	21.3
28.327	3.15055	704	43.8
29.155	3.06303	144	8.9
29.356	3.04246	151	9.4

Form 1 converted to a hydrate of bendamustine hydrochloride (Form 2) during 2 months of storage at 25°C / 94%RH. The aqueous solubility was 4.5mg/ml with a solution pH of 2.16, but significant degradation occurred to the sample in this experiment. The pKa values found for this material by UV in aqueous conditions were 0.88 (Base), 4.17 (Acid) and 6.94 (Base). The LogP value found was 1.10 with a LogD at pH7.4 of 0.68. The single crystal structure of this form was obtained:

A View of the Single Crystal Structure of Form 1 of Bendamustine HCl



Unit Cell Data and Final Residuals for Bendamustine Hydrochloride Form 1

Crystal Data		Form 1
Chemical Formula		$[C_{16}H_{22}N_3O_2]^+ Cl^-$
Molecular weight		394.7
Crystal system		monoclinic
Space group		C2/c
	-193°C	22°C
a (Å)	23.0847(4)	23.080(5)
b (Å)	6.80560(10)	6.882(2)
c (Å)	25.5054(5)	25.504(6)
beta (°)	114.2480(10)	114.09(1)
volume ($Å^3$)	3653.52(11)	3693.8(4)
Z	8	
Density (calculated) (g/ml)	1.435	1.419
R(Fobs)	0.0382	
wR(all, Fsq)	0.1392	
S	1.006	

Form 1 was shown to be more stable to degradation in light, as compared to Form 2.

Form 2, a monohydrate, was characterized as a white powder consisting of rod shaped particles. Form 2 was crystalline by XRPD and the purity was 98.3%. The XRPD data is depicted below.

XRPD Data for Bendamustine HCl Form 2

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
10.169	8.69836	167	8.5
10.638	8.31653	1274	64.6
11.443	7.73271	155	7.9
12.46	7.10378	162	8.2
13.662	6.48137	186	9.4
15.055	5.88491	234	11.9
18.828	4.71319	631	32
19.724	4.50101	206	10.5
20.115	4.41437	955	48.4
20.451	4.34275	1017	51.6
20.95	4.24033	654	33.2
21.45	4.14261	371	18.8
22.15	4.01325	301	15.3
23.105	3.84943	1972	100
23.449	3.79375	373	18.9
23.859	3.72952	236	12
24.101	3.6926	271	13.7
24.511	3.6317	317	16.1
24.849	3.58309	290	14.7
25.204	3.53342	434	22
25.498	3.49344	320	16.2
25.843	3.44749	257	13
26.538	3.35877	788	40
27.248	3.27289	382	19.4
27.695	3.22103	402	20.4
28.018	3.18459	243	12.3
28.256	3.15834	248	12.6
28.487	3.13331	297	15
29.046	3.07423	352	17.9
29.255	3.0527	244	12.4

Thermal analysis showed a broad endotherm with onset at 37°C due to water loss. This corresponded with a 5.2% weight loss on heating between ambient and 100°C, equating to loss of 1.2 equivalents of water, and a conversion to Form 4. The sample showed a 4% uptake between 10 and 15%RH during GVS analysis, equating to 1 mole of water. On XRPD re-analysis after the GVS cycles a peak at 14° 2θ was observed. This peak is indicative of the presence of Form 1, suggesting that partial conversion occurred during the GVS experiment. A similar XRPD trace was obtained after storing pure Form 1 at 25°C / 94%RH for one month as the sample was in the process of converting to Form 2.

There were no significant changes to the sample by XRPD after one month of storage at 40°C / 75%RH, but the sample became less crystalline during one month at 40°C / 11%RH. A significant decrease in crystallinity and purity was observed during light stability experiments.

A review of the prior art indicates that a monohydrate of bendamustine hydrochloride has been prepared previously. *See*, W. Ozegowski and D. Krebs, *supra*. That monohydrate has a reported melting point of 152-56 °C. This melting point is similar to that observed with bendamustine hydrochloride Form 2, which has an observed melting point of 153-157 °C. While not conclusive, it is possible that Form 2 and the bendamustine hydrochloride monohydrate reported in the prior art are the same polymorph. But as no further characterization details, for example XRPD, have been reported or are available for the bendamustine hydrochloride monohydrate reported in the prior art, it is not known whether the monohydrate reported previously was Form 2 bendamustine hydrochloride.

Storage of Form 1, Form 2 and 1:1 mixtures for up to 6 weeks only showed a conversion of Form 1 to 2 after storage at high humidity (60°C/ 95%RH, 25°C 94%RH and possibly 4°C / 88%RH for 6, 6 and 2 weeks respectively). No conversion of Form 2 to Form 1 was noted in these studies after 6 weeks. Kinetic factors make it very difficult to determine the absolute thermodynamic stability in the 6 weeks studied and both forms were kinetically stable for 6 weeks at 4°C/ 34 to 76%RH, 25°C/ 43 to 75%RH and 60°C/ 11 to 75%RH.

Form 3 was characterized as a white powder which was partially crystalline by XRPD. No significant changes were observed on XRPD re-analysis after 1 month of storage under ambient conditions, but conversion to Form 2 occurred during 1 week at 40°C / 75%RH. The purity was 95.9%. XRPD data for Form 3 is shown below.

XRPD Data for Bendamustine HCl Form 3

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
3.85	22.95248	13.6	2.1
5.384	16.41406	16.3	2.5
5.75	15.37009	12.1	1.9
7.892	11.20261	40.4	6.2
10.575	8.36538	177	27.2
13.426	6.59478	30.1	4.6
13.636	6.49389	10.9	1.7
13.993	6.32893	36.3	5.6
14.7	6.0261	7.62	1.2
15.547	5.69958	121	18.6

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
15.734	5.63243	41.4	6.4
17.35	5.1112	25	3.8
17.608	5.0369	14.1	2.2
18.594	4.77186	55.1	8.5
18.85	4.70772	85.8	13.2
19.428	4.56899	80.2	12.3
19.749	4.49541	436	67
19.995	4.44068	173	26.6
21.3	4.17144	216	33.3
22.11	4.02037	233	35.8
23.328	3.81319	409	63
25.449	3.49996	393	60.5
25.571	3.48361	355	54.6
25.733	3.46204	294	45.3
26.083	3.41636	650	100
26.394	3.37675	305	46.9
26.61	3.34983	279	43
27.852	3.2032	393	60.5
27.977	3.1892	403	62
28.109	3.17455	392	60.3
29.039	3.07492	195	30

Form 4 was characterized as a white powder which was crystalline by XRPD. Thermal analysis showed an endotherm due to melting at 153°C. (Peak = 157 °C). Form 4 converted to Form 2 during 24 hours under ambient conditions. XRPD data for Form 4 is depicted below.

XRPD Data for Bendamustine HCl Form 4

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
3.86	22.88824	63.2	4.6
7.794	11.34336	120	8.8
10.267	8.61623	293	21.4
10.831	8.16867	1297	95
11.624	7.61314	149	10.9
11.804	7.4972	134	9.8
12.806	6.91286	169	12.4
14.077	6.29121	209	15.3
15.521	5.70899	376	27.5
16.038	5.5262	135	9.9
18.748	4.73313	168	12.3
19.636	4.52097	455	33.3
20.447	4.34345	1021	74.7
20.734	4.28411	793	58.1
21.227	4.18563	557	40.8
21.865	4.06498	202	14.8
22.263	3.99311	198	14.5
23.1	3.85031	306	22.4

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
23.579	3.77323	1366	100
23.95	3.71555	513	37.5
24.39	3.64947	250	18.3
24.548	3.62633	237	17.3
25.477	3.49624	266	19.5
25.81	3.45184	659	48.3
26.559	3.35619	258	18.9
27.101	3.29025	363	26.6
27.627	3.22885	818	59.9
28.415	3.14102	364	26.6

Amorphous bendamustine hydrochloride had a glass transition temperature of about 50°C and became gummy during 24 hours under ambient conditions, showing it is hygroscopic. Also, partial crystallization occurred during 1 week at 40 °C / 75%RH, possibly to a mixture of Forms 2 and 3. After subjection to GVS humidity cycle, amorphous bendamustine hydrochloride converted to Form 2.

Preferred pharmaceutical compositions of the invention comprise amorphous bendamustine hydrochloride. The bendamustine hydrochloride may be provided as compositions consisting primarily of an amorphous form of bendamustine hydrochloride or as compositions comprising amorphous bendamustine hydrochloride as well as a crystalline form, such as crystalline bendamustine hydrochloride Form 1, Form 2, Form 3, Form 4, or mixtures thereof. Preferred pharmaceutical compositions of the invention comprise bendamustine hydrochloride substantially free from crystalline bendamustine hydrochloride.

In preferred embodiments, pharmaceutical compositions comprising at least one of Form 1, Form 2, Form 3, Form 4, and amorphous bendamustine hydrochloride, as well as at least one pharmaceutically acceptable excipient, are provided. Preferably, the pharmaceutical compositions comprise at least one of Form 1, Form 3, Form 4, and amorphous bendamustine hydrochloride, as well as at least one pharmaceutically acceptable excipient. More preferred are pharmaceutical compositions that comprise amorphous bendamustine hydrochloride, Form 4, and at least one pharmaceutically acceptable excipient.

Pharmaceutically acceptable excipients are known in the art and include those described in, for example, U.S. Application No. 11/267,010, the content of which is incorporate herein in its entirety. These pharmaceutical compositions may be prepared as

injectables, either as liquid solutions or suspensions, as well as solid forms, for example, capsules, tablets, lozenges, pastilles, powders, suspensions, and the like.

In preferred embodiments, the pharmaceutical compositions are sublimed, preferably freeze-dried or lyophilized, compositions. Methods of preparing such sublimed, preferably freeze-dried or lyophilized, preparations of bendamustine hydrochloride that contain Form 1, Form 2, Form 3, Form 4, or a mixture thereof, are also within the scope of the invention. Methods of preparing such sublimed, preferably freeze-dried or lyophilized, preparations of bendamustine hydrochloride that contain Form 1, Form 3, Form 4, amorphous bendamustine hydrochloride, or a mixture thereof, are also within the scope of the invention. Methods of preparing such sublimed, preferably freeze-dried or lyophilized, preparations of bendamustine hydrochloride that further contain Form 2, are also within the scope of the invention.

Lyophilization involves the addition of water to a compound, followed by freezing of the resultant suspension or solution, and sublimation of the water from the compound. In preferred embodiments, at least one organic solvent is added to the suspension/solution. In other preferred embodiments, the suspension/solution further comprises a lyophilization excipient. The lyophilized preparations of bendamustine hydrochloride of the present invention may further comprise amorphous bendamustine hydrochloride.

In a typical lyophilization procedure, water, a pharmaceutically acceptable lyophilizing excipient, an organic solvent, and a compound are combined to form a solution, which is then sterilized, preferably using sterile filtration methodology. This solution is then lyophilized using standard lyophilization equipment and techniques.

While preferred embodiments of the present invention include lyophilization of bendamustine hydrochloride, it is envisioned that other sublimation techniques may also be used. For example, one of more of the described forms of bendamustine hydrochloride may be dissolved, dispersed or suspended in a solvent, the resulting mixture (be it a solution, dispersion or suspension) frozen, and the solvent removed by sublimation.

A lyophilization excipient can be any pharmaceutically acceptable excipient that, when used during the lyophilization process, results in a lyophilzed product that has improved properties, for example, improved handling properties, solubility properties, and the like. A lyophilization excipient can be, for example, a bulking agent; suitable bulking agents are known in the art. Examples of suitable lyophilization excipients include, for example, sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose,

hetastarch, or mixtures thereof. A lyophilization excipient may also comprise a pharmaceutically acceptable antioxidant, such as, for example, ascorbic acid, acetylcysteine, cysteine, sodium hydrogen sulfite, butyl-hydroxylanisole, butyl-hydroxytoluene, or alpha-tocopherol acetate. A preferred lyophilization excipient is mannitol.

Solvents for use in the present invention include water and organic solvents that form stable solutions with bendamustine hydrochloride without appreciably degrading the bendamustine, and which are capable of being evaporated/sublimed through lyophilization. Examples of suitable organic solvents include, for example, methanol, ethanol, n-propanol, iso-propanol, n-butanol, tert-butanol, or mixtures thereof. A preferred organic solvent is tert-butanol.

In one embodiment of the invention are methods of preparing lyophilized compositions that comprise at least one crystalline form of bendamustine hydrochloride. Preferably, the crystalline form of bendamustine hydrochloride is bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 2, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, or a mixture thereof. In other embodiments of the invention, the lyophilized compositions further comprise amorphous bendamustine hydrochloride. More preferred methods of the invention produce lyophilized compositions comprising a mixture of bendamustine Form 4 and amorphous bendamustine hydrochloride.

Preferred methods of preparing lyophilized compositions comprising at least one crystalline form of bendamustine hydrochloride comprise combining bendamustine hydrochloride with at least one solvent to form a solution and then lyophilizing the solution. In some embodiments, the solution further comprises at least one lyophilization excipient. Preferred lyophilization excipients include, for example, sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof. More preferably, the pharmaceutically acceptable excipient is mannitol. In some embodiments, the solvent is water, an organic solvent, or a mixture thereof. Preferably, the organic solvent is methanol, ethanol, n-propanol, iso-propanol, n-butanol, tert-butanol, or a mixture thereof. More preferably, the organic solvent is tert-butanol. In certain embodiments, the solvent is a mixture of water and an organic solvent, for example, a mixture having a ratio of water to organic solvent of from about 1:1 to about 3:1 (v/v), preferably about 7:3 (v/v).

Lyophilized compositions produced according to any of the methods described herein are also within the scope of the invention. An X-ray Powder Diffractogram of one such composition, prepared in accordance with the lyophilization procedures described herein and comprising amorphous bendamustine hydrochloride, bendamustine hydrochloride Form 4, and mannitol is shown in Figure 14. The XPRD data corresponding to this Diffractogram is shown below.

Angle (2-Theta)	d value (Angstrom)	Intensity (Counts)	Intensity (%)
7.98	11.07642	231	6.3
9.75	9.06671	1710	47.0
10.58	8.35697	751	20.7
13.68	6.46585	30	0.8
15.43	5.73932	286	7.9
18.69	4.74293	91	2.5
19.48	4.55224	474	13.1
19.64	4.51705	799	22.0
19.89	4.45920	416	11.5
20.45	4.33901	3635	100.0
21.12	4.20296	1052	29.0
21.30	4.16740	545	15.0
22.15	4.01060	1349	37.1
22.76	3.90380	95	2.6
23.34	3.80874	293	8.1
24.72	3.59834	1153	31.7
25.30	3.51781	1396	38.4
25.43	3.50023	899	24.7
25.91	3.43569	454	12.5
27.95	3.19006	534	14.7
29.39	3.03627	35	1.0
29.73	3.00276	40	1.1
30.64	2.91594	38	1.1
31.20	2.86471	39	1.1
32.22	2.77642	109	3.0
33.65	2.66154	37	1.0
35.00	2.56159	287	7.9
35.34	2.53782	117	3.2
36.11	2.48539	682	18.8
36.23	2.47719	538	14.8
36.58	2.45430	105	2.9
38.04	2.36363	27	0.8
39.53	2.27806	36	1.0

Also within the scope of the invention are methods of treating diseases, such as, for example, chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, or breast cancer, with a pharmaceutical composition of the present invention. Preferably, the solid forms of the invention are used to treat chronic

lymphocytic leukemia. Also preferred are methods of using the solid forms of the invention to treat indolent B-cell non-Hodkin's lymphoma, in particular, indolent B-cell non-Hodgkin's lymphoma that has progressed during or within six months of treatment with, for example, rituximab or a rituximab-containing regimen. In certain embodiments, the method comprises administering a therapeutically effective amount of a pharmaceutical composition of the present invention directly to the patient (for example, when the pharmaceutical composition is a tablet or capsule). In other embodiments, the method comprises modifying a pharmaceutical composition of the present invention before administration, such as by dissolving the composition in water or another solvent prior to administration. In these embodiments, the method comprises administering to the patient a therapeutically effective amount of a preparation prepared from a pharmaceutical composition of the present invention. Preferably, the preparation is an injectable preparation. The injectable preparation may be administered subcutaneously, intracutaneously, intravenously, intramuscularly, intra-articularly, intrasynovially, intrasternally, intrathecally, intralesionally, intracranially or via infusion. Other conditions amenable to treatment utilizing the compositions and injectable preparations of the present invention include small cell lung cancer, hyperproliferative disorders, and autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and lupus.

Preferably, the dose administered is about 100 mg/m^2 or about 120 mg/m^2, administered intravenously. Dosages of about 25 mg/m^2, 60 mg/m^2, 50 mg/m^2 and 90 mg/m^2, administered intravenously, are also within the scope of the invention. Preferably, the dosage is administered intravenously over about 30 minutes or over about 60 minutes. Also preferred are methods of administration wherein the dosage is administered on days 1 and 2 of a 28-day cycle. In some embodiments, the dosage is administered in from 1 to 6 or from 1 to 8 cycles.

The injectable preparations described herein are in the form of a sterile injectable preparation, for example, as a sterile, injectable aqueous or oleaginous suspension or solution formulated according to techniques known in the art. Typically, the pharmaceutical compositions of the present invention, containing at least one of Form 1, Form 2, Form 3, Form 4, or amorphous bendamustine hydrochloride, preferably at least one of Form 1, Form 3, Form 4, or amorphous bendamustine hydrochloride, are formulated as lyophilized powders which may be provided, for example, in vials containing 100 mg of drug per 50 mL or 20 mL vial. The injectable preparation may be prepared by reconstitution of a freeze-dried or lyophilized composition with Sterile Water

for Injection and then further dilution with a pharmaceutically acceptable intraveneous solution, such as, for example, 0.9% sodium Chloride, 5% dextrose in water (D5W), Lactated Ringers solution, or 0.45% Sodium Chloride/2.5% dextrose.

Preferably, the pharmaceutical compositions of bendamustine hydrochloride described herein are reconstituted into an injectable preparation, for example, with sterile water, in less than about 20 minutes. More preferably, reconstitution occurs in less than about 10 minutes, most preferably about 5 minutes.

A typical reconstitution process would include reconstituting, preferably aseptically, 100 mg bendamustine hydrochloride with 20 mL Sterile Water for Injection. This yields a clear, colorless to pale yellow solution having a bendamustine HCl concentration of 5 mg/mL. If lyophilized bendamustine hydrochloride is being reconstituted, the bendamustine hydrochloride should completely dissolve in about 5 minutes. The volume needed for the required dose (based on 5 mg/mL concentration) can be aseptically withdrawn and transferred to a 500 mL infusion bag of 0.9% Sodium Chloride (or other pharmaceutically acceptable intravenous solution) for injection. Preferably, the reconstituted solution is transferred to the infusion bag within 30 minutes of reconstitution. After transfer, the contents of the infusion bag are thoroughly mixed. Administration by intravenous infusion is typically provided over a time period of from about 30 to about 60 minutes.

It is envisioned that the pharmaceutical compositions of the present invention can be administered in combination with one or more anti-neoplastic agents where the anti-neoplastic agent is given prior to, concurrently with, or subsequent to the administration of the composition of the present invention. Pharmaceutically acceptable anti-neoplastic agents are known in the art. Preferred anti-neoplastic agents are those disclosed in co-pending U.S. Application No. 11/330,868, filed January 12, 2006, the entirety of which is incorporated herein by reference.

Therapeutically effective amounts of bendamustine can be readily determined by an attending diagnostician by use of conventional techniques. The effective dose can vary depending upon a number of factors, including type and extent of progression of the disease or disorder, overall health of a particular patient, biological efficacy of bendamustine, formulation of bendamustine, and route of administration of the forms of bendamustine. Bendamustine can also be administered at lower dosage levels with gradual increases until the desired effect is achieved.

TERMINOLOGY

The term "anti-solvent," as used herein, means a solvent in which a compound is substantially insoluble.

The term "crystalline," as used herein, means having a regularly repeating arrangement of molecules or external face planes.

The term "crystalline composition," as used in herein, refers to a solid chemical compound or mixture of compounds that provides a characteristic pattern of peaks when analyzed by x-ray powder diffraction; this includes, but is not limited to, polymorphs, solvates, hydrates, co-crystals, and desolvated solvates.

The term "isolating" as used herein, means separating a compound from a solvent, anti-solvent, or a mixture of solvent and anti-solvent to provide a solid, semisolid or syrup. This is typically accomplished by means such as centrifugation, filtration with or without vacuum, filtration under positive pressure, distillation, evaporation or a combination thereof. Isolating may or may not be accompanied by purifying during which the chemical, chiral or chemical and chiral purity of the isolate is increased. Purifying is typically conducted by means such as crystallization, distillation, extraction, filtration through acidic, basic or neutral alumina, filtration through acidic, basic or neutral charcoal, column chromatography on a column packed with a chiral stationary phase, filtration through a porous paper, plastic or glass barrier, column chromatography on silica gel, ion exchange chromatography, recrystallization, normal-phase high performance liquid chromatography, reverse-phase high performance liquid chromatography, trituration and the like.

The term "pharmaceutically acceptable excipient," as used herein, includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art, such as in *Remington: The Science and Practice of Pharmacy*, 20th ed.; Gennaro, A. R., Ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2000. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The term "solution," as used herein, refers to a mixture containing at least one solvent and at least one compound that is at least partially dissolved in the solvent.

The term "solvate," as used herein, means a crystalline composition of variable stoichiometry formed by a solute and an organic solvent as defined herein.

The term "solvent," as used herein, means a substance, typically a liquid, that is capable of completely or partially dissolving another substance, typically a solid. Solvents for the practice of this invention include, but are not limited to, water, acetic acid, acetone, acetonitrile, benzene, chloroform, carbon tetrachloride, dichloromethane, dimethylsulfoxide, 1,4-dioxane, ethanol, ethyl acetate, butanol, tert-butanol, N,N-dimethylacetamide, N,N-dimethylformamide, formamide, formic acid, heptane, hexane, isopropanol, methanol, methyl ethyl ketone (butanone), 1-methyl-2-pyrrolidinone, mesitylene, nitromethane, polyethylene glycol, propanol, 2-propanone, propionitrile, pyridine, tetrahydrofuran, toluene, xylene, mixtures thereof and the like.

The term "sublimation," as used herein, refers to the transition from the solid phase to the gas phase with no intermediate liquid stage.

The term "substantially free," as used herein with regard to compositions that contain a particular form of bendamustine hydrochloride while being "substantially free" of other forms of the compound, means that the recited form is associated with less than 10%, preferably less than 5%, in particular less than 2% and most preferably less than 1% of the other recited forms of bendamustine hydrochloride.

The term "therapeutically effective amount," as used herein, refers to the amount determined to be required to produce the physiological effect intended and associated with a given drug, as measured according to established pharmacokinetic methods and techniques, for the given administration route. Appropriate and specific therapeutically effective amounts can be readily determined by the attending diagnostician, as one skilled in the art, by the use of conventional techniques. The effective dose will vary depending upon a number of factors, including the type and extent of progression of the disease or disorder, the overall health status of the particular patient, the relative biological efficacy of the compound selected, the formulation of the active agent with appropriate excipients, and the route of administration.

INSTRUMENTATION

X-Ray Powder Diffraction (XRPD)

The novel crystalline forms of bendamustine hydrochloride have been characterized by XRPD which produces a fingerprint of the particular crystallite form. Measurements of 2θ values typically are accurate to within ±0.2 degrees.

Bruker AXS/Diemens D5000

X-Ray Powder Diffraction patterns were collected on a Siemens D5000 diffractometer using CuKα radiation (40kV, 40mA), θ–θ goniometer, automatic divergence and receiving slits, a graphite secondary monochromator and a scintillation counter. The instrument is performance checked using a certified corundum standard (NIST 1976).

Ambient conditions - Samples run under ambient conditions were prepared as flat plate specimens. Approximately 35mg of the sample was gently packed into a cavity cut into polished, zero-background (510) silicon wafer and a Mylar cover was placed over the sample. The sample was rotated in its own plane during analysis.

Bruker AXS C2 GADDS

X-Ray Powder Diffraction patterns were collected on a Bruker AXS C2 GADDS diffractometer using Cu Kα radiation (40kV, 40mA), automated XYZ stage, laser video microscope for autosample positioning and a HiStar 2-dimensional area detector. X-ray optics consists of a single Göbel multilayer mirror coupled with a pinhole collimator of 0.3mm.

The beam divergence, i.e. the effective size of the X-ray beam on the sample, was approximately 5mm. A θ–θ continuous scan mode was employed with a sample - detector distance of 20cm which gives an effective 2θ range of 3.2° – 29.7°. Typically, the sample would be exposed to the X-ray beam for 120 seconds.

Ambient conditions - Samples run under ambient conditions were prepared as flat plate specimens using powder without grinding. Approximately 1-2mg of the sample was lightly pressed on a glass slide to obtain a flat surface.

Non-ambient conditions - Samples run under non-ambient conditions were mounted on a silicon wafer with heatconducting compound. The sample was then heated to the appropriate temperature at ca. 20°C.min^{-1} and subsequently held isothermally for ca 1 minute before data collection was initiated.

Single Crystal X-Ray Diffraction (SCXRD)

The crystals chosen were coated with paratone oil and flash frozen on a (Bruker SMART CCD diffractometer. Data were collected on a Bruker AXS 1K SMART CCD

diffractometer equipped with an Oxford Cryosystems Cryostream cooling device. Structures were solved using either the SHELXS or SHELXD programs and refined with the SHELXL program as part of the Bruker AXS SHELXTL suite. Unless otherwise stated, hydrogen atoms attached to carbon were placed geometrically and allowed to refine with a riding isotropic displacement parameter. Hydrogen atoms attached to a heteroatom were located in a difference Fourier synthesis and were allowed to refine freely with an isotropic displacement parameter.

^{1}H NMR

^{1}H NMR spectra were collected on a Bruker 400MHz instrument equipped with an auto-sampler and controlled by a DRX400 console. Automated experiments were acquired using ICON-NMR v4.0.4 (build 1) running with Topspin v 1.3 (patch level 6) using the standard Bruker loaded experiments. For non-routine spectroscopy, data were acquired through the use of Topspin alone. Samples were prepared in d6-DMSO, unless otherwise stated. Off-line analysis was carried out using ACD SpecManager v 9.09 (build 7703).

Differential Scanning Calorimetry (DSC)

DSC data were collected on a TA Instruments Q1000 equipped with a 50 position auto-sampler. The instrument was calibrated for energy and temperature calibration using certified indium. Typically 0.5-2mg of each sample, in a pin-holed hermetically sealed aluminium pan, was heated at 10°C.min-1 from 25°C to 200°C. A nitrogen purge at 50ml.min-1 was maintained over the sample. The instrument control software was Thermal Advantage v4.6.6 and the data were analyzed using Universal Analysis v4.3A.

Thermo-Gravimetric Analysis (TGA)

TGA data were collected on a TA Instruments Q500 TGA, equipped with a 16 position autosampler. The instrument was temperature calibrated using certified Alumel. Typically 1-2mg of each sample was loaded into a pin-holed hermetically sealed aluminum DSC pan on a pre-tared platinum crucible, and was heated at 10°C.min^{-1} from ambient temperature to 200°C. A nitrogen purge at 60ml.min^{-1} was maintained over the sample. The instrument control software was Thermal Advantage v4.6.6 and the data were analyzed using Universal Analysis v4.3A.

Purity Analysis

Purity analysis was performed on an Agilent HP1100 series system equipped with a diode array detector and using ChemStation software vB.02.01-SR1.

Type of method	Normal Phase		Reverse Phase	√

	Isocratic		Gradient	√
Column:	Zorbax Bonus-RP C14, 150 x 4.6mm, 5µm			
Column Temperature (°C):	30			
Test Sample Make-Up:	NMP / mobile phase A 1:1			
Injection (µl):	2			
Detection: Wavelength, Bandwidth(nm):	254, 8			
Flow Rate (ml.min-1):	1.0			
Phase A:	0.1% TFA in water			
Phase B:	0.1% TFA in acetonitrile			
Timetable:	Time (min)	% Phase A	% Phase B	
	0	93	7	
	5	93	7	
	13	73	27	
	16	73	27	
	25	43	57	
	26	10	90	
	31	10	90	

Thermodynamic Aqueous Solubility by HPLC

Aqueous solubility was determined by suspending sufficient compound in 0.25ml of water to give a maximum final concentration of ≥10mg.ml^{-1} of the parent free-form of the compound. The suspension was equilibrated at 25°C for 24 hours (unless otherwise stated) after which the pH was measured. The suspension was then filtered through a glass fibre C filter into a 96 well plate. The filtrate was then diluted by a factor of 100 times. Quantitation was by HPLC with reference to a standard solution of approximately 0.1mg.ml-1 in DMSO. Different volumes of the standard, diluted and undiluted sample solutions were injected. The solubility was calculated using the peak areas determined by integration of the peak found at the same retention time as the principal peak in the standard injection.

Type of method:	Reverse phase with gradient elution		
Column:	Phenomenex Luna, C18 (2) 5µm 50 x 4.6mm		
Column Temperature (°C):	25		
Injection (µl):	5, 8 and 50		
Detection: Wavelength, Bandwidth (nm) :	260, 80		
Flow Rate (ml.min-1):	2		
Phase A:	0.1% TFA in water		
Phase B:	0.085% TFA in acetonitrile		
Timetable:	Time (min)	% Phase A	% Phase B
	0.0	95	5
	1.0	80	20

	2.3	5	95
	3.3	5	95
	3.5	95	5
	4.4	95	5

Gravimetric Vapor Sorption (GVS)

Sorption isotherms were obtained using a Hiden IGASorp moisture sorption analyser, controlled by CFRSorp software. The sample temperature was maintained at 25°C by a Huber recirculating water bath. The humidity was controlled by mixing streams of dry and wet nitrogen, with a total flow rate of 250ml.min-1. The relative humidity was measured by a calibrated Vaisala RH probe (dynamic range of 0-95%RH), located near the sample. The weight change, (mass relaxation) of the sample as a function of %RH was constantly monitored by themicrobalance (accuracy ±0.001mg). Typically 1-3mg of sample was placed in a tared mesh stainless steel basket under ambient conditions. The sample was loaded and unloaded at 40%RH and 25°C (typical room conditions). A moisture sorption isotherm was performed as outlined below (2 scans giving 1 complete cycle). The standard isotherm was performed at 25°C at 10%RH intervals over a 0-90%RH range.

Parameter	Values
Adsorption -Scan 1	40 -90
Desorption / Adsorption -Scan 2	85 -Dry, Dry -40
Intervals (%RH)	10
Number of Scans	2
Flow rate ($ml.min^{-1}$)	250
Temperature (°C)	25
Stability ($°C.min^{-1}$)	0.05
Minimum Sorption Time (hours)	1
Maximum Sorption Time (hours)	4
Mode	AF2
Accuracy (%)	98

The software uses a least squares minimization procedure together with a model of the mass relaxation, to predict an asymptotic value. The measured mass relaxation value must be within 5% of that predicted by the software before the next %RH value is selected. The minimum equilibration time was set to 1 hour and the maximum to 4 hours.

pKa Determination and Prediction

Data were collected on a Sirius GlpKa instrument with a D-PAS attachment. Measurements were made at 25 °C in aqueous solution by UV. The compound was

initially dissolved in DMSO at 5mg/ml of which 50µl (0.25mg) was used for the titration from pH 1.3 to 9.0. The titration media was ionic-strength adjusted (ISA) with 0.15 M KCl (aq). The data were refined using Refinement Pro software v1.0. Prediction of pKa values was made using ACD pKa prediction software v9.

Log P Determination

Data were collected by potentiometric titration on a Sirius GlpKa instrument using three ratios of octanol : ionic-strength adjusted (ISA) water to generate Log P, Log Pion, and Log D values. The data were refined using Refinement Pro software v1.0. Prediction of Log P values was made using ACD v9 and Syracuse KOWWIN v1.67 software.

Preparation of Bendamustine Hydrochloride (Crude)

Step 1: 4-{5-[Bis-(2-hydroxy-ethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}-butyric acid ethyl ester (27.0 kg) was dissolved in 270 kg chloroform. After cooling to 0 to 5 °C, 19.2 kg thionyl chloride was added over about 1 hour. The mixture was warmed to 25 °C ± 5 °C and stirred for 20 to 24 hours. 75.6 kg hydrochloric acid (32 % aqueous solution) was then added. After phase separation, the organic (lower) phase was removed. The product remained in the aqueous phase.

Step 2: A suspension of activated charcoal in hydrochloric acid was added to the aqueous phase obtained in step 1. The mixture was heated over 1 hour to 85 to 90 °C and stirred for 4 to 5 hours at reflux. The suspension was then filtered and rinsed with aqueous hydrochloric acid. The solvent was distilled off under reduced pressure at a temperature not exceeding 65 °C. 108 kg to 324 kg (108 kg preferred) of warm (35 to45 °C) deionized water was added to induce crystallization.

After crystallization, the mixture was cooled to 20 C ± 5 °C and stirred for an additional 1 to 2 hours or overnight. The product was collected by filtration on a filter dryer, washed with three portions each of 108 to 324 kg (108 kg preferred) deionized water and 108 to 216 kg (108 kg preferred) of cold acetone. The crude product was treated four times each with 54 to 108 kg (54 kg preferred) acetone at reflux for at least 1 hour, in the filter dryer. The suspension was filtered and the product dried at a temperature not higher than 40 °C under reduced pressure, to give 21.4 kg ± 2.1 kg bendamustine hydrochloride crude (70% ± 10%, calculated as dried substance).

Step 3 (optional): The product obtained from step 2 was dissolved in hydrochloric acid (32% aqueous solution) and heated to reflux (85 to 90 °C) for at least 4 hours. To improve color, activated charcoal can be added to the hydrochloric acid and the mixture heated to reflux (85 to 90°C) for at least 4 hours. With activated charcoal, the suspension

was filtered and rinsed with aqueous hydrochloric acid. Solvent was distilled off under reduced pressure at a temperature not exceeding 65 °C. The mixture was then diluted with deionized water. If no crystallization occurred within 15 min, the mixture was seeded. After crystallization, the suspension was stirred at 40 °C ± 5 °C for one hour, then cooled to 20 °C ± 5 °C. After stirring an additional 1 to 2 hours at 20 °C ± 5 °C, the product was collected by filtration, washed three times with cold deionized water, and at least three times with cold acetone. The crude product was treated four times with acetone at reflux for at least 1 hour. The suspension was filtered and the product dried at a temperature not higher than 40 °C, under reduced pressure. Yield was of crude bendamustine hydrochloride was 80% ± 10%.

Preparation of Purified Bendamustine Hydrochloride

Bendamustine HCl crude (15.0 kg) was suspended with 0.45 kg activated charcoal in ethanol / water (vol/vol = 97/3) at room temperature. The mixture was quickly warmed to 75 to 80 °C and stirred for not more than 10 min under reflux conditions. The mixture was filtered to remove the activated charcoal. After filtration, 33.0 kg of filtered acetone was added quickly at 40-50 °C to induce crystallization.

After crystallization, the mixture was stirred for 30 to 60 min at 40-50 °C, then cooled to 0 to 5 °C, and stirred for at least an additional 30 min or overnight. The product was collected by filtration and washed with three 45 kg of cold acetone. After that, the crude product was treated 4 times each with 30 kg acetone at reflux for at least 1 hour. The suspension was filtered and the product dried at a temperature not higher than 40 °C under reduced pressure providing 11.3 ± 1.5 kg bendamustine hydrochloride (75% ± 10%).

Preparation of Bulk Solution (1 L) of Bendamustine Hydrochloride

Under sterile conditions, Water for Injection ("WFI," ~ 65% of total batch size) was transferred to a stainless steel compounding vessel equipped with a mixer. The temperature of the WFI in the compounding tank was adjusted to 15 to 25 °C. Mannitol (25.5 g) was added to the compounding vessel and mixed at for a minimum of 5 minutes while maintaining the solution temperature at 15 to 25 °C. Tertiary butyl alcohol ("TBA," 234.2 g) was added to the compounding vessel. The solution was mixed for a minimum of 5 minutes at 15 to 25 °C. Purified bendamustine HCl (15.0 g) was added to the compounding vessel and mixed for a minimum of 10 minutes while maintaining the solution temperature between 15 to 25 °C. Water for Injection, USP, sufficient to bring

the batch to 1 L was added and mixed for a minimum of 10 minutes. The bulk solution was sterilized by filtration through a 0.22 μm filter using nitrogen at 1-2 bar.

Lyophilization of Filtered Bulk Solution of Bendamustine Hydrochloride

Step 1: The formulated, sterile filtered bendamustine HCl bulk solution was filled by a fully automated filling/stoppering machine. The vials continued to the stoppering station, where they were partially stoppered with pre-sterilized stoppers. Bendamustine HCl drug product was filled to approximately 6.47 g (6.67 mL) in a 20-cc Type I borosilicate tubing glass amber vial. Filled and partially stoppered vials were transferred to the lyophilizer located in the lyophilization area.

Step 2: The filled and partially stoppered vials from step 1 are transferred to the lyophilizer equipped with eight shelves that can be loaded with product-filled trays. The filled and partially stoppered drug product vials were lyophilized. A summary of the freeze drying cycle used during lyophilization of bendamustine HCl drug product is provided in the Table 1 below.

Table 1: Lyophilization Cycle for Bendamustine HCl

Process parameters	Target Setpoint
Loading temperature	5°C
Freezing temperature	Hold at -50°C for 4 hours
Primary drying vacuum	150 microns
Primary drying temperature	Hold at -15°C for 27 hours
Intermediate drying temperature	Hold at -12°C for 7 hours
Secondary drying vacuum	50 microns
Secondary drying temperature	Hold at 40°C for 15 hours

At the end of the lyophilization cycle, the chamber pressure was raised to ~0.6 bar with sterile filtered nitrogen. The vials were hydraulically stoppered by adjusting the shelves to the stoppering position under sterile filtered nitrogen atmosphere. After the vials were stoppered, the shelves were raised, and the chamber was backfilled with sterile filtered air to atmospheric pressure for unloading. This procedure results in about 100 mg of bendamustine HCl/vial.

Preparation of Solutions of Bendamustine Hydrochloride

50 mg of bendamustine hydrochloride Form 1 was weighed into a screw-top vial. Solvent was added in aliquots (with heating to 50°C) until a clear solution was obtained. Observations are recorded in Table 2.

Table 2: Solubility of Bendamustine Hydrochloride

Solvent	Volume Added	Solution Obtained?
Ethanol	1ml	Yes (50°C)
Acetic acid	1ml	Yes (50°C)
Methanol	100µl	Yes (50°C)
Formamide	1ml	Yes (50°C)
DMF	500µl	Yes (50°C)
DMSO	100µl	Yes (50°C)
DMA	500µl	Yes (50°C)

Maturation Experiment

Approximately 10mg of Form 1 bendamustine hydrochloride was slurried in the solvents list in Table 3. The slurries were shaken for 48 hours with alternating 4 hour periods at 50 C and ambient temperature. Any solid material was then isolated by filtration and analyzed by XRPD. Solutions were allowed to evaporate. Results are shown in Table 3 below.

Table 3. Assignment of XRPD Results from Maturation of Bendamustine Hydrochloride

Solvent	XRPD Analysis	Solvent	XRPD Analysis
Ethanol	Form 1	DCM	Form 1
Ethyl acetate	Form 1		
TBME	Form 1	Methyl acetate	Form 1
IPA	Form 1	DMF	Hydrate (Form 2)
Isopropyl acetate	Form 1		
Acetone	Form 1	Dioxane	Form 1
THF	Form 1	Diethyl ether	Form 1
Acetonitrile	Form 1	Anisole	Form 1
Heptane	Form 1	MIBK	Form 1
Water	degradant	Nitromethane	Form 1
Toluene	Form 1	DIPE	Form 1

Methanol	Mix of Form 1 and hydrate (Form 2)	DMA	Hydrate (Form 2)

Crystallization of Bendamustine by Fast Evaporation

Solutions of Bendamustine Hydrochloride in ethanol, acetic acid, methanol, formamide, DMF, DMSO, and DMA were allowed to evaporate under ambient conditions by allowing the uncapped vials of solution to evaporate to dryness (referred to herein as "rapid evaporation"). Resulting solids were analyzed by XRPD. Results are shown in Table 4.

Table 4 Assignment of XRPD Results from Crystallization of Bendamustine Hydrochloride by Fast Evaporation

Solvent	XRPD Analysis
Ethanol	Form 1
Acetic acid	Hydrate (Form 2)
Methanol	Mix of Form 1 and hydrate (Form 2)
DMF	Form 1
DMSO	Form 1
DMA	Form 1

Crystallization of Bendamustine by Slow Evaporation

Solutions of Bendamustine Hydrochloride in ethanol, acetic acid, methanol, formamide, DMF, DMSO, and DMA were allowed to evaporate under ambient conditions by allowing the capped vials of solution, the vial caps having pinholes, to evaporate to dryness under ambient conditions. The rate of evaporation was constrained by use of air tight film covers containing small holes. Resulting solids were analyzed by XRPD. Results are shown in Table 5.

Table 5. Assignment of XRPD Results from Crystallization of Bendamustine Hydrochloride

Solvent	XRPD Analysis
Ethanol	Form 1
Acetic acid	Form 1

Methanol	Mix of Form 1 and hydrate (Form 2)
Formamide	No solid obtained
DMF	Insufficient material
DMSO	Form 1*
DMA	No solid obtained

* Single crystal data presented herein for Form 1 was obtained from a sample recrystallized from DMSO

Cystallization by Anti-Solvent

Toluene was added as anti-solvent to solutions of Bendamustine Hydrochloride in ethanol, acetic acid, methanol, formamide, DMF, DMSO, and DMA to encourage crystallization The volume of toluene added and observations on anti-solvent addition are recorded in Table 6. Solids were isolated by filtration. The Resulting solids were analyzed by XRPD. Results are shown in Table 6.

Table 6. Assignment of XRPD Results from Crystallization of Bendamustine Hydrochloride by Anti-Solvent Addition

Solvent	Anti-Solvent Used	Volume of Anti-solvent	Observations	XRPD Analysis
Ethanol	Toluene	10ml	No precipitate - evaporated	Form 1
Acetic acid	Toluene	0.5ml	Precipitate	Form 1
DMF	Toluene	0.5ml	Precipitate	Form 1
DMSO	Toluene	1ml	Precipitate	Form 1
DMA	Toluene	0.5ml	Precipitate	Form 1

Preparation of Form 2 from Form 1 of Bendamustine Hydrochloride

One mL of water was added 30 mg of bendamustine hydrochloride Form 1 and the mixture warmed to 25 °C to provide a clear solution. After about 4 minutes, Form 2 precipitated from solution as a white solid. The solid was collected by filtration.

Stability of Forms 1 and 2 of Bendamustine Hydrochloride

10 mg of bendamustine hydrochloride Form 1 (A), bendamustine hydrochloride Form 2 (B), and a 1:1 mixture of Forms 1 and 2 (C) were stored under the conditions listed in Table 7. Samples were analyzed by XRPD at 1 day, 2 week, and 6 week time points. The results are shown in Table 7A. Under high humity conditions (~90%RH), conversion of Form 1 of bendamustine hydrochloride to Form 2 was observed. The rate of this conversion appears to increase with temperature. The purity of Forms 1 and 2 after storage

at 4°C / 87%RH (5) and 60°C / 75%RH (13) for 6 weeks was measured. No large purity decreases were observed.

Table 7. Bendamustine Hydrochloride Stability Study Conditions

Condition	Temperature (°C)	Relative Humidity (%RH)
1	4	33.6 (Magnesium Chloride)
2	4	43.1 (Potassium Carbonate)
3	4	58.9 (Magnesium Nitrate)
4	4	75.7 (Sodium Chloride)
5	4	87.7 (Potassium Chloride)
6	25	43.2 (Potassium Carbonate)
7	25	57.6 (Sodium Bromide)
8	25	75.3 (Sodium Chloride)
9	25	93.6 (Potassium Nitrate)
10	60	11.0 (Lithium Chloride)
11	60	29.3 (Magnesium Chloride)
12	60	~ 43 (Potassium Carbonate)
13	60	74.5 (Sodium Chloride)
14	60	~ 95 (Potassium Sulphate)

5

Table 7A. XRPD Analysis of Stability Study Samples of Bendamustine Hydrochloride

Condition	XRPD Analysis after 1 Day	XRPD Analysis after 2 Weeks	XRPD Analysis after 6 Weeks
1	No changes	No changes	No changes
2	No changes	No changes	No changes
3	No changes	No changes	No changes
4	No changes	No changes	No changes
5	No changes	C) Fully converted to Form 2	C) Some Form 1 now present
6	No changes	No changes	No changes
7	No changes	No changes	No changes
8	No changes	No changes	No changes
9	No changes	A) Partially converted to Form 2 C) Fully converted to Form 2	A) Partially converted to Form 2 C) Fully converted to Form 2
10	No changes	No changes	No changes
11	No changes	No changes	No changes

Condition	XRPD Analysis after 1 Day	XRPD Analysis after 2 Weeks	XRPD Analysis after 6 Weeks
12	No changes	No changes	No changes
13	Not performed	No changes	No changes
14	Not performed	A) Partially converted to Form 2 B) Sample deliquesced C) Fully converted to Form 2	A) Fully converted to Form 2 B) Sample deliquesced C) Fully converted to Form 2

Light Stability of Bendamustine Hydrochloride

Samples of Form 1 and Form 2 of Bendamustine Hydrochloride were stressed in a Suntest Light Box with a light intensity of 250 watts/m2 for 1 week with the black body temperature set to 25 °C. A blank of each sample, wrapped in foil for protection, was also included in the experiment. After the experiment, samples were analyzed by XRPD and the purity was determined by HPLC. A significant decrease in both crystallinity and purity was observed for Form 2 during the light stress test. In contrast, Form 1 showed only a slight decrease in purity. *See* Table 8.

Table 8. XRPD and Purity Analysis of Stability Study Samples of Bendamustine Hydrochloride

Sample	XRPD	Purity (%)
Form 1 blank	No change	97.3
Form 1	No change (sample brown in colour)	95.9
Form 2 blank	No change	95.6
Form 2	Less crystalline (sample brown in colour)	68.7

In certain embodiments, the invention is directed to a pharmaceutical composition comprising bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 2, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, or a mixture thereof. The invention is also directed to those pharmaceutical compositions wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 1. The invention is also directed to those pharmaceutical compositions wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 2. The invention is also directed to those pharmaceutical compositions wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 3. The invention is also directed to those pharmaceutical compositions wherein the bendamustine hydrochloride is bendamustine hydrochloride

Form 4. The invention is also directed to those pharmaceutical compositions, further comprising amorphous bendamustine hydrochloride.

Other embodiments of the invention are directed to a crystalline form of bendamustine hydrochloride that is bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 2, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, or a mixture thereof. The invention is also directed to crystalline forms, wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 1 The invention is also directed to crystalline forms, wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 2. The invention is also directed to crystalline forms, wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 3. The invention is also directed to crystalline forms, wherein the bendamustine hydrochloride is bendamustine hydrochloride Form 4.

Other embodiments of the invention are directed to a crystalline form of bendamustine hydrochloride that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 25.12, 24.85, 22.92, 21.97, and/or 14.05 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride that produce an X-ray powder diffraction pattern further comprising one or more of the following reflections: 16.82, 17.51, 18.45, 24.85, and/or 28.33 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride having an X-ray powder diffraction pattern substantially as depicted in FIG. 2. The invention is also directed to pharmaceutical compositions comprising the crystalline form of bendamustine hydrochloride as set forth herein.

Other embodiments of the invention are directed to a crystalline form of bendamustine hydrochloride that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 10.64, 20.12, 20.45, and/or 23.11 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride that produce an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.17, 15.06, 18.82, 20.95, 25.20, 26.54, and/or 29.05 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride having an X-ray powder diffraction pattern substantially as depicted in FIG. 6. The

invention is also directed to pharmaceutical compositions comprising the crystalline form of bendamustine hydrochloride as set forth herein.

Other embodiments of the invention are directed to a crystalline form of bendamustine hydrochloride that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 26.08, 27.85, and/or 28.11 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride that produce an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.58, 15.55, and/or 19.75 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride having an X-ray powder diffraction pattern substantially as depicted in FIG. 10. The invention is also directed to pharmaceutical compositions comprising the crystalline form of bendamustine hydrochloride as set forth herein.

Other embodiments of the invention are directed to a crystalline form of bendamustine hydrochloride that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 10.83, 15.52, 20.45, and/or 23.58 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride that produce an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.27, 19.64, 20.73, 21.23, 25.81 and/or 27.63 ± 0.2 degrees 2θ. The invention is also directed to crystalline forms of bendamustine hydrochloride having an X-ray powder diffraction pattern substantially as depicted in FIG. 11. The invention is also directed to pharmaceutical compositions comprising the crystalline form of bendamustine hydrochloride as set forth herein.

Other embodiments of the invention are directed to a lyophilized composition comprising bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 2, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, or a mixture thereof. In certain embodiments, the bendamustine hydrochloride is bendamustine Form 1. In other embodiments, the bendamustine hydrochloride is bendamustine Form 2. In other embodiments, the bendamustine hydrochloride is bendamustine Form 3. In other embodiments, the bendamustine hydrochloride is bendamustine Form 4. The invention is also directed to lyophilized compositions described herein further comprising amorphous bendamustine hydrochloride.

A preferred embodiment of the invention includes a lyophilized composition as described herein, comprising amorphous bendamustine hydrochloride, bendamustine hydrochloride Form 2, and a pharmaceutically acceptable excipient.

Also within the scope of the invention is a method for preparing a lyophilized composition comprising a crystalline form of bendamustine hydrochloride comprising the steps of combining bendamustine hydrochloride with at least one solvent to form a mixture; and
lyophilizing the mixture. Preferably, methods of the invention include those wherein the solution further comprises a lyophilization excipient. Preferably, the lyophilization excipient is sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof. More preferably, the lyophilization excipient is mannitol. Preferably, methods of the invention include those wherein the solvent is water, an organic solvent, or a mixture thereof. Preferably, the organic solvent is methanol, ethanol, n-propanol, iso-propanol, n-butanol, tert-butanol, or a mixture thereof. More preferably, the organic solvent is tert-butanol. In other methods of the invention, the solvent is a mixture of water and an organic solvent. In preferred methods of the invention, the ratio of the water to the organic solvent is about 1:1 (v/v). In preferred methods of the invention, the ratio of the water to the organic solvent is about 2:1 (v/v) In preferred methods of the invention, the ratio of the water to the organic solvent is about 3:1 (v/v) In preferred methods of the invention, the ratio of the water to the organic solvent is about 7:3 (v/v).

In preferred methods of the invention, the crystalline form of bendamustine hydrochloride is Form 1. In other preferred methods of the invention, the crystalline form of bendamustine hydrochloride is Form 2. In still other preferred methods of the invention, the crystalline form of bendamustine hydrochloride is Form 3. In yet other preferred methods of the invention, the crystalline form of bendamustine hydrochloride is Form 4. Other preferred methods of the invention include those wherein the lyophilized composition further comprises amorphous bendamustine hydrochloride.

Also within the scope of the invention are method of treating chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma or breast

cancer comprising administering to a patient in need thereof a therapeutically effective amount of a preparation prepared from a composition as described herein.

Also within the scope of the invention are methods of preparing Form 1 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in ethanol, ethyl acetate, tert-butyl methyl ether, iso-propyl alcohol, isopropyl acetate, dichloromethane, methyl acetate, acetone, tetrahydrofuran, acetonitrile, heptane, toluene, methanol, dioxane, diethyl ether, anisole, nitromethane, or di-isopropyl ether, and evaporating the solution under ambient conditions.

Also within the scope of the invention are methods of preparing Form 1 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in ethanol, methanol, dimethylformamide, dimethylsulfoxide, or dimethylamine, and rapidly evaporating the solution to dryness under ambient conditions.

Also within the scope of the invention are methods of preparing Form 1 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in ethanol, acetic acid, methanol, or dimethylsulfoxide, and slowly evaporating the solution to dryness under ambient conditions.

Also within the scope of the invention are methods of preparing Form 1 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in acetic acid, formamide, dimethylformamide, dimethylsulfoxide, or dimethylamine, and adding a sufficient quantity of toluene to induce crystallization.

Also within the scope of the invention are methods of preparing Form 2 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in dimethylformamide, methanol, or dimethylamine and evaporating the solution under ambient conditions.

Also within the scope of the invention are methods of preparing Form 2 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in acetic acid or methanol, and rapidly evaporating the solution to dryness under ambient conditions.

Also within the scope of the invention are methods of preparing Form 2 bendamustine hydrochloride comprising providing a solution of bendamustine hydrochloride in methanol and slowly evaporating the solution to dryness under ambient conditions.

Also within the scope of the invention are methods of preparing Form 2 bendamustine hydrochloride comprising providing an amount of Form 1 bendamustine hydrochloride and storing the amount at a relative humidity of at least about 88% for a period of time sufficient to convert Form 1 to Form 2.

Also within the scope of the invention are methods of preparing Form 2 bendamustine hydrochloride comprising combining bendamustine hydrochloride Form 1 with water to form a solution and allowing Form 2 to precipitate from the solution.

Also within the scope of the invention are methods of preparing Form 3 bendamustine hydrochloride comprising providing an amount of amorphous bendamustine hydrochloride and storing the amount at about 40 °C and about 75% relative humidity for a period of time sufficient to convert amorphous bendamustine hydrochloride to Form 3.

Also within the scope of the invention are methods of preparing Form 4 bendamustine hydrochloride comprising providing an amount of Form 2 bendamustine hydrochloride and heating Form 2 to about 100 °C for a period of time sufficient to convert Form 2 to Form 4.

Also within the scope of the invention are methods of preparing a pharmaceutical composition of bendamustine hydrochloride comprising the steps of:
preparing bendamustine hydrochloride Form 1; and combining the Form 1 with a pharmaceutically acceptable excipient.

Also within the scope of the invention are methods of preparing a pharmaceutical composition of bendamustine hydrochloride comprising the steps of:
preparing bendamustine hydrochloride Form 2; and combining the Form 2 with a pharmaceutically acceptable excipient.

Also within the scope of the invention are methods of preparing a pharmaceutical composition of bendamustine hydrochloride comprising the steps of: preparing bendamustine hydrochloride Form 3; and combining the Form 3 with a pharmaceutically acceptable excipient

Also within the scope of the invention are methods of preparing a pharmaceutical composition of bendamustine hydrochloride comprising the steps of: preparing bendamustine hydrochloride Form 4; and combining the Form 4 with a pharmaceutically acceptable excipient

Also within the scope of the invention are methods of preparing a lyophilized composition of bendamustine hydrochloride comprising the steps of combining Form 1 bendamustine hydrochloride with a solvent to form a mixture; and lyophilizing the mixture. According to the invention, the Form 1 bendamustine hydrochloride is prepared according to any of the methods described herein.

Also within the scope of the invention are methods of preparing a lyophilized composition of bendamustine hydrochloride comprising the steps of combining Form 2 bendamustine hydrochloride a solvent to form a mixture; and lyophilizing the mixture. According to the invention, the Form 1 bendamustine hydrochloride is prepared according to any of the methods described herein.

Also within the scope of the invention are methods of preparing a lyophilized composition of bendamustine hydrochloride comprising the steps of combining Form 3 bendamustine hydrochloride with a solvent to form a mixture; and lyophilizing the mixture. In certain methods of the invention, the Form 3 bendamustine hydrochloride is prepared by providing an amount of amorphous bendamustine hydrochloride and storing the amount at about 40 °C and about 75% relative humidity for a period of time sufficient to convert amorphous bendamustine hydrochloride to Form 3.

Also within the scope of the invention are methods of preparing a lyophilized composition of bendamustine hydrochloride comprising the steps of: combining Form 4 bendamustine hydrochloride with a solvent to form a mixture; and lyophilizing the mixture. In certain methods of the invention, the Form 4 bendamustine hydrochloride is

prepared by providing an amount of Form 2 bendamustine hydrochloride and heating Form 2 to about 100 °C for a period of time sufficient to convert Form 2 to Form 4.

Also within the scope of the invention are lyophilized compositions comprising amorphous bendamustine hydrochloride, wherein said composition is substantially free of any crystalline bendamustine hydrochloride.

In preferred methods of preparing a lyophilized composition of bendmustine hydrochloride, the described mixtures further comprise a lyophilization excipient. Preferably, the lyophilization excipient is sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof. In more preferred methods, the lyophilization excipient is mannitol.

In preferred methods of preparing a lyophilized composition of bendmustine hydrochloride, the solvent is water, an organic solvent, or a mixture thereof. Preferably, the organic solvent is methanol, ethanol, n-propanol, iso-propanol, n-butanol, tert-butanol, or a mixture thereof. In more preferred methods, the organic solvent is tert-butanol.

In preferred methods of preparing a lyophilized composition of bendmustine hydrochloride, the solvent is a mixture of water and an organic solvent. Preferably, the ratio of the water to the organic solvent is about 1:1 (v/v). Also preferred are those methods wherein the ratio of the water to the organic solvent is about 2:1 (v/v). In other preferred methods, the ratio of the water to the organic solvent is about 3:1 (v/v). In other preferred methods, the ratio of the water to the organic solvent is about 7:3 (v/v).

As those skilled in the art will appreciate, numerous modifications and variations of the present invention are possible in view of the above teachings. It is therefore understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described herein, and the scope of the invention is intended to encompass all such variations.

What is Claimed:

1. A solid form of bendamustine hydrochloride that comprises at least one of bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, amorphous bendamustine hydrochloride, or a mixture thereof.

2. The solid form of bendamustine hydrochloride according to claim 1, comprising bendamustine hydrochloride Form 1.

3. The solid form of bendamustine hydrochloride according to claim 1, comprising bendamustine hydrochloride Form 3.

4. The solid form of bendamustine hydrochloride according to claim 1, comprising bendamustine hydrochloride Form 4.

5. The solid form of bendamustine hydrochloride according to claim 1, comprising amorphous bendamustine hydrochloride.

6. The solid form of bendamustine hydrochloride according to any one of the preceding claims further comprising bendamustine hydrochloride Form 2.

7. The solid form of bendamustine hydrochloride according to claim 1 that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 25.1, 24.9, 22.9, 22.0, and/or 14.1 ± 0.2 degrees 2θ.

8. The solid form of bendamustine hydrochloride according to claim 7 that produces an X-ray powder diffraction pattern further comprising one or more of the following reflections: 16.8, 17.5, 18.5, 24.9, and/or 28.3 ± 0.2 degrees 2θ.

9. The solid form of bendamustine hydrochloride according to claim 1 that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 26.1, 27.9, and/or 28.1 ± 0.2 degrees 2θ.

10. The solid form of bendamustine hydrochloride according to claim 9 that produces an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.6, 15.6, and/or 19.8 ± 0.2 degrees 2θ.

11. The solid form of bendamustine hydrochloride according to claim 1 that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 10.8, 15.5, 20.5, and/or 23.6 ± 0.2 degrees 2θ.

12. The solid form of bendamustine hydrochloride according to claim 11 that produces an X-ray powder diffraction pattern further comprising one or more of the following reflections: 10.3, 19.6, 20.7, 21.2, 25.8 and/or 27.6 ± 0.2 degrees 2θ.

13. A composition comprising the solid form of bendamustine hydrochloride according to any one of the preceding claims.

14. A composition comprising the solid form of bendamustine hydrochloride according to any one of claims 1 through 12, wherein the composition is substantially free of other solid forms of bendamustine hydrochloride.

15. A composition according to claim 13 or 14 wherein the composition is a pharmaceutical composition and further comprises at least one pharmaceutically acceptable excipient.

16. The composition of claim 15 wherein the pharmaceutically acceptable excipient is sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof.

17. The composition of claim 16 wherein the excipient is mannitol.

18. A lyophilized composition comprising the solid form of bendamustine hydrochloride according to any one of claims 1 to 12.

19. The lyophilized composition according to claim 18, wherein the composition is substantially free of other solid forms of bendamustine hydrochloride.

20. The lyophilized composition according to claim 18, comprising a mixture of amorphous bendamustine hydrochloride and bendamustine hydrochloride Form 4.

21. The lyophilized composition according to claim 20 that produces an X-ray powder diffraction pattern comprising one or more of the following reflections: 7.98, 10.58, 15.43, 19.64, and/or 19.89 ± 0.2 degrees 2θ.

22. The composition or lyophilized composition according to any one of claims 13 to 21 for use in treating chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma or breast cancer.

23. The composition or lyophilized composition according to claim 22 wherein the non-Hodgkin's lymphoma is indolent B-cell non-Hodgkin's lymphoma.

24. A method for preparing a lyophilized composition that comprises at least one crystalline form of bendamustine hydrochloride, said method comprising the steps of:

combining bendamustine hydrochloride with at least one solvent to form a solution; and

lyophilizing the solution.

25. The method according to claim 24, wherein the solution further comprises at least one lyophilization excipient.

26. The method according to claim 25, wherein the lyophilization excipient is sodium phosphate, potassium phosphate, citric acid, tartaric acid, gelatin, glycine, mannitol, lactose, sucrose, maltose, glycerin, dextrose, dextran, trehalose, hetastarch, or a mixture thereof.

27. The method according to claim 26, wherein the lyophilization excipient is mannitol.

28. The method according to claim 24, wherein the solvent is water, an organic solvent, or a mixture thereof.

29. The method according to claim 28, wherein the organic solvent is methanol, ethanol, n-propanol, iso-propanol, n-butanol, tert-butanol, or a mixture thereof.

30. The method according to claim 29, wherein the organic solvent is tert-butanol.

31. The method according to claim 24, wherein the solvent is a mixture of water and an organic solvent.

32. The method according to claim 31, wherein the ratio of the water to the organic solvent is about 7:3 (v/v).

33. The method according to any one of claims 24 to 32, wherein said crystalline form of bendamustine hydrochloride is bendamustine hydrochloride Form 1, bendamustine hydrochloride Form 2, bendamustine hydrochloride Form 3, bendamustine hydrochloride Form 4, or a mixture thereof.

34. The method according to claim 33, wherein said lyophilized composition further comprises amorphous bendamustine hydrochloride.

35. The method according to claim 33, wherein said lyophilized composition comprises a mixture of bendamustine hydrochloride Form 4 and amorphous bendamustine hydrochloride.

36. The method according to claim 35, wherein said lyophilized composition further comprises mannitol.

37. A lyophilized composition prepared according to the method of any one of claims 24 to 36.

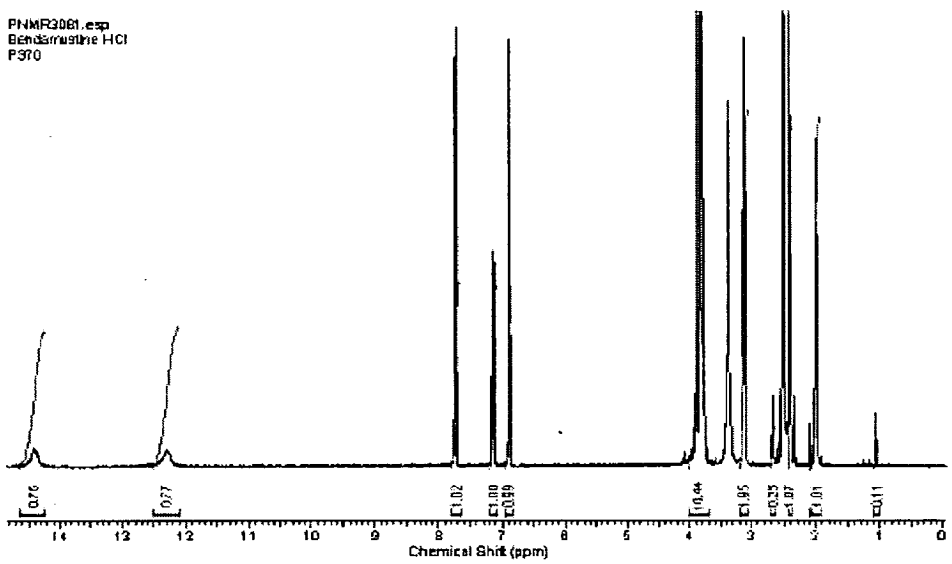


FIG. 1

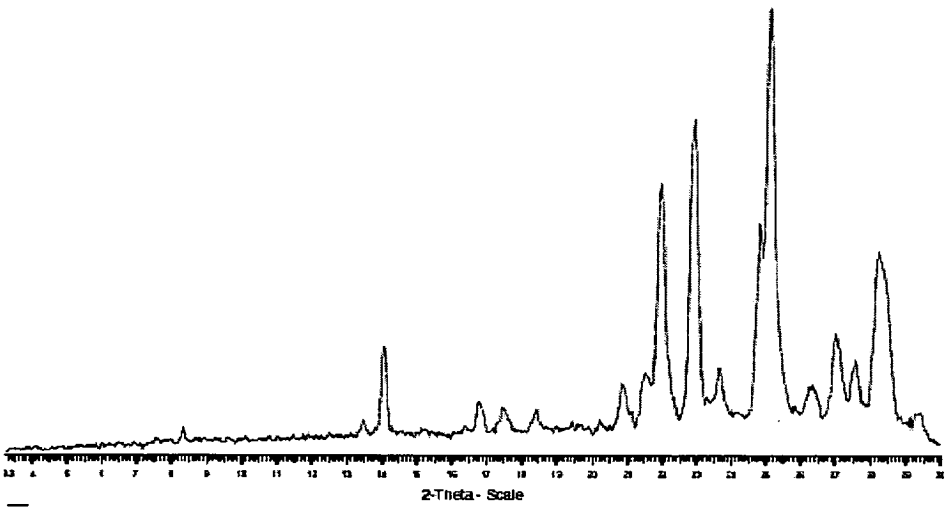


FIG. 2

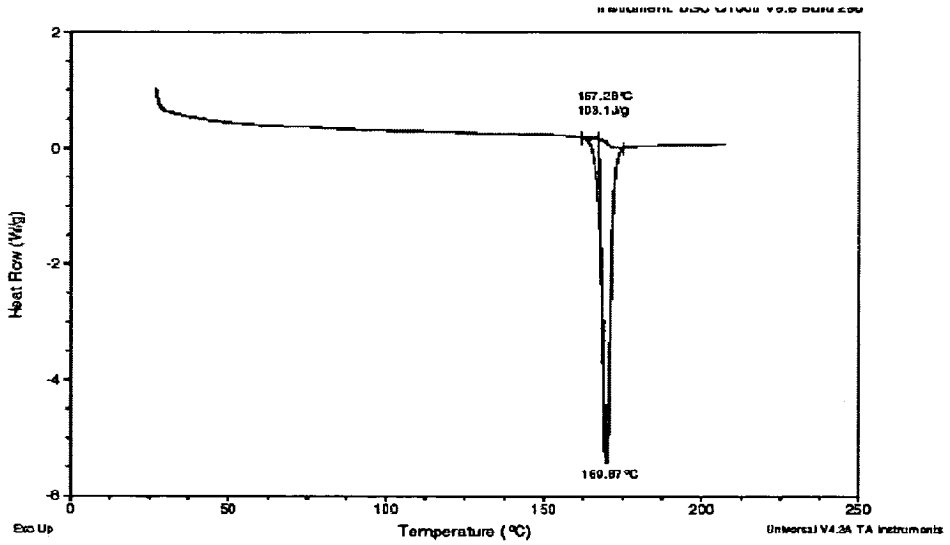


FIG. 3

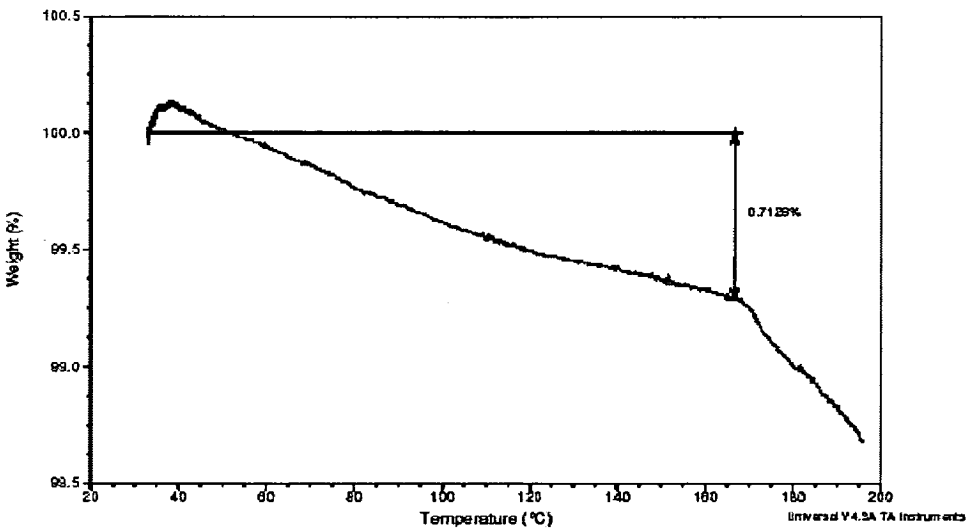


FIG. 4

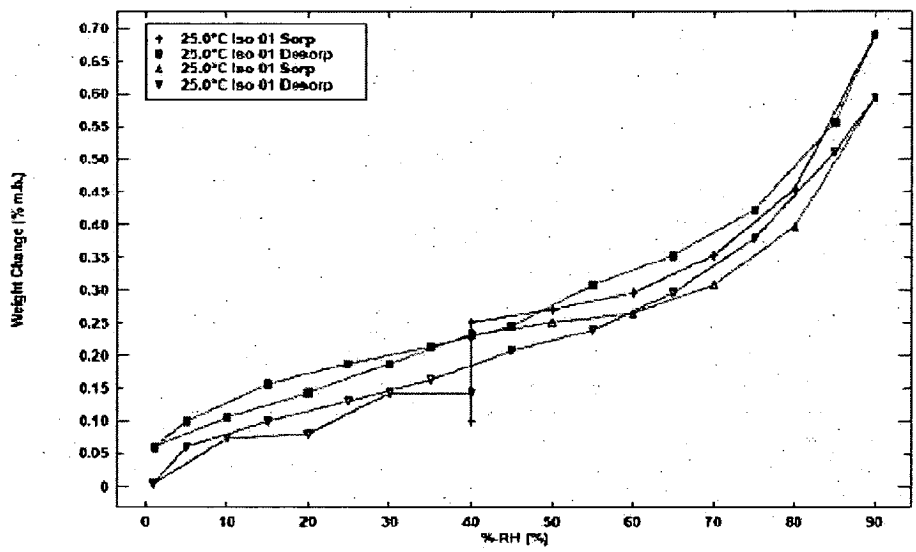


FIG. 5

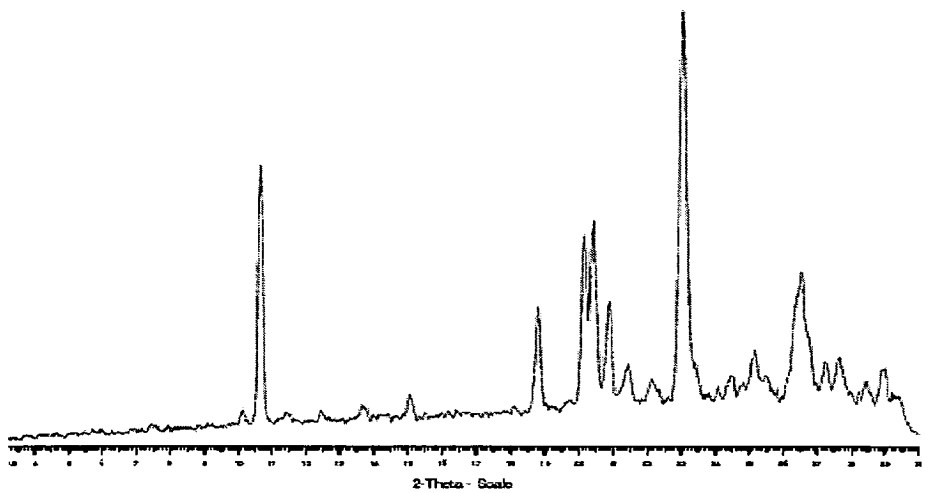


FIG. 6

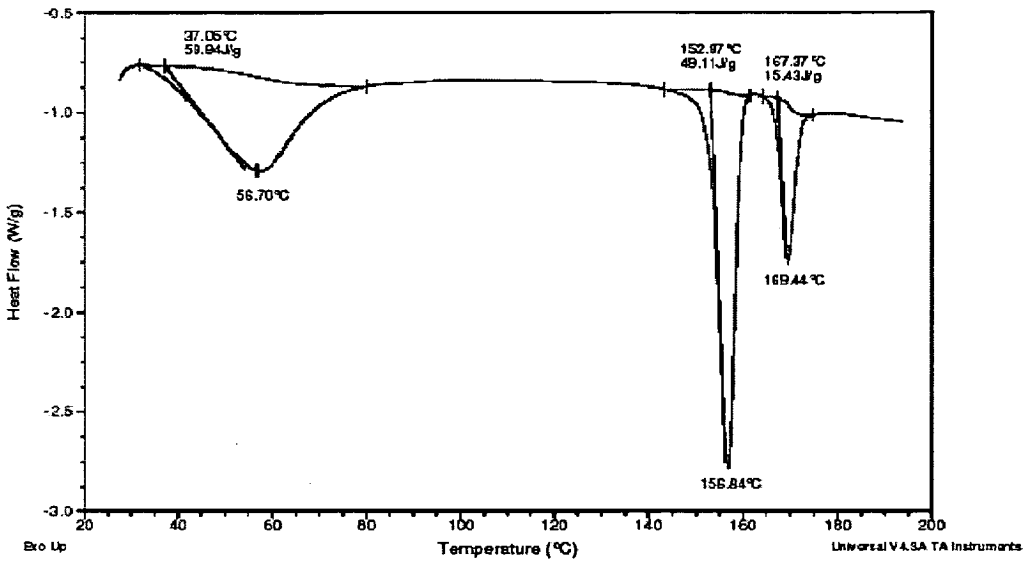


FIG. 7A

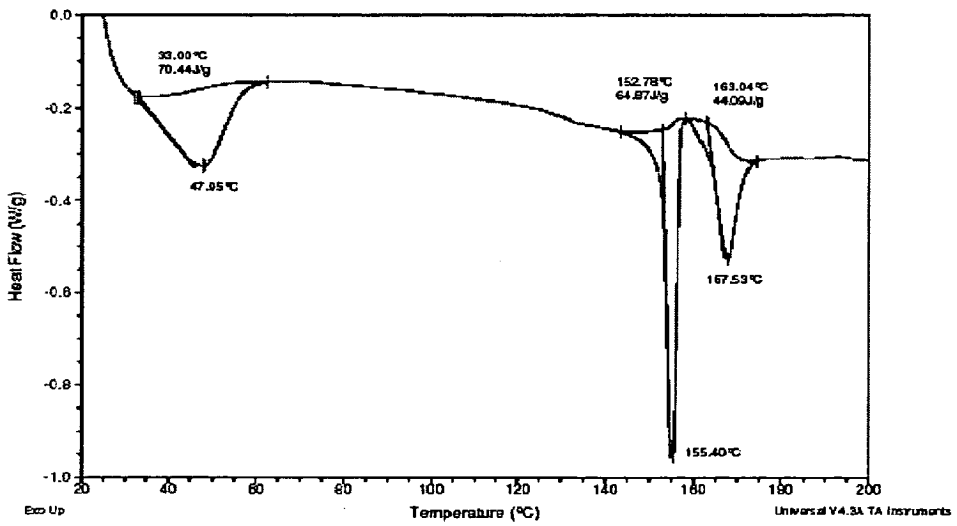


FIG. 7B

(2 °C/minute heating rate)

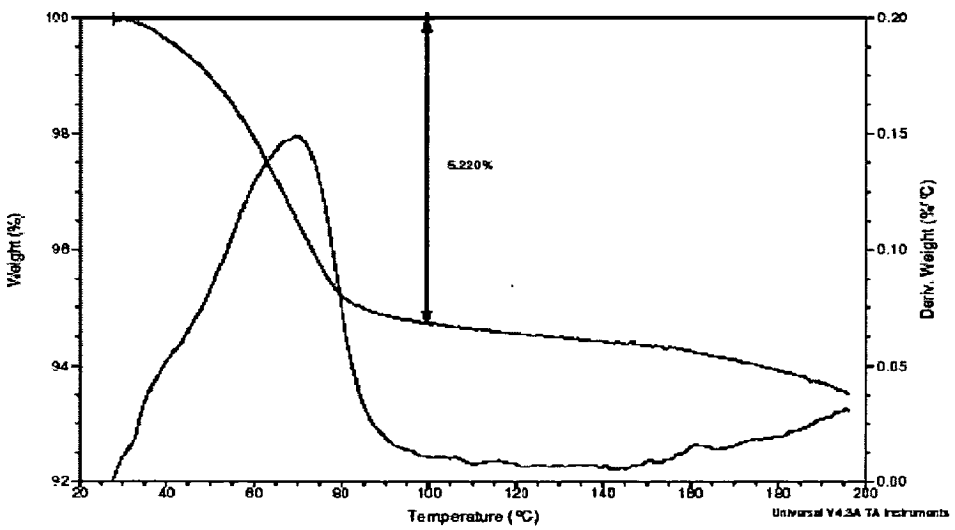


FIG. 8

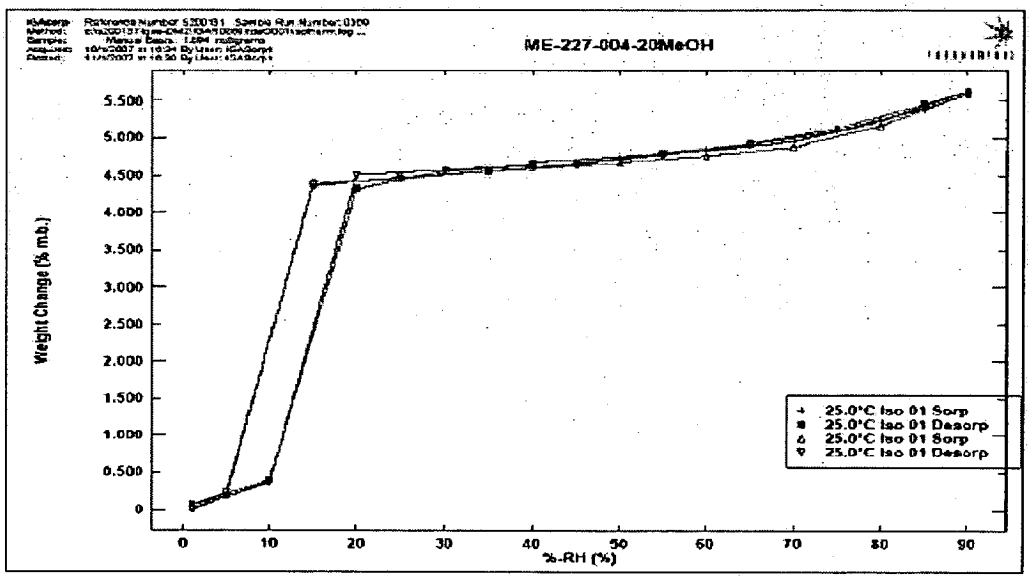


FIG. 9

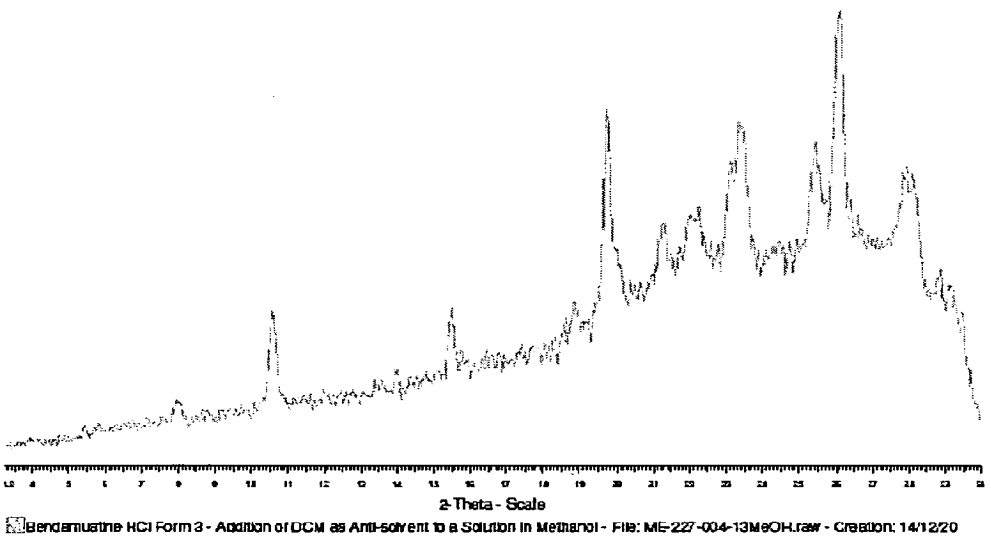


FIG. 10

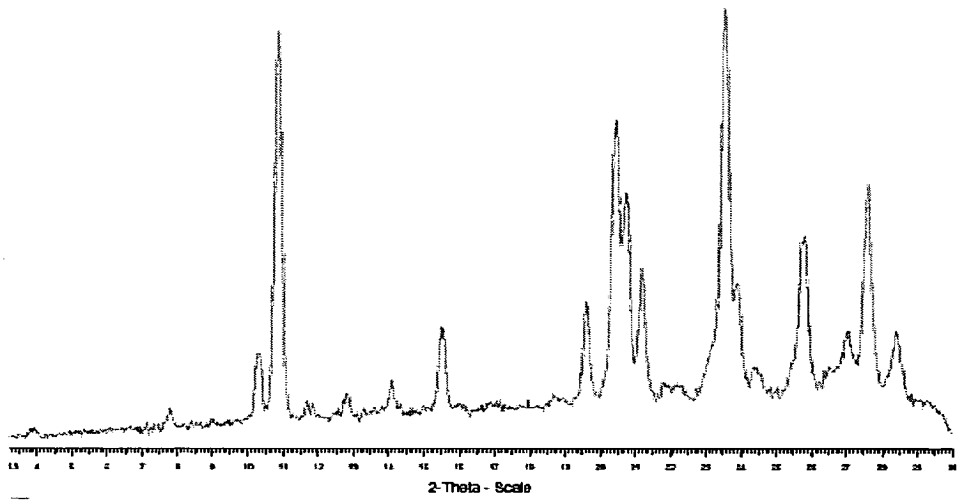


FIG. 11

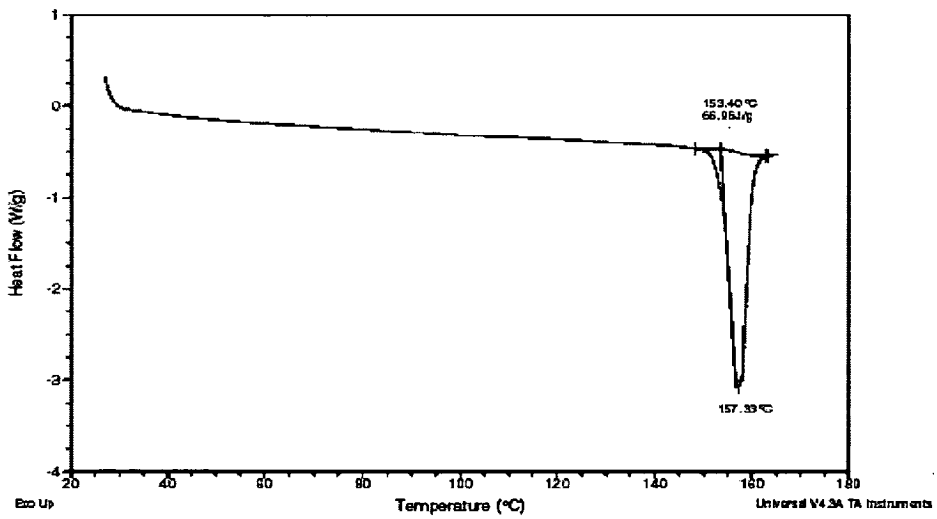


FIG. 12

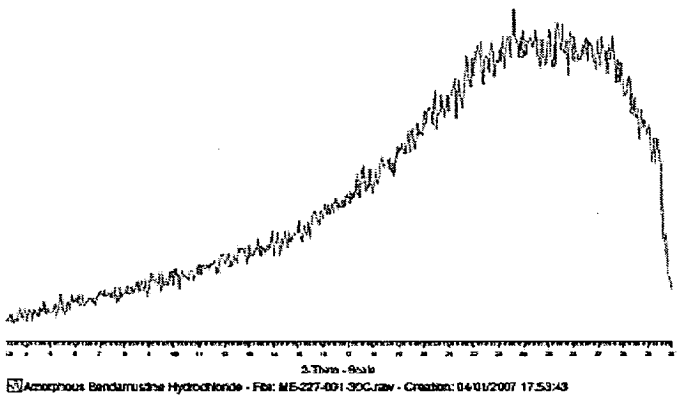


FIG. 13

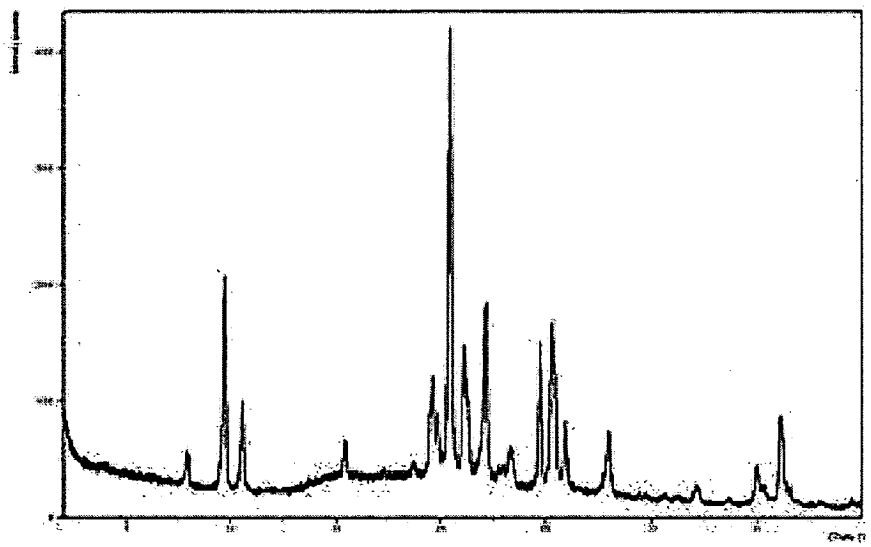


FIG. 14

Electronic Acknowledgement Receipt

EFS ID:	12895427
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	Bendamustine pharmaceutical compositions
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/Viantinna Campana Bordas
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	30-MAY-2012
Filing Date:	12-JAN-2006
Time Stamp:	17:06:20
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	Transmittal Letter	CEPH-4391_SIDS_Trans_05-30-12.PDF	104232 09e7234c3235bb79191e8d169c473445224f69d3	no	3

Warnings:	
Information:	

2	Information Disclosure Statement (IDS) Form (SB08)	CEPH-4391_SIDS_1449_05-30-12.PDF	130456 2f051a7af1b8e5e8888b7d96066f3a41127ba8d1	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	DD_34727_wEngTrans.PDF	479859 1b73f52edcafc99211628a2b582611992ebef2c2	no	24
Warnings:					
Information:					
4	Foreign Reference	WO_2006-076620.PDF	3298762 5969d0d5d177547a996634ffbb705e9ba905be711	no	67
Warnings:					
Information:					
5	Foreign Reference	WO_2009-120386.PDF	2222154 4444b188386414701576e2af951680c49a36feb3	no	57
Warnings:					
Information:					
6	Non Patent Literature	Berge_JPharmSci_1-19.PDF	2852035 3b634dd466773385e3b019c1614173df46df4259	no	19
Warnings:					
Information:					
7	Non Patent Literature	Byrn_PharmaceuticalResearch_1995_945-954.PDF	1890299 db74edf553479e230930197947fbb8f0570e161e	no	10
Warnings:					
Information:					
8	Non Patent Literature	ECSafetySheet_Ribomustin_1998_8pgs.PDF	310513 5e1907141bbba8c37dc24d814abdc82112b69467	no	8
Warnings:					
Information:					
9	Non Patent Literature	Goodman_ThePharmacologicalBasisofTherapeutics_1985_7th edition.PDF	3156798 47a6c4001abdf70d014d9ed5f5e62ea573f5002f	no	16
Warnings:					
Information:					
10	Non Patent Literature	Ni_IntJofPharmaceutics_2001_39-46.PDF	360496 85fb5884e7ca8992960792e952048c2f9a82f8f0	no	8

Warnings:					
Information:					
11	Non Patent Literature	Ozegowski_ZblPharm_1971_1013-1019_w-EnglishTranslation.PDF	578826 d30df05808d204c44244d2f2b86d638b269fb315	no	15
Warnings:					
Information:					
12	Non Patent Literature	Remington_PharmaceuticalSciencees_1990.PDF	111441 c60dcf607ae807cf50aca957fe79d28888b86b7b	no	4
Warnings:					
Information:					
13	Non Patent Literature	Schwanen_Leukemia_2002_2096-2105.PDF	264866 5eda34f8f87b80d1816d05527857ea391a2a9512	no	10
Warnings:					
Information:					
14	Non Patent Literature	Weidmann_AnnalsofOncology_2002_1285-1289.PDF	59944 9eda1333d9af06116daaee03cdb204b54cd8f0dd	no	5
Warnings:					
Information:					
			Total Files Size (in bytes):	15820681	

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.

DOCKET NO.: CEPH-4391 / CP391 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Jason Edward Brittain **Confirmation No.: 9998**

Application No.: 11/330,868 **Group Art Unit: 1617**

Filing Date: January 12, 2006 **Examiner: Ali Soroush**

For: Bendamustine pharmaceutical compositions

Filed Via EFS

INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 CFR § 1.56 and in accordance with 37 CFR §§ 1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as an admission that this information is material as that term is defined in 37 CFR § 1.56(b).

☒ **IDS Filed Under 37 CFR 1.97(b)**

In accordance with § 1.97(b), since this Information Disclosure Statement is being filed either within three months of the filing date of the above-identified application, within three months of the date of entry into the national stage of the above identified application as set forth in § 1.491, before the mailing date of a first Office Action on the merits of the above-identified application, or before the mailing date of a first Office Action after the filing of request for continued examination under § 1.114, no additional fee is required.

☐ **IDS filed Under 37 CFR 1.97(c)**

In accordance with § 1.97(c), this Information Disclosure Statement is being filed after the period set forth in § 1.97(b) above but before the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311, or before an action that otherwise closes prosecution in the application, therefore:

☐ Certification in Accordance with § 1.97(e) is attached; or

☐ The fee of **$180.00** as set forth in § 1.17(p) is attached.

☐ **IDS filed Under 37 CFR 1.97(d)**

In accordance with § 1.97(d), this Information Disclosure Statement is being filed after the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311 but before, or simultaneously with, the payment of the Issue Fee, therefore included are: Certification in Accordance with § 1.97(e); and the submission fee of **$180.00** as set forth in § 1.17(p).

CONTENT OF IDS PURSUANT TO 37 CFR 1.98

☒ Copies of reference numbers 79-82 listed on the attached Form PTO-1449 or Substitute Form PTO-1449 are not required to be submitted pursuant to 37 CFR § 1.98(a)(2)(iii).

☒ Copies of reference numbers 83-94 listed on the attached Form PTO-1449 or Substitute Form PTO-1449 are enclosed herewith.

☐ Copies of reference numbers are not being submitted because they were previously cited by or submitted to the U.S. Patent and Trademark Office in patent application number , filed for which a claim for priority under 35 U.S.C. § 120 has been made in the instant application.

☒ The month of publication for reference numbers 89 and 91-92 is not available. However, the year of publication for these references is sufficiently earlier than the effective US filing date and any foreign priority date so that the particular month of publication is not in issue pursuant to 37 CFR § 1.98(b).

REFERENCES IN A LANGUAGE OTHER THAN ENGLISH

☐ The following documents are not in the English language. Accordingly, a concise explanation of the relevance of the document was incorporated in the specification passages identified below, the document was identified in a foreign communication as identified below or an English language counterpart application has been provided as indicated below.

Foreign Language Document	Cite No.	Pages of Reference in Specification or Relevance of Document

Foreign Language Document	Cite No.	English Language Counterpart	Cite No.

☐ **CERTIFICATION IN ACCORDANCE WITH § 1.97(e)**

I hereby certify that:

☐ Each item of information contained in this 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 this information disclosure statement.

☐ No item of information contained in this 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 this information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of this information disclosure statement.

Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

Date: May 30, 2012

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

WOODCOCK WASHBURN LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

DOCKET NO.: CEPH-4391 / CP391 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Jason Edward Brittain **Confirmation No.: 9998**

Application No.: 11/330,868 **Group Art Unit: 1617**

Filing Date: January 12, 2006 **Examiner: Ali Soroush**

For: Bendamustine pharmaceutical compositions

Filed Via EFS

INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 CFR § 1.56 and in accordance with 37 CFR §§ 1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as an admission that this information is material as that term is defined in 37 CFR § 1.56(b).

☒ **IDS Filed Under 37 CFR 1.97(b)**

In accordance with § 1.97(b), since this Information Disclosure Statement is being filed either within three months of the filing date of the above-identified application, within three months of the date of entry into the national stage of the above identified application as set forth in § 1.491, before the mailing date of a first Office Action on the merits of the above-identified application, or before the mailing date of a first Office Action after the filing of request for continued examination under § 1.114, no additional fee is required.

☐ **IDS filed Under 37 CFR 1.97(c)**

In accordance with § 1.97(c), this Information Disclosure Statement is being filed after the period set forth in § 1.97(b) above but before the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311, or before an action that otherwise closes prosecution in the application, therefore:

☐ Certification in Accordance with § 1.97(e) is attached; or

☐ The fee of **$180.00** as set forth in § 1.17(p) is attached.

☐ **IDS filed Under 37 CFR 1.97(d)**

In accordance with § 1.97(d), this Information Disclosure Statement is being filed after the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311 but before, or simultaneously with, the payment of the Issue Fee, therefore included are: Certification in Accordance with § 1.97(e); and the submission fee of **$180.00** as set forth in § 1.17(p).

CONTENT OF IDS PURSUANT TO 37 CFR 1.98

☒ Copies of reference numbers 79-82 listed on the attached Form PTO-1449 or Substitute Form PTO-1449 are not required to be submitted pursuant to 37 CFR § 1.98(a)(2)(iii).

☒ Copies of reference numbers 83-94 listed on the attached Form PTO-1449 or Substitute Form PTO-1449 are enclosed herewith.

☐ Copies of reference numbers are not being submitted because they were previously cited by or submitted to the U.S. Patent and Trademark Office in patent application number , filed for which a claim for priority under 35 U.S.C. § 120 has been made in the instant application.

☒ The month of publication for reference numbers 89 and 91-92 is not available. However, the year of publication for these references is sufficiently earlier than the effective US filing date and any foreign priority date so that the particular month of publication is not in issue pursuant to 37 CFR § 1.98(b).

REFERENCES IN A LANGUAGE OTHER THAN ENGLISH

☐ The following documents are not in the English language. Accordingly, a concise explanation of the relevance of the document was incorporated in the specification passages identified below, the document was identified in a foreign communication as identified below or an English language counterpart application has been provided as indicated below.

Foreign Language Document	Cite No.	Pages of Reference in Specification or Relevance of Document

Foreign Language Document	Cite No.	English Language Counterpart	Cite No.

☐ **CERTIFICATION IN ACCORDANCE WITH § 1.97(e)**

I hereby certify that:

☐ Each item of information contained in this 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 this information disclosure statement.

☐ No item of information contained in this 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 this information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of this information disclosure statement.

Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

Date: May 30, 2012

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

WOODCOCK WASHBURN LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

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
11/330,868	01/12/2006	

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))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT 05/30/2012

	(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))	* 9	Minus	** 78	= 0	X $ =		OR	X $60=	0
Independent (37 CFR 1.16(h))	* 1	Minus	***21	= 0	X $ =		OR	X $250=	0
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT

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

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

46347 7590 08/29/2012
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

EXAMINER
SOROUSH, ALI

ART UNIT	PAPER NUMBER
1617	

DATE MAILED: 08/29/2012

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

TITLE OF INVENTION: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1740	$300	$0	$2040	11/29/2012

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

46347 7590 08/29/2012
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

TITLE OF INVENTION: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1740	$300	$0	$2040	11/29/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
SOROUSH, ALI	1617	548-304700

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

46347 7590 08/29/2012
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

EXAMINER	
SOROUSH, ALI	
ART UNIT	PAPER NUMBER
1617	

DATE MAILED: 08/29/2012

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 802 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 802 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1617	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *05/30/2012*.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *83-91*.
4. ☐ 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 the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 05302012
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/ALI SOROUSH/
Examiner, Art Unit 1617

DETAILED ACTION

Claim Status

Claims 83-91 are pending.

Claims 31, 32, and 78-82 are cancelled and 1-30 and 33-77 were previously cancelled.

Claims 83-91 have been examined.

Claims 83-91 are rejected.

Priority

Priority to application 60/644,354 filed on 01/14/2005 is acknowledged.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on 05/30/2012 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement has been considered by the examiner.

Withdrawn Claim Rejections - 35 USC § 103

Response to Applicant's Arguments

The rejection of claims 31, 32 and 78-82 under 35 U.S.C. 103(a) as being unpatentable over Battelli et al. (US Patent 4670262, Published 06/02/1987) in view of Klaveness et al. (US Patent 2002/0102215 A1, Published 08/01/2002) is moot since the claims have been cancelled.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance: the prior art teaches a formulation of bendamustine and mannitol to be lyophilized. The prior art also teach a combination of mannitol, tertiary-butyl alcohol, water, and an anti-neoplastic agent can be lyophilized. The prior art suggests using a combination of mannitol and tertiary-butyl alcohol with bendamustine to produce a formulation to be lyophilized. However, Applicant has unexpectedly found that the addition of tertiary-butyl alcohol stabilizes the formulation such that bendamustine degradation is negiligable (no more than 0.5% formation of bendamustine ethyl ester). Therefore, claims 83-91 are allowed.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Claims 83-91 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALI SOROUSH whose telephone number is (571)272-9925. The examiner can normally be reached on M-F (9am-6pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Fereydoun G. Sajjadi can be reached on (571)272-3311. 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.

/ALI SOROUSH/
Examiner, Art Unit 1617

August 20, 2012

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

SEARCHED

Class	Subclass	Date	Examiner
34	284	08/20/2012	AS
548	304.7	08/20/2012	AS

SEARCH NOTES

Search Notes	Date	Examiner
see search history printouts	08/20/2012	AS
Inventor/Assignee search EAST/PALM (Jason Edward Brittain, Joe Craig Franklin, Cephalon, Inc.)	08/20/2012	AS

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
34	284	08/20/2012	AS
548	304.7	08/20/2012	AS

/ALI SOROUSH/ Examiner.Art Unit 1617	

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 9998

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/330,868	01/12/2006	514	1617	CP391

APPLICANTS

Jason Edward Brittain, El Cajon, CA;
Joe Craig Franklin, Tulsa, OK;

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

This appln claims benefit of 60/644,354 01/14/2005

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

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

02/27/2006

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /ALI SOROUSH/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	6	78	21

ADDRESS

WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891
UNITED STATES

TITLE

Bendamustine pharmaceutical compositions

FILING FEE RECEIVED		
7630	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

Substitute for 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(use as many sheets as necessary)*				***Complete if Known***	
				Application Number	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
				Examiner Name	Ali Soroush
Sheet	1	of	2	**Attorney Docket Number**	CEPH-4391 / CP391

U. S. PUBLICATION AND PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number Number – Kind Code (if known)	Publication or Grant Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document
/A.S./	**79**	2004/0072889	04-15-2004	Masferrer
	80	2006/0128777	06-15-2006	Bendall et al.
	81	2009/0264488	10-22-2009	Cooper et al.
	82	2012/0071532	03-22-2012	Cooper et al.

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
/A.S./	**83**	DD 34727	12-28-1964	Krebs Dietrich	X
	84	WO 2006/076620	07-20-2006	Cephalon, Inc.	
	85	WO 2009/120386	10-01-2009	Cephalon, Inc.	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author, 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
/A.S./	**86**	Berge et al., "Pharmaceutical Salts", Journal of pharmaceutical sciences, January 1977, 66(1), 1-19	
	87	Byrn et al., "Pharmaceutical Solids: A Strategic Approach to Regulatory Consideration", Pharmaceutical Research, July 1995, 12(7), 945-954	
	88	EC Safety Data Sheet: Ribomustin® in http://www.docstoc.com/docs/22323231/EC-Safety-Data-Sheet-Bendamustin (published: July 3, 1998; updated March 1, 2007), 8 pages	
	89	Goodman et al., The Pharmacological Basis of Therapeutics, 1985, 7th edition, Macmillan publishing company, New York	
	90	Ni et al., "Use of pure t-butanol as a solvent for freeze-drying: a case study", International Journal of Pharmaceutics, September 2001, 226(1-2), 39-46	

Examiner Signature	/Ali Soroush/	**Date Considered**	08/20/2012

Substitute for 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(use as many sheets as necessary)*				***Complete if Known***	
				Application Number	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
				Examiner Name	Ali Soroush
Sheet	2	of	2	**Attorney Docket Number**	CEPH-4391 / CP391

NON PATENT LITERATURE DOCUMENTS			
/A.S./	**91**	Ozegowski et al., "IMET 3393, gamma-(1-methyl-5-bis-(ß-chloräthyl)-amino-benzimidazolyl(2)-buttersäure-hydrochlorid, ein neues Zytostatikum aus der Reihe der Benzimidazol-Loste", Zbl Pharm., 1971;110, Heft 10, 1013–1019 (Translation Included)	X
	92	Remington: Pharmaceutical Sciences, 1990, Mack Publishing company, Easton, Pennsylvania	
	93	Schwanen et al., "In Vitro Evaluation of Bendamustine Induced Apoptosis in B-Chronic Lymphocytic Leukemia", Leukemia, October 2002, 16(10), 2096-2105	
	94	Weidmann et al., "Bendamustine is Effective in Relapsed or Refractory Aggressive non-Hodgkin's Lymphoma", Annals of Oncology, August 2002, 13(8), 1285-1289	

Examiner Signature	/Ali Soroush/	**Date Considered**	08/20/2012

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
11330868	BRITTAIN ET AL.
Examiner	**Art Unit**
ALI SOROUSH	1617

ORIGINAL		INTERNATIONAL CLASSIFICATION														
CLASS	SUBCLASS	CLAIMED									NON-CLAIMED					
548	304.7	C	0	7	D	235 / 04 (2006.01.01)										

CROSS REFERENCE(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
34	284				

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	83														
2	84														
3	85														
4	86														
5	87														
6	88														
7	89														
8	90														
9	91														

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	9	
/ALI SOROUSH/ Examiner.Art Unit 1617	08/20/2012	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	12	bendamustine "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105"	EPO	OR	ON	2012/08/20 17:07
L2	1158	bendamustine "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:09
L3	17	L2 near5 water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:09
L4	15	L2 near5 (mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:10
L5	19	L2 with (mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:10
L6	203678	"tert-Butanol" "2-methyl-2-propanol" "tertiary-butyl alcohol" "2-Methylpropan-2-ol" "Dimethylethanol" "1,1-Dimethylethanol" ""tert-butyl alcohol" "t-butyl alcohol" ""1,1-Dimethyl ethanol" "trimethyl carbinol" "t-butyl hydroxide" "trimethyl methanol" "dimethyl ethanol" "methyl-2-propanol"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:15
L7	165922	(mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:15
L8	24	L6 near5 L7	US-PGPUB; USPAT;	OR	ON	2012/08/20 17:16

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
L9	107	L6 with L7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
L10	2	L9 and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
L11	364	L6 same L7	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
L12	7	L11 and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
L13	7	L2 near5 L6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
L14	8	L2 with L6	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:17
L15	183540	Freeze$1drying lyophilisation lyophilization cryodesiccation lyophilized lyophilize	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:22
L16	516	L15 and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:23

L17	22	L15 same L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:23
L18	93	Mundipharma.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:29
L19	0	Mundipharma.as. and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:30
L20	34	L2 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:36
L21	1160	bendamustine "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105" "IMET 3393"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:53
L22	273	34/284.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
L23	0	34/284.ccls. and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
L24	273	34/284.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
L25	2	"5977129".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2012/08/20 18:39

			DERWENT; IBM_TDB			
L26	904	548/304.4.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:00
L27	11	L26 and (nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:01
L28	593	548/304.7.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:06
L29	14	L28 and (nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:06
L30	9	(brittain.in. franklin.in. cephalon.as.) and bendamustine.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:08
S1	2	treanda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
S2	0	bendamustine same (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
S3	10	bendamustine and (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
S4	46	bendamustine and (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR;	OR	ON	2010/08/14 19:42

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S5	3	bendamustine same (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
S6	88851	lyophilize lyophilization freeze$dry freeze$dried free$1drying	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:56
S7	22	S6 same (alkylating adj agent)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:57
S8	2	bendamustine same (aqueous adj solution) same unstable	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:03
S9	0	"cephalon.in"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:04
S10	563	cephalon.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
S11	11	S10 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
S12	4	bendamustine same (aqueous adj solution)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
S13	458	bendamustine	US-PGPUB;	OR	ON	2010/08/14

			USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			20:06
S14	30	bendamustine adj hydrochloride	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
S15	58	bendamustine same injection	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:07
S16	18	bendamustine same solid	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:12
S17	2	bendamustine same unstable	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:13
S18	2	"0656211"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:29
S19	0	"0656211"	EPO	OR	ON	2010/08/14 20:29
S20	610	ku.in.	EPO	OR	ON	2010/08/14 20:29
S21	1	S20 and thiotepa	EPO	OR	ON	2010/08/14 20:30
S22	0	"5330835".pn.	EPO	OR	ON	2010/08/17 12:07
S23	2	"5330835".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/17 12:08
S24	3	"4145400".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2010/08/17 12:10

			JPO; DERWENT; IBM_TDB			
S25	3	"4145440".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/17 12:10
S26	1	10/417631.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/24 13:13
S27	0	benadmustine with mannitol with alcohol	EPO	OR	ON	2011/04/22 20:07
S28	0	benadmustine	EPO	OR	ON	2011/04/22 20:07
S29	11	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" "16506-27-7"	EPO	OR	ON	2011/04/22 20:20
S30	775	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" "16506-27-7"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:20
S31	10	S30 with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S32	13	S30 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S33	13	S30 with alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S34	22	S30 same alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO;	OR	ON	2011/04/22 20:22

			DERWENT; IBM_TDB			
S35	23	S30 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:24
S36	345	S30 and mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:36
S37	52	S36 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:38
S38	108	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) with (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:44
S39	31	S38 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:44
S40	2	"5362718".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:52
S41	1	S30 same (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55
S42	15	S30 and (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55

S43	18	S30 with rapamycin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:56
S44	23	S30 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
S45	6	S30 same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
S46	132	S30 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
S47	299	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02
S48	7	S47 and S30	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02
S49	65	cyclophosphamide with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07
S50	17	S49 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07

S51	0	S50 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:12
S52	17166	(nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S53	113050	S52 sme (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S54	6	S52 same (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S55	2335	S52 and (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S56	4	S35 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:15
S57	3	S30 same tablet	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:18
S58	60242	(t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
S59	81388	lyophilization lyophilize freeze$1dry freeze$1drying	US-PGPUB; USPAT;	OR	ON	2011/04/22 21:22

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S60	477	S58 same S59	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
S61	52	S60 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:23
S62	7	chlorambucil same lyophilization	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:41
S63	49972	freeze$1dry freez$1drying lyophilisation lyophilization cryodesiccation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
S64	82	S63 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
S65	6	S38 and S64	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:46
S66	13	S30 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:48
S67	10	fishman.in. and K4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50

S68	0	fishman.in. and S30	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50
S69	2	"20020102215"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:53
S70	986	brittain.in. franklin.in. and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 22:53
S71	2	(brittain.in. franklin.in.) and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 22:54
S72	0	"4670262".pn.	EPO	OR	ON	2011/04/25 11:15
S73	2	"4670262".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:15
S74	626	jenapharm.as. ribosepharm.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:43
S75	0	S74 and (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:44
S76	28	S74 and (powder)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:44
S77	396	GlOlA.in.	US-PGPUB; USPAT; USOCR;	OR	ON	2011/04/25 15:35

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S78	0	S77 and dinitroalanine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 15:35
S79	4	S77 and dinitroaniline	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 15:35

8/20/2012 7:15:12 PM
C:\Users\asoroush\Documents\EAST\Workspaces\11330868.wsp

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	11/330,868	Filing Date	2006-01-12	Docket Number (if applicable)	CP391/CEPH-4391	Art Unit	1617
First Named Inventor	Jason Edward Brittain			Examiner Name	Ali Soroush		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☐ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☒ Other WO2006065392

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 233050

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

☐ Applicant Signature

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Signature of Registered U.S. Patent Practitioner			
Signature	/Stephanie A. Barbosa/	Date (YYYY-MM-DD)	2012-11-15
Name	Stephanie A. Barbosa	Registration Number	51430

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Substitute for 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1616
(use as many sheets as necessary)				**Examiner Name**	Ali Soroush
Sheet	1	of	1	**Attorney Docket Number**	CEPH-4391 (CP391US)

U. S. PUBLICATION AND PATENT DOCUMENTS				
Examiner Initials	Cite No.	Document Number Number – Kind Code (if known)	Publication or Grant Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document
	1	5,192,743	03-09-1993	Hsu et al.
	2	5,183,746	02-02-1993	Shaked et al.

FOREIGN PATENT DOCUMENTS					
Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
	3	WO 2006/065392	06-22-2006	Cephalon, Inc.	

Examiner Signature		**Date Considered**	

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 June 2006 (22.06.2006)

PCT

(10) International Publication Number
WO 2006/065392 A2

(51) International Patent Classification:
A61K 31/4184 (2006.01)

(21) International Application Number:
PCT/US2005/040068

(22) International Filing Date:
4 November 2005 (04.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/625,193 5 November 2004 (05.11.2004) US
60/660,226 10 March 2005 (10.03.2005) US

(71) Applicant *(for all designated States except US)*: **CEPHALON, INC.** [US/US]; 41 Moores Road, P.O. Box 4011, Frazer, PA 19355 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BENDALL, Heather, Helene** [US/US]; 3082 Portofino Drive, Del Mar, California 92014 (US). **ELLIOTT, Gary, T.** [US/US]; 12433 Kingspine Avenue, San Diego, California 92131 (US). **LEONI, Lorenzo, M.** [CH/CH]; Via Campagna, CH-6527 Lodrino (CH). **NIEMEYER, Christina, Carol** [US/US]; 12439 Holland Road, Poway, California 92064 (US). **MULTANI, Pratik, S.** [US/US]; 10486 Harvest View Way, San Diego, California 92128 (US).

(74) Agent: **WOODCOCK WASHBURN LLP**; One Liberty Place, 46th Floor, Philadelphia, PA 19103 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, 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.

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

Published:
— *without international search report and to be republished upon receipt of that report*

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

WO 2006/065392 A2

(54) Title: CANCER TREATMENTS

(57) Abstract: Methods and compositions for treating cancers characterized by death-resistant cancer cells are described. In general, such methods involve administration of a therapeutically effective amount of a compound that induces mitotic catastrophe in the some, and preferably most or all, of the cancerous cells. Methods for assessing the efficacy of such treatments are also provided.

CANCER TREATMENTS

FIELD OF THE INVENTION

[0001] This invention relates generally to cancer treatment, particularly cancers resistant to drug-induced apoptosis.

BACKGROUND OF THE INVENTION

1. Introduction.

[0002] This application claims the benefit of, and priority to, each of the following U.S. provisional patent applications: serial numbers 60/625,193, entitled "Cancer Treatments" and filed November 5, 2004; and 60/660,266, entitled "Cancer Treatments" and filed March 10, 2005. Each of these applications is incorporated herein by reference in its entirety, including figures, tables, and claims.

[0003] The following description includes information that may be useful in understanding the present invention. It is not an admission that any such information is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

2. Background.

[0004] Cancer is now the second leading cause of death in the United States and over 8,000,000 persons in the United States have been diagnosed with cancer. In 1995, cancer accounted for 23.3% of all deaths in the United States. See U.S. Dept. of Health and Human Services, National Center for Health Statistics, Health United States 1996-97 and Injury Chartbook 117 (1997).

[0005] Cancer is not fully understood on the molecular level. It is known that exposure of a cell to a carcinogen such as certain viruses, certain chemicals, or radiation, leads to DNA alteration that inactivates a "suppressive" gene or activates an "oncogene".

Suppressive genes are growth regulatory genes, which upon mutation, can no longer control cell growth. Oncogenes are initially normal genes (called proto-oncogenes) that by mutation or altered context of expression become transforming genes. The products of transforming genes cause inappropriate cell growth. More than twenty different normal cellular genes can become oncogenes by genetic alteration. Transformed cells differ from normal cells in many ways, including cell morphology, cell-to-cell interactions, membrane content, cytoskeletal structure, protein secretion, gene expression and mortality (transformed cells can grow indefinitely).

[0006] A neoplasm, or tumor, is an abnormal, unregulated, and disorganized proliferation of cell growth, and is generally referred to as cancer. A neoplasm is malignant, or cancerous, if it has properties of destructive growth, invasiveness, and metastasis. Invasiveness refers to the local spread of a neoplasm by infiltration or destruction of surrounding tissue, typically breaking through the basal laminas that define the boundaries of the tissues, thereby often entering the body's circulatory system. Metastasis typically refers to the dissemination of tumor cells by lymphatics or blood vessels. Metastasis also refers to the migration of tumor cells by direct extension through serous cavities, or subarachnoid or other spaces. Through the process of metastasis, tumor cell migration to other areas of the body establishes neoplasms in areas away from the site of initial appearance.

[0007] Cancer is now primarily treated with one or a combination of three types of therapies: surgery; radiation; and chemotherapy. Surgery involves the bulk removal of diseased tissue. While surgery is sometimes effective in removing tumors located at certain sites, for example, in the breast, colon, and skin, it cannot be used in the treatment of tumors located in other areas, such as the backbone, nor in the treatment of disseminated neoplastic conditions such as leukemia. Radiation therapy involves the exposure of living tissue to ionizing radiation causing death or damage to the exposed cells. Side effects from radiation therapy may be acute and temporary, while others may be irreversible. Chemotherapy involves the disruption of cell replication or cell metabolism. It is used most often in the treatment of breast, lung, and testicular cancer.

[0008] The adverse effects of systemic chemotherapy used in the treatment of neoplastic disease are most feared by patients undergoing treatment for cancer. Of these adverse effects, nausea and vomiting are the most common. Other adverse side effects include cytopenia, infection, cachexia, mucositis in patients receiving high doses of chemotherapy with bone marrow rescue or radiation therapy; alopecia (hair loss); cutaneous complications such as pruritis, urticaria, and angioedema; neurological complications; pulmonary and cardiac complications; and reproductive and endocrine complications. Chemotherapy-induced side effects significantly impact the quality of life of the patient and may dramatically influence patient compliance with treatment. As such, improved methods of treatment are needed.

3. Definitions.

[0009] An "alkylating agent" refers to a chemotherapeutic compound that chemically modifies DNA and disrupts its function. Some alkylating agents cause formation of cross links between nucleotides on the same strand, or the complementary strand, of a double-stranded DNA molecule, while still others cause base-pair mismatching between DNA strands.. Exemplary alkylating agents include bendamustine, busulfan, carboplatin, carmustine, cisplatin, chlorambucil, cyclophosphamide, dacarbazine, hexamethylmelamine, ifosphamide, lomustine, mechlorethamine, melphalan, mitotane, mytomycin, pipobroman, procarbazine, streptozocin, thiotepa, and triethylenemelamine.

[00010] An "anti-metabolite" refers to a chemotherapeutic agent that interferes with the synthesis of biomolecules, including those required for DNA synthesis (*e.g.*, nucleosides and nucleotides) needed to synthesize DNA. Examples of anti-metabolites include capecitabine, chlorodeoxyadenosine, cytarabine (and its activated form, ara-CMP), cytosine arabinoside, dacabazine, floxuridine, fludarabine, 5-fluorouracil, gemcitabine, hydroxyurea, 6-mercaptopurine, methotrexate, pentostatin, trimetrexate, and 6-thioguanine.

[00011] An "anti-mitotic" refers to a chemotherapeutic agent that interferes with mitosis, typically through disruption of microtubule formation. Examples of anti-mitotic compounds include navelbine, paclitaxel, taxotere, vinblastine, vincristine, vindesine, and vinorelbine.

[00012] In the context of this invention, a "chemotherapeutic agent" refers to a chemical intended to destroy malignant cells and tissues. Chemotherapeutic agents include small molecules, nucleic acids (*e.g.*, anti-sense molecules, ribozymes, small interfering RNA molecules, *etc.*), and proteins (e.g., antibodies, antibody fragments, cytokines, enzymes, and peptide hormones) that have anti-tumor effects when administered to a patient in order to prevent or treat a cancer or other malignancy. Chemotherapeutic agents are often divided classes based on mechanism of action, *e.g.*, alkylating agents, anti-metabolites, and anti-mitotic agents.

[00013] The term "combination therapy" refers to a therapeutic regimen that involves the provision of at least two distinct therapies to achieve an indicated therapeutic effect. For example, a combination therapy may involve the administration of two or more chemically distinct active ingredients, for example, a fast-acting chemotherapeutic agent and a myeloprotective agent. Alternatively, a combination therapy may involve the administration of one or more chemotherapeutic agents as well as the delivery of radiation therapy and/or surgery or other techniques to either improve the quality of life of the patient or to treat the cancer. In the context of the administration of two or more chemically distinct active ingredients, it is understood that the active ingredients may be administered as part of the same composition or as different compositions. When administered as separate compositions, the compositions comprising the different active ingredients may be administered at the same or different times, by the same or different routes, using the same of different dosing regimens, all as the particular context requires and as determined by the attending physician. Similarly, when one or more chemotherapeutic agents are combined with, for example, radiation and/or surgery, the drug(s) may be delivered before or after surgery or radiation treatment.

[00014] An "intercalating agent" refers to a chemotherapeutic agent that inserts itself between adjacent base pairs in a double-stranded DNA molecule, disrupting DNA structure and interfering with DNA replication, gene transcription, and/or the binding of DNA binding proteins to DNA.

[00015] "Monotherapy" refers to a treatment regimen based on the delivery of one therapeutically effective compound, whether administered as a single dose or several doses over time.

[00016] In the context of the commercialization of pharmaceuticals, the terms "promotion", "promote", "promoting", and the like refer to any and all informational, persuasive, and scientific activities conducted by or on behalf of a manufacturer, distributor, or other entity involved in the discovery, research, development, and/or commercialization of the particular pharmaceutical compound, composition, or treatment regimen intended, directly or indirectly, to induce the prescription, supply, purchase, and/or use of the compound, composition, or treatment regimen. Such activities may be directed toward anyone in the in the supply and distribution chain, including, without limitation, medical professionals (e.g., physicians and nurses), pharmacists, health care administrators, insurance company or government representatives, and patients (including potential patients). In other words, the primary aim of promotion is to stimulate the sale or use of, and/or interest in, a particular pharmaceutical compound, composition, or treatment regimen, and thus any activity intended to serve this aim constitutes "promotion" of the particular pharmaceutical compound, composition, or treatment regimen.

[00017] A "patentable" composition, process, machine, or article of manufacture according to the invention means that the subject matter satisfies all statutory requirements for patentability at the time the analysis is performed. For example, with regard to novelty, non-obviousness, or the like, if later investigation reveals that one or more claims encompass one or more embodiments that would negate novelty, non-obviousness, *etc.*, the claim(s), being limited by definition to "patentable" embodiments, specifically exclude the unpatentable embodiment(s). Also, the claims appended hereto are to be interpreted both to provide the broadest reasonable scope, as well as to preserve their validity. Furthermore, if one or more of the statutory requirements for patentability are amended or if the standards change for assessing whether a particular statutory requirement for patentability is satisfied from the time this application is filed or issues as a patent to a time the validity of one or more of the appended claims is questioned, the

claims are to be interpreted in a way that (1) preserves their validity and (2) provides the broadest reasonable interpretation under the circumstances.

[00018] The term "pharmaceutically acceptable salt" refers to salts which retain the biological effectiveness and properties of the compounds of this invention and which are not biologically or otherwise undesirable. In many cases, the compounds of this invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto. Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids, while pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. For a review of pharmaceutically acceptable salts see Berge, *et al.* ((1977) *J. Pharm. Sci.*, vol. 66, 1). The expression "non-toxic pharmaceutically acceptable salts" refers to non-toxic salts formed with nontoxic, pharmaceutically acceptable inorganic or organic acids or inorganic or organic bases. For example, the salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, fumaric, methanesulfonic, and toluenesulfonic acid and the like. Salts also include those from inorganic bases, such as ammonia, hydroxyethylamine and hydrazine. Suitable organic bases include methylamine, ethylamine, propylamine, dimethylamine, diethylamine, trimethylamine, triethylamine, ethylenediamine, hydroxyethylamine, morpholine, piperazine, and guanidine.

[00019] A "plurality" means more than one.

[00020] The term "rituximab refractory" means prior treatment with rituximab, but inappropriate for further treatment due to disease refractory to rituximab therapy, given either as a single agent or in combination (defined as no response, or progression within 6 months of completing rituximab treatment), and/or untoward reaction to prior rituximab therapy, making further treatment unwarranted, as determined by the physician or treating specialist.

[00021] The term"anti-CD20 refractory" means prior treatment with an agent that interacts with the CD20 antigen, but inappropriate for further treatment due to disease refractory to the anti-CD20 agent given either as a single agent or in combination (defined as not response, or progression within 6 months of completing the anti-CD20 treatment), and/or untoward reaction to prior anti-CD20 therapy, making further treatment unwarranted, as determined by the physician or treating specialist.

[00022] The "S phase" of the cell cycle refers to the phase in which the chromosomes are replicated.

[00023] The term "species" is used herein in various contexts, *e.g.*, a particular species of chemotherapeutic agent. In each context, the term refers to a population of chemically indistinct molecules of the sort referred in the particular context.

[00024] A "subject" or "patient" refers to an animal in need of treatment that can be effected by molecules of the invention. Animals that can be treated in accordance with the invention include vertebrates, with mammals such as bovine, canine, equine, feline, ovine, porcine, and primate (including humans and non-humans primates) animals being particularly preferred examples.

[00025] A "therapeutically effective amount" refers to an amount of an active ingredient sufficient to effect treatment when administered to a subject in need of such treatment. In the context of cancer therapy, a "therapeutically effective amount" is one that produces an objectively measured change in one or more parameters associated with cancer cell survival or metabolism, including an increase or decrease in the expression of one or more genes correlated with the particular cancer, reduction in tumor burden, cancer cell lysis, the detection of one or more cancer cell death markers in a biological sample (*e.g.*, a biopsy and an aliquot of a bodily fluid such as whole blood, plasma, serum, urine, *etc.*), induction of induction apoptosis or other cell death pathways, *etc.* Of course, the therapeutically effective amount will vary depending upon the particular subject and condition being treated, the weight and age of the subject, the severity of the disease condition, the particular compound chosen, the dosing regimen to be followed, timing of administration, the manner of administration and the like, all of which can readily be determined by one of ordinary skill in the art. It will be appreciated that in the

context of combination therapy, what constitutes a therapeutically effective amount of a particular active ingredient may differ from what constitutes a therapeutically effective amount of the active ingredient when administered as a monotherapy (*i.e.*, a therapeutic regimen that employs only one chemical entity as the active ingredient).

[00026] The term "treatment" or "treating" means any treatment of a disease or disorder, including preventing or protecting against the disease or disorder (that is, causing the clinical symptoms not to develop); inhibiting the disease or disorder (*i.e.*, arresting or suppressing the development of clinical symptoms; and/or relieving the disease or disorder (*i.e.*, causing the regression of clinical symptoms). As will be appreciated, it is not always possible to distinguish between "preventing" and "suppressing" a disease or disorder since the ultimate inductive event or events may be unknown or latent. Accordingly, the term "prophylaxis" will be understood to constitute a type of "treatment" that encompasses both "preventing" and "suppressing". The term "protection" thus includes "prophylaxis".

SUMMARY OF THE INVENTION

[00027] One object of this invention is to provide patentable methods of treating cancers characterized by death-resistant cancer cells by administration of a compound (*e.g.*, bendamustine) that induces mitotic catastrophe in the cancer cells, alone or in conjunction with other compounds and/or treatments. In preferred embodiments, these methods involve determining whether a patient has a cancer characterized by death-resistant cancer cells, and, if so, then administering to the patient a therapeutically effective amount of bendamustine. Still another object of the invention concerns methods of assessing the efficacy of cancer treatments based on the detection of a cancer cell death marker in a biological sample taken from a patient at one or more periods during or after the administration of a cancer therapy.

[00028] Thus, one aspect of the invention relates to patentable methods of treating cancer patients whose cancers are characterized by death-resistant cancer cells, *i.e.*, cancer cells that resist apoptosis or other programmed cell death pathways, as well as cells that exhibit multi-drug resistance (MDR), as may be induced, for example, by administration of one or more alkylating agents, alone or in conjunction with an anti-

CD20 agent, *e.g.*, rituximab. These methods comprise administering to a patient a therapeutically effective amount of a compound that induces mitotic catastrophe in the death-resistant cancer cells. Such cells include those that are resistant to drug-induced apoptosis. Examples of such cells include those that have a p53 deficiency, typically as a result of a mutation of, including deletions in or of, a gene encoding p53. Representative examples of such cancers include non-Hodgkin's lymphoma ("NHL") and chronic lymphocytic leukemia ("CLL"). A particularly preferred compound for inducing mitotic catastrophe is the alkylating agent bendamustine. Thus, a related aspect concerns methods of treatment that involve characterization of the cells of a particular cancer as death-resistant cancer cells, followed by treatment with a compound (*e.g.*, bendamustine) that induces mitotic catastrophe in such cells, alone or in conjunction with other chemotherapeutic agents, adjuvants, surgery, and/or radiation. In addition, the efficacy of such treatment regimens can be monitored to assess whether the particular monotherapy or combination therapy treatment is achieving the desired effect.

[00029] Another aspect of the invention concerns certain related patentable methods for treating a cancer, particularly cancers characterized by death-resistant cancer cells. These methods comprise the administration to a patient of a therapeutically effective amount of a compound at a time when at least a portion of the cells comprising the cancer are in the S phase of the cell cycle. In some embodiments, at least a portion of the patient's cancerous cells are driven into the S phase as a result of administering to the patient a compound that drives cells into the S phase. Bendamustine is a particularly preferred compound for driving cancer cells into the S phase. Because bendamustine is useful in driving cancer cells into the S phase, additional preferred embodiments involve the subsequent administration of one or more other chemotherapeutic agent species that are more active (i.e., exert a greater therapeutic effect, for example, cytotoxicity, when cells are in the S-phase of the cell cycle. In such methods, the subsequent administration of one or more other chemotherapeutic agents preferably occurs at least about 10 minutes, and preferably at least about 30 to about 60 minutes or more after bendamustine administration, although it is preferred that the administration of such other agent(s) occurs within about 72 hours, preferably about 48 hours or less, after bendamustine is administered. In some of these preferred embodiments, the other chemotherapeutic

agent(s) is(are) given within about 30 minutes to about 36 hours after the administration of bendamustine, preferably within about 30 minutes to 24 hours after administration of bendamustine, and in some cases, within about 30 minutes to six to about twelve hours after administration of bendamustine. Related methods involve reducing toxicity associated with a cancer therapy. Such methods comprise administering a plurality of doses of therapeutically effective amounts bendamustine to a cancer patient. The first dose may well result in an undesired toxicity. In such event, the administration of the second (or other subsequent doses) may be delayed until after the undesired toxicity begins to subside. In some cases, the doses of bendamustine administered at different times may also vary.

[00030] Yet another aspect of the invention thus relates to patentable methods for assessing the efficacy of a cancer treatment based on the administration of an alkylating agent (*e.g.*, bendamustine), either during the course of or after completion of the treatment, be it a monotherapy or a combination therapy. When the assessment is performed after administration of a therapeutic regimen that involves administration of an alkylating agent (*e.g.*, bendamustine), preferably a sufficient period is allowed to elapse so that the alkylating agent can exert its intended, or desired, therapeutic effect. In such methods, a marker of cancer cell death (*i.e.*, a molecule (*e.g.*, a protein, carbohydrate, lipid, nucleic acid, or other molecule) produced by or released from a dying or dead cancer cell, as well as a phenotype such as a lack of cell viability, inability to proliferate, senescence, etc.) that correlates with treatment efficacy is detected in a biological sample obtained from the patient to determine if the treatment with was efficacious. Preferred markers of cell death include adenylate kinase activity levels, the level of PARP cleavage products, and reduced cell viability. Depending on the marker, such detection may be qualitative, semi-quantitative, or quantitative. The presence, or level, of the marker detected indicates whether the treatment is, or has been, efficacious.

[00031] In still another aspect of the invention, the invention concerns treatments for cancer based on administering bendamustine to patients who have a cancer resistant, or refractory, to one or more alkylating agents and an anti-CD20 agent (for example, rituximab). Preferably, these methods are deployed against cancers characterized by death-resistant cancer cells. A related aspect of the invention concerns methods of doing

business in the treatment of such cancers, which involve promoting bendamustine use to treat a refractory cancer or a cancer characterized by death-resistant cancer cells, particularly a cancer refractory to treatment with a combination of one or more alkylating agents and an anti-CD20 agent, *e.g.*, rituximab. Still another aspect concerns whether a patient's cancer is amenable to bendamustine treatment. As will be appreciated, any suitable assessment of bendamustine susceptibility can be employed. In some preferred embodiments of these methods, some or all of a cell sample from cancerous tissue taken from a patient is exposed to bendamustine under growth conditions which, in the absence of a compound that is toxic to cancer cells, allows the cancer cells to proliferate. The assessment of susceptibility is then made based on the results of the assay. For example, reduced proliferation, as compared to controls, would indicate that the cells, and hence the patient's cancer, are susceptible to a bendamustine-based therapy. In contrast, no effect on (or enhanced proliferation) would indicate a lack of susceptibility.

[00032] Yet another aspect of the invention relates to the use of bendamustine in the manufacture of a medicament for treatment of a cancer characterized by death-resistant cancer cells or for treatment of a refractory cancer, particularly a cancer refractory to treatment with a combination of one or more alkylating agents and an anti-CD20 agent *e.g.*, rituximab. Preferably, such medicaments include a therapeutically effective amount of bendamustine.

BRIEF DESCRIPTION OF THE DRAWINGS

[00033] This patent application contains at least one figure executed in color. Copies of this patent application with color drawing(s) will be provided upon request and payment of the necessary fee.

[00034] Figure 1 has two panels, A and B, each which show gene expression profiles. The panels show changes in gene expression measured in the Non-Hodgkin's Lymphoma cell line, SU-DHL-1, using an Affymetrix gene chip (U133A) containing more than 12,000 known genes. Bendamustine was tested at IC_{50} (25 μM; lane 1) and IC_{90} (35 μM; lane 2). Chlorambucil (5 μM; lane 3) and phosphoramide mustard, a cyclophosphamide

metabolite (50 μM; lane 4), were tested at IC_{90}. Isolation of mRNA was performed 8h after exposure. *A.* The clustergram shown represents the top 100 most modulated genes as compared to a control (diluent, DMSO). The red color represents the genes that were up-modulated; blue represents the genes that were down-regulated. *B.* The clustergram represents genes that are concomitantly induced by all three tested drugs.

[00035] Figure 2 has three bar graphs, 2A, 2B, and 2C. Q-PCR analysis was performed as described in the Methods section, below, in SU-DHL-1 cells exposed to equitoxic concentrations of bendamustine, phosphoramide mustard, and chlorambucil. The levels of input cDNA were normalized using an assay for 18s RNA, and the level of transcripts in the untreated sample was set to 1. Figure 2 A shows the relative RNA levels of two representative p53-dependent genes, p21 and NOXA. Figure 2 B shows the RNA levels of four genes involved in the M-phase cell cycle checkpoint, polo-like-kinase 1 (PLK-1), the aurora kinases A and B, and cyclin B1. Figure 2 C shows the relative RNA levels of genes involved in DNA-repair mechanisms, EXO1 and Fen1. The columns represents the mean +/- SE of the fold changes from DMSO-treated controls. The results were obtained from three independent experiments.

[00036] Figure 3 shows several immunoblots that demonstrate that enhanced apoptotic effect of bendamustine (50 μM) as compared to cyclophosphamide (50 μM) and chlorambucil (4 μM) in NHL cells (SU-DHL-1). To generate these immunoblots, cell lysates were prepared after 20 hours exposure as described in the Methods section, below. Probing the membrane with ß-actin served as a loading control and is shown below the regulated proteins. The top-left panel represents the expression of Ser15-phosphorylated p53, detected using a phospho-specific antibody. The middle-left panel shows total p53 and p21 expression. The lower-left panel represents the expression of Bax. The right panels shows the expression of the full-length PARP (top) and the caspase-cleaved fragment of PARP using an antibody that recognizes the specific caspase-cleavage site.

[00037] Figure 4 consists of two graphs, A and B that represent functional analyses of selected DNA repair mechanisms. Figure 4 A shows that bendamustine, but not cyclophosphamide, leads to DNA damage repair via base excision repair (BER). The role of the repair enzyme Ape-1, an apurininc endonuclease that plays a critical role in the

BER pathway in the cytotoxic activity of bendamustine and a cyclophosphamide metabolite, phosphoramide mustard (PM), was assessed using the Ape-1 inhibitor methoxyamine (MX). The left shift of the curve observed with bendamustine and MX shows that DNA damage produced by bendamustine is repaired by BER. Figure 4 B shows that inhibition of MGMT repair activity does not affect bendamustine cytotoxicity. The role of the repair enzyme MGMT (O^6-methylguanine-DNA methyltransferase) in the cytotoxic activity of bendamustine was assessed using the MGMT inhibitor O^6-benzylguanine (O^6-BG). The addition of O^6-benzylguanine did not significantly change the IC_{50} of bendamustine, so it is unlikely that bendamustine induces O^6-alkylguanine DNA adducts. In contrast, O^6-benzylguanine significantly sensitizes cells to other nitrogen mustards such as carmustine and phosphoramide mustard (PM).

[00038] Figure 5 illustrates that bendamustine efficiently enters tumor cells and induces prolonged and extensive DNA damage, which results in the initiation of at least three signaling pathways: 1) activation of "canonical" p53-dependent stress pathway resulting in a strong activation of intrinsic apoptosis, probably mediated by pro-apoptotic BCL-2 family members such as NOXA and Bax; 2) activation of a DNA repair mechanism, such as the base-excision repair machinery, that are not activated by other alkylating agents frequently used in NHL or CLL patients; and 3) inhibition of several mitotic checkpoints, such as the kinases PLK-1 and Aurora A and B. While not wishing to be bound to a particular theory, the concomitant induction of DNA damage and inhibition of mitotic checkpoints presumably prevents tumor cells exposed to bendamustine from efficiently repairing DNA damage before undergoing mitosis. Cells thus enter mitosis with damaged DNA, or cells that can not proceed to "conventional" p53-dependent apoptosis, will undergo death by mitotic catastrophe. This alternative programmed cell death pathway, together with the strong activation of traditional apoptosis, is believed to be why bendamustine is very effective in killing drug-resistant cancer cells *in vitro*, as well as in patients having chemo-refractory tumors.

[00039] Figure 6 is a histogram that shows the results of adenylate kinase assays performed in the course of several of the "wash-out" experiments described in Example 3, below. In these experiments, SU-DHL-1 cells were treated with either 50 μM bendamustine, 20 μM phosphoramide mustard, or 2 μM chlorambucil for either 30, 60, or

90 minutes. After the timed drug incubation, the cells were washed in 1X PBS to "wash out" the particular chemotherapeutic agent and then fresh medium was added. Cells were then cultured for 48 hours, after which time adenylate kinase assays were performed on the cell supernatants. The pink bars represent zero minutes of drug (or no drug) incubation. The green bars represent 30 minute incubations, the orange bars represent 60 minute incubations, and the purple bars represent 120 minute incubations. The results plot the level of adenylate kinase activity in the supernatants versus the three drugs and a "no drug" control. Standard deviation are represented at the top of each bar on the graph.

[00040] Figure 7, like Figure 6, is a histogram that shows the results of adenylate kinase assays performed in the course of several of the "wash-out" experiments described in Example 3, below. The difference between the results depicted in Figures 6 and 7 is that the data represented in Figure 6 concerns 48 hours of cell culture after each of the drugs was "washed out" of the culture, whereas the data in Figure 7 concerns 72 hours of cell culture post "washing out" the particular drug.

[00041] As those in the art will appreciate, the following description describes certain preferred embodiments of the invention in detail, and is thus only representative and does not depict the actual scope of the invention. Before describing the present invention in detail, it is understood that the invention is not limited to the particular molecules, systems, and methodologies described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the invention defined by the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

[00042] The present invention is based on the surprising discovery that the alkylating agent bendamustine exerts very rapid cytotoxic effects on a number of cancer cell types, including those refractory to conventional chemotherapeutic regimens. It has also been discovered that bendamustine exerts its toxic effects through distinct modes of action, as compared to other anti-cancer drugs, as described in detail below.

[00043] Bendamustine, 4-{5-[bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl}, is a chemotherapeutic agent of the nitrogen mustard class. Bendamustine primarily

exhibits alklyating activity, *i.e.*, it is a DNA-damaging agent. When administered to humans (typically by bolus intravenous infusion), bendamustine has a short serum half-life, on the order of 2 hours. Thus, it is rapidly cleared from a patient's system. Surprisingly, it has been discovered that, after cell uptake, bendamustine rapidly exerts its durable cytotoxic effects. Indeed, as reported in Example 3, below, the vast majority of the compound's cytotoxic effects are exerted upon exposing cancer cells to the agent for as little as about 30 minutes.

[00044] Current protocols for bendamustine treatment typically involve the delivery of three separate bolus intravenous infusions each containing an equivalent amount of bendamustine. The second infusion is generally given one day after the first infusion, followed by the third infusion three weeks after the first infusion. This regimen has been used due toxicities related to bendamustine treatment, including myelosuppression. Given the short serum half-life of bendamustine and its fast-acting nature, drug-related toxicity can be reduced by delaying the second and subsequent administrations. Indeed, because extensive and perhaps lethal tumor lysis has been occasionally been reported in connection with bendamustine treatment of non-Hodgkin's lymphoma, greater spacing of the multiple administrations of the drug may serve to reduce the incidence of tumor lysis. In addition to reducing unwanted toxicity, greater spacing of bendamustine administrations in a particular treatment regimen will also serve to increase the therapeutic window, *i.e.*, the time period over which the drug is exerting its intended therapeutic benefit.

[00045] The composition(s) used in the practice of the invention may be processed in accordance with conventional methods of pharmaceutical compounding techniques to produce medicinal agents (*i.e.*, medicaments or therapeutic compositions) for administration to subjects, including humans and other mammals, *i.e.*, "pharmaceutical" and "veterinary" administration, respectively. *See*, for example, the latest edition of Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, PA). Typically, a compound such as bendamustine is combined as a composition with a pharmaceutically acceptable carrier. The composition(s) may also include one or more of the following: preserving agents; solubilizing agents; stabilizing agents; wetting agents; emulsifiers; sweeteners; colorants; odorants; salts; buffers; coating agents; and antioxidants.

[00046] The drugs used in the practice of the invention may be prepared as free acids or bases, which are then preferably combined with a suitable compound to yield a pharmaceutically acceptable salt. The expression "pharmaceutically acceptable salts" refers to non-toxic salts formed with nontoxic, pharmaceutically acceptable inorganic or organic acids or inorganic or organic bases. For example, the salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, fumaric, methanesulfonic, and toluenesulfonic acid and the like. Salts also include those from inorganic bases, such as ammonia, hydroxyethylamine and hydrazine. Suitable organic bases include methylamine, ethylamine, propylamine, dimethylamine, diethylamine, trimethylamine, triethylamine, ethylenediamine, hydroxyethylamine, morpholine, piperazine, and guanidine.

[00047] In any event, the therapeutic compositions are preferably made in the form of a dosage unit containing a given amount of a desired therapeutic agent (*e.g.*, bendamustine) and a carrier (*i.e.*, a physiologically acceptable excipient). What constitutes a therapeutically effective amount of any such molecule for a human or other mammal (or other animal) will depend on a variety of factors, including, among others, the type of disease or disorder, the age, weight, gender, medical condition of the subject, the severity of the condition, the route of administration, and the particular compound employed. Thus, dosage regimens may vary widely, but can be determined routinely using standard methods. In any event, an "effective amount" of chemotherapeutic agent is an amount that elicits the desired cytotoxic. The quantity of such a therapeutic molecule required to achieve the desired effect will depend on numerous considerations, including the particular molecule itself, the disease or disorder to be treated, the capacity of the subject's cancer to respond to the molecule, route of administration, *etc.* Precise amounts of the molecule required to achieve the desired effect will depend on the judgment of the practitioner and are peculiar to each individual subject. However, suitable dosages may range from about several nanograms (ng) to about several milligrams (mg) of active ingredient per kilogram body weight per day.

[00048] The preparation of therapeutic compositions is well understood in the art. Typically, such compositions are prepared as injectable, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients that are physiologically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water for injection, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, anti-pyretics, stabilizing agents, thickening agents, suspending agents, anesthetics, preservatives, antioxidants, bacteriostatic agents, analgesics, pH buffering agents, *etc.* that enhance the effectiveness of the active ingredient. Such components can provide additional therapeutic benefit, or act towards preventing any potential side effects that may be posed as a result of administration of the pharmaceutical composition.

[00049] The compositions of the invention may be administered orally, parentally, by inhalation spray, rectally, intranodally, intrathecally, or topically in dosage unit formulations containing conventional carriers, adjuvants, and vehicles. In the context of therapeutic compositions intended for human administration, pharmaceutically acceptable carriers are used. The terms "pharmaceutically acceptable carrier" and "physiologically acceptable carrier" refer to molecular entities and compositions that are physiologically tolerable and do not typically produce an unintended allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a subject.

[00050] For oral administration, the composition may be of any suitable form, including, for example, a capsule, tablet, lozenge, pastille, powder, suspension, or liquid, among others. Liquids may be administered by injection as a composition with suitable carriers including saline, dextrose, or water. The term "parenteral" includes infusion (including continuous or intermittent infusion) and injection via a subcutaneous, intravenous, intramuscular, intrasternal, or intraperitoneal route. Suppositories for rectal administration can be prepared by mixing the active ingredient(s) with a suitable non-irritating excipient such as cocoa butter and/or polyethylene glycols that are solid at ordinary temperatures but liquid at physiological temperatures.

[00051] The compositions may also be prepared in a solid form (including granules, powders or suppositories). The compositions may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers *etc.* Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert excipient such as sucrose, lactose, or starch. Such dosage forms may also comprise additional substances other than inert diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting sweetening, flavoring, and perfuming agents.

[00052] Injectable preparations, such as sterile injectable aqueous or oleaginous suspensions, may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Suitable vehicles and solvents that may be employed are water for injection, Ringer's solution, and isotonic sodium chloride solution, among others. In addition, sterile, fixed oils can be employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[00053] For topical administration, a suitable topical dose of a composition may be administered one to four, and preferably two or three, times daily. The dose may also be administered with intervening days during which no dose is applied. Suitable compositions for topical delivery often comprise from 0.001% to 10% w/w of active ingredient, for example, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation. Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through

the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes), and drops suitable for administration to the eye, ear, or nose.

[00054] Exemplary methods for administering the compositions of the invention (*e.g.*, so as to achieve sterile or aseptic conditions) will be apparent to the skilled artisan. Certain methods suitable for such purposes are set forth in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th Ed. (1985). The administration to the patient can be intermittent; or at a gradual, continuous, constant, or controlled rate.

[00055] Typical therapeutically effective doses for bendamustine for the treatment of non-Hodgkin's lymphoma can be from about 60-120 mg/m^2 given as a single dose on two consecutive days, or with several days between doses. The cycle can be repeated about every three to four weeks. For the treatment of chronic lymphocytic leukemia (CLL) bendamustine can be given at about 80-100 mg/ m^2 on days 1 and 2. The cycle can be repeated after about 4 weeks. For the treatment of Hodgkin's disease (stages II-IV), bendamustine can be given in the "DBVBe regimen" with daunorubicin 25 mg/ m^2 on days 1 and 15, bleomycin 10 mg/ m^2 on days 1 and 15, vincristine 1.4 mg/ m^2 on days 1 and 15, and bendamustine 50 mg/ m^2 on days 1-5 with repetition of the cycle about every 4 weeks. For breast cancer, bendamustine (120 mg/ m^2) on days 1 and 8 can be given in combination with methotrexate 40 mg/ m^2 on days 1 and 8, and 5-fluorouracil 600 mg/ m^2 on days 1 and 8 with repetition of the cycle about every 4 weeks. As a second-line of therapy for breast cancer, bendamustine can be given at about 100-150 mg/ m^2 on days 1 and 2 with repetition of the cycle about every 4 weeks.

[00056] The methods of the invention involve both monotherapy and combination therapy. In the context of combination therapy, the invention envisions the administration of two or more chemotherapeutic agents. A wide variety of chemotherapeutic agents are known in the art. Some of these compounds have already been approved for use in treating one or more cancer indications. Others are in various stages of pre-clinical and clinical development. Examples of chemotherapeutic agents useful in the practice of combination therapies according to the invention include the alkylating agents busulfan, carboplatin, carmustine, cisplatin, chlorambucil, cyclophosphamide, dacarbazine, hexamethylmelamine, ifosphamide, lomustine, mechlorethamine, melphalan, mitotane,

mytomycin, pipobroman, procarbazine, streptozocin, thiotepa, and triethylenemelamine. Preferred anti-metabolites for use in conjunction with bendamustine include capecitabine, chlorodeoxyadenosine, cytarabine (and its activated form, ara-CMP), cytosine arabinoside, dacabazine, floxuridine, fludarabine, 5-fluorouracil, gemcitabine, hydroxyurea, 6-mercaptopurine, methotrexate, pentostatin, trimetrexate, and 6-thioguanine. Preferred anti-mitotic compounds that can be used in combination therapies with bendamustine include navelbine, paclitaxel, taxotere, vinblastine, vincristine, vindesine, and vinorelbine.

[00057] Other classes of chemotherapeutic agents include topoisomerase I inhibitors (*e.g.*, camptothecin, irinotecan. topotecan, *etc.*); topoisomerase II inhibitors such as daunorubicin, doxorubicin, etoposide, idarubicin, mitoxantrone, and teniposide; angiogenesis inhibitors (*e.g.*, dalteparin, suramin, *etc.*); antibodies, including alemtuzumab, bevacizumab, bexarotene, epratuzumab, gemtuzumab ozogamicin, ibritumomab tiuxetan, imatinib mesylate, raltitrexed, revlimid, rituximab, trastuzumab; tyrosine kinase inhibitors; intercalating agents; and hormones, such as anastrozole, estrogen, anti-estrogen (*e.g.*, fulvestrant and tamoxifen), exemestane, flutamide, goserelin, leuprolide, nilutamide, levimasole, letrozole, prednisone, and toremifene. Other chemotherapeutic agents include proteins such as angiostatin, asparaginase, denileukin diftitox, endostatin, imiquimod, interferon, interleukin-11, and pegaspargase. Still other chemotherapeutic agents include molecules such as alitretinoin, altretamine, amifostine, amsacrine, arsenic trioxide, bleomycin, capecitabine, carboxyamidotriazole, celecoxib, dactinomycin, epirubicin, geldanmycin, 17-Allylamino-17-demethoxygeldanamycin (17 AAG), irinotecan, 2-methoxyestradiol, mithramycin, mytomycin C, oxaliplatin, squalamine, temozolamide, thalidomide, tretinoin triapine, and valrubicin. As those in the art will appreciate, these and other chemotherapeutic agents now known or later developed may be used in combination with bendamustine to treat various neoplasias, including cancers.

EXAMPLES

[00058] The following examples are provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. These examples are in no way to be considered to limit the scope of the invention in any manner.

Example 1

Molecular Analysis of the Mechanism of Action of Bendamustine

A. Introduction.

[00059] Bendamustine (Treanda™, Salmedix, Inc. CA; Ribomustin™ (Ribosepharm GmbH, Munich Germany)) is an anti-tumor agent with demonstrated preclinical and clinical activity against various human cancers, such as Non-Hodgkin's Lymphomas (NHL), chronic lymphocytic leukemias, solid tumors, breast and small cell lung cancers, and multiple myelomas, including those refractory to conventional DNA-damaging agents. Bendamustine, 4-{5-[bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl} butyric acid hydrochloride, was originally synthesized with the intention of producing an agent with low toxicity and both alkylating and anti-metabolite properties. It has three sub-structural elements: a 2-chloroethylamine alkylating group; a benzimidazole ring; and a butyric acid side-chain. The 2-chloroethylamine alkylating group is shared with other nitrogen mustards, such as cyclophosphamide, chlorambucil, and melphalan. The benzimidazole central ring system is a unique feature of bendamustine, although the butyric acid side chain is present in chlorambucil. This multi-faceted structure may contribute to its unique anti-neoplastic activity profile and distinguishes it from conventional alkylating agents.

[00060] DNA alkylating agents are extremely useful in the chemotherapy armamentarium. Such drugs may possess unexpected mechanisms of action, such as a capacity of some of these compounds to induce programmed necrosis and the capacity of others (*e.g.*, platins) to induce apoptosis even in cells deprived of nuclei. In the case of the "nitrogen mustards", major differences exist in their profile of activity as reflected by their differentiated use in various indications: cyclophosphamide, which is used primarily in treating NHL; chlorambucil, which is used in treating chronic lymphocytic leukemia; and melphalan, which is used in treating multiple myeloma.

[00061] The main anti-tumor action of bendamustine, in common with other alkylating agents, results from the formation of cross-links between the paired strands of DNA, although other modes of action may also be involved. Thus, the anti-tumor action of bendamustine may derive from mechanisms which are more complex than simply classic

alkylation activity, as DNA double-strand breaks caused by bendamustine are significantly more durable than those caused by cyclophosphamide or BNCU, bendamustine shows activity against cell lines which are resistant *in vitro* and *ex vivo* to other alkylating agents, and unique pro-apoptotic activity has been demonstrated by bendamustine as a single agent and in combination with other anti-cancer agents in several *in vitro* tumor models. Detailed molecular studies on the exact mechanism of action of bendamustine remain sparse. For this reason, state-of-the art molecular tools were used to fully dissect the mechanism of action of bendamustine. This example presents results derived from pharmacogenomic assays to analyze the gene expression profile changes induced by bendamustine in NHL cell lines. These pharmacogenomic analyses were validated by functional assays dealing with the initiation of apoptotic signaling, the mechanism of DNA repair, and the modulation of mitotic checkpoints. Finally, bendamustine has been profiled in the National Cancer Institute's human tumor 60 cell line in vitro screen, and its comparative activity against a library of other alkylating agents (*i.e.*, chlorambucil and phosphoramide mustard (the metabolite of cyclophosphamide)) was studied. Results were also generated using pharmacogenomic assays to analyze the gene expression profile changes induced by bendamustine in NHL cell lines. These pharmacogenomic analyses were validated by Q-PCR and functional assays dealing with the initiation of apoptotic signaling, mechanisms of DNA repair, and the modulation of mitotic checkpoints. Together, these results demonstrate that bendamustine possesses multiple mechanisms of action that are distinct from other DNA alkylating drugs, explaining bendamustine's activity in patients having tumors refractory to conventional therapy.

B. Materials and Methods.

a. Cells.

[00062] SU-DHL-1 cells were obtained from the University California San Diego. Cells were grown in RPMI 1640 (Hyclone) supplemented with 10% FBS (Invitrogen) and 100 units/ml penicillin/streptomycin.

b. Reagents.

[00063] Bendamustine hydrochloride was obtained from Fujisawa Deutschland (Munich, Germany). Phosphoramide mustard cyclohexylamine salt (PM, NSC69945), an active metabolite of cyclophosphamide, was obtained from the synthetic repository of the Developmental Therapeutics Program (DTP) at the National Cancer Institute (NCI). All other reagents were obtained from commercial sources such as Sigma-Aldrich.

c. Drug Treatments.

[00064] For most of the assays presented in this example, the concentrations used for bendamustine, phosphoramide mustard (the active metabolite of cyclophosphamide), and chlorambucil were selected based on their cytotoxic activity measured with the MTT assay over a period of three days. Drugs were prepared in DMSO and then diluted in culture medium.

d. Preparation of RNA Samples and Analysis of Expression Data.

[00065] Cells were harvested (5×10^6 cells) in 1mL TRIZOL solution (Invitrogen, San Diego, CA) and total RNA was isolated as per manufacturer's instructions. Biotin-labeled cDNA (15 μg) was hybridized to each GeneChip array (Affymetrix, Santa Clara). Briefly, the procedure to prepare material for hybridization to the chips involved multiple steps. Total RNA was isolated and quantified by optical density. cDNA was generated using a specific primer that recognizes the poly A tail coupled with a T7 promoter (dT7-(T)24) with dNTP, DTT, and Superscript II to generate the first strand cDNA. This approach alleviated the need to isolate poly-A(+) mRNA. The second strand was synthesized by adding dNTPs with DNA ligase, DNA pol I, and RNAse H, and incubating for 2 h at 16°C before adding T4 DNA polymerase for an additional 5 min. cDNA was column purified and quantified. In vitro transcription (IVT) was performed prior to hybridization to the high-density oligonucleotide arrays. The starting material for this reaction was 1 μg of cDNA to which NTPs were added with 25 % less CTP and UTP to be compensated by adding 10 mM biotinylated-11-CTP and 10 mM biotinylated-16-UTP. The final addition of T7 enzyme in the appropriate buffer for 6 h at 37°C yielded the biotinylated IVT RNA which was then column purified (RNeasy, Qiagen).

Chemically fragmented IVT RNA (15 μg) was mixed with control oligonucleotides, standards (including a housekeeping gene), and salmon sperm DNA in the appropriate buffer, heated to 95°C for 5 minutes, and hybridized to the chip for 16 h at 42°C. Non-hybridized material was washed off with 2XSSPE and phycoerythrin-labeled avidin was then added to the reaction. The excess fluorochrome was washed off and the chip was then scanned for intensity of fluorescence in each synthesis feature (synthesis features are 7.5 square microns).

e. Bioinformatics Analysis.

[00066] A strategy and a process for the analysis of gene expression data was developed, which involved the use of the CORGON method to analyze scanned images of Affymetrix GeneChips. CORGON is freely available software, whose core statistical method is known (Sasik, *et al.* (2002), Bioinformatics, vol. 18, no. 12:1633-40). Only genes that were present at $p < 0.05$ (95% confidence level) in at least one of the conditions were considered for further analysis. A comparison of CORGON with the Affymetrix Microarray Suite (AMS) 5.0 software revealed a 4.4% false positive error rate for CORGON as compared to 29% for AMS 5.0. The genes selected were sorted according to the average or peak magnitude of modulation. The top 100 most modulated genes were chosen for clustering based on the similarity of their expression pattern. Hierarchical clustering methods were used. This initial classification was extremely useful in determining what were the primary genes and pathways modulated by the process under investigation. Clusters of genes that appeared to be co-regulated were subjected to promoter analysis. The next step was GO3 analysis, an unbiased and unsupervised tool for finding statistically significant terms in the Gene Ontology database (website: www.geneontology.org) related to the process. GO3 facilitates the process of identifying the critical components of the system that were modulated significantly. There were three ontologies in the database: molecular function; biological process; and cellular component. The analysis was performed at the UCSD Center for AIDS Research Genomics Core Facility.

f. Quantitative PCR Analysis.

[00067] The expression levels of specific transcripts were determined using quantitative PCR (Q-PCR). Total RNA from each treated SU-DHL-1 cell pellet was isolated using an RNeasy mini-prep kit (Qiagen, Valencia, CA). cDNAs were made using a ThermoScript reverse-transcriptase kit (Invitrogen) and oligo-dT primers according to the manufacturer's protocol. Q-PCR amplification and quantitation was carried out using an iCycler machine (Bio-RAD, Hercules, CA). Sample amplification was performed in a volume of 25 µL containing 12.5 µL of 2 x IQ SybrGreen™ Mix (Bio-Rad), 1µM of each primer, and a volume of cDNA corresponding to 80 ng of total RNA. Cycling conditions were: 95°C for 5 seconds; 30 seconds at the appropriate annealing temperature for each primer; and 72°C for 30 seconds. Target specificity of the assays was validated by melt curve analysis. The expression of each gene was normalized relative to 18s expression levels for each sample. The expression of each gene relative to untreated control was then calculated per the method of Livak and Schmittgen ((2001), Methods, vol. 25:402-408). Primers were designed using Beacon Designer™ (Premier Biosoft, Palo Alto, CA) or designed based on the literature. Primer sequences and annealing temperatures are as follows (each primer is written 5' to 3', followed by its SEQ ID NO):

Gene ID	Forward Primer	Reverse Primer	Anneal Temp
18s	CGCCGCTAGAGGTGAAATTC (1)	TTGGCAAATGCTTTCGCT (2)	55°C
p21	CCTCATCCCGTGTTCTCCTTT (3)	GTACCACCCAGCGGACAAGT (4)	57°C
Noxa	ATTTCTTCGGTCACTACACAA (5)	AACGCCCAACAGGAACAC (6)	55°C
PLK-1	CTCAACACGCCTCATCCT (7)	GTGCTCGCTCATGTAATTGC (8)	57°C
Aurora A	TCCTTGTCAGAATCCATTACCTGT (9)	GAATGCGCTGGGAAGAATTTG (10)	55°C
Aurora B	AGAGTGCATCACACAACGAGA (11)	CTGAGCAGTTTGGAGATGAGGTC (12)	56°C
Cyclin B1	AGTGTGACCCAGACTGCCTC (13)	CAAGCCAGGTCCACCTCCTC (14)	57°C
Exo1	TTGGTCTGGAGGTCTTGGAGA (15)	GAATCGCTCTTTCTTCGGAACTG (16)	57°C

g. COMPARE Analysis.

[00068] Bendamustine was tested in the NCI's *in vitro* anti-tumor screen consisting of 60 human tumor cell lines. Testing involved a minimum of five concentrations at 10-fold dilutions, and each screen was repeated twice. A 48 hour continuous drug exposure

protocol was used. A Sulforhodamine B protein assay estimated cell viability or growth. The COMPARE method and associated data are freely available on the Developmental Therapeutics Program (DTP) website (website: dtp.nci.nih.gov). The NCI assigned bendamustine the number: NSC138783.

h. Western Blot Analysis.

[00069] SU-DHL-1 cells were incubated with 50 μM bendamustine, 2 μM chlorambucil, or 20 μM phosphoramide mustard for 20 hours. Cells were washed twice with 1 x PBS and lysed for 1 hour with ice cold lysis buffer (1 M Tris-HCl (pH 7.4), 1 M KCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholine, with 1mM sodium orthovanidate, 1 mM sodium fluoride, protease inhibitor cocktail (Roche, Nutley, NJ), and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)) added directly before lysis. Non-soluble membranes, DNA, and other precipitants were pelleted and the protein supernatant obtained. Protein concentrations were determined using the Bradford assay (Pierce, Rockford, IL). 20 μg of lysate were separated by gel electrophoresis on a 4-12% polyacrylamide gel, transferred to nitrocellulose membranes (Invitrogen), and detected by immunoblotting using the following primary monoclonal antibodies: anti-p53, anti-phosphorylated p53 (Ser15-specific), anti-p21, and anti-cleaved PARP (caspase-specific cleavage site), which were all purchased from Cell Signaling (Beverly, MA); anti-Bax and anti-PARP, which were purchased from BD Pharmingen (San Diego, CA), and anti-beta-actin, used for a loading control, which was purchased from Sigma (St. Louis, MO). Primary antibodies were incubated overnight at 4°C with gentle shaking. Membranes were washed three times with 1 x PBS and incubated with Alexa Flour 680 goat anti-mouse secondary antibody (1:4000) (Molecular Probes, Eugene, OR) for 2 hours at room temperature with gentle shaking. Blots were washed three times with 1 x PBS and scanned on a LiCor Odyssey scanner.

i. In vitro cell based Ape-1 and AGT assays.

[00070] Cells were pre-incubated for 30 minutes with either 6 mM methoxyamine (Sigma) or 50 μM O^6-benzylguanine (Sigma), inhibitors of Ape-1 base excision repair enzyme and alkylguanyl transferase (AGT) enzyme, respectively. The cells were then exposed to various concentrations of the indicated agents for 72 hrs. Cytotoxicity was

evaluated by the MTT assay (13) and an IC_{50} was measured as the drug concentration that inhibited by 50% the value of the untreated control. Analyses were performed using GraphPad Prism version 3.00 GraphPad Software (San Diego, CA).

j. Cell cycle analyses.

[00071] SU-DHL-1 cells were incubated with equitoxic (IC_{50}) concentrations of bendamustine (50 μM), chlorambucil (4 μM), or phosphoramide mustard (50 μM) for 8 hours. Cells were washed with PBS and fixed in 70% ethanol 20° C for at least one hour. Fixed cells were re-hydrated by washing with PBS. Cells were resuspended in a propidium iodide staining solution consisting of 10 μg/ml propidium iodide (Calbiochem, La Jolla, CA), 10 μg/ml RNAse A (DNase free, Novagen, Madison, WI), and 10 μl/ml Triton-X (Sigma) in PBS. Samples were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). Analyses of cell cycle distribution were performed using DNA ModFit LT (Verity House Software, Inc. Sunnyvale, CA) modeling software.

k. H2AX foci formation.

[00072] Cell were grown on Lab-Tek chamber slides (Nalge Nunc Intl., Naperville, IL) in RPMI 1640 media supplemented with 10% FBS. After allowing the cells to attach for at least one day, cells were treated in media with either DMSO or 50 μM bendamustine. The cells were incubated for 30 minutes at 37°C and then washed two times with PBS. They were incubated for an additional 4 hours at 37°C. The cells were then washed twice with 1 x PBS and incubated 10 minutes in -20°C 100% methanol to fix the cells. They were then washed three times for five minutes each with 1 x PBS. They were incubated at room temperature for 1 hour in blocking buffer (10% FBS in 1 x PBS, 1% BSA). The slides were incubated at 4°C with rocking overnight with the primary polyclonal anti-H2AX antibody (R & D Systems, Minneapolis, MN). The antibody was diluted in blocking buffer at a ratio of 1:10,000. Slides were washed three times with 1 x PBS and incubated with Alexa Flour 488 goat anti-rabbit secondary antibody (1:4000) (Molecular Probes, Eugene, OR) for 45 minutes at room temperature with gentle shaking. Slides were washed three times with 1 x PBS and then the chambers removed and SlowFade Light Antifade with DAPI (Molecular Probes) was added to the cells and coverslips sealed on the slides. Analysis was performed using a motorized Zeiss

AxioPlan 2e imaging microscope with DIC optics and fluorescence, a Zeiss AxioCam HRm camera and Zeiss Axiovision software Version 4.2.

1. Phosphorylation of H2AX at residue Ser139 immunoblot.

[00073] Cell lines were grown to confluency in RPMI 1640 media supplemented with 10% FBS. The cells were then washed twice with 1 x PBS and lysed for 1 hour with ice cold lysis buffer (1 M Tris-HCl (pH 7.4), 1 M KCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholine, with 1mM sodium orthovanidate, 1 mM NaF, protease inhibitor cocktail (Roche, Nutley, NJ), and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)) added directly before lysis. Non-soluble membranes, DNA, and other precipitants were pelleted and the protein supernatant obtained. Protein concentrations were determined using the Bradford assay (Pierce, Rockford, IL). Twenty micrograms of lysate were separated by gel electrophoresis on a 4-12% polyacrylamide gel, transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA), and detected by immunoblotting using a polyclonal anti-H2AX antibody (R & D Systems, Minneapolis, MN). The antibody was diluted in blocking buffer at a ratio of 1:2000, and the membranes were incubated for 2 hours at room temperature with gentle shaking. Membranes were washed three times with 1 x PBS and incubated with Alexa Flour 680 goat anti-rabbit secondary antibody (1:5000) (Molecular Probes, Eugene, OR) for 2 hours at room temperature with gentle shaking. Blots were washed three times with 1 x PBS and scanned on a LiCor Odyssey scanner.

C. Results.

a. Gene expression profiling identifies signature genes that are regulated by bendamustine that are distinct from chlorambucil or cyclophosphamide.

[00074] Equitoxic concentrations for bendamustine, chlorambucil, and phosphoramide mustard (the active metabolite of cyclophosphamide) were determined by measuring cell viability after three days exposure to drug. For the assays presented in this study, the concentrations used for bendamustine, phosphoramide mustard, and chlorambucil were selected based on this data (Table 1, below). These concentrations also reflect the clinically achievable levels for each drug. Affymetrix GeneChip analysis was used to

compare the expression levels of over 12,000 genes in drug-treated SU-DHL-1, a non-Hodgkin's lymphoma cell line, cells compared to control cells. SU-DHL-1 cells were incubated with bendamustine at the IC_{50} concentration (25 μM) and at the IC_{90} concentration (35 μM). Chlorambucil and the cyclophosphamide metabolite phosphoramide mustard were tested at IC_{90}, *i.e.*, 5 μM and 50 μM, respectively. Gene expression was monitored following 8 hours treatment with drug to identify the proximal events of this early stress response.

[00075] The genomic analysis revealed that the majority of the genes are similarly regulated between the three tested drugs, as demonstrated by the clustergram of the top 100 modulated genes (Figure 1A). Most genes were upregulated (red color) upon exposure to the drugs. A subset of genes was transcriptionally repressed following drug treatment (blue color). Importantly, a group of genes was identified that displayed differential regulation by bendamustine compared to the other two drugs tested.

[00076] Many of the induced genes (Figure 1B) were known to possess p53-response elements in their promoter regions and are considered p53-dependent. Examples of these genes are: p21 (p53-induced cell division kinase inhibitor); wip1 (p53-induced protein phosphatase 1); NOXA (p53-induced pro-apoptotic Bcl-2 family member); DR5/KILLER (p53-regulated DNA damage-inducible cell death receptor); and BTG2. Interestingly, four members of the tumor necrosis factor receptor superfamily (members 6, 9, 10, and 10b) were identified in the top-100 modulated genes. Several of these genes have been shown to play a critical role in the regulation of the extrinsic apoptotic pathway (REF, TRAIL/TNF apoptosis). Several other genes display an opposite trend between bendamustine and the other two compounds (data not shown). These genes were upregulated by bendamustine, at both concentrations, but were down-regulated by both chlorambucil and phosphoramide mustard.

[00077] To assess the pharmacogenomic differences between bendamustine, chlorambucil, and phosphoramide mustard, the results from the gene profiling were re-analyzed with the GO3 software, an unbiased and unsupervised tool for finding statistically significant terms in the Gene Ontology (GO) database (website: www.geneontology.org) related to the process. Genes significantly up- or down-

regulated in bendamustine-treated cells and at least 1.5-fold above or below levels of expression in control-treated cells were connected to biological process annotations provided by the Gene Ontology (GO) consortium. Based on the hierarchical structure of the GO annotations, the probability that each immediate daughter term (a P value) be linked to the number of selected genes by chance was calculated. The results of the GO analysis comparing the DMSO-treated control and the bendamustine–treated cells (at IC_{90} dose) are reported in Table 2, below. In Table 2, below, the first column represents general categories, the second and third columns are the number and name of the specific biological process, and the last column is the p value for each process. The p value was calculated using the GO3 software. Four major functional groups were found be statistically modulated by bendamustine: (1) DNA-damage, stress response, apoptosis; (2) DNA metabolism, DNA repair, transcription; (3) cell proliferation, cell cycle, mitotic checkpoint; and (4) cell regulation. Each of these groups encompasses several biological processes that were found to be significantly modulated by bendamustine. The biological processes that provided the lowest p values and therefore were the most statistically significant were: response to DNA damage stress (GO6974); DNA metabolism (GO6259); and cell proliferation (GO8283).

[00078] A similar analysis performed with chlorambucil and phosphoramide mustard suggested that little overlap exists between the profile obtained with bendamustine and chlorambucil. Some similarities in gene modulation were observed between bendamustine and phosphoramide mustard, although these were limited to the "DNA metabolism, DNA repair, and transcription" group. These results provided the basis for the selection of specific gene products for the quantitative validation of the gene array results and more definitive differentiation of bendamustine.

b. Validation of genomic analysis by real-time quantitative Q-PCR analysis.

[00079] Confirmation and validation of the array data was performed by real-time quantitative PCR analysis (Q-PCR). Several genes involved in p53-signaling, apoptosis, DNA repair, and cell cycle/mitotic checkpoints were all differentially regulated when comparing bendamustine to the other alkylating agents tested.

[00080] Two examples of "canonical" p53-dependent genes selected for Q-PCR validation were p21 (Cip1/Waf1), the cyclin-dependent kinase inhibitor 1A, and the pro-apoptotic BH3-only Bcl-2 family member, NOXA. Both genes were found to be induced in SU-DHL-1 cells, 8 hours after exposure to bendamustine. Both genes were also induced by equitoxic concentrations of phosphoramide mustard and chlorambucil, but to a much lower extent (Figure 2A).

[00081] One of the most striking results that emerged from the validation analysis was the differential regulation of several mitosis-related genes, including polo-like kinase 1 (PLK-1), the Aurora Kinases A and B, and cyclin B1. These genes are considered to play an important in mitotic checkpoint regulation. Treatment with bendamustine led to a 60 to 80% down-regulation of the mRNA expression of all these genes. In contrast, phosphoramide mustard or chlorambucil only exerted a minor effect on the transcripts of these genes, with possibly the exception of the Aurora kinases (Figure 2B).

[00082] Differences also emerged in the analysis of the mRNA expression of the DNA-repair gene exonuclease-1 (EXO1). Bendamustine induced a slightly stronger (2.5-fold) up-regulation of Exo1 expression (Figure 2C) compared with that observed with phosphoramide mustard (1.5-fold) or chlorambucil (1.8-fold). Fen1 (flap endonuclease 1) was also upregulated by bendamustine, and phosphoramide mustard upregulated this gene to the same level when used at equitoxic concentrations (Figure 2C).

c. Apoptosis signaling by bendamustine in NHL cells.

[00083] To dissect the molecular events involved in bendamustine-induced programmed cell death in NHL cells, expression of key apoptotic proteins was monitored by immunoblot analysis. The results clearly showed that bendamustine can efficiently and rapidly trigger the classical p53-dependent apoptotic pathway. One of the initial or apical events is the induction of p53 phosphorylation, as detected using antibodies that specifically recognize phosphorylation of the serine-15 residue. An 8-fold up-regulation of Ser-15-phosphorylated p53 was observed in SU-DHL-1 cells exposed to bendamustine, while only a minor up-regulation was seen in phosphoramide mustard treated cells, and no changes were observed in chlorambucil-treated cells (Figure 3, top-left panel).

[00084] In parallel with the induction of phosphorylated p53, a strong increase in the expression of total p53 was seen in bendamustine-treated cells. Chlorambucil-treated cells displayed a small increase in total p53, while exposure to phosphoramide mustard induced no change in p53 levels. The changes observed in p21 protein expression were minor for each of the drugs when compared to changes in protein expression levels of p53. An increase in the protein expression of Bax, a key BH3-only pro-apoptotic Bcl-2 family member, was observed only in bendamustine-treated SU-DHL-1 cells (Figure 3, low-left panel).

[00085] The most striking difference observed in comparing the effect of bendamustine with phosphoramide mustard and chlorambucil was found when the expression of PARP, poly-ADP-ribose polymerase-1, was compared. PARP is a critical NAD-requiring enzyme important in DNA-repair mechanisms. PARP is also an "early" substrate of the pro-apoptotic proteolytic caspase enzymes. SU-DHL-1 cells treated with bendamustine showed a dramatic reduction of PARP protein expression (Figure 3, top-right panel). The reason for the reduction of PARP expression was its cleavage by caspases, as demonstrated by the appearance of proteolytic cleavage products recognized by a "cleavage-specific" antibody (Figure 3, middle-right panel). Notably, no changes in the expression of PARP were detected in NHL cells treated by equitoxic concentrations of phosphoramide mustard or chlorambucil. Similar results were observed when using double the equitoxic doses of phosphoramide mustard (40 μM) and chlorambucil (4 μM) while maintaining the dose of bendamustine (50 μM) (data not shown). Thus, an assessment of PARP expression levels can be used for various purposes. For example, a PARP assay can be to provide an indication as to the efficacy of a particular therapeutic regimen, wherein reduced PARP expression (preferably measured at the protein level, for example by PARP activity, for the presence of PARP cleavage products, *etc.*) indicates that the administered drug is having the desired effect. In addition, a PARP assay can be used prognostically to determine, for example, if cells of a tissue (for example, cells derived from a biopsy or other biological sample) are likely to respond to a particular therapy (*e.g.*, bendamustine monotherapy or a combination therapy wherein one of the therapies utilizes bendamustine).

d. Inhibition of base excision repair, but not O^6-methylguanine-DNA methyltransferase repair, blocks bendamustine activity.

[00086] The role of the repair enzyme Ape-1, an apurininc endonuclease that plays a critical role in the base excision repair (BER) pathway in the cytotoxic activity of bendamustine and the cyclophosphamide metabolite, phosphoramide mustard, was assessed using the Ape-1 inhibitor methoxyamine. The IC_{50} of bendamustine was reduced approximately four-fold (from approximately 50 μM to approximately 12 μM) with methoxyamine addition (Figure 4A). In contrast, the IC_{50} of phosphoramide mustard only changed slightly when methoxyamine was added. The results suggest that BER may play an important role in the repair of bendamustine-induced DNA damage, but not in the repair of the damage induced by cyclophosphamide.

[00087] The effect of O^6-benzylguanine, a known inhibitor of O^6-alkylguanine-DNA alkyltransferase (AGT) on the anti-tumor activity of bendamustine, was also tested in the SU-DHL-1 cells. The results demonstrated that the cytotoxic potency of bendamustine was not enhanced by adding O^6-benzylguanine. Opposite results were obtained with cyclophosphamide, suggesting that unlike cyclophosphamide, bendamustine does not rely appreciably on the O^6-methylguanine-DNA methyltransferase DNA repair mechanism (Figure 4B).

e. Bendamustine HCl rapidly induces the formation of double-strand breaks resulting in unique cell cycle alterations.

[00088] To investigate the capacity of bendamustine HCl to induce double-strand breaks (DSBs), two biochemical markers were analyzed: nuclear localization of gamma-H2AX histone by immunofluorescence; and phosphorylation of H2AX at residue Ser139 by immunoblot analysis. Results confirmed that bendamustine HCl potently and rapidly induced DSBs in a variety of tumor cells, including multidrug-resistant and p53 deficient lines. Incubation with 50 μM bendamustine HCl leads to the formation of intranclear foci detectable after as few as 30 minutes. Time-course analysis showed that Ser139 phosphorylation of gamma-H2AX was detectable after 24 hours of continuous exposure to bendamustine HCL as well as after a very short exposure to the drug (30 minutes), followed by drug removal (washout). Bendamustine HCl induced phosphorylation of

H2AX occurred earlier than with other 2-chloroethylamino DNA alkylators such as cyclophosphamide. Cell-cycle analysis of SU-DHL-1 lymphoma cells exposed for eight hours to 50 μM bendamustine HCl showed an average S-phase distribution increase of over 40% without an attendant G2M arrest. Exposure to equitoxic concentrations of chlorambucil and cyclophosamide increased S-phase distribution by approximately 20% and 15% respectively. These findings illustrate that bendamustine HCl can induce DNA double-strand breaks, even after a transient 30 minute exposure.

f. Bendamustine displays a unique profile of activity using the NCI COMPARE analysis.

[00089] Bendamustine cytotoxicity was evaluated in the 60 human cell lines of the National Cancer Institute's preclinical anti-tumor drug discovery screen (NCI screen). The NCI screen is useful for comparing relative potency of potential anti-neoplastic agents with known therapeutic agents from an extensive database of more than 45,000 compounds and natural products. The COMPARE analysis was run using the GI50 results generated with bendamustine as a "seed". Compounds with high Pearson correlation coefficients (PCC) often have similar mechanisms of action. Bendamustine did not demonstrate a strong correlation (>0.8) in the NCI screen with any agent (Table 3, below). Out of the six top matches with bendamustine, only the methylating agent DTIC (dacarbazine) showed approximately an 80% correlative agreement (r value). In contrast, a total of 25 compounds with correlation coefficients over 0.83 were identified for melphalan, chlorambucil, or the active metabolite of cyclophosphamide. In addition, direct comparison of melphalan, chlorambucil, and cyclophosphamide sensitivity patterns in this screen demonstrated high correlation coefficients between the three drugs (0.762-0.934, data not shown). These data show a statistical agreement in sensitivity profile of the agents and a high likelihood of a common mechanism of action. The lack of correlation between bendamustine and other members of the nitrogen mustard class is compelling and reveals that bendamustine has a distinct pattern of anti-tumor activity.

D. Discussion.

[00090] The results of these experiments, obtained using a variety of biological and analytical tools, demonstrate that bendamustine possesses a distinct mechanism of action

when compared to other clinically used compounds that share the same "nitrogen mustard" active moiety, such as cyclophosphamide and chlorambucil.

[00091] One of the tools employed in this study was a pharmacogenomic approach, which allows the simultaneous analysis and monitoring of expression levels of thousands of fully characterized genes upon incubation of target cell lines with a selected drug, has been successfully used to elucidate the mechanism of action of other anticancer drugs. Its major advantage was the generation of unbiased information that led to the identification of a distinct mechanism of action for bendamustine, differentiating it from other DNA-alkylating agents.

[00092] With this approach, a strong classical p53-dependent stress-response "signature" was detected for bendamustine, and present, but at a greatly reduced intensity, in phosphoramide mustard- and chlorambucil-treated cells. Q-PCR analysis confirmed the gene-array analysis, validating the up-regulation of genes containing p53-responsive elements, such as p21 (Waf/Cip1) and NOXA. As an inhibitor for cyclin-dependent kinases, particularly those that function during the G_1 phase of the cell cycle, *p21/Waf1/Cip1* is believed to mediate, at least in part, p53-induced G_1 arrest. The mechanisms leading to p53-induced cell cycle arrest and apoptosis have been extensively investigated and reported. Noxa encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins. NOXA was shown to be a target of p53-mediated transactivation and to function as a mediator of p53-dependent apoptosis through mitochondrial dysfunction. Mouse embryonic fibroblasts deficient in Noxa showed notable resistance to oncogene-dependent apoptosis in response to DNA damage.

[00093] Activation of the p53 pro-apoptotic pathway was then confirmed by immunoblot analysis, with the detection of phosphorylated p53 (Ser15), as well as with the up-regulation of Bax. Although other nitrogen mustards have been previously reported to induce a p53-mediated stress response, bendamustine provides a stronger and more rapidly induced signal when compared to equitoxic doses of the cyclophosphamide metabolite (PM) or chlorambucil. Bendamustine was also found to induce a rapid and extensive cleavage of PARP, an enzyme that catalyzes poly(ADP-ribosylation) of a variety of proteins. Although bendamustine induces PARP cleavage, the difference

between the ability of the three drugs to cause PARP cleavage in SU-DHL-1 cells was striking. This rapid induction of PARP cleavage may play a critical role in the mechanism of action of bendamustine, given the importance of PARP for DNA repair mechanisms. Indeed, in response to DNA damage, cells initially activate PARP, resulting in an increase of the accessibility of DNA to DNA repair enzymes and transcription factors. In addition, PARP has been implicated in initiating cell death by either apoptosis or necrosis.

[00094] Another major difference that emerged from the pharmacogenomic profiling of bendamustine and the other tested nitrogen mustards was the effect on expression levels of polo-like kinase 1 (PLK-1), Aurora kinases (A and B), and Cyclin B1. The mitotic checkpoint kinases PLK-1 and Aurora are involved in many aspects of cell cycle regulation, such as activation and inactivation of CDK/cyclin complexes, centrosome assembly and maturation, and activation of the anaphase-promoting complex (APC) during the metaphase-anaphase transition, and cytokinesis. Interestingly, when these checkpoint regulators are inhibited using siRNA or using targeted small molecules, potentiation of the effect of DNA-damaging drugs is observed, together with the appearance of mitotic catastrophe. Mitotic catastrophe is a form of cell death that occurs during metaphase and is morphologically distinct from apoptosis. Mitotic catastrophe can occur in absence of functional p53 or in cells where conventional caspase-dependent apoptosis is suppressed. For this reason, initiation of mitotic catastrophe is an appealing mechanism of tumor cell death, since it may also function in tumor cells that have been selected by several rounds of chemotherapy using conventional chemotherapeutic drugs. The extensive and durable DNA-damage elicited by bendamustine and concomitant inhibition of M-phase-specific checkpoints by bendamustine may trigger mitotic catastrophe in the treated cells. This may explain the clinically documented activity of bendamustine in patients refractory to cyclophosphamide and chlorambucil-containing regimens.

[00095] Efficient DNA-repair mechanisms have been demonstrated to play a critical role in the mechanism of action of DNA-alkylating drugs. Activation of discrete DNA-repair mechanisms may also confer a distinct profile of activity to drugs that share similar chemical features. The pharmacogenomic analysis described herein identified DNA-

repair genes differentially regulated by bendamustine compared to phosphoramide mustard and chlorambucil. One such gene, exonuclease 1 (Exo1), is a 5'-3' exonuclease that interacts with MutS and MutL homologs and has been implicated in the excision step of DNA mismatch repair and in the processing and repair of double-strand breaks. Exo1 has been involved in somatic hypermutation and class-switch recombination and is therefore very important in B cell function and the generation of antibodies.

[00096] To investigate further the differences in the repair mechanisms between bendamustine, cyclophosphamide, and chlorambucil, functional assays were performed. Two major mechanisms were investigated: the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT); and the apurinic/apyrimidinc endonuclease Ape-1. AGT, a ubiquitous enzyme, removes the O^6-alkylguanine DNA adduct caused by several alkylating agents, including nitrosureas and triazenes. Clinical evidence suggests that brain tumors that express high levels of AGT, and may thus be more resistant to some DNA-alkylators such as temozolomide. The nucleoside O^6-benzylguanine (O^6-BG) provides a means to effectively inactivate the AGT protein. In some cell lines, benzylguanine clearly enhanced the toxicity of the activated from of cyclophosphamide. As shown here, the cytotoxic potency of cyclophosphamide, but not bendamustine, was enhanced by adding O^6-benzylguanine, indicating that bendamustine does not induce O^6-alkylguanine DNA adducts which can be repaired by AGT.

[00097] Ape-1/Ref-1 is an apurinic/apyrimidinic endonuclease that plays a critical role in the base excision repair (BER) pathway. BER is activated by damage induced by a variety of DNA-damaging drugs, including DNA alkylators and DNA-methylating agents, such as temozolomide. The role of Ape-1 was tested using the compound methoxyamine (MX), a specific inhibitor of its enzymatic activity. The cytotoxic activity of bendamustine was enhanced by the inhibition of Ape-1 by MX, indicating a role for BER. No changes were observed using the cyclophosphamide metabolite, underlying a major difference between the DNA-repair mechanisms activated by these drugs.

[00098] The NCI Human Tumor 60 Cell line *In Vitro* Screen is useful in comparing relative potency of potential anti-neoplastic agents with other known therapeutic agents. It has also been demonstrated in many cases that when pairs of compounds are found to

have a high correlation coefficient between their screening results using the panel, as evaluated by the COMPARE statistical analysis program, the agents often have similar mechanisms of action. The high correlation observed for the nitrogen mustards melphalan, chlorambucil, and cyclophosphamide are all with known alkylating agents, confirming the ability of the COMPARE analysis to find common mechanisms of action. Out of the six top matches with bendamustine, only the methylating agent DTIC (dacarbazine) showed approximately an 80% correlative agreement (r value). These results reveal that bendamustine displays a distinct mechanism of action in relationship to other known alkylating agents.

[00099] Based on the results presented in this example, the deduced mechanism of action of bendamustine is illustrated in Figure 5. Bendamustine can efficiently enter tumor cells and induce prolonged and extensive DNA alkylation and fragmentation, probably due to the high chemical stability of the aziridinium transition state ring conferred by bendamustine's benzimidazole ring system. Bendamustine treatment results in the initiation of three main signaling pathways: 1) activation of the "canonical" p53-dependent stress pathway, resulting in strong activation of intrinsic apoptosis, which is mediated by pro-apoptotic BCL-2 family members such as NOXA and Bax; 2) activation of DNA repair mechanisms, such as the base-excision repair machinery, that are not activated by other nitrogen mustards frequently used in NHL or CLL patients; and 3) inhibition of several mitotic checkpoints, such as the kinases PLK-1 and Aurora A and B. The concomitant induction of DNA damage and inhibition of mitotic checkpoints may not allow the tumor cells exposed to bendamustine to efficiently repair the DNA damage before undergoing mitosis. Cells entering mitosis with extensively damaged DNA, or cells that cannot proceed to the "conventional" p53-dependent apoptosis, will undergo death by mitotic catastrophe. This alternative programmed cell death pathway, together with the strong activation of traditional apoptosis, indicates why bendamustine is effective in drug-resistant cells *in vitro*, as well as in patients carrying chemo-refractory tumors. Consequently, bendamustine treatment will represent an important addition to the armamentarium of the clinician for the treatment of patients with indolent non-Hodgkin's lymphoma and other hematologic cancers, among others.

Example 2

Bendamustine Activity in NHL Cells Induces the Mitotic Catastrophe Death Pathway

[00100] As described in Example 1 above, bendamustine is an alkylating agent with a distinct mechanism of action, and is undergoing clinical trials in NHL and CLL patients refractory to traditional DNA-damaging agents. Bendamustine induces unique changes in gene expression in NHL cells and displays a lack of cross-resistance with other 2-chloroethylamine alkylating agents. Quantitative PCR analysis confirmed that the G 2/M checkpoint regulators Polo-like kinase 1 (PLK-1) and Aurora A kinase (AurkA) are down-regulated in the NHL cell line SU-DHL-1 after 8 hours of exposure to clinically relevant concentrations of the drug. No changes in these same genes were observed when cells were exposed to equi-toxic doses of chlorambucil or an active metabolite of cyclophosphamide.

[00101] The ability of bendamustine to induce cytotoxicity in cells unable to undergo classical caspase-mediated apoptosis was investigated. Multi-drug resistant MCF-7/ADR cells and p53 deficient RKO-E6 colon adenocarcinoma cells were exposed for two or three days to either 50 μM bendamustine alone or 50 μM bendamustine and 20 μM pan-caspase inhibitor zVAD-fmk. Although zVAD-fmk was able to inhibit bendamustine-induced increases in Annexin-V-positive cells, microscopic analysis of nuclear morphology using the DNA stain DAPI in cells treated with either bendamustine alone or in combination with zVAD-fmk showed increased incidence of micronucleation. Multi/micro-nucleation and abnormal chromatin condensation are both hallmarks of mitotic catastrophe and have been observed in tumor cells exposed to microtubule-binding drugs such as the vinca alkaloids and taxanes. Activation of mitotic catastrophe may amplify the cytotoxicity of bendamustine and its activity in tumor cells where classical apoptotic pathways were inhibited.

Example 3

Fast-Acting Bendamustine Activates Potent Apoptosis and Cell Death in Lymphoma and Leukemia Cells

[00102] As described above, the alkylating agent bendamustine exhibits chemotherapeutic activity against drug-resistant cancers, among others, and possesses a

unique mechanism of action when compared to other related anti-tumor agents. As is the case with other anti-neoplastic nitrogen mustards, bendamustine has a relatively short serum half-life in humans (approximately 2 hours), and is administered clinically by bolus intravenous infusion. The purpose of the work reported in this example was to assess the capacity of bendamustine to induce cell death and apoptosis when exposed for brief periods to cancer cells in vitro. The activity of bendamustine in such experimental models was compared to other structurally-related agents. The results obtained indicate that bendamustine exerts maximal anti-tumor activity after a brief (30 minute) exposure to cells. To obtain these results, the NHL cell line SU-DHL-1 was exposed to 50 μM bendamustine for brief periods ranging from 30 minutes to 4 hours, washed, and allowed to recover for 20 hours in drug-free media. Cells exposed to bendamustine for as few as 30 minutes displayed extensive loss of viability as measured by a variety of biological assays, including measurement of intracellular ATP and release of adenylate kinase into the supernatant at 48 and 72 hours post drug exposure (Figures 6 and 7). In contrast, cells treated with other members of this class of alkylating agents (here, chlorambucil, melphalan, and the cyclophosphamide metabolite phosphoramide mustard; data shown for chlorambucil and phosphoramide mustard) experienced minimal loss of viability when exposed to these agents for 30, 60, and 120 minutes. These other nitrogen mustards required a much longer exposure period (at least 4 hours) to induce a cytotoxic effect comparable to bendamustine in these assays. These findings were confirmed using an MTT-based assay in which bendamustine had a similar IC_{50} in SU-DHL-1 and HL-60 cells at 72 hours following exposure to drug for 30 minutes, 4 hours, or 72 hours. By comparison, chlorambucil, melphalan, and phosphoramide mustard exhibited 10- to 20-fold higher IC_{50}s when incubated with these same cell lines for 30 minutes compared to continuous (72 hour) exposure.

[00103] Intracellular ATP levels were assayed using the following luciferase-based ATP assay. 10 mL of CellTiter-Glo® reagent was mixed with the appropriate amount of CellTiter-Glo substrate (per the manufacturer's instructions; Promega Corp.), and the mixture was allowed to equilibrate for ten minutes. 100 μL of this solution was then combined with 100 μL of cell-containing culture medium, and the mixture was allowed to incubate for ten minutes. Luminescence was detected using a CCD-based plate reader.

[00104] An adenylate kinase (ADK) assay was selected because as a cell membrane of a treated cell looses integrity, ADK is released into the culture medium (or, in the context of a biological sample, into the extracellular space, blood, etc. To perform the ADK assays in 96-well plates, in each test well 20 μL of supernatant from an aliquot of culture medium briefly centrifuged to pellet cells was mixed with 100 μL of the ADK reagent (20 mL Cambrex ToxiLight reagent plus the appropriate amount of Cambrex ToxiLight substrate per the manufacturer's instructions; Cambrex Corp., NJ) that had just been prepared and allowed to equilibrate for 15 min. The reaction mixture was then incubated for two minutes to allow the kinase reaction to occur. Luminescence from the samples was then read immediately in a plate reader.

[00105] Cell viability was also assessed by mixing 20 μL aliquots of the particular cell culture with180 μL Guava ViaCount Reagent (Guava Technologies, Hayward, CA), diluted 1:10 dilution just prior to use. Each mixture was then incubated for five minutes. A ViaCount cell counting assay was then performed using a Guava PC Flow Cytometer, which allows the number of live cells per 1,000 total cells to be determined. Live versus dead cells were distinguished using the dye 7AAD, which can diffuse into dead or dying cells through their deteriorating cell membranes.

[00106] As described in Example 1, rapid induction of PARP (poly [ADP-ribose] polymerase) cleavage is a hallmark of bendamustine-induced cell death in NHL cells. Maximal PARP cleavage was observed in SU-DHL-1 cells exposed for as few as 30 minutes to 50 μM SDX-105 and, following drug washout, further incubated for 8 hours. No PARP cleavage was observed in cells treated in a similar manner for 30 minutes with 40 μM phosphoramide mustard, 4 μM chlorambucil, or 2 μM melphalan. The concentrations of each drug used represents equitoxic concentrations when compared to 50 μM bendamustine as measured by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay after a period of 72 hours of drug exposure.

[00107] MTT assays were performed to titrate doses of the various drugs to determine the effective concentrations required to kill 50% of the treated cells. These assays were performed in 96-well plates. Concentrations ranged up to a maximum of 500 μM. In each assay, controls included untreated cells and kill control. For plates used to test cells

in the "wash-out" experiments, plates were centrifuged for 5 minutes to pellet cells. Medium was then removed, the cell pellets were rinsed once with 1X PBS, and then resuspended in fresh medium. Cells were incubated with the particular dosage of drug for 3 days at 37°C in an atmosphere containing 5.0% CO_2. After three days, 10 µL of MTT (12 mM) Reagent (5 mg/mL MTT (Promega) dissolved in fresh culture medium, filter-sterilized, stored at 2-8°C) was added to each well. Following a four-hour incubation, 100 µL of lysis buffer (20% SDS, 0.015M HCl) was added to each well. The mixtures were placed overnight at 37°C in an atmosphere containing 5.0% CO_2 to allow cells to lyse. The next morning, the degree of cell lysis was determined using a multiwell scanning spectrophotometer reading at 595 nm.

[00108] Comparable results were obtained by treating the human cancer cell line HL-60 with 100 µM bendamustine or 12 µM chlorambucil. Periods of exposure to the drug were 30 minutes, 1 hour, or 2.5 hours, wherein the culture medium containing drug was removed after the noted time period and replaced with fresh medium containing no drug.

[00108] Taken together, these results illustrate the unique capacity of bendamustine to activate an irreversible cell death pathway following even brief incubation with cancer cells, which distinguishes it from other related alkylating agents. Such fast-acting cytotoxicity confirms bendamustine's potent clinical activity, and indicates that it will be useful for treating various cancers, including those that are refractory to conventional chemotherapy.

Example 4
Clinical Data

[00109] This study evaluated the efficacy and toxicity of bendamustine in patients with NHL who have relapsed or are refractory to previous chemotherapy regimens. Patients refractory to rituximab had disease progression within 6 months of treatment.

[00110] **Methods:** This Phase II multicenter trial enrolled patients with relapsed indolent or transformed rituximab-refractory B-cell NHL from 17 sites in the US and Canada. Indolent histologic phenotype was seen in 84% of patients, while 16% had transformed disease. Median age of patients was 63 years (range: 38-84) and 88% had

Stage III/IV disease. Patients received bendamustine 120 mg/m^2 IV over 30-60 minutes, days 1 and 2, every 21 days for up to 6 cycles. Response was measured using the International Working Group criteria.

[00111] **Results:** The intent-to-treat (ITT) population consisted of 75 heavily pretreated patients with a median of 2 prior chemotherapies. The overall objective response rate (ORR) in the ITT population was 74%; 25% had a complete response, 49% had a partial response, 12% had stable disease, and 14% had disease progression. Of 15 patients who were refractory to prior alkylator treatment (patients who progressed after at least one prior alkylator-containing therapy), 10 (67%) experienced an objective response to bendamustine. The median duration of response was 6.6 months for all patients, 9.3 months for indolent patients, and 2.4 months for transformed patients.

[00112] **Conclusions:** Single-agent bendamustine produced durable objective responses with acceptable toxicity, despite unfavorable prognostic features, in heavily pretreated rituximab-refractory indolent and transformed NHL patients, including those patients who were also refractory to prior alkylator treatment.

* * *

[00113] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the appended claims.

[00114] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the spirit and scope of the invention as defined by the appended claims.

[00115] All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications, including those to which priority or another benefit is claimed, are herein incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[00116] The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

What it claimed is:

1. A method of treating cancer, comprising determining that a patient has a cancer characterized by death-resistant cancer cells, followed by administering to the patient a therapeutically effective amount of bendamustine.

2. A method according to claim 1, wherein the cancer is resistant to apoptosis.

3. A method according to claim 1, wherein the death-resistant cancer cells comprise a p53 deficiency.

4. A method according to claim 1, wherein the cancer is selected from the group consisting of non-Hodgkin's lymphoma and chronic lymphocytic leukemia.

5. A method of treating a cancer patient comprising administering bendamustine, waiting for at least about 30 minutes but not longer than about 48 hours, and administering another chemotherapeutic agent or agents that are more active when cells are in the S-phase of the cell cycle.

6. A method according to claim 5, where the chemotherapeutic agent is given about 30 minutes to about 36 hours after the administration of bendamustine.

7. A method according to claim 5, wherein the chemotherapeutic agent is given about 30 minutes to 24 hours after administration of bendamustine.

8. A method according to claim 5, wherein the chemotherapeutic agent is given about 30 minutes to twelve hours after administration of bendamustine.

9. A method according to claim 5, wherein the chemotherapeutic is given about 30 minutes to six hours after administration of bendamustine.

10. A method according to claim 5, wherein the patient has a cancer characterized by death-resistant cancer cells.

11. A method of assessing efficacy of a cancer treatment, comprising determining whether a level of a marker of cancer cell death in a biological sample taken from a cancer patient correlates with treatment efficacy, wherein the determination is made during or following administration of a therapeutic regimen intended to treat the cancer, wherein the therapeutic regimen comprises administration of an alkylating agent.

12. A method according to claim 11, wherein the alkylating agent is bendamustine.

13. A method of assessing efficacy of a cancer treatment, comprising:

a. treating a cancer with a therapeutically effective amount of bendamustine;

b. waiting a sufficient period of time to allow bendamustine to exert a desired therapeutic effect; and

c. determining a level of a marker of cancer cell death to determine if treatment with bendamustine was efficacious.

14. A method of reducing toxicity associated with a cancer therapy that comprises administering a plurality of doses of bendamustine to a cancer patient, comprising administering a first dose of a therapeutically effective amount of bendamustine to the patient, which first bendamustine dose results in an undesired toxicity, and delaying administration of a second dose of a therapeutically effective amount of bendamustine to the patient until after the undesired toxicity begins to subside.

15. A method of assessing whether a patient's cancer is susceptible to bendamustine, comprising:

a. exposing at least a portion of a cell sample from cancerous tissue of a patient to bendamustine under growth conditions which, in the absence of a compound that is toxic to cancer cells, allows the cancer cells to proliferate; and

b. assessing whether the cancer is susceptible to bendamustine exposure.

16. A method according to claim 15 wherein the assessment of whether the cancer is susceptible to bendamustine exposure comprises determining a level of a marker of cancer cell death.

17. A method according to claim 16 wherein the marker of cancer cell death is selected from the group consisting of a level of adenylate kinase activity, , viability of the cells, and a level of a PARP cleavage product.

18. A method of treating cancer, comprising determining that a patient has a cancer characterized as resistant to one or more alkylating agents and an anti-CD20 agent, comprising administering to said patient a therapeutically effective amount of bendamustine.

19. A method according to claim 18 wherein the cancer is Non-Hodgkin's lymphoma.

20. A method according to claim 18, wherein the anti-CD20 agent is rituximab.

21. A method of doing business in connection with the treatment of a cancer characterized by death-resistant cancer cells, comprising promoting bendamustine for use to treat a cancer characterized by death-resistant cancer cells.

22. A method according to claim 21 wherein the cancer is a cancer refractory to a treatment comprises a combination of one or more alkylating agents and an anti-CD20 agent.

23. A method of doing business in connection with the treatment of a refractory cancer, comprising promoting bendamustine use to treat a refractory cancer.

24. A method according to claim 23 wherein the refractory cancer is a cancer refractory to treatment with a combination of one or more alkylating agents and an anti-CD20 agent.

25. Use of bendamustine in the manufacture of a medicament for treatment of a cancer characterized by death-resistant cancer cells.

26. Use of bendamustine in the manufacture of a medicament for treatment of a refractory cancer.

27. A use according to claim 26 wherein the refractory cancer is a cancer refractory to treatment with a combination of one or more alkylating agents and an anti-CD20 agent.

Figure 1A Bendamustine gene expression profile clustering: top 100 modulated genes

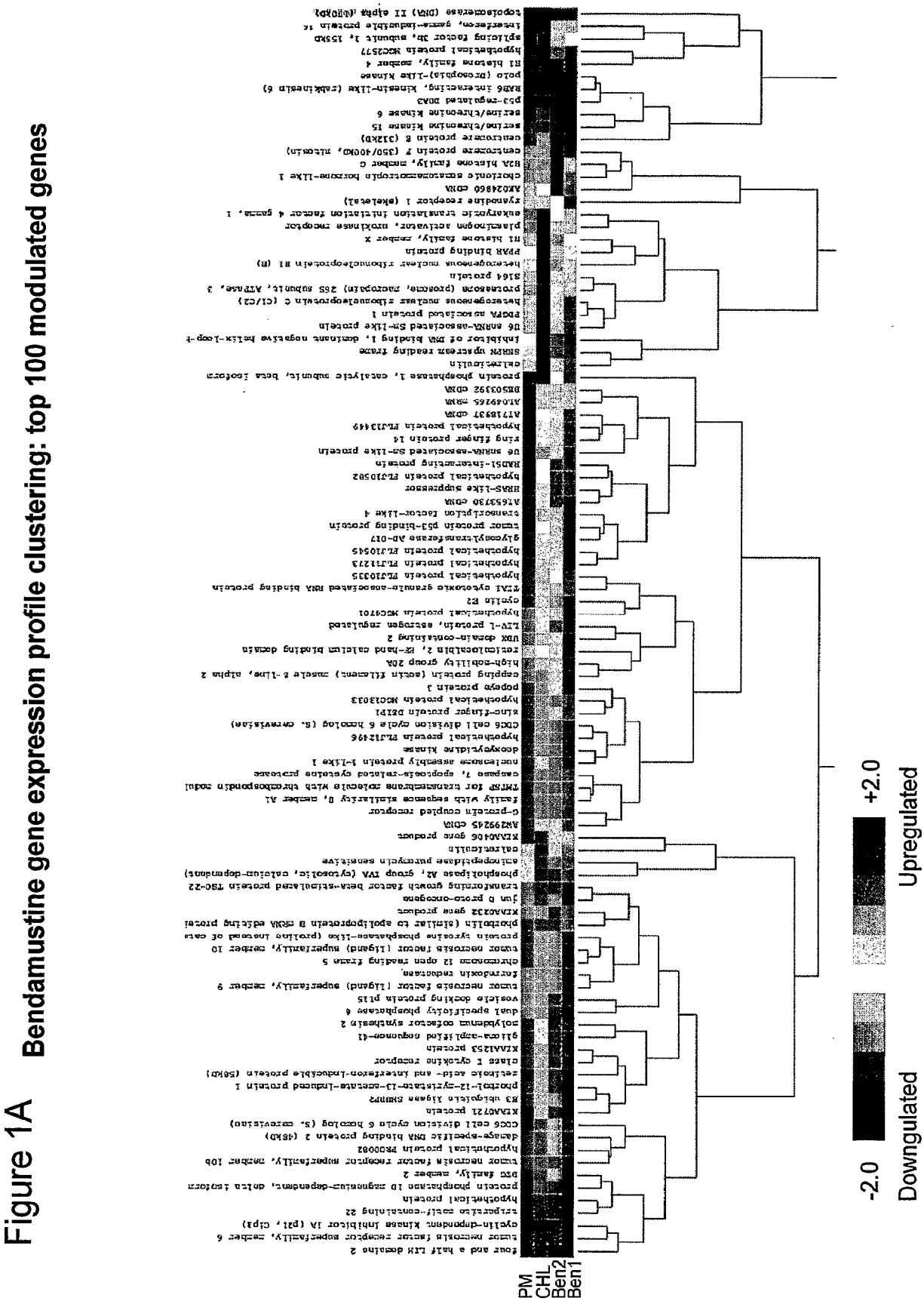
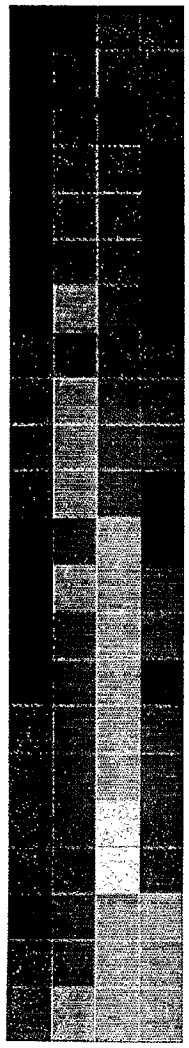


Figure 1B: Top genes up-regulated by the three drugs tested



NM_001450	four and a half LIM domains 2
AA164751	tumor necrosis factor receptor superfamily, member 6
NM_000389	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
AA083478	tripartite motif-containing 22
NM_016617	hypothetical protein
NM_003620	protein phosphatase 1D magnesium-dependent, delta isoform
NM_006763	BTG family, member 2
AF016266	tumor necrosis factor receptor superfamily, member 10b
NM_018590	hypothetical protein PRO0082
NM_000107	damage-specific DNA binding protein 2 (48kD)
NM_001254	CDC6 cell division cycle 6 homolog (S. cerevisiae)
AL050331	KIAA0721 protein
AY014180	E3 ubiquitin ligase SMURF2
NM_021127	phorbol-12-myristate-13-acetate-induced protein 1
N47725	retinoic acid- and interferon-inducible protein (58kD)
AI983115	class I cytokine receptor
AF164794	KIAA1253 protein
NM_006530	glioma-amplified sequence-41
NM_004531	molybdenum cofactor synthesis 2
BC003143	dual specificity phosphatase 6
BE875592	vesicle docking protein p115
NM_003811	tumor necrosis factor (ligand) superfamily, member 9

Figure 2A: Q-PCR validation of selected p53-dependent and pro-apoptotic genes

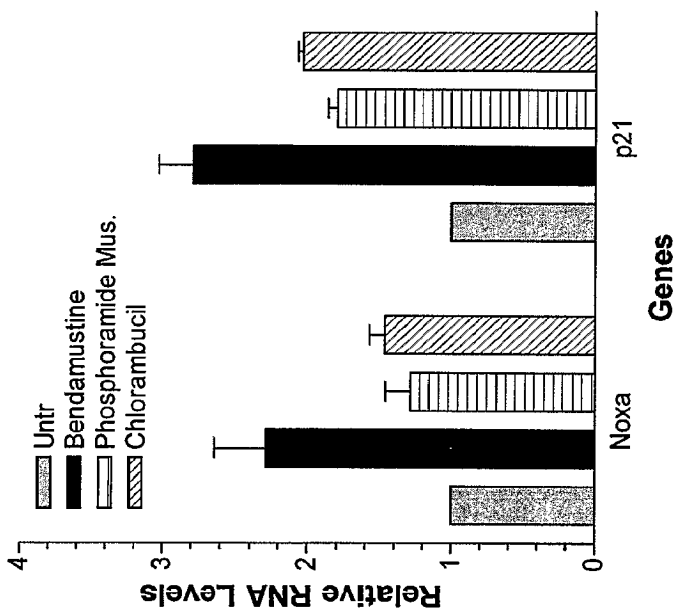


Figure 2B: Q-PCR validation of selected mitotic checkpoint genes

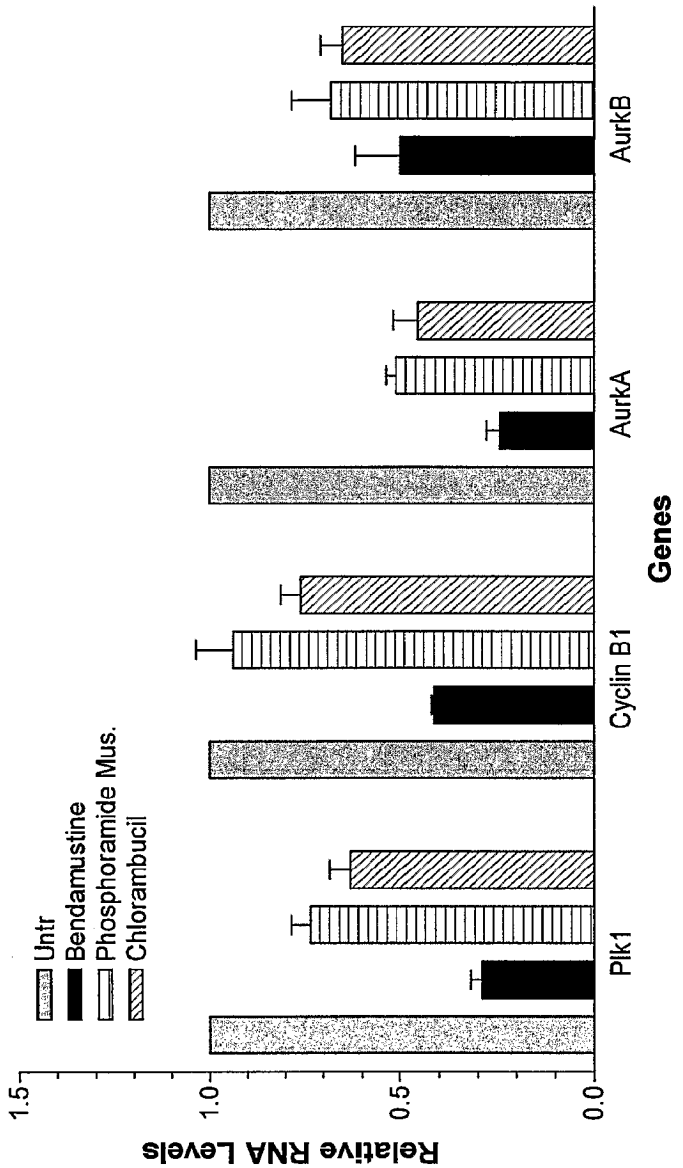


Figure 2C: Q-PCR validation of selected DNA-repair genes

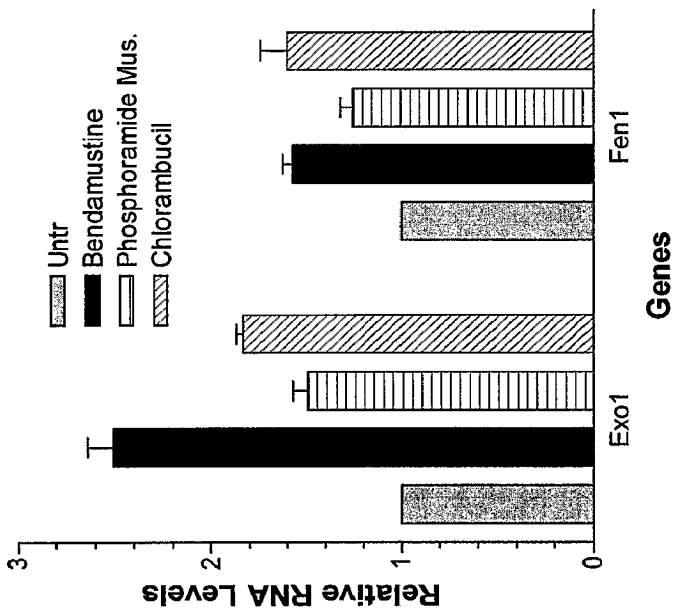


Figure 3

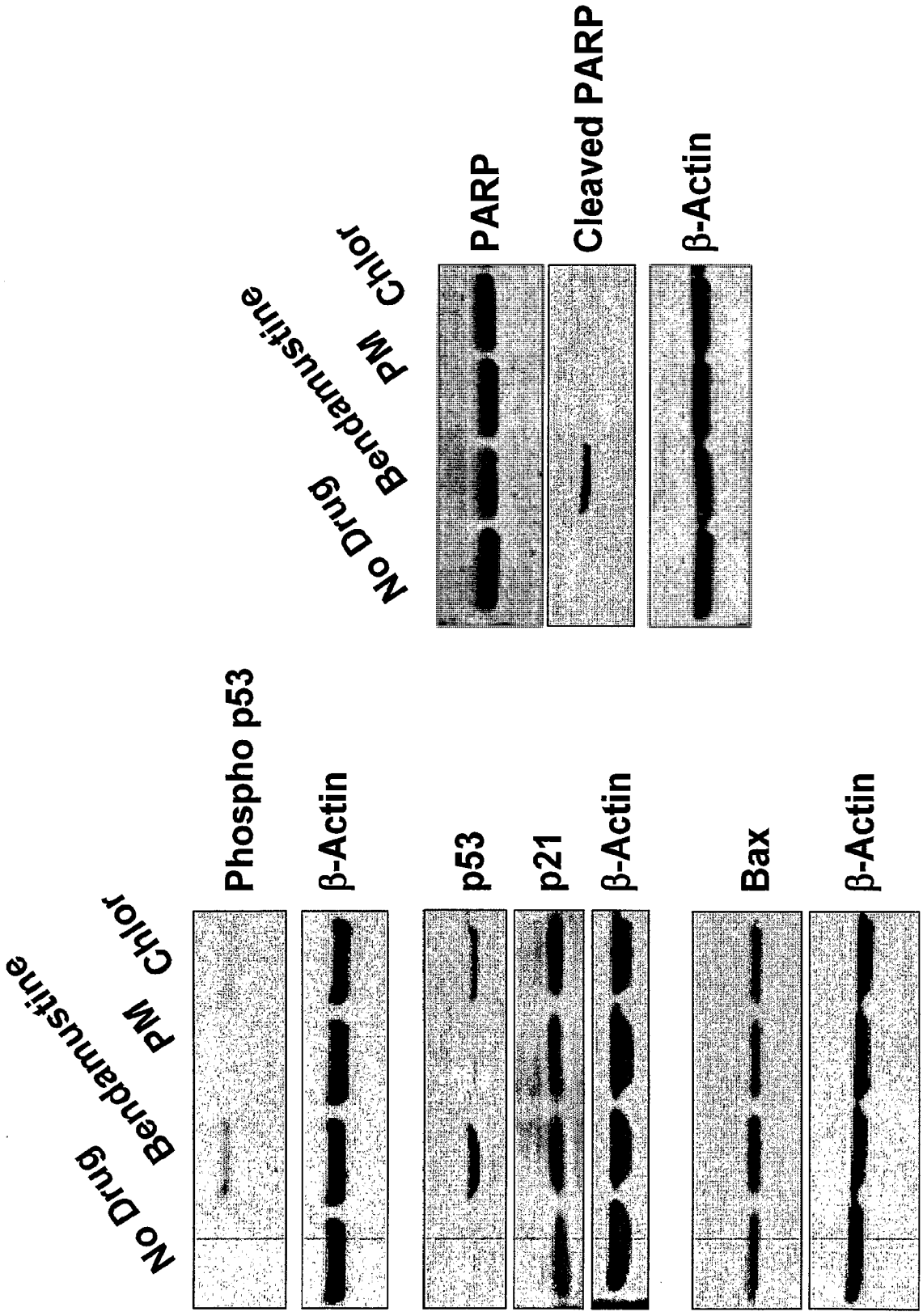


Figure 4A: Effect of MX (Ape-1 inhibitor) on bendamustine activity vs. cyclophosphamide activity

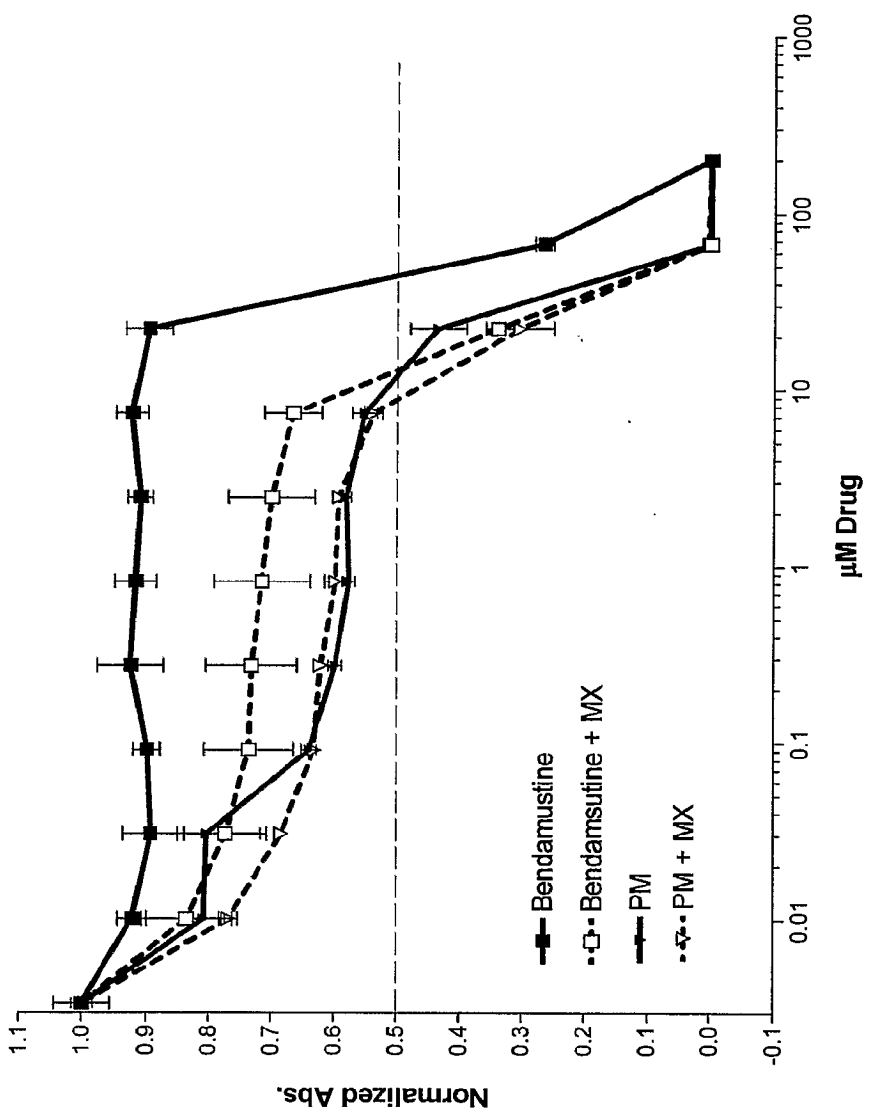


Figure 4B: Effect of O6-Benzylguanine on bendamustine, cyclophosphamide, and carmustine

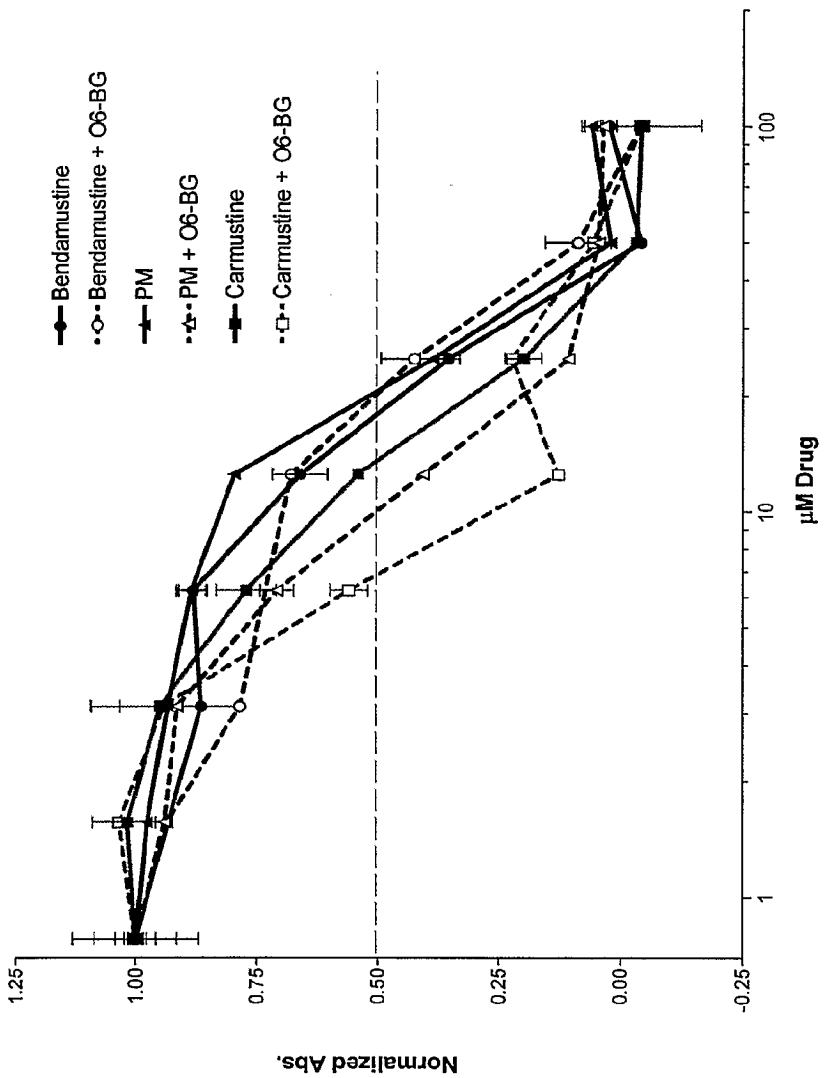


Figure 5

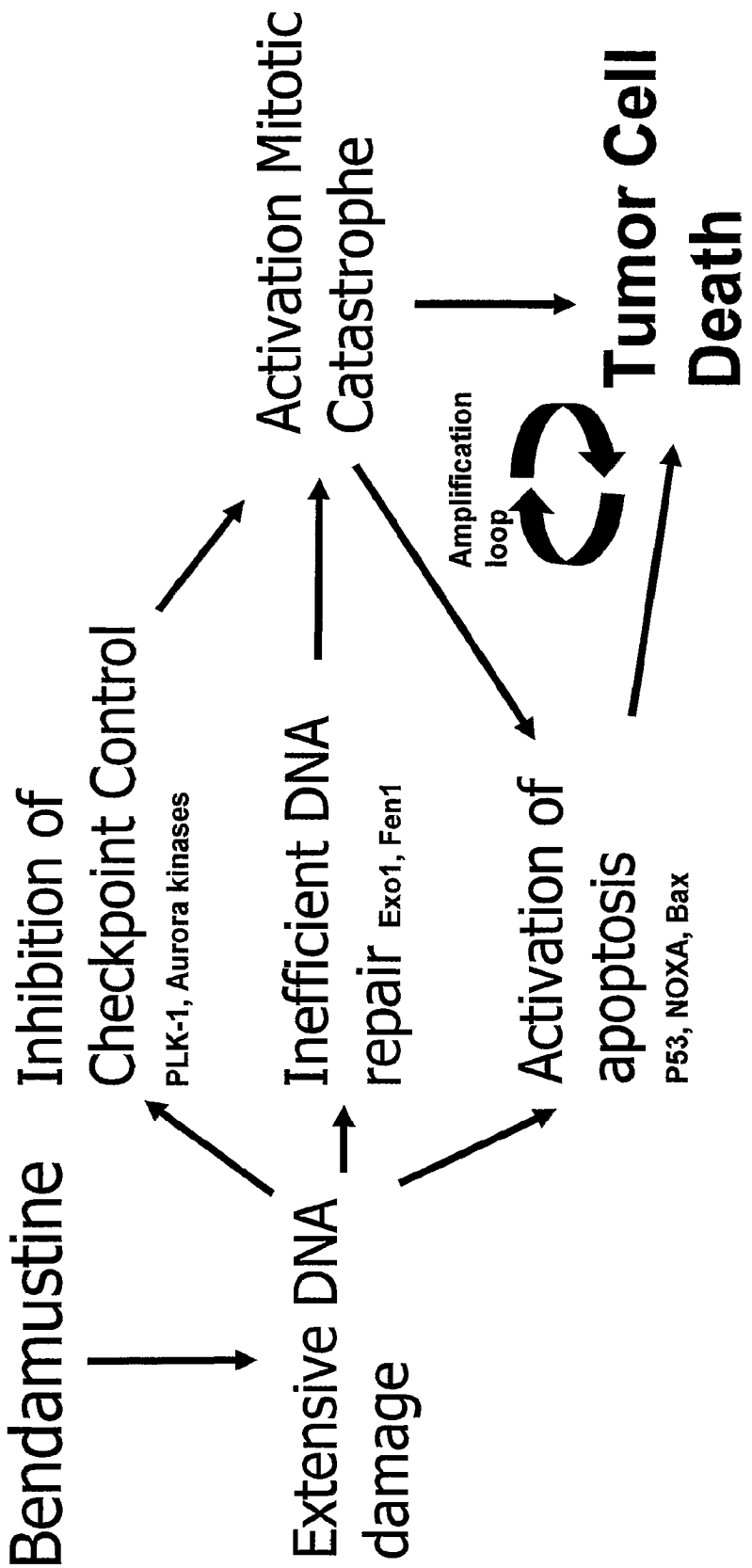


Figure 6

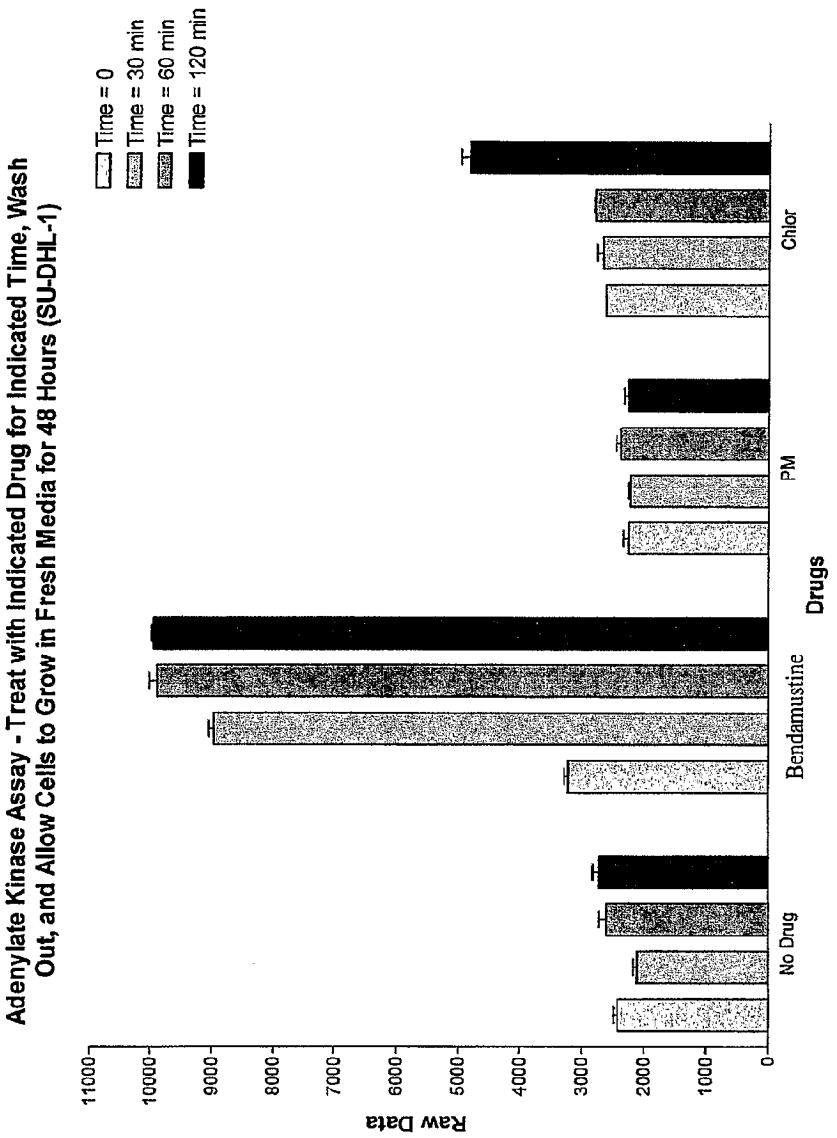


Figure 7

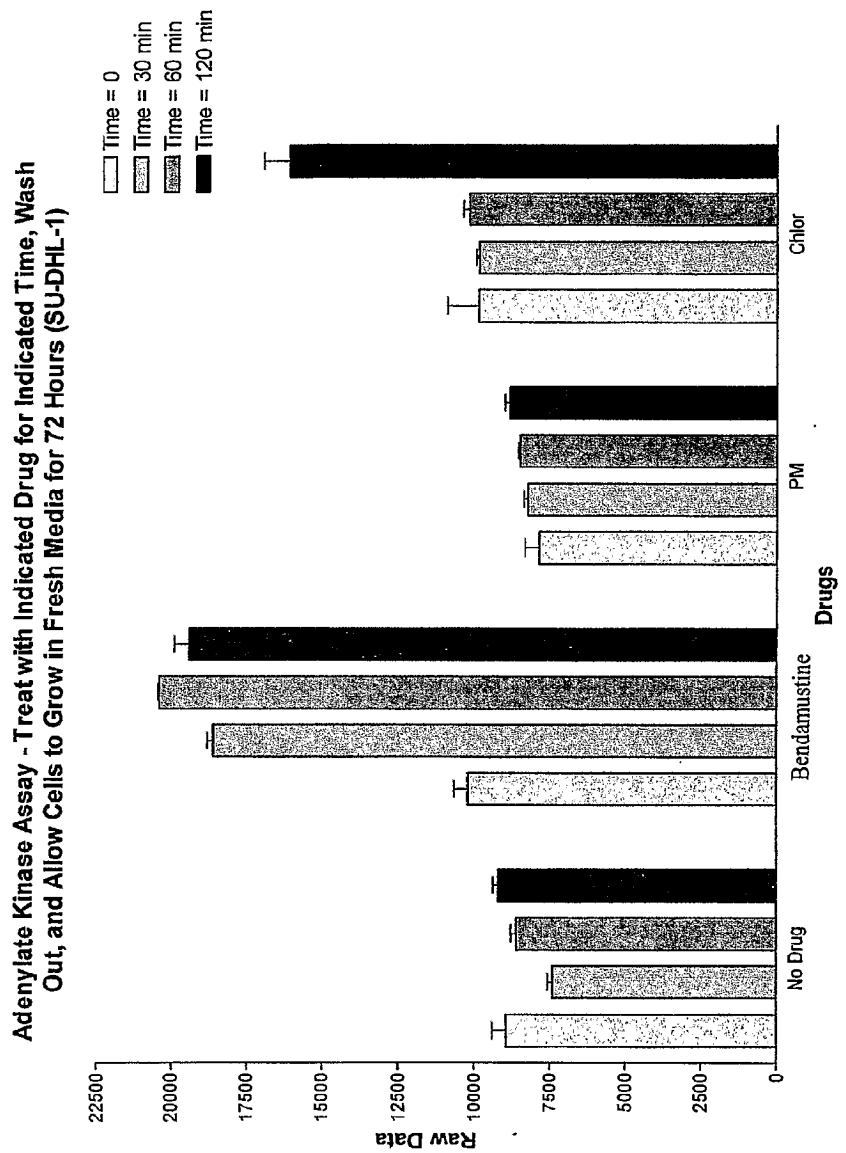


Table 1: IC50s of Bendamustine, PM, Chlorambucil, in SU-DHL-1 cells

Cell Line	Drug	Ave IC50 (μM)	STDV	Ave IC90 (μM)	STDV
SU-DHL-1	**Bendamustine**	33.2	10.6	56.3	16.1
	Chlorambucil	3.4	1.1	6.2	1.3
	Phosporamide Mustard	21.3	7.6	33.0	6.2

Table 2: Results from GO-clustering analysis from bendamustine-indced gene changes in SU-DHL-1 cells (see Figure 2C)

Functional Groups	GO number	GO Description: Biological Process	P value
DNA-damage, stress response, apoptosis	**6974**	**Response to DNA damage stress**	**0.00001**
	6950	Response to stress	0.0003
	16265	Death	0.0482
DNA metabolism, DNA repair, transcription	**6259**	**DNA metabolism**	**0.00003**
	6139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	0.0004
	6357	Regulation of transcription from Pol II promoter	0.0003
	6366	Transcription from Pol II promoter	0.0068
Cell proliferation, cell cycle, mitotic checkpoint	**8283**	**Cell proliferation**	**0.00001**
	8151	Cell growth and/or maintenance	0.0041
	6275	Regulation of DNA replication	0.0101
	278	Mitotic cell cycle	0.0334
	79	Regulation of CDK activity	0.0192
	7078	Mitotic metaphase plate congression	0.0470
	50790	Regulation of enzyme activity	0.0363
Cell regulation	**50789**	**Regulation of biological process**	**0.00004**
	50794	Regulation of cellular process	0.0035
	9987	Cellular process	0.0379

GO clustering analysis performed as described in Methods section. The table represents the terms identified from the Gene Ontology database (http://www.geneontology.org/) that are the most statistically-significantly modulated between untreated control and SU-DHL-1 treated with IC50 dose of bendamustine.

Table 3: Closest compounds to bendamustine by NCI COMPARE Analysis

Compound	Mechanism of Action	Correlation (PCC) GI50, TGI, or LC50
0 compounds show a PCC>0.800		
DTIC, Dacarbazine	DNA Alkylator, Methylating agent	0.792 (LC50)
TOPO1B	Topoisomerase I inhibitor	0.619 (TGI)
Daunomycin analog	Anthracycline, DNA intercalator	0.574 (TGI)
Melphalan	DNA Alkylator, Nitrogen mustard	0.550 (GI50)
YOSHI 864	DNA Alkylator	0.542 (GI50)
Ara-AC (Fazarabine)	Antimetabolite, DNA methylation inhibitor	0.524 (TGI)

Electronic Patent Application Fee Transmittal

Application Number:	11330868
Filing Date:	12-Jan-2006
Title of Invention:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
First Named Inventor/Applicant Name:	Jason Edward Brittain
Filer:	Stephanie A. Barbosa/D. McCarty
Attorney Docket Number:	CP391

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($)
Miscellaneous:				
Request for continued examination	1801	1	930	930
		Total in USD ($)		**930**

Electronic Acknowledgement Receipt

EFS ID:	14234022
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/D. McCarty
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	15-NOV-2012
Filing Date:	12-JAN-2006
Time Stamp:	10:48:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$930
RAM confirmation Number	9912
Deposit Account	233050
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 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance 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	Request for Continued Examination (RCE)	CEPH-4391-Request-for-Continued-Examination.PDF	697815 3651fc99edcb3775e7feeebcece8d5c39e1a459f	no	3
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	CEPH-4391-1449-SB08.PDF	118926 3294cc37639a83da6d151771c4d769cdea8247c3	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	CEPH-4391-WO2006065392.PDF	3615895 fc562911721188028 43abd382a57c479043dc2b3	no	63
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30511 573b20af41b9144082924535373 36c7f3e6d999e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	4463147		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

Substitute for 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
(use as many sheets as necessary)				**Examiner Name**	Soroush, Ali
Sheet	1	of	1	**Attorney Docket Number**	CEPH-4391 / CP391

FOREIGN PATENT DOCUMENTS					
Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
	95	EP 0780386	06-25-1997	F. Hoffmann-La Roche AG	
	96	WO 97/08174	03-06-1997	Smithkline Beecham Corporation	

NON PATENT LITERATURE DOCUMENTS			
Examiner Initials	Cite No.	Include name of the author, 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
	97	Department of Health and Human Services, Food and Drug Administration, "International Conference on Harmonisation; Guidance on Impurities: Residual Solvents," Federal Register, December 24, 1997, 62(247), 67377-67388	

Examiner Signature		**Date Considered**	

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 780 386 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
25.06.1997 Bulletin 1997/26

(21) Application number: **96119780.3**

(22) Date of filing: **10.12.1996**

(51) Int. Cl.[6]: **C07D 309/08**, C07D 211/54, C07D 213/64, C07D 401/06, C07D 407/12, C07D 413/12, C07D 405/12, A61K 31/35, A61K 31/445

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **20.12.1995 US 8939**
07.08.1996 US 22439
04.12.1996 US 32096

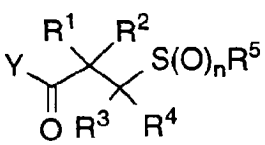
(71) Applicants:
- **F. HOFFMANN-LA ROCHE AG**
  4070 Basel (CH)
- **AGOURON PHARMACEUTICALS, INC.**
  La Jolla, CA 93057 (US)

(72) Inventors:
- **Bender, Steven Lee**
  CA 92054 (US)
- **Broka, Chris Allen**
  CA 94404 (US)
- **Campbell, Jeffrey Allen**
  Fremont, CA 94555 (US)
- **Castelhano, Arlindo Lucas**
  NY 10956 (US)
- **Fisher, Lawrence Emerson**
  CA 94040 (US)
- **Hendricks, Robert Than**
  Palo Alto, CA 94306 (US)
- **Sarma, Keshab**
  CA 94087 (US)

(74) Representative: **Mezger, Wolfgang, Dr.**
F.Hoffmann-La Roche AG
Patent Department (PLP),
124 Grenzacherstrasse
4070 Basel (CH)

(54) Matrix metalloprotease inhibitors

(57) Compounds of the formula:



I

wherein:

n is 0, 1 or 2;

Y is hydroxy or XONH-, where X is hydrogen or lower alkyl;

R^1 is hydrogen or lower alkyl;

R^2 is hydrogen, lower alkyl, heteroalkyl, aryl, aralkyl, arylheteroalkyl, cycloalkyl, cycloalkylalkyl, heteroaryl, heteroaralkyl, heteroarylheteroalkyl, heterocyclo, heterocylo-lower alkyl, heterocyclo-lower heteroalkyl or $-NR^6R^7$, wherein:

R^6 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl, aryl, heteroaryl and heteroaralkyl;

R^7 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, $-C(O)R^8$, $-C(O)NR^8R^9$, $-SO_2NR^8R^9$, $-SO_2R^{10}$, aryloxycarbonyl, or alkoxycarbonyl; or R^6 and R^7 together with the nitrogen atom to which they are attached represent a heterocyclo group; wherein

R^8 and R^9 are independently hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl or heteroalkyl; and

R^{10} is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, heteroalkyl or heterocyclo; or

R^1 and R^2 together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group;

R^3 is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl,

Printed by Rank Xerox (UK) Business Services
2.14.8/3.4

(Cont. next page)

heteroaralkyl, heteroalkyl or lower alkoxy;

R^4 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl; or

R^2 and R^3 together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or

R^3 and R^4 together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and

R^5 is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl;

or pharmaceutically acceptable salts or esters thereof exhibit useful pharmacological properties, in particular for use as matrix metalloprotease inhibitors, particularly for interstitial collagenases.

Description

The present invention relates to compounds of formula I and their pharmaceutically acceptable salts and esters thereof, that inhibit matrix metalloproteases, particularly interstitial collagenases, and are therefore useful in the treatment of mammals having disease states alleviated by the inhibition of such matrix metalloproteases.

Matrix metalloproteases ("MMPs") are a family of proteases (enzymes) involved in the degradation and remodeling of connective tissues. Members of this family of endopeptidase enzymes are present in various cell types that reside in or are associated with connective tissue, such as fibroblasts, monocytes, macrophages, endothelial cells, and invasive or metastatic tumor cells. MMP expression is stimulated by growth factors and cytokines in the local tissue environment, where these enzymes act to specifically degrade protein components of the extracellular matrix, such as collagen, proteoglycans (protein core), fibronectin and laminin. These ubiquitous extracellular matrix components are present in the linings of joints, interstitial connective tissues, basement membranes, and cartilage. Excessive degradation of extracellular matrix by MMPs is implicated in the pathogenesis Of many diseases, including rheumatoid arthritis, osteoarthritis, multiple sclerosis, chronic obstructive pulmonary disease, cerebral hemorrhaging associated with stroke, periodontal disease, aberrant angiogenesis, tumor invasion and metastasis, corneal ulceration, and in complications of diabetes. MMP inhibition is, therefore, recognized as a good target for therapeutic intervention.

The MMPs share a number of properties, including zinc and calcium dependence, secretion as zymogens, and 40-50% amino acid sequence homology. The MMP family currently consists of at least eleven enzymes, and includes collagenases, stromelysins, gelatinases, matrilysin, metalloelastase, and membrane-type MMP, as discussed in greater detail below.

Interstitial collagenases catalyze the initial and rate-limiting cleavage of native collagen types I, II, and III. Collagen, the major structural protein of mammals, is an essential component of the matrix of many tissues, for example, cartilage, bone, tendon and skin. Interstitial collagenases are very specific matrix metalloproteases which cleave these collagens to give two fragments which spontaneously denature at physiological temperatures and therefore become susceptible to cleavage by less specific enzymes. Cleavage by the collagenases results in the loss of structural integrity of the target tissue, essentially an irreversible process. There are currently three known human collagenases. The first is human fibroblast-type collagenase (HFC, MMP-1, or collagenase-1) that is produced by a wide variety of cells including fibroblasts and macrophages. The second is human neutrophil-type collagenase (HNC, MMP-8, or collagenase-2) that has so far only been demonstrated to be produced by neutrophils. The most recently discovered member of this group of MMPs is human collagenase-3 (MMP-13) which was originally found in breast carcinomas, but has since shown to be produced by chondrocytes. The only collagenase known to exist in rodents is the homolog of human collagenase-3.

The gelatinases include two distinct, but highly related, enzymes: a 72-kD enzyme (gelatinase A, HFG, MMP-2) secreted by fibroblasts and a wide variety of other cell types, and a 92-kD enzyme (gelatinase B, HNG, MMP-9) released by mononuclear phagocytes, neutrophils, corneal epithelial cells, tumor cells, cytotrophoblasts and keratinocytes. These gelatinases have been shown to degrade gelatins (denatured collagens), collagen types IV (basement membrane) and V, fibronectin and insoluble elastin.

Stromelysins 1 and 2 have been shown to cleave a broad range of matrix substrates, including laminin, fibronectin, proteoglycans, and collagen types IV and IX in their non-helical domains.

Matrilysin (MMP-7, PUMP-1) has been shown to degrade a wide range of matrix substrates including proteoglycans, gelatins, fibronectin, elastin, and laminin. Its expression has been documented in mononuclear phagocytes, rat uterine explants and sporadically in tumors. Other less characterized MMPs include macrophage metalloelastase (MME, MMP-12), membrane type MMP (MMP-14), and stromelysin-3 (MMP-11).

Inhibitors of MMPs provide useful treatments for diseases associated with the excessive degradation of extracellular matrix, such as arthritic diseases (rheumatoid arthritis and osteoarthritis), multiple sclerosis, bone resorptive diseases (such as osteoporosis), the enhanced collagen destruction associated with diabetes, chronic obstructive pulmonary disease, cerebral hemorrhaging associated with stroke, periodontal disease, corneal or gastric ulceration, ulceration of the skin, tumor invasion and metastasis, and aberrant angiogenesis. The involvement of individual collagenases in the degradation of tissue collagens probably depends markedly on the tissue. The tissue distribution of human collagenases suggests that collagenase-3 is the major participant in the degradation of the collagen matrix of cartilage, while collagenase-1 is more likely to be involved in tissue remodeling of skin and other soft tissues. Thus, inhibitors selective for collagenase-3 over collagenase-1 are preferred for treatment of diseases associated with cartilage erosion, such as arthritis, etc.

Inhibitors of MMP also are known to substantially inhibit the release of tumor necrosis factor (TNF) from cells, and which therefore may be used in the treatment of conditions mediated by TNF. Such uses include, but are not limited to, the treatment of inflammation, fever, cardiovascular effects, hemorrhage, coagulation and acute phase response, cachexia and anorexia, acute infections, shock states, restinosis, aneurysmal disease, graft versus host reactions and autoimmune disease.

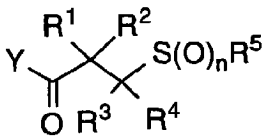
In addition to these effects on the release of TNF from cells, MMP inhibitors have also been shown to inhibit the

release of other biologically active molecules from cells, including soluble receptors (CD30 and receptors for TNF (p55 and p75), IL-6, IL-1 and TSH), adhesion molecules (*e.g.*, L-selection, ICAM-1, fibronectin) and other growth factors and cytokines, including Fas ligand, TGF-a, EGF, HB-EGF, SCF and M-CSF. Inhibition of the release or shedding of such proteins may be of benefit in a number of disease states, including rheumatoid arthritis, multiple sclerosis, vascular disease, Type II diabetes, HIV, cachexia, psoriasis, allergy, hepatitis, inflammatory bowel disease, and cancer.

Since non-specific inhibition of the shedding enzymes (sheddases) may have opposite pharmacological effects, selectivity will be a particular advantage, *e.g.*, the inhibition of TNF release without the concurrent inhibition of TNF receptor release.

The design and uses of MMP inhibitors is described, for example, in *J. Enzyme Inhibition,* **2**, 1-22 (1987); *Drug News & Prospectives,* **3**(8), 453-458 (1990); *Arthritis and Rheumatism,* **36**(2), 181-189 (1993); *Arthritis and Rheumatism,* **34**(9), 1073-1075 (1991); *Seminars in Arthritis and Rheumatism,* **19**(4), Supplement 1 (February), 16-20 (1990); *Drugs of the Future,* **15**(5), 495-508 (1990); and *J. Enzyme Inhibition,* **2**, 1-22 (1987). MMP inhibitors are also the subject of various patents and patent applications, for example, U.S. Patent Nos. 5,189,178 and 5,183,900, European Published Patent Applications 438 223, 606 426, and 276 436, and published Patent Cooperation Treaty International Applications WO 92/21360, WO 92/06966, WO 92/09563, and WO 94/25434.

One aspect of the invention concerns compounds represented by Formula I:



I

wherein:

n is 0, 1 or 2;

Y is hydroxy or XONH-, where X is hydrogen or lower alkyl;

R^1 is hydrogen or lower alkyl;

R^2 is hydrogen, lower alkyl, heteroalkyl, aryl, aralkyl, arylheteroalkyl, cycloalkyl, cycloalkylalkyl, heteroaryl, heteroaralkyl, heteroarylheteroalkyl, heterocyclo, heterocylo-lower alkyl, heterocyclo-lower heteroalkyl or $-NR^6R^7$, wherein:

R^6 is hydrogen, lower alkyl, cycloalkyl or cycloalkyl alkyl, aryl, heteroaryl and heteroaralkyl;

R^7 is hydrogen, lower alkyl, cycloalkyl or cycloalkyl alkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, $-C(O)R^8$, $-C(O)NR^8R^9$, $-SO_2NR^8R^9$, $-SO_2R^{10}$, aryloxycarbonyl, or alkoxycarbonyl; or

R^6 and R^7 together with the nitrogen atom to which they are attached represent a heterocyclo group; wherein

R^8 and R^9 are independently hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl or heteroalkyl; and

R^{10} is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, heteroalkyl or heterocyclo; or

R^1 and R^2 together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group;

R^3 is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, heteroalkyl or lower alkoxy;

R^4 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl; or

R^2 and R^3 together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or

R^3 and R^4 together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and

R^5 is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl; or heteroaralkyl;

or a pharmaceutically acceptable salt or ester thereof.

A second aspect of this invention relates to pharmaceutical compositions containing a therapeutically effective amount of a compound of Formula I or a pharmaceutically acceptable salt or ester thereof admixed with at least one pharmaceutically acceptable excipient.

A third aspect of this invention relates to methods for treating mammals having a disease state alleviated by the inhibition of matrix metalloproteases, by administering an effective amount of a compound of Formula I, or a pharmaceutical composition thereof, to the mammal. Such disease states include arthritic diseases, multiple sclerosis, bone

resorption disease (such as osteoporosis), the enhanced collagen destruction associated with diabetes, chronic obstructive pulmonary disease, cerebral hemorrhaging associated with stroke, periodontal disease, corneal or gastric ulceration, ulceration of the skin, and tumor metastasis.

A fourth aspect of this invention relates to methods for preparing compounds of Formula I.

Among the family of compounds of the present invention as defined above, a particular family of compounds of formula I consists of n is 0, 1 or 2; Y is hydroxy or XONH-, where X is hydrogen or lower alkyl; R^1 is hydrogen or lower alkyl; R^2 is hydrogen, lower alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, heterocyclo, or $-NR^6R^7$; or R^1 and R^2 together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group; in which R^6 is hydrogen, lower alkyl, or phenyl; and R^7 is hydrogen, lower alkyl, benzyl, $-C(O)R^8$, $-C(O)NR^8R^9$, $-SO_2NR^8R^9$, $-SO_2R^{10}$, benzyloxycarbonyl, or alkoxycarbonyl; or R^6 and R^7 together with the nitrogen atom to which they are attached represent a heterocyclo group; wherein R^8 and R^9 are independently hydrogen or lower alkyl; and R^{10} is lower alkyl, aryl, heteroaryl, or heterocyclo; R^3 is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aralkyl, heteroaralkyl, or lower alkoxy; R^4 is hydrogen or lower alkyl; or R^2 and R^3 together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or R^3 and R^4 together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and R^5 is lower alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl.

Within these families a preferred category includes compounds where n is 2 and Y is -NHOH.

Within this category, one preferred group includes the compounds where R^1 is hydrogen and R^5 is aryl. One preferred subgroup within this group includes the compounds where R^2 is hydrogen and R^3 is aralkyl, especially benzyl, and R^4 is hydrogen and R^5 is optionally substituted phenyl or naphthyl, more especially where R^5 is 4-methoxyphenyl, phenylthiophenyl, phenoxyphenyl, or biphenyl.

Another preferred subgroup within this group includes the compounds where R^3 and R^4 together with the carbon to which they are attached form a cycloalkyl group, especially cyclopentyl and cyclcohexyl, more especially in combination where R^5 is 4-methoxyphenyl or 4-phenoxyphenyl.

Yet another preferred subgroup within this group includes the compounds where R^3 and R^4 together with the carbon to which they are attached form a heterocyclo group, in particular optionally substituted piperidinyl or tetrahydropyranyl, especially piperidin-4-yl, 1-methylpiperidin-4-yl, 1-(cyclopropylmethyl)piperidin-4-yl, or tetrahydropyranyl, more especially in combination where R^5 is 4-phenoxyphenyl, 4-(4-chlorophenoxy)phenyl, 4-bromophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

Another preferred group within this category includes the compounds where R^2 is $-NR^6R^7$, R^1, R^3 and R^4 are hydrogen, and R^5 is aryl. One preferred subgroup within this group includes the compounds where R^5 is 4-phenoxyphenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl, especially where R^6 is hydrogen and R^7 is CBZ-valinamido, valinamido or dimethylaminosulfonyl.

Another preferred group within this category includes the compounds where R^1 and R^2 together with the carbon to which they are attached form a heterocyclo group. A preferred subgroup within the group includes compounds where R^3 and R^4 are hydrogen and R^1 and R^2 together with the carbon to which they are attached form a heterocyclo group, in particular optionally substituted piperidinyl or tetrahydropyranyl, especially piperidin-4-yl, 1-methylpiperidin-4-yl, 1-(cyclopropylmethyl)piperidin-4-yl, or most preferably tetrahydropyranyl, more especially in combination where R^5 is 4-phenoxyphenyl, 4-(4-chlorophenoxy)phenyl, 4-(4-bromophenoxy)phenyl, 4-(4-fluorophenoxy)phenyl, 4-(thiophen-2-yl)phenoxy)phenyl, 4-(thiophen-3-yl)phenoxy)phenyl, 4-(thiazol-2-yl)phenoxy)phenyl, 4-(2-pyridyloxy)phenyl, or 4-(5-chloro-2-pyridyloxy)phenyl.

Another preferred group within this category includes compounds wherein R^1 and R^2 are both alkyl, R^3 and R^4 are hydrogen. One preferred subgroup includes compounds wherein R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

Another group within this category includes compounds wherein R^2 and R^3 together with the carbons to which they are attached form a cycloalkyl group and R^5 is aryl. Preferably, the cycloalkyl group is cyclopentyl or cyclohexyl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

Preferred compounds are:

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide;
2-{4-[4-(4-chlorophenoxy)-phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide;
2-{4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;
2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-[1-methyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide;
N-hydroxy-2-{1-methyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-acetamide;
N-hydroxy-2-{1-methyl-4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-acetamide;
2-[1-cyclopropylmethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-*N*-hydroxyacetamide;
2-{1-cyclopropylmethyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;

2-{1-cyclopropylmethyl-4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-[4-(4-phenoxyphenylsulfinyl)-tetrahydropyran-4-yl]-acetamide;
2-{4-[4-(4-chlorophenoxy)-phenylsulfinyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
2-{4-[4-(4-fluorophenoxy)-phenylsulfinyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetamide;
2-{4-[4-(4-chlorophenoxy)-phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
2-{4-[4-(4-fluorophenoxy)-phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-bromophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-fluorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
3-[4-(4-chlorophenoxy)phenylsulfonyl]-2,2-dimethyl-*N*-hydroxypropionamide;
4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-(cyclopropylmethyl)piperidine-4-(*N*-hydroxycarboxamide);
4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-(nicotinoyl)piperidine-4-(*N*-hydroxycarboxamide);
4-[4-(phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(thiophen-2-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(thiophen-3-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(furan-2-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(benzofuran-2-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(thiazol-2-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(thiazol-4-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(thiazol-5-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(imidazol-1-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(4-(imidazol-2-yl)-phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
4-[4-(5-chloro-2-pyridyloxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide);
3-[4-(5-chloro-2-pyridyloxy)phenylsulfonyl]-2,2-dimethyl-*N*-hydroxypropionamide;
(R)-2-(CBZ-valinamido)-N-hydroxy-3-(4-phenoxyphenylsulfonyl)propionamide;
(R)-N-hydroxy-2-valinamido-3-(4-phenoxyphenylsulfonyl)-propionamide;
(R)-2-dimethylamino-N-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide;
(R)-2-dimethylaminosulfonamido-N-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide

and pharmaceutically acceptable salts thereof.

Definitions

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

"Alkyl" means a branched or unbranched saturated hydrocarbon chain containing 1 to 8 carbon atoms, such as methyl, ethyl, propyl, *tert*-butyl, *n*-hexyl, *n*-octyl and the like.

"Lower alkyl" means a branched or unbranched saturated hydrocarbon chain containing 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, *tert*-butyl, *n*-butyl, *n*-hexyl and the like, unless otherwise indicated.

The term "heteroalkyl" refers to a branched or unbranched, cyclic or acyclic saturated organic radical containing carbon, hydrogen and one or more heteroatom containing substituents independently selected from OR^a, NR^aR^b, and $S(O)_nR^a$ (where n is 0, 1 or 2) and R^a is hydrogen, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl or acyl, R^b is hydrogen, alkyl, cycloalkyl, aryl, aralkyl, acyl, alkylsulfonyl, carboxamido, or mono- or di-alkylcarbamoyl. Representative examples include hydroxyalkyl, aminoalkyl, alkoxyalkyl, aryloxymethyl, N-acylaminoalkyl, thienylthiomethyl and the like.

"Acyl" refers to the group -C(O)-R', where R' is lower alkyl.

"Alkylene" refers to a straight chain or branched chain divalent radical consisting solely of carbon and hydrogen, containing no unsaturation and having from one to six carbon atoms, *e. g.,* methylene, ethylene, propylene, 2-methylpropylene, butylene, 2-ethylbutylene, hexylene, and the like.

"Lower alkoxy" means the group -O-R', where R' is lower alkyl.

"Alkoxycarbonyl" means the group RO-C(O)- where R is alkyl as herein defined.

"Alkoxycarbonylalkyl" means the group $ROC(O)(CH_2)_n$- where R is alkyl as herein defined and n is 1, 2 or 3.

"Aryl" refers to a monovalent aromatic carbocyclic radical having a single ring (*e.g.,* phenyl) or two condensed rings (*e.g.,* naphthyl), which can optionally be mono-, di- or tri-substituted, independently, with hydroxy, carboxy, lower alkyl, cycloalkyl, cycloalkyloxy, lower alkoxy, chloro, fluoro, trifluoromethyl and/or cyano. The ring(s) can alternatively be optionally monosubstituted with the group R^a-Z-, where Z is oxygen, sulfur, -CH=CH-, $-CH_2$, carbonyl, a covalent bond, or nitrogen optionally substituted with lower alkyl, and R^a is a monovalent aromatic carbocyclic, heteroaryl or heterocyclo radical, or a combination thereof, having 1 or 2 rings, for example phenyl, pyridyl, thienyl, imidazolyl, furanyl, pyrimidinyl, benzothiophene, azanaphthalene, indolyl, phenyl-(furan-2-yl), phenyl-(thien-2-yl), phenyl-(thien-3-yl), phenyl-

(imidazol-2-yl), phenyl-(thiazol-2-yl), phenyl-(morpholin-2-yl), and phenyl-(oxazol-2-yl), (the ring(s) represented by R^a being optionally mono-or disubstituted by hydroxy, carboxy, lower alkyl, lower alkoxy, halo, trifluoromethyl and/or cyano). Examples of aryl substituted by R^a-Z- are benzoyl, diphenylmethane, biphenyl, 6-methoxybiphenyl, 4-(4-methylphenoxy)phenyl, 4-phenoxyphenyl, 2-thiophenoxyphenyl, 4-pyridethenylphenyl, 4-(thiophen-2-yl)phenoxyphenyl, 4-(thiophen-3-yl)phenoxyphenyl, 4-(2-pyridyloxy)phenyl, 4-(5-chloro-2-pyridyloxy)phenyl, 4-(thiazol-5-yl)phenoxyphenyl, 4-(imidazol-2-yl)phenoxyphenyl, and the like.

"Heteroaryl" refers to a monovalent aromatic carbocyclic radical having one or two rings incorporating one, two or three heteroatoms (chosen from N, O or S) within the ring(s), such as thiazole, oxazole, imidazole, thiophene, quinolyl, benzofuranyl, pyridyl, and indolyl, which can optionally be mono-, di- or tri-substituted, independently, with OH, COOH, lower alkyl, lower alkoxy, halo, trifluoromethyl and/or cyano.

"Aralkyl" refers to a radical of the formula R^b-R^c-, wherein R^b is aryl as defined above and R^c is alkylene as defined above, for example benzyl, phenylethylene, 3-phenylpropyl, biphenylpropyl.

"Benzyloxycarbonyl" refers to a radical of the formula $R^dCH_2OC(O)$-, where R^d is phenyl. "Benzyloxycarbonylamino" refers to a radical of the formula $R^dCH_2OC(O)NH$-, where R^d is phenyl.

"Cycloalkyl" means a saturated monovalent monocyclic hydrocarbon radical containing 3-8 carbon atoms, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

"Cycloalkylalkyl" means cycloalkyl as defined above attached to an alkylene radical as defined above.

"Halo" refers to bromo, chloro or fluoro.

"Heteroaralkyl" refers to a radical of the formula R^eR^c-, where R^e is heteroaryl as defined above and R^c is alkylene as defined above.

"Heterocyclo" refers to a monovalent saturated carbocyclic radical, consisting of either a 5 to 7 membered monocyclic ring or a 9 to 14 membered bicyclic ring, substituted by one, two or three heteroatoms chosen from N, O, or S, optionally fused to a substituted or unsubstituted benzene ring. Examples of heterocyclo radicals are morpholino, piperazinyl, piperidinyl, pyrrolidinyl, tetrahydrothiopyranyl, tetrahydrothiopyranyl-1,1-dioxide, tetrahydropyranyl, and the like, which can be optionally substituted by one or more substituents independently selected from lower alkyl, lower alkoxy, alkylamino, alkylaminoalkyl, acyl valyl, alkylsulfonyl, dialkylamino, heteroaroyl, alkoxycarbonylalkyl, and an amino protecting group where appropriate (*e.g.* CBZ, for example, 1-CBZ-piperidin-4-yl). However, the definition "R^6 and R^7 together with the nitrogen to which they are attached represent a heterocyclo group" clearly can refer only to a heterocyclo group containing at least one nitrogen atom.

"Hydroxylamino" refers to the group -NHOH.

"BOC" refers to *tert*-butoxycarbonyl.

"CBZ" refers to benzyloxycarbonyl.

"DCC" refers to 1,3-dicyclohexylcarbodiimide.

"Valine amide" refers to the radical $(CH_3)_2CHCH(NH_2)C(O)NH$-.

"Optional" or "optionally" means that the subsequently described event of circumstances may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not. For example, "optionally substituted phenyl or aryl" means that the phenyl or aryl moiety may or may not be substituted and that the description includes both substituted and unsubstituted phenyl. The phrase "optional pharmaceutical excipients" indicates that a composition or dosage form so described may or may not include pharmaceutical excipients other than those specifically stated to be present, and that the formulation or dosage form so described includes instances in which optional excipients are present and instances in which they are not.

"Amino-protecting group" as used herein refers to those organic groups intended to protect nitrogen atoms against undesirable reactions during synthetic procedures, and includes, but is not limited to, benzyl, acyl, benzyloxycarbonyl (carbobenzyloxy), *p*-methoxybenzyloxy-carbonyl, *p*-nitrobenzyloxycarbonyl, *tert*-butoxycarbonyl, trifluoroacetyl, and the like.

"Base" as used here includes both strong inorganic bases such as sodium hydroxide, lithium hydroxide, ammonium hydroxide, potassium carbonate and the like, and organic bases such as pyridine, diisopropylethylamine, 4-methylmorpholine, triethylamine, dimethylaminopyridine and the like.

"Pharmaceutically acceptable salt" refers to those salts which retain the biological effectiveness and properties of the free bases or free acids and which are not biologically or otherwise undesirable. If the compound exists as a free base, the desired acid salt may be prepared by methods known to those of ordinary skill in the art, such as treatment of the compound with an inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like; or with an organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid, and the like. If the compound exists as a free acid, the desired base salt may also be prepared by methods known to those of ordinary skill in the art, such as the treatment of the compound with an inorganic base or an organic base. Salts derived from inorganic bases include, but are not limited to, the sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Salts derived from organic bases include, but are not limited to, salts of

primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethylaminoethanol, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, *N*-ethylpiperidine, polyamine resins and the like.

"Pharmaceutically acceptable ester" as used herein refers for example to those non-toxic esters of a compound of Formula I where R^1 is hydroxy, and are formed by reaction of such compounds, by means well known in the art, with an appropriate alkanol of 1-8 carbon atoms, for example methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, *tert*-butanol, *i*-butanol (or 2-methylpropanol), *n*-pentanol, *n*-hexanol, and the like.

The terms "inert organic solvent" or "inert solvent" mean a solvent inert under the conditions of the reaction being described in conjunction therewith, including, for example, benzene, toluene, acetonitrile, tetrahydrofuran ("THF"), *N,N*-dimethylformamide ("DMF"), chloroform ("$CHCl_3$"), methylene chloride (or dichloromethane or "CH_2Cl_2"), diethyl ether, ethyl acetate, acetone, methylethyl ketone, methanol, ethanol, propanol, isopropanol, *tert*-butanol, dioxane, pyridine, and the like. Unless specified to the contrary, the solvents used in the reactions of the present invention are inert solvents.

The compounds of this invention may possess one or more asymmetric centers; such compounds can therefore be produced as mixtures of stereoisomers or as individual (*R*)- or (*S*)- stereoisomers. The individual enantiomers may be obtained by resolving a racemic or non-racemic mixture of an intermediate at some appropriate stage of the synthesis. It is understood that the individual (*R*)- or (*S*)-stereoisomers as well as racemic mixtures and other mixtures of stereoisomers are encompassed within the scope of the present invention.

The use of the symbol "(*R*)" or "(*S*)" preceding a substituent designates the absolute stereochemistry of that substituent according to the Cahn-Ingold-Prelog rules [see Cahn et al., *Angew. Chem. Inter. Edit.*, **5**, 385 (1966), ertata p. 511; Cahn et al., *Angew. Chem.*, **78**, 413 (1966); Cahn and Ingold, *J. Chem. Soc.*, (London), 612 (1951); Cahn et al., *Experientia*, **12**, 81 (1956); Cahn J., Chem. Educ., **41**, 116 (1964)]. Because of the interrelation of the designated substituent with the other substituents in a compound having a or β prefixes, the designation of the absolute configuration of one substituent fixes the absolute configuration of all substituents in the compound and thus the absolute configuration of the compound as a whole.

"Stereoisomers" are isomers that differ only in the way the atoms are arranged in space.

"Enantiomers" are a pair of stereoisomers that are non-superimposable mirror images of each other. Enantiomers rotate the plane of polarized light in opposite directions. The enantiomer that rotates the plane to the left is called the levo isomer, and is designated (-). The enantiomer that rotates the plane to the right is called the dextro isomer, and is designated (+).

"Diastereoisomers" are stereoisomers which are not mirror-images of each other.

"Racemic mixture" means a mixture containing equal parts of individual enantiomers. "Non-racemic mixture" is a mixture containing unequal parts of individual enantiomers.

"Mammal" includes humans and all domestic and wild animals, including, without limitation, cattle, horses, swine, sheep, goats, dogs, cats, and the like.

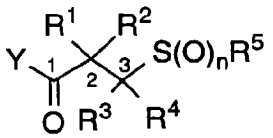
"Treating" or "treatment" as used herein cover the treatment of a disease-state in a mammal, particularly in a human, and include:

(i) preventing the disease-state from occurring in a mammal, in particular, when such mammal is predisposed to the disease-state but has not yet been diagnosed as having it;
(ii) inhibiting the disease-state, *i.e.*, arresting its development; or
(iii) relieving the disease-state, *i.e.*, causing regression of the disease-state.

The term "therapeutically effective amount" refers to that amount of a compound of Formula I that is sufficient to effect treatment, as defined above, when administered to a mammal in need of such treatment. The therapeutically effective amount will vary depending on the subject and disease state being treated, the severity of the affliction and the manner of administration, and may be determined routinely by one of ordinary skill in the art.

Nomenclature

The compounds of Formula I, illustrated below, will be named using the indicated numbering system:



A compound of Formula I wherein is Y is *N*-hydroxylamino; R^1 and R^2 are hydrogen; R^3 is benzyl; R^4 is hydrogen; R^5 is 4-methoxyphenyl; and n is 2, is named 3-benzyl-3-(4-methoxyphenylsulfonyl)-*N*-hydroxypropionamide.

A compound of Formula I wherein Y is *N*-hydroxylamino; R^1 and R^2 are hydrogen; R^3 and R^4 together with the carbon to which they are attached represent tetrahydropyran-4-yl; R^5 is 4-(4-fluorophenoxy)phenyl; and n is 2, is named as an acetic acid derivative, *i.e.*, 2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxy-acetamide.

A compound of Formula I wherein Y is hydroxy; R^1 is hydrogen; R^2 is methyl; R^3 and R^4 together with the carbon to which they are attached represent 1-methylpiperidin-4-yl; R^5 is biphenyl; and n is 1, is named 2-[4-(biphenyl-4-sulfinyl)-1-methylpiperidin-4-yl]-propionic acid.

A compound of Formula I wherein Y is *N*-hydroxylamino; R^1 and R^2 together with the carbon to which they are attached represent tetrahydropyran-4-yl, R^3 and R^4 are hydrogen, R^5 is 4-(4-chlorophenoxy)-phenyl; and n is 2, is named 4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide).

Synthetic Reaction Parameters

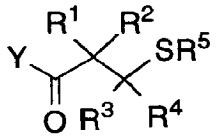
Unless specified to the contrary, the reactions described herein take place at atmospheric pressure within a temperature range from 5°C to 100°C (preferably from 10°C to 50°C; most preferably at "room" or "ambient" temperature, *e.g.*, 20°C). Further, unless otherwise specified, the reaction times and conditions are intended to be approximate, *e.g.*, taking place at about atmospheric pressure within a temperature range of about 5°C to about 100°C (preferably from about 10°C to about 50°C; most preferably about 20°C) over a period of about 1 to about 10 hours (preferably about 5 hours). Parameters given in the Examples are intended to be specific, not approximate.

Amide couplings used to form the compounds of Formula I are generally performed by the carbodiimide method with reagents such as 1,3-dicyclohexylcarbodiimide or *N'*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride or alternatively 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), in the presence of 1-hydroxybenzotriazole hydrate (HOBT) in an inert solvent such as *N,N*-dimethylformamide (DMF) or methylene chloride (CH_2Cl_2). Other methods of forming the amide or peptide bond include, but are not limited to, synthetic routes via an acid chloride, acyl azide, mixed anhydride or activated ester such as a *p*-nitrophenyl ester. Typically, solution phase amide couplings with or without peptide fragments are performed.

The selection of amino protecting groups used in the preparation of compounds of Formula I is dictated in part by the particular amide coupling conditions, and in part by the components involved in the coupling. Amino-protecting groups commonly used include those which are well-known in the art, for example, benzyloxycarbonyl (carbobenzyloxy) (CBZ), *p*-methoxybenzyloxycarbonyl, *p*-nitro-benzyloxycarbonyl, *N-tert*-butoxycarbonyl (BOC), and the like. It is preferred to use either BOC or CBZ as the protecting group for the a-amino group because of the relative ease of removal by mild acids in the case of BOC, *e.g.*, by trifluoroacetic acid (TFA) or hydrochloric acid in ethyl acetate; or removal by catalytic hydrogenation in the case of

PREPARATION OF COMPOUNDS OF FORMULA I

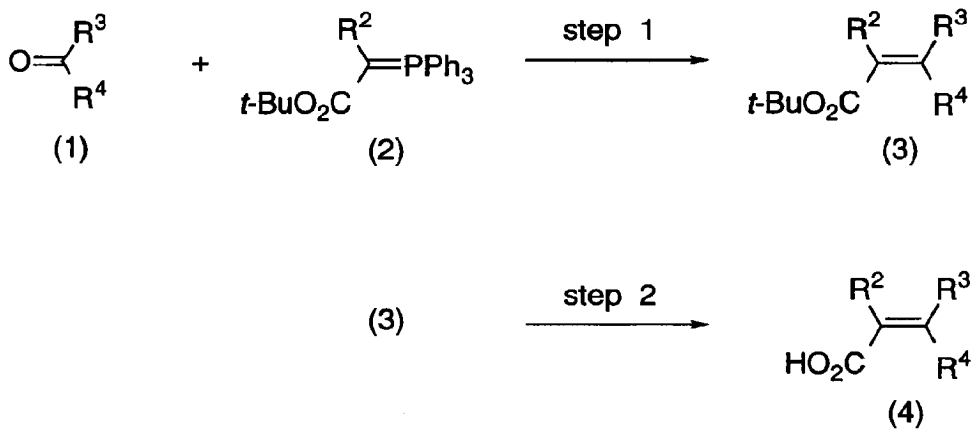
One method for preparing a compound of the Formula I, in particular wherein n is 1 or 2; Y is hydroxy or XONH-, where X is hydrogen or lower alkyl; R^1 is hydrogen or lower alkyl; R^2 is hydrogen, lower alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, or heterocyclo; or R^1 and R^2 together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group; R^3 is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aralkyl, heteroaralkyl, or lower alkoxy; R^4 is hydrogen or lower alkyl; or R^2 and R^3 together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or R^3 and R^4 together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and R^5 is lower alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl; comprises contacting a compound of the Formula:



with an oxidizing agent. Suitable oxidation conditions are outlined in the description of reaction scheme VIII below.

One method of preparing compounds of Formula I where n is 0, R^1 is hydrogen and R^2 is not $-NR^6R^7$ is from the corresponding unsaturated acid of Formula (4), the preparation of which is shown below in Reaction Scheme I:

REACTION SCHEME I



Starting Materials

Aldehydes and ketones of Formula (1) are commercially available, for example from Aldrich Chemical Co., or may be prepared as shown below, or prepared according to methods well known to those skilled in the art. The ylides of Formula (2) are commercially available, for example, (*tert*-butoxycarbonylmethylene)triphenylphosphorane is available from Aldrich, or may be prepared by standard methods known to those skilled in the art, for example by reacting the appropriate bromo derivative of formula $R^2CHBrCO_2$-(*tert*-butyl) with triphenylphosphine, and reacting the resulting triphenylphosphonium bromide derivative with a strong base.

Step 1 - Preparation of Compounds of Formula (3)

In general, a solution of an aldehyde or ketone compound of Formula (1) is reacted in an inert organic solvent, for example benzene, with a compound of Formula (2) (or alternatively, the corresponding phosphonate, for example trimethyl phosphonoacetate) for a period of 8 to 48 hours at 15°C to 30°C (aldehydes), preferably 20°C, or 70°C to 90°C (ketones), preferably 80°C, until starting material is consumed. The reaction product, an enoic ester of Formula (3), is isolated and purified by conventional means.

Step 2 - Preparation of Compounds of Formula (4)

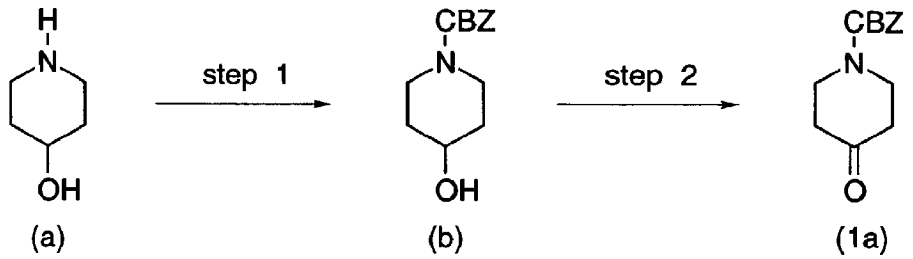
The compound of Formula (3) is then hydrolyzed under acidic conditions, optionally in the presence of an inert solvent, *e.g.,* treatment with trifluoroacetic acid in methylene chloride for about 20 minutes to 3 hours. The reaction is carried out at a temperature range from about 0°C to 40°C, preferably at about room temperature. In the case where trimethyl phosphonoacetate is used in Step 1, a methyl ester is produced which may be hydrolyzed conventionally

under basic conditions, for example sodium hydroxide in aqueous methanol or ethanol. The reaction product, an enoic acid of Formula (4), is isolated and purified by conventional means.

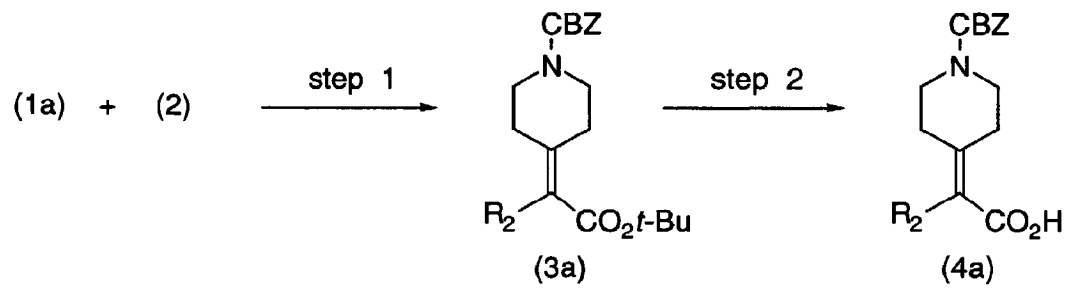
Preparation of Compounds of Formula (4) where R^3 and R^4 together with the Carbon to which they are attached represent a Piperidine Derivative

The preparation of compounds of Formula (4) where R^3 and R^4 together with the carbon to which they are attached represent a piperidine derivative, represented below as a compound of Formula (4a), in general requires the protection of the NH group. An example is shown below in Reaction Scheme II.

REACTION SCHEME II



(a) (b) (1a)



(3a) (4a)

Step 1 - Preparation of Compounds of Formula (b)

In general, a solution of a hydroxypiperidine compound of Formula (a) is protected by reaction of (a) in an inert organic solvent, for example tetrahydrofuran, in the presence of an excess of a tertiary base, for example triethylamine, with an equimolar amount of benzyl chloroformate. The reaction is carried out in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 10 to 30 hours, preferably about 18 hours. The reaction product of Formula (b) is isolated and purified by conventional means.

Step 2 - Preparation of Compounds of Formula (1a)

A compound of Formula (1a) is a compound of Formula (1) where R^3 and R^4 together with the carbon to which they are attached represent a protected piperidine derivative.

In general, a solution of a compound of Formula (b) is oxidized to a ketone of Formula (1a) by reaction of (b) in an inert organic solvent, for example methylene chloride, with an oxidizing agent, for example pyridinium chlorochromate, preferably in the presence of an inert support, for example Celite. The reaction is carried out in the temperature range

from about 0°C to 40°C, preferably at about 25°C, for about 10 to 30 hours, preferably about 18 hours. The reaction product of Formula (1a) is isolated and purified by conventional means.

Alternatively, reaction of commercially available 4-piperidone monohydrate hydrochloride with benzyl chloroformate under Schotten-Baumann conditions gives a compound of Formula (1a) in a single step.

Preparation of Compounds of Formula (4) where R^3 and R^4 Together with the Carbon to which they are attached Represent a Piperidine Derivative

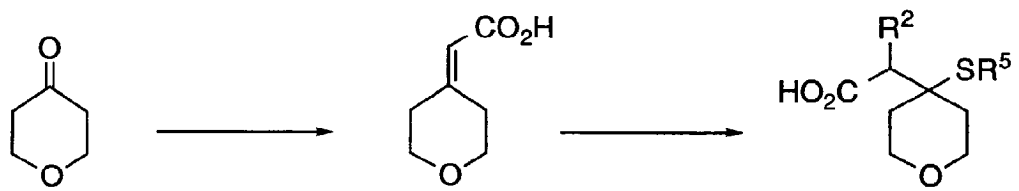
A compound of Formula (4) where R^3 and R^4 together with the carbon to which they are attached represent a piperidine derivative is represented as a compound of Formula (4a).

The protected piperidine ketone of Formula (1a) is converted to (3a), which is hydrolyzed to (4a) as described in Reaction Scheme I, Steps 1 and 2. The compound of Formula (4a) is then converted to a compound of Formula I where n is 0 as described in Reaction Scheme III below. The benzyloxycarbonyl (CBZ) protecting group is removed by catalytic hydrogenation, to give a compound of Formula I where R^3 and R^4 together with the carbon to which they are attached represent piperidine.

Preparation of Compounds of Formula (4) where R^3 and R^4 Together with the Carbon to which they are attached Represent a Pyran Derivative

Compounds of Formula (4) where R^3 and R^4 together with the carbon to which they are attached represent a tetrahydropyran derivative, represented as Formula (4b), are prepared similarly to the procedure shown above, starting from the corresponding 4-oxotetrahydropyran. The reaction is shown below in Reaction Scheme III and described in Example 3.

REACTION SCHEME III



The tetrahydropyran derivative of Formula (4b) is then converted to the corresponding compound of Formula I, *i.e.*, a compound of Formula I where n is 0, as described in Reaction Scheme VII.

Preparation of Compounds of Formula (4) where R^3 and R^4 Together with the Carbon to which they are Attached represent a Tetrahydrothiopyran-1,1-dioxide Derivative

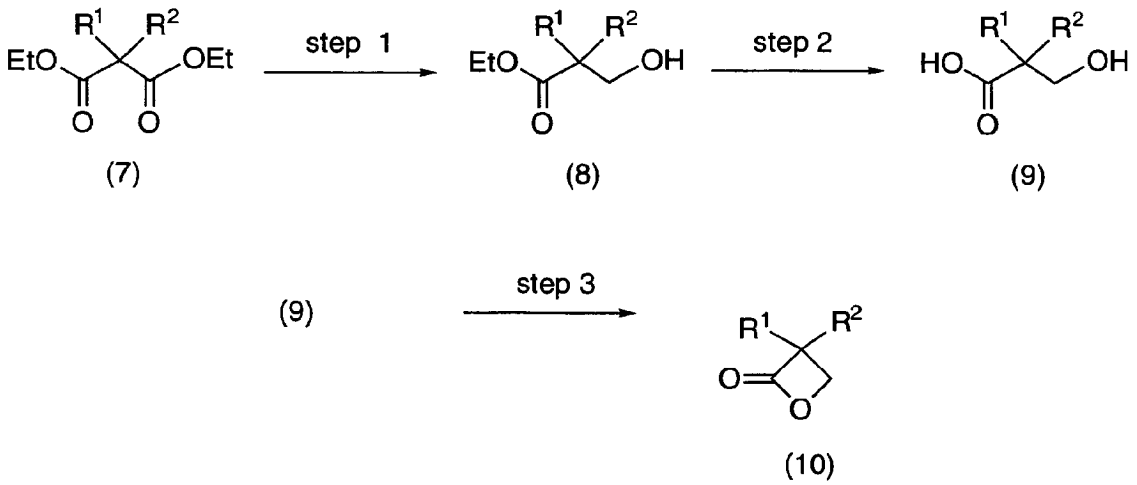
Compounds of Formula (4) where R^3 and R^4 together with the carbon to which they are attached represent a tetrahydrothiopyran1,1-dioxide derivative are prepared similarly to the procedure shown above, starting from the corresponding 4-oxotetrahydrothiopyran.

The tetrahydrothiopyran-1,1-dioxide derivative of Formula (4) is then converted to the corresponding compound of Formula I where n is 0 as described in Reaction Scheme III.

Alternative Preparation of Compounds of Formula I

Another method of preparing compounds of Formula I where R^2 is not $-NR^6R^7$ and R^3 and R^4 are both hydrogen is from the corresponding lactone of Formula (10), the preparation of which is shown below in Reaction Scheme IV.

REACTION SCHEME IV



Step 1 - Preparation of Compounds of Formula (8)

The starting compounds of Formula (7) are commercially available, or may be prepared by means well known in the art starting from diethyl malonate, *e.g.*, Gibson and Johnson, *J. Chem. Soc.*, p2525 (1930), (other diesters may be employed in place of the diethyl ester if desired). In general, a solution of a compound of Formula (7) is dissolved in an inert aromatic solvent, preferably benzene or toluene, and cooled to about -40° to -20°C, preferably about -30°C. To this cold solution is added a suitable hindered reducing agent, preferably diisobutylaluminum hydride in an inert aromatic solvent, maintaining the temperature at no higher than about 25°C. After the addition is complete, the reaction is maintained at about 15°C until all the starting material is consumed. After about 10 minutes the reaction is quenched by addition of a protic solvent, preferably ethanol, maintaining the temperature at no higher than about -15°C. Sodium borohydride is optionally added, but preferably the reaction is simply allowed to warm to about room temperature. The reaction product of Formula (8) is isolated and purified by conventional means.

Step 2 - Preparation of Compounds of Formula (9)

In general, the compound of Formula (8) is hydrolysed with a base to form the hydroxymethyl acid of Formula (9).

The compound of Formula (8) is dissolved in an aqueous protic solvent, preferably aqueous methanol, and reacted with about 3 molar equivalents of a base, for example potassium hydroxide or lithium iodide, followed by sodium cyanide. The reaction is carried out in the temperature range from about 80°C to 120°C, preferably at about the reflux temperature of the solvent mixture, for about 8 hours. The reaction product of Formula (9) is isolated and purified by conventional means.

Step 3 - Preparation of Compounds of Formula (10)

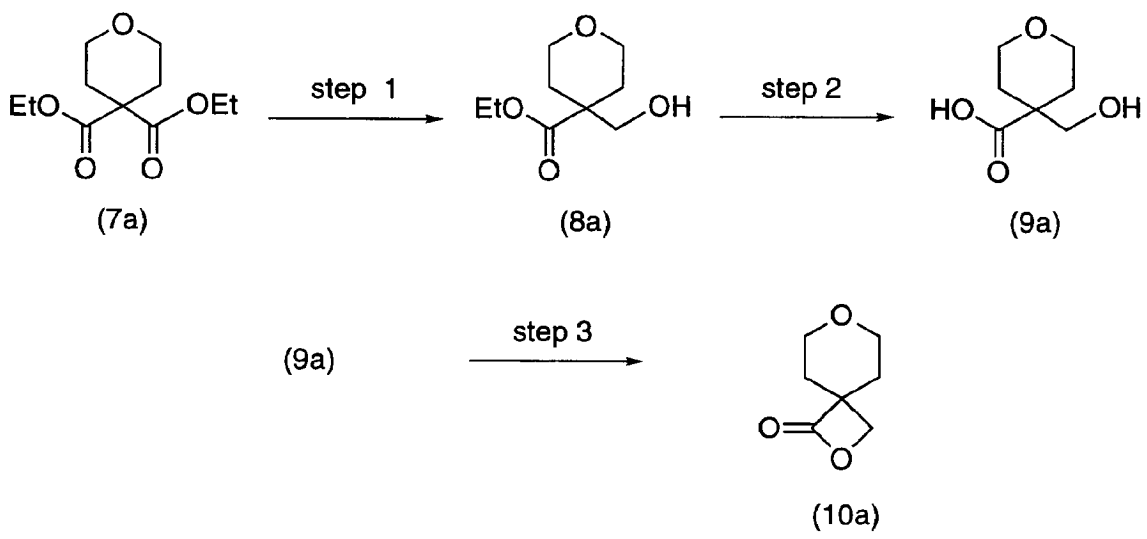
In general, the compound of Formula (9) is dehydrated to form a lactone of Formula (10).

To a mixture of the compound of Formula (9) and about 2 molar equivalents of a tertiary base, preferably triethylamine, optionally in the presence of 4-dimethylaminopyridine, in an inert solvent, for example, diethyl ether or dichloromethane, at about -20°C, is added about 1 molar equivalent of a dehydrating agent, for example trifluoromethanesulfonic anhydride, methanesulfonic anhydride, methanesulfonyl chloride, *p*-toluenesulfonyl chloride, benzenesulfonyl chloride, preferably benzenesulfonyl chloride. The reaction is carried out at about -10°C, for about 10 minutes to 4 hours, preferably about 30 minutes. The reaction product of Formula (10) is isolated by conventional means synthesis without further purification.

Preparation of Compounds of Formula (10) where R^1 and R^2 together with the Carbon to which they are attached Represent a Tetrahydropyran Derivative

To give a specific example, the preparation of a compound of Formula (10) where R^1 and R^2 together with the carbon to which they are attached represent a tetrahydropyran derivative (represented as Formula (10a)) is shown below in Reaction Scheme V, and described in Example 5.

REACTION SCHEME V

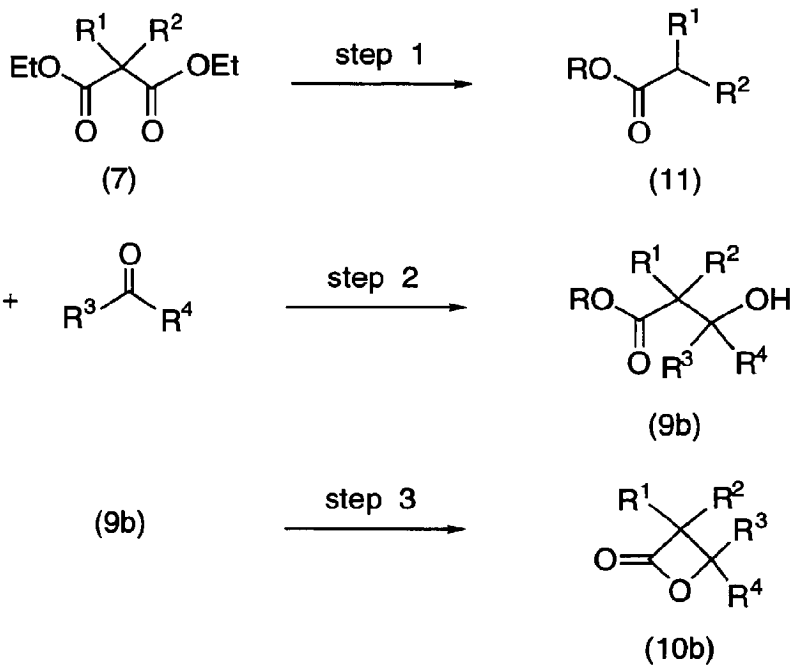


The starting compound of Formula (7a) is either commercially available or may be prepared as shown in Example 31A. Steps 1-3 are carried out in the same manner as shown in Reaction Scheme IV.

Preparation of Compounds of Formula (10) where R^3 and R^4 are as Defined in the compounds of formula I

The preparation of a compound of Formula (10) where R^3 and R^4 are as defined in the compounds of formula I, represented as Formula (10b), is shown below in Reaction Scheme VI, and described in Example 5.

REACTION SCHEME VI



Step 1 - Preparation of Compounds of Formula (11)

The compound of Formula (11), where R is Et, may be prepared from the compound of Formula (7) by decarboxylation. In general, the diester is reacted with a mixture of lithium iodide and sodium cyanide at about 130° to 140°C in a suitable solvent, for example *N, N*-dimethylformamide, for about 24 hours.

Step 2 - Preparation of Compounds of Formula (9b)

In general, an anion of a compound of Formula (11), where R is H or lower alkyl, is reacted with a compound of the formula $R^3R^4C=O$ to form a hydroxy acid or hydroxy ester, respectively, of Formula (9b).

A solution of the compound of Formula (11) in an anhydrous ethereal solvent, preferably tetrahydrofuran, is added to about 1.1 molar equivalent (when R is lower alkyl) or about 2 molar equivalents (when R is hydrogen) of a hindered base, preferably lithium diisopropylamide, in an anhydrous ethereal solvent, preferably tetrahydrofuran, at about 0°C. When the addition is complete, a small quantity of a polar solvent is optionally added, preferably hexamethylphosphoramide. To this mixture is added an excess of a compound of the formula $R^3R^4C=O$. The addition is carried out at a temperature range of about -78 to 10°C, preferably at about -78°C when R^3 and R^4 are hydrogen, or preferably 0°C for ketones, followed by reaction at room temperature for about 2-24 hours, preferably about 10 hours. Where R in the starting material of Formula (11) is hydrogen, the reaction product of Formula (9b) is isolated and purified by conventional means. Where R in the starting material of Formula (11) is lower alkyl, the reaction product of Formula (9b), where R = H, is obtained by hydrolyzing the ester product using a base, preferably lithium hydroxide, as described above, then isolating and purifying (9b) by conventional means.

Step 3 - Preparation of Compounds of Formula (10b)

The compound of Formula (9b) is then converted to a compound of Formula (10b) in the same manner as described in Reaction Scheme IV.

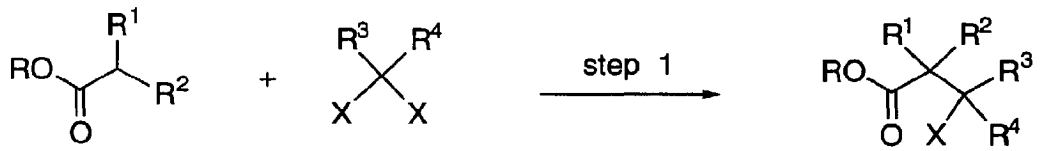
The method of Reaction Scheme VI can be used, for example, to prepare compounds of Formula (10) where R^1 and R^2 taken together with the carbon to which they are attached is tetrahydropyran-4-yl, by starting with 4-carboxytet-

rahydropyran or an ester thereof, for example, the ethyl ester. Similarly, compounds of Formula (10) where R^1 and R^2 taken together with the carbon to which they are attached is piperidin-4-yl or derivatives thereof, may be prepared by starting with 1-benzyloxycarbonyl-4-carboxypiperidine, *N*-(*tert*-butoxycarbonyl)-4-carboxypiperidine, or an ester thereof, for example, the ethyl ester.

Alternative Preparation of Compounds of Formula I

Compounds of Formula I can also be prepared from compounds of Formula (13), the preparation of which is shown below in Reaction Scheme VIa, and described in Example 5A.

REACTION SCHEME VIA



(11) (13)

where R is hydrogen or lower alkyl, and X is halo or -*p*-tosyl.

Step 1 - Preparation of Compounds of Formula (13) from (11)

The starting compounds of Formula (13) are commercially available, for example, an ester of commercially available chloropivalic acid may be prepared conventionally, or compounds of Formula (13) may be prepared by means well known in the art, for example, Gibson and Johnson, *J. Chem. Soc.*, p2525 (1930). In general, an anion of a compound of Formula (11) is reacted with an alkyl dihalide to form a halo-substituted hydroxy acid ester of Formula (13).

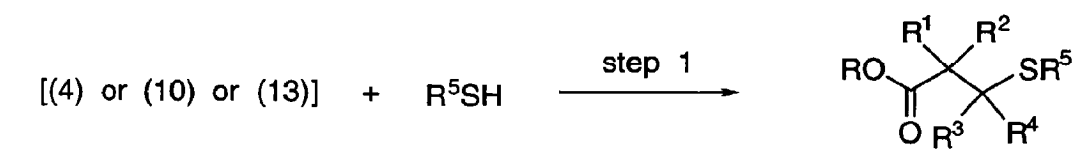
A solution of the compound of Formula (11) in an anhydrous ethereal solvent, preferably tetrahydrofuran, is added to about 1.1 molar equivalent (when R is lower alkyl) or about 2 molar equivalents (when R is hydrogen) of a hindered base, preferably lithium diisopropylamide, in an anhydrous ethereal solvent, preferably tetrahydrofuran, at about -100 to 0°C, preferably at about -78°C. To this mixture is added an excess of an alkyl dihalide, preferably diiodomethane. The addition is carried out a temperature range of about -5° to 50°C for about 1-5 hours. The reaction product of Formula (13) is isolated by conventional means, and preferably used in the next step of the synthesis without further purification.

It should be noted that a compounds of Formula (13) where X is *p*-tosyl, are obtained by tosylation by conventional means of compounds of Formula (8) or (9b).

Preparation of Compounds of Formula I

The intermediates of Formulae (4), (10), and (13) may be converted to compounds of Formula I where Y is hydroxy and n is 0, designated as compounds of Formula Ia, as shown in Reaction Scheme VII below.

REACTION SCHEME VII



Ia

where R is hydrogen or lower alkyl.

Compounds of Formula (4) are either commercially available, for example from Aldrich, or may be prepared according to methods known to those skilled in the art, for example, as described by Mannich and Rister, *Chem. Ber.*, **57**, 1116

(1924) for acids where R^3 and R^4 are each hydrogen, or may be prepared as described above, or as described in Example 3. Compounds of Formula (5) are commercially available, for example from Aldrich, Fluka, etc.), or may be prepared according to methods known to those skilled in the art, *e.g.,* as described below in Example 4.

Step 1 - Preparation of Compounds of Formula Ia from (4)

Compounds of Formula I where n is 0 and Y is hydroxy, designated as compounds of Formula Ia, may be prepared by heating an enoic acid of Formula (4) with an equimolar amount of a thiol of Formula (5) in the presence of an approximately equimolar amount of a secondary amine, preferably piperidine. The reaction is carried out in the temperature range from about 70°C to 120°C, preferably at about 100°C, for about 1 to 24 hours, preferably about 3 hours. The sulfide reaction product, a compound of Formula Ia, is isolated and purified by conventional means.

Step 1 - Preparation of Compounds of Formula Ia from (10)

Compounds of Formula I where n is 0 and Y is hydroxy, designated as compounds of Formula Ia, may be prepared by reacting a lactone of Formula (10) with about 1.1 molar equivalents of an anion of a thiol of Formula (5) (generated by reaction of (5) with an alkaline metal hydride, preferably sodium hydride in a polar solvent, preferably *N, N*-dimethylformamide). The reaction is carried out in a polar solvent, preferably *N, N*-dimethylformamide, at a temperature range of about 0°C to 70°C, preferably at about 0° to 25°C. The sulfide reaction product, a compound of Formula Ia, is isolated and purified by conventional means.

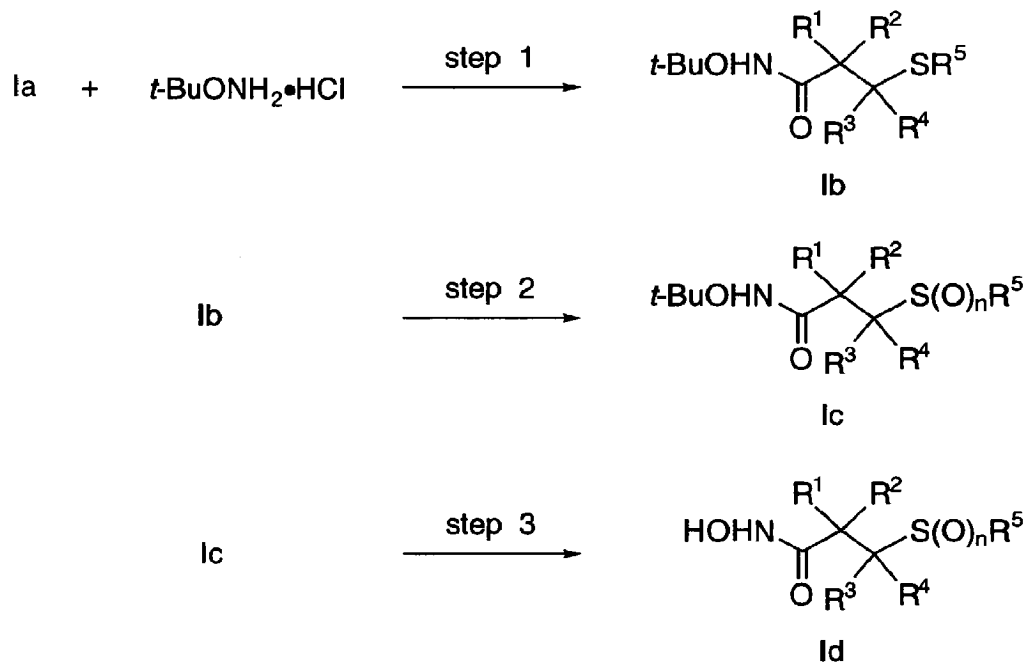
Step 1 - Preparation of Compounds of Formula Ia from (13)

Compounds of Formula I where n is 0 and Y is hydroxy or lower alkoxy, designated as compounds of Formula Ia, may be prepared by reacting an enoic acid ester of Formula (13) with about 1.1 molar equivalents of an anion of a thiol of Formula (5) (generated by reaction of (5) with an alkaline metal hydride, preferably sodium hydride in a polar solvent, preferably *N,N*-dimethylformamide). The reaction is carried out in a polar solvent, preferably *N,N*-dimethylformamide, at a temperature range of about 30°C to 120°C, preferably at about 80°C, for about 10 minutes. The sulfide reaction product, a compound of Formula Ia, is isolated and purified by conventional means.

Conversion of Compounds of Formula Ia to other Compounds of Formula I

One method of converting compounds of Formula Ia to other compounds of Formula I is shown below in Reaction Scheme VIII.

REACTION SCHEME VIII



Step 1 - Preparation of Compounds of Formula Ib

In general, compounds of Formula I where n is 0 and Y is *tert*-BuONH-, designated as compounds of Formula Ib, are prepared by reacting a compound of Formula Ia with an excess of a *O*-(*tert*-butyl)-hydroxylamine hydrochloride and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (or other carbodiimide derivatives, for example 1,3-dicyclohexylcarbodiimide), in the presence of 1-hydroxybenzotriazole hydrate and a tertiary base, for example dimethylaminopyridine, triethylamine, 4-methylmorpholine, pyridine, or a mixture of such bases. The reaction is carried out in an inert solvent, preferably methylene chloride, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 10 to 30 hours, preferably about 18 hours. The *N*-*tert*-butoxy reaction product, a compound of Formula Ib, is isolated and purified by conventional means.

Step 2 - Preparation of Compounds of Formula Ic where n is 1

In general, compounds of Formula I where n is 1 and Y is *tert*-BuONH-, (*i.e.,* sulfoxides), designated as compounds of Formula Ic, are prepared from compounds of Formula Ib by reaction with a mild oxidizing agent, for example sodium periodate or one equivalent of "OXONE"™ (potassium peroxymonosulfate, Aldrich Chemical Co.), until starting material can no longer be detected. The reaction is carried out in an inert solvent, preferably aqueous acetone, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 10 minutes to 4 hours, preferably about 30 minutes. The sulfoxide product, a compound of Formula Ic where n is 1, is isolated and purified by conventional means.

Step 2 - Preparation of Compounds of Formula Ic where n is 2

In general, compounds of Formula I where n is 2, Y is *tert*-BuONH-, and R^1 is hydrogen (*i.e.,* sulfones), designated as compounds of Formula Ic, are prepared from compounds of Formula Ib by reaction with about 1-3 molar equivalents, preferably about 1.5 molar equivalents, of a strong oxidizing agent, for example, *m*-chloroperbenzoic acid or OXONE. The reaction is carried out in an inert solvent, preferably a protic solvent, preferably aqueous methanol, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 10 minutes to 4 hours, preferably about 2 hours. The sulfone product, a compound of Formula Ic where n is 2, is isolated and purified by conventional means.

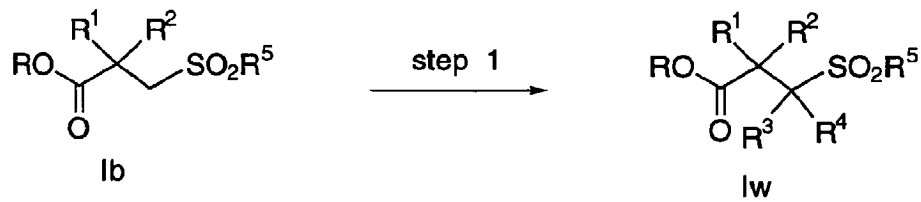
Step 3 - Preparation of Compounds of Formula Id

In general, compounds of Formula I where Y is HONH-, designated as compounds of Formula Id, are prepared by hydrolysing an *N-tert*-butoxy compound of Formula Ib or Ic under acid conditions under conditions similar to that shown for the preparation of compounds of Formula (4) above, or using hydrochloric acid gas in a sealed tube in an inert solvent, for example, 1,2-dichloroethane. The hydroxyamino reaction product, a compound of Formula Id where Y is HONH-, is isolated and purified by conventional means.

Alternative Method of Introduction of R^3 and R^4 into Compounds of Formula I

An alternative method of introducing the groups R^3 and R^4 into compounds of Formula I is shown below in Reaction Scheme VIIIA.

REACTION SCHEME VIIIA



Ib Iw

where R is hydrogen or lower alkyl.

Step 1- Preparation of Compounds of Formula I where n is 2, and R^3 is as defined in the compounds of formula I but is other than Hydrogen

The compounds of Formula I where n is 2, Y is hydroxy or alkoxy, R^3 is as defined in the compounds of formula I other than hydrogen, and R^1, R^2, and R^4 are defined in the compounds of formula I, designated as compounds of Formula Iw are prepared by the alkylation of compounds of Formula I where both R^3 and R^4 are hydrogen.

A solution of the compound of Formula Iw in an anhydrous ethereal solvent, preferably tetrahydrofuran, is added to a hindered base, preferably lithium diisopropylamide, in a manner similar shown above in Reaction Scheme VIA. To this mixture is added about 1 molar equivalent of an alkyl or aralkyl halide. The reaction addition is stirred for about 1-3 hours, then stirred stirred for an additional 1-5 hours, preferably 3 hours, at about room temperature. The reaction product is isolated and purified by conventional means.

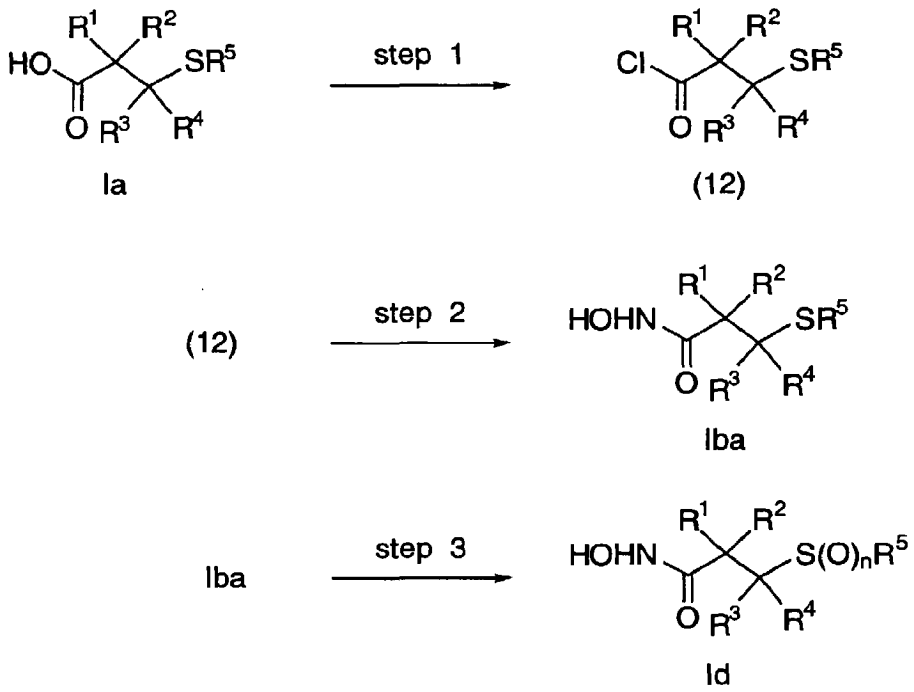
R^4 may be introduced in the same manner as shown above.

Compounds of Formula Iw can be converted to other compounds of Formula I as shown previously.

Preferred Procedure for Preparing Compounds of Formula Id from Compounds of Formula Ia

A preferred method of converting compounds of Formula Ia to other compounds of Formula I is shown below in Reaction Scheme IX.

REACTION SCHEME IX



Step 1 - Preparation of Compounds of Formula Iba

In general, an acid halide of a compound of Formula Ia, designated as compounds of Formula (12), is prepared by reacting a compound of Formula Ia with a halogenating agent.

The compound of Formula Ia is reacted with an excess of a halogenating agent, for example oxalyl chloride, oxalyl bromide, phosphorous oxychoride, phosphorous trichloride, phosphorous pentachloride, thionyl chloride, preferably oxalyl chloride in the presence of a small amount of *N, N*-dimethylformamide as a catalyst. The reaction is carried out in an inert solvent, preferably methylene chloride, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 10 to 30 hours, preferably about 18 hours. The acid halide reaction product, a compound of Formula (12), is isolated by conventional means.

Step 2 - Preparation of Compounds of Formula Iba

Compounds of Formula I where n is 0 and Y is HONH-, designated as compounds of Formula Iba, may be prepared by reacting a compound of Formula (12) with about 1-5 molar equivalents, preferably about 3.5 molar equivalents, of *N, O*-bis(trimethylsilyl)-hydroxylamine, or more preferably aqueous hydroxylamine dissolved in a suitable solvent, for example a mixture of *tert*-butanol/tetra-hydrofuran. The reaction is carried out in an inert solvent, preferably methylene chloride, in the temperature range from about 0°C to 25°C, preferably at about 25°C, for about 1-10 hours, preferably about 3 hours for *N, O*-bis(trimethylsilyl)hydroxylamine, or about 1.5 hours for aqueous hydroxylamine. The *N*-hydroxamic acid product, a compound of Formula Iba, is isolated and purified by conventional means.

Step 3 - Preparation of Compounds of Formula Id

The compound of Formula Iba is converted to a compound of Formula Id where n is 1 or 2 in the same manner as shown in Reaction Scheme VIII, steps 2 or 3, above.

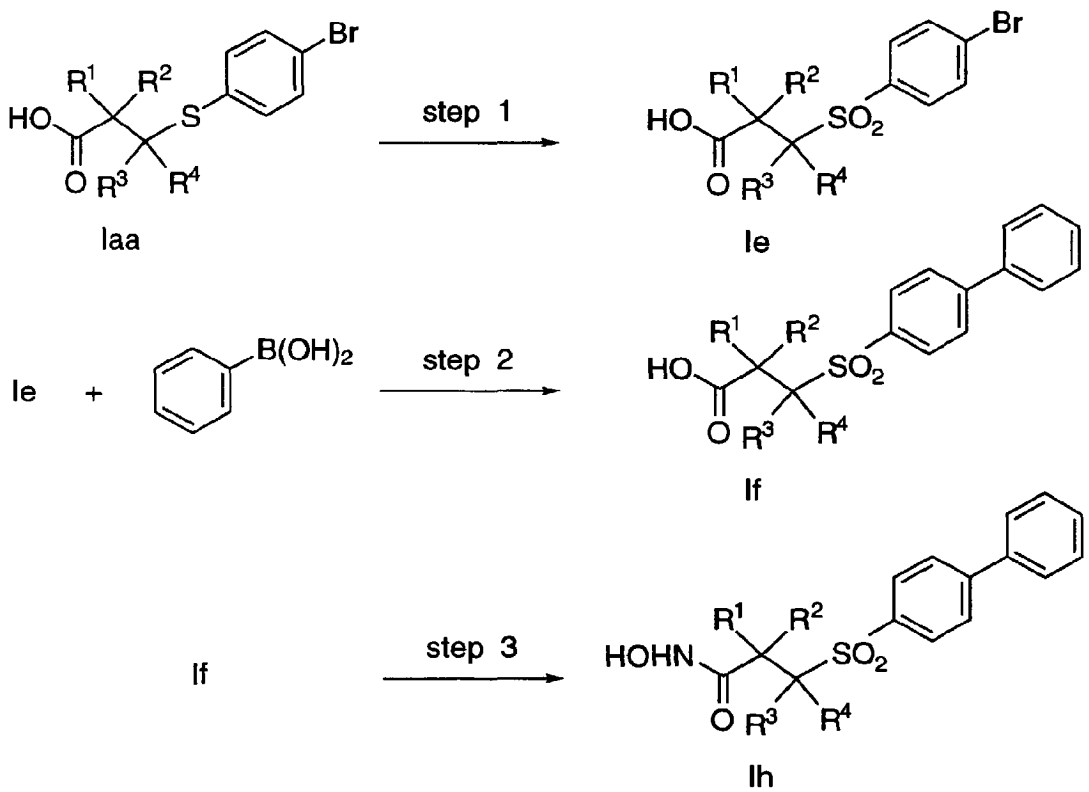
Alternative Preparation of Compounds of Formula I

It should be noted that the sequence of the steps in the above Reaction Schemes for the preparation of compounds of Formula Id may be changed. That is, a compound of Formula Ia may be oxidized first to a sulfone, followed by conversion of the carboxy group to hydroxyamino as shown above, if so desired.

Preparation of Compounds of Formula I where R^5 is Biphenyl

Compounds of Formula I where R^5 is optionally substituted biphenyl are preferably prepared from compounds of Formula Ia where R^5 is optionally substituted bromophenyl. For example, compounds where R^5 is 4-biphenyl can be prepared from compounds of Formula Ia where R^5 is 4-bromophenyl, represented below as a compound of Formula Iaa, as shown below in Reaction Scheme X.

REACTION SCHEME X



Step 1 - Preparation of Compounds of Formula Ie

In general, compounds of Formula I where n is 2, Y is hydroxy, R^5 is 4-bromophenyl, and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula Ie, are prepared from compounds of Formula Iaa by reaction with a strong oxidizing agent in the same manner as shown above in Reaction Scheme VIII, Step 2.

Step 2 - Preparation of Compounds of Formula If

In general, compounds of Formula I where n is 2, Y is hydroxy, R^5 is biphenyl, and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula If, are prepared by reacting a compound of Formula Ie with phenylboronic acid and zero-valent palladium catalysts, preferably tetrakis(triphenylphosphine)palladium.

The reaction is carried out in a protic solvent, preferably a mixture of ethanol and benzene, in the temperature range from about 30°C to 100°C, preferably at about 80°C. When the desired temperature is reached, aqueous 2M sodium carbonate is added, and refluxing continued for about 1-8 hours, preferably about 2 hours. The reaction product, a compound of Formula If, is isolated by conventional means and preferably purified using preparative TLC.

Step 3 - Preparation of Compounds of Formula Ih

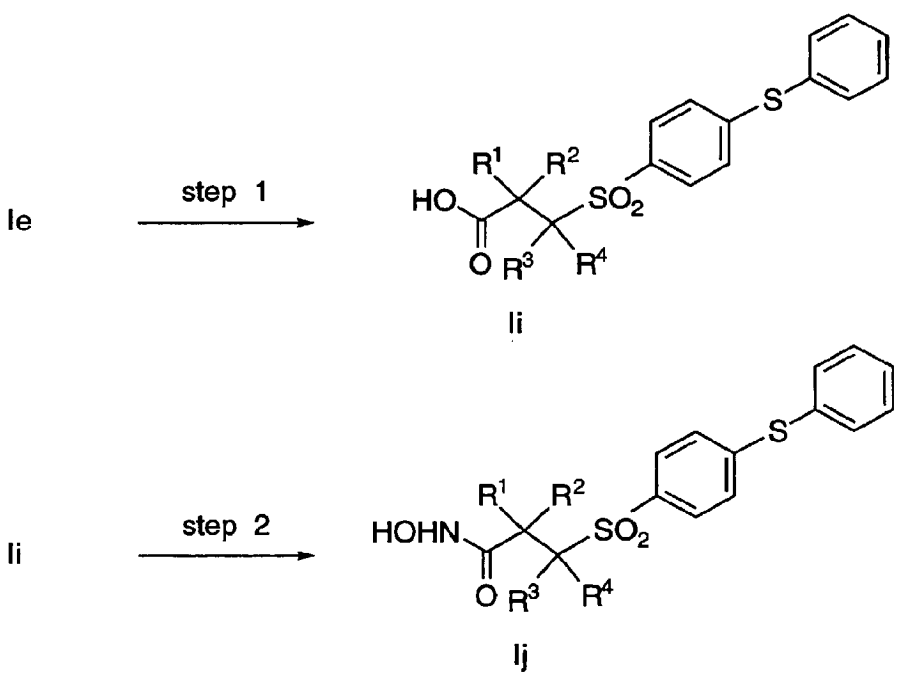
In general, compounds of Formula I where n is 2, Y is HONH-, R^5 is biphenyl, and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula Ih, may be prepared from the corresponding compounds of Formula If in the same manner as shown above in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

To prepare compounds of Formula I where R^5 is substituted biphenyl, a compound of Formula Iaa optionally substituted on the 4-bromophenyl ring is reacted with an optionally substituted boronic acid in the same manner as shown above.

Preparation of Compounds of Formula I where R^5 is Diphenylsulfide

Compounds of Formula I where R^5 is optionally substituted diphenylsulfide are preferably prepared from the corresponding compounds of Formula Ie, *i.e.,* compounds of Formula I in which R^5 is optionally substituted 4-bromophenyl, prepared as in Reaction Scheme X. For example, compounds where R^5 is 4-diphenylsulfide can be prepared from compounds of Formula Ie as shown below in Reaction Scheme XI.

REACTION SCHEME XI



Step 1 - Preparation of Compounds of Formula Ii

In general, compounds of Formula I where n is 2, Y is hydroxy, R^5 is 4-diphenylsulfide, and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula Ii, are prepared from compounds of Formula Ie by heating an anion of thiophenol (preferably prepared *in situ,* for example, by treatment of thiophenol with sodium or potassium hydride, preferably potassium hydride, in a polar solvent, preferably *N,N*-dimethylformamide. The

reaction is carried out in a polar solvent, preferably *N,N*-dimethylformamide, in the temperature range from about 30°C to 100°C, preferably at about 75°C, for about 4-48 hours, preferably about 18 hours. The reaction product, a compound of Formula Ii, is isolated by conventional means and preferably purified using preparative TLC.

Step 2 - Preparation of Compounds of Formula Ij

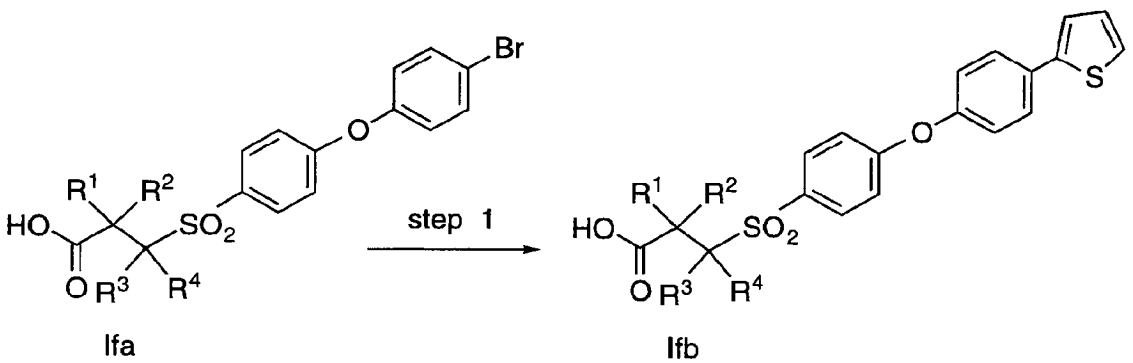
In general, compounds of Formula I where n is 2, Y is HONH-, R^5 is 4-diphenylsulfide, and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula Ij, are prepared from the corresponding compounds of Formula Ii in the same manner as shown above in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

To prepare compounds of Formula I where R^5 is substituted 4-diphenylsulfide, a compound of Formula Ie optionally substituted on the 4-bromophenyl ring is reacted with an optionally substituted anion of thiophenol in the same manner as shown above.

Preparation of Compounds of Formula I where R^5 is 4-[4-(thiophen-2-yl)phenoxy]phenyl

Compounds of Formula I where R^5 is optionally substituted 4-[4-(4-thiophen-2-yl)phenoxy]phenyl are prepared from the corresponding compounds of Formula I where R^5 is optionally substituted 4-(4-bromophenoxy)phenyl. This reaction is shown in Reaction Scheme XIA.

SCHEME XIA



Preparation of Compounds of Formula Ifb

The 4-bromo group of the compound of Formula (Ifa), which may be prepared by methods analogous to those previously shown, or as described in Example 16D, is displaced to give a compound of Formula Ifb, using the same procedure as described in Reaction Scheme X, step 2.

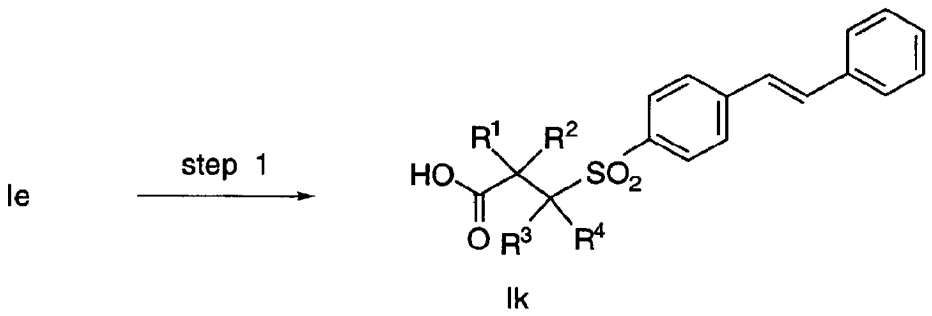
The compound of Formula (Ifa) is reacted similarly in order to introduce other aryl or heteroaryl groups.

Reduction of a compound of Formula Ifa with palladium and hydrogen replaces the bromo group by hydrogen.

Preparation of Compounds of Formula I where R^5 is 1,2-Diphenylethene

Compounds of Formula I where R^5 is optionally substituted 1,2-diphenylethene are preferably prepared from the corresponding compounds of Formula I where R^5 is optionally substituted 4-bromophenyl, as prepared in Reaction Scheme X. For example, compounds where R^5 is 4-diphenylethene can be prepared from compounds of Formula Ie as shown below in Reaction Scheme XII.

REACTION SCHEME XII



Ik

Step 1 - Preparation of Compounds of Formula Ik

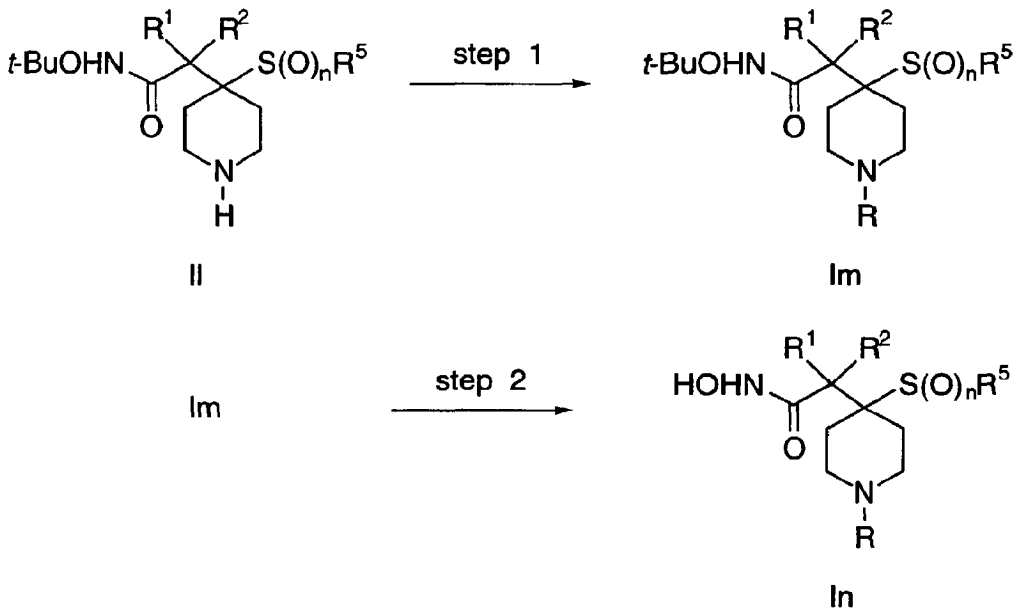
In general, compounds of Formula I where Y is hydroxy, R^5 is 4-(1,2-diphenylethene), and R^1, R^2, R^3, and R^4 are as defined in the compounds of formula I, designated as compounds of Formula Ik, are prepared by reacting a compound of Formula Ie with an optionally substituted styrene in the presence of a hindered tertiary organic base, for example diisopropylethylamine, and palladium diacetate, and trimethylphenylphosphine or other triphenylphosphine derivatives, preferably trimethylphenylphosphine or tetrakis(triphenylphosphine)-palladium(0). The reaction is carried out in the absence of solvent, in the temperature range from about 30°C to 100°C, preferably at about 80°C, for about 4-48 hours, preferably about 16 hours. The reaction product, a compound of Formula Ik, is isolated by conventional means and preferably purified using preparative TLC.

Conversion of the carboxylic acid of Formula Ik to its hydroxyamino equivalent is carried out in the same manner as shown above in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

Preparation of Compounds of Formula I where R^3 and R^4 together with the Carbon to which they are attached represent an *N*-Substituted Piperidine Derivative

The preparation of compounds of Formula I where R^1 and R^2 or R^3 and R^4 together with the carbon to which they are attached represent an *N*-substituted piperidine derivative are prepared from the corresponding unsubstituted piperidine derivative. This procedure is exemplified by reference to a compound of Formula I where R^3 and R^4 together with the carbon to which they are attached represent an *N*-substituted piperidine derivative, designated as compounds of Formula II, as shown below in Reaction Scheme XIII.

REACTION SCHEME XIII



Step 1 - Preparation of Compounds of Formula Im

Compounds of Formula I where Y is *t*-BuONH-, R^1 and R^2 are as defined in the compounds of formula I, and R^3 and R^4 together with the carbon to which they are attached represent an *N*-substituted piperidine derivative, are designated as compounds of Formula Im.

In general, compounds of Formula Im are prepared by reacting a compound of Formula II with a compound of the formula RX, where R is lower alkyl, cycloalkylalkyl, acyl, alkoxycarbonylalkyl, picolyl, $-SO_2R^a$, where R^a is lower alkyl or $-NR^bR^c$, where R^b and R^c are independently hydrogen or lower alkyl; and the like, and X is chloro, bromo or iodo; for example, RX may be methyl iodide, cyclopropylmethyl bromide, 3-picolyl chloride, ethyl bromoacetate, bromoacetamide, acetyl chloride, dimethylaminosulfonyl chloride, in the presence of a base, for example triethylamine or potassium carbonate. The reaction is carried out in a polar solvent, preferably *N,N*-dimethylformamide, in the temperature range from about 0°C to 50°C, preferably at about 25°C, for about 4 to 48 hours, preferably about 16 hours. The reaction product, a compound of Formula Im, is isolated by conventional means and preferably used with no further purification.

Alternatively, a reductive alkylation may be carried out on a compound of Formula II to give a compound of Formula Im. For example, reducing a compound of Formula II in acetone in the presence of a catalyst, for example palladium on carbon, under hydrogen gives an *N*-isopropyl derivative of Formula Im.

Step 2 - Preparation of Compounds of Formula In

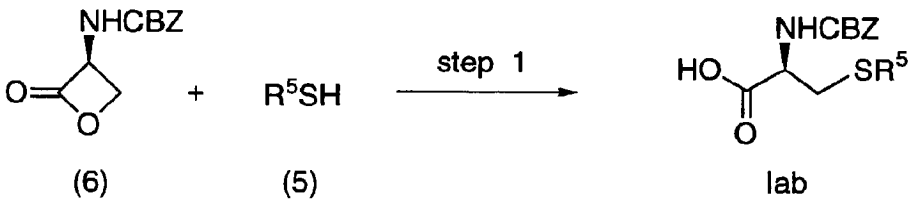
Compounds of Formula I where Y is HONH-, R^1 and R^2 are as defined in the compounds of formula I, and R^3 and R^4 together with the carbon to which they are attached represent an *N*-substituted piperidine derivative, are designated as compounds of Formula In.

In general, compounds of Formula In are prepared from a compound of Formula Im by reaction with a strong acid, preferably hydrochloric acid. The reaction is carried out in a sealed tube in an inert solvent, preferably 1,2-dichloroethane, in the temperature range from about 0°C to 45°C, preferably at about 20°C, for about 10 to 72 hours, preferably about 48 hours. The reaction product, a compound of Formula In, is isolated and purified by conventional means, preferably by chromatography.

Preparation of Compounds of Formula I where R^2 is $-NR^6R^7$

Compounds of Formula I where R^2 is $-NR^6R^7$, in which R^6 is hydrogen and R^7 is CBZ, where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, shown below, for example, as Formulae Ip and Iq, are prepared by a different route, as shown in Reaction Schemes XIV, XV, and XVI. This route provides compounds of Formula Iab, optically pure or as racemic mixtures, depending upon the chirality of the starting lactone.

REACTION SCHEME XIV



(6) (5) Iab

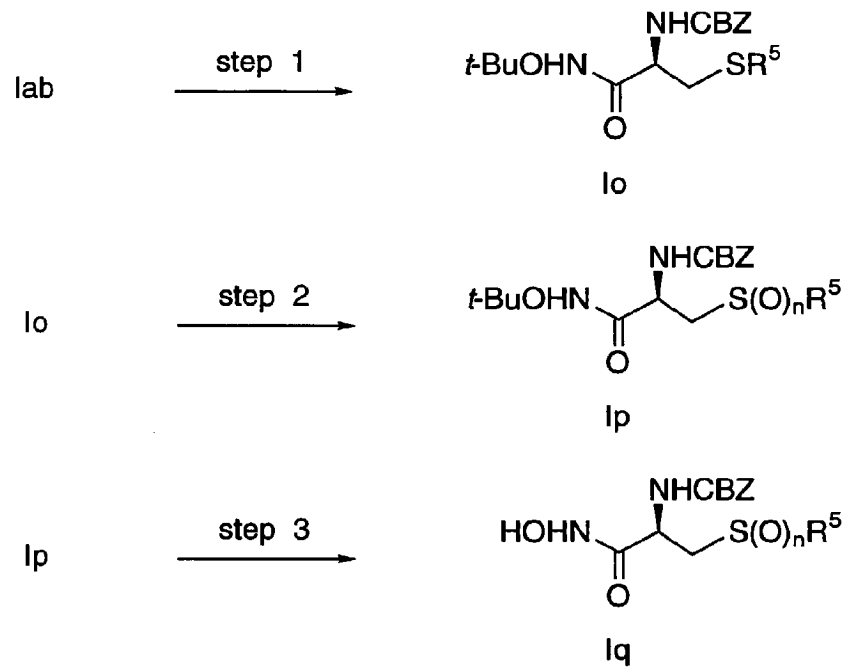
Step 1 - Preparation of Compounds of Formula Iab

In general, compounds of Formula Ia where Y is hydroxy, R^2 is $-NR^6R^7$, in which R^6 is hydrogen and R^7 is CBZ, where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula Iab, are prepared by treating an anion of a thiol of Formula (5) (preferably prepared *in situ*, for example, by treatment of Formula (5) with sodium or potassium hydride, preferably potassium hydride, in a polar solvent, preferably *N, N*-dimethylformamide) with a lactone of Formula (6). The reaction is carried out in a polar solvent, preferably *N, N*-dimethylformamide, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 5 minutes to 10 hours, preferably about 30 minutes to 6 hours. The sulfide reaction product, a compound of Formula Iab, is isolated by conventional means and preferably used directly in the next step.

Preparation of Compounds of Formula I where R^2 is $-NR^6R^7$

Compounds of Formula I where R^2 is $-NR^6R^7$, in which R^6 is hydrogen and R^7 is CBZ, where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, are prepared from compounds of Formula Iab as shown below in Reaction Scheme XV.

REACTION SCHEME XV



Step 1 - Preparation of Compounds of Formula Io

Compounds of Formula I where Y is *tert*-BuONH-, R^2 is -NHCBZ where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula Io, are prepared as shown in the same manner as shown in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

Step 2 - Preparation of Compounds of Formula Ip

Compounds of Formula Ip where n is 2, Y is *tert*-BuONH-, R^2 is -NHCBZ where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of the Formula Ip, are prepared in the same manner as shown in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

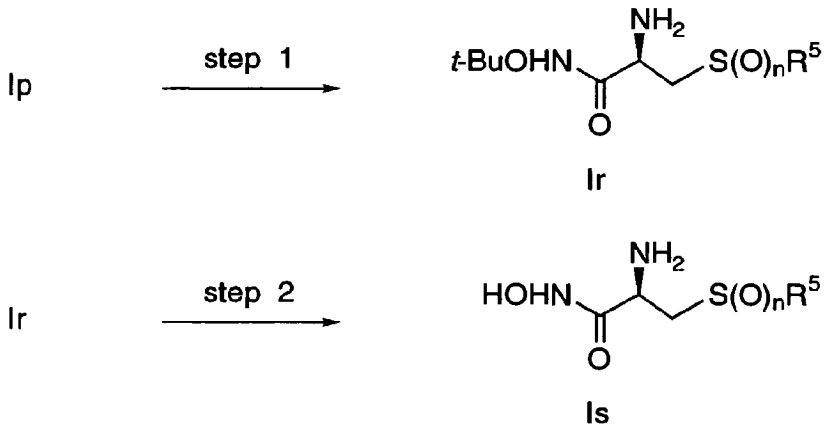
Step 3 - Preparation of Compounds of Formula Iq

Compounds of Formula I where n is 2, Y is HONH-, R^2 is -NHCBZ where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are as defined in the compounds of formula I, designated as compounds of the Formula Iq, are prepared by hydrolyzing a compound of Formula Ip in the same manner as shown above in Reaction Scheme VIII, or preferably as shown in Reaction Scheme IX or X.

Preparation of Compounds of Formula I where R^2 is -NR^6R^7

Compounds of Formula I where R^2 is -NR^6R^7, in which R^6 and R^7 are both hydrogen, and R^1, R^3 and R^4 are hydrogen, are prepared from compounds of Formula Ip as shown below in Reaction Scheme XVI.

REACTION SCHEME XVI



Step 1 - Preparation of Compounds of Formula Ir

In general, compounds of Formula I where n is 2, Y is *tert*-BuONH-, R^2 is -NH_2, and R^1 , R^3 and R^4 are hydrogen, designated as compounds of Formula Ir, are prepared by reducing a compound of Formula Ip using a metal catalyst, preferably palladium on carbon. The reaction is carried out under hydrogen at about 1 atmosphere, in a protic solvent, preferably ethanol, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 4 to 48 hours, preferably about 18 hours. The *N-tert*-butoxy reaction product, a compound of Formula Ir, is isolated and purified by conventional means.

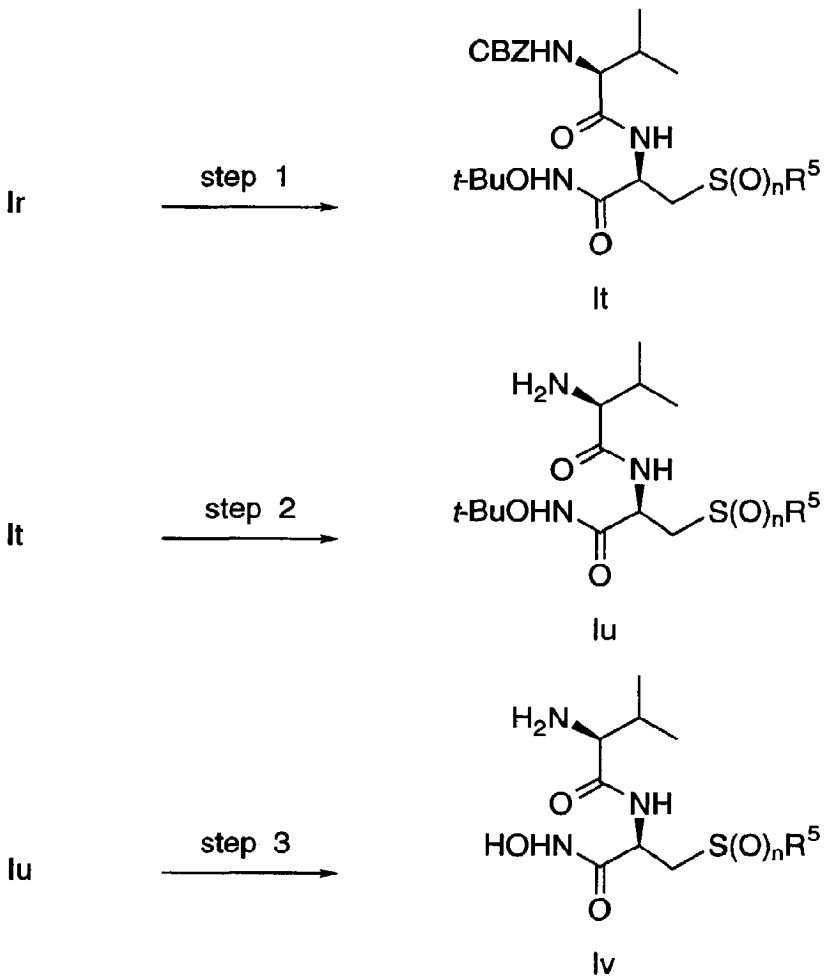
Step 2 - Preparation of Compounds of Formula Is

In general, compounds of Formula I where n is 2, Y is HONH-, R^2 is -NH_2, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula Is, are prepared by reacting a compound of Formula Ir with a strong acid, preferably hydrochloric acid. The reaction is carried out in a sealed tube in an inert solvent, preferably 1,2-dichloroethane, in the temperature range from about -10°C to 40°C, preferably at about 25°C, for about 4 to 48 hours, preferably about 18 hours. The hydroxyamino reaction product, a compound of Formula Is, is isolated and purified by conventional means, preferably as its hydrochloride salt.

Preparation of Compounds of Formula I where R^2 is -NR^6R^7

Alternatively, the compound of Formula Ir can be used to produce other compounds of Formula I where R^6 and/or R^7 are as defined in the Summary of the invention, but not both hydrogen. For example, the preparation of a compound of Formula I where R^2 is valine amide is shown below in Reaction Scheme XVII.

REACTION SCHEME XVII



Step 1 - Preparation of Compounds of Formula It

In general, compounds of Formula I where n is 2, Y is *tert*-BuONH-, R^2 is 2-(*S*)-CBZ-valine amide, *i.e.,* where R^6 is hydrogen and R^7 is 2-(*S*)-CBZ-3-methyl-1-butanoyl, where CBZ represents benzyloxycarbonyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula It, are prepared by reacting a compound of Formula Ir with CBZ-(*S*)-valine in the presence of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide and 1-hydroxybenzotriazole and a slight excess of a tertiary amine, preferably triethylamine. The reaction is carried out in an inert solvent, preferably methylene chloride, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 6-48 hours, preferably about 16 hours. The reaction product, a compound of Formula It, is isolated by conventional means, and is preferably used in the next step without further purification.

Step 2 - Preparation of Compounds of Formula Iu

In general, compounds of Formula I where n is 2, Y is *tert*-BuONH-, R^2 is 2-(*S*)-amino-valine amide, *i.e.,* where R^6 is hydrogen and R^7 is 2-(*S*)-amino-3-methyl-1-butanoyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula It, are prepared by reducing a compound of Formula It using a metal catalyst, preferably palladium on carbon. The reaction is carried out under hydrogen at about 1 atmosphere, in a protic solvent, preferably a mixture of methanol

and ethanol, in the temperature range from about 0°C to 40°C, preferably at about 25°C, for about 1 to 8 hours, preferably about 3 hours. The reaction product, a compound of Formula Iu, is isolated and purified by conventional means, preferably chromatography.

Step 3 - Preparation of Compounds of Formula Iv

In general, compounds of Formula I where n is 2, Y is HONH-, R^2 is 2-(*S*)-amino-valine amide, *i.e.*, where R^6 is hydrogen and R^7 is 2-(*S*)-amino-3-methyl-1-butanoyl, and R^1, R^3 and R^4 are hydrogen, designated as compounds of Formula Iv, are prepared by reacting a compound of Formula Iu with a strong acid, preferably hydrochloric acid. The reaction is carried out in a sealed tube in an inert solvent, preferably 1,2-dichloroethane, in the temperature range from about -20°C to 40°C, preferably at about 25°C, for about 4 to 48 hours, preferably about 24 hours. The hydroxyamine reaction product, a compound of Formula Iv, is isolated and purified by conventional means, preferably as its hydrochloride salt.

Preparation of Compounds of Formula I where R^2 is $-NR^6R^7$

In a manner similar to that shown above, compounds of Formula I where R^2 is $-NR^6R^7$, in which R^6 and R^7 are both methyl, are prepared by reacting a compound of Formula Ir in a polar solvent, preferably *N,N*-dimethylformamide, with about two equivalents of methyl iodide in the presence of a base, preferably potassium carbonate, then treating the product with hydrochloric acid gas as shown in Step 3 above.

Preparation of Compounds of Formula I where R^2 is $-NR^6R^7$

In a manner similar to that shown above, compounds of Formula I where where R^2 is $-NR^6R^7$, in which R^6 is hydrogen and R^7 is $-NHSO_2N(CH_3)_2$, are prepared by reacting a compound of Formula Ir with about one equivalent of dimethylsulfamoyl chloride in an inert solvent, preferably methylene chloride, in the presence of a base, preferably pyridine, then treating the product with hydrochloric acid gas as shown in Step 3 above.

Similarly, the compound of Formula Ir can be used to produce other compounds of Formula I where R^6 and/or R^7 are as defined in the Summary of the invention, but not both hydrogen, in the same manner as shown in Reaction Scheme XVII above.

Isolation and Purification of the Compounds

Isolation and purification of the compounds and intermediates described herein can be effected, if desired, by any suitable separation or purification procedure such as, for example, filtration, extraction, crystallization, column chromatography, thin-layer chromatography, thick-layer chromatography, preparative low or high-pressure liquid chromatography or a combination of these procedures. Specific illustrations of suitable separation and isolation procedures can be had by reference to the Examples hereinbelow. However, other equivalent separation or isolation procedures could, of course, also be used.

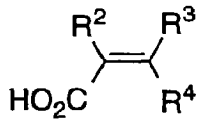
Salts of Compounds of Formula I

Some of the compounds of Formula I may be converted to a corresponding acid addition salt by virtue of the presence of basic nitrogen atoms. The conversion is accomplished by treatment with at least a stoichiometric amount of an appropriate acid, such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid and the like. Typically, the free base is dissolved in an inert organic solvent such as diethyl ether, ethyl acetate, chloroform, ethanol or methanol and the like, and the acid added in a similar solvent. The temperature is maintained at 0° to 50°C. The resulting salt precipitates spontaneously or may be brought out of solution with a less polar solvent.

In summary, the compounds of the present invention are made by the procedures outlined below:

1. A process for preparing compounds of Formula I where R^1 is hydrogen comprises:

reacting a compound of the formula:



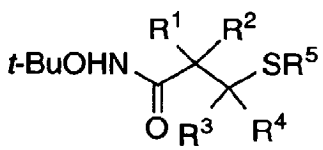
(4)

where R^2, R^3 and R^4 are as defined in the compounds of formula I, except that R^2 cannot be $-NR^6R^7$;

with a compound of the formula R^5SH, where R^5 is as defined in the compounds of formula I, in the presence of a secondary base.

2. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

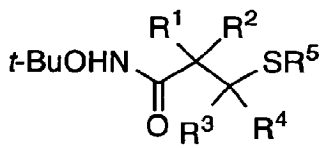


where R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

with a mild oxidizing agent, for example, sodium periodate.

3. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

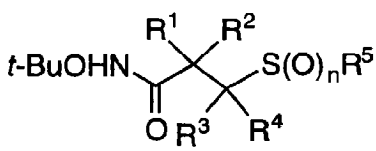


where R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

with a strong oxidizing agent, for example, OXONE or m-chloroperbenzoic acid.

4. Alternatively, a process for preparing compounds of Formula I where n is 2 comprises:

reacting a compound of the formula:

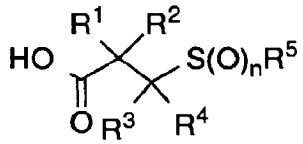


where R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

with a strong oxidizing agent, for example, OXONE or m-chloroperbenzoic acid.

5. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

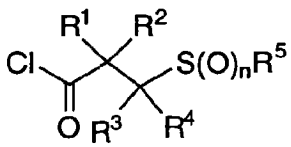


where n, R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

with *O*-(*tert*-butyl)hydroxylamine hydrochloride in the presence of a carbodiimide, for example, *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and a tertiary amine.

6. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

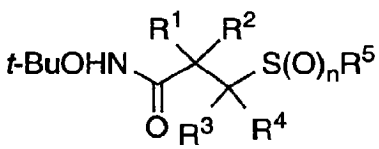


where n, R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

with hydroxylamine or *N,O*-bistrimethylsilyl hydroxylamine.

7. Alternatively, a process for preparing compounds of Formula I comprises:

hydrolysing a compound of the formula:

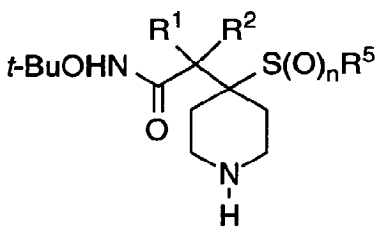


where n, R^1, R^2, R^3, R^4 and R^5 are as defined in the compounds of formula I,

under acid conditions, for example, with hydrochloric acid or trifluoroacetic acid.

8. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

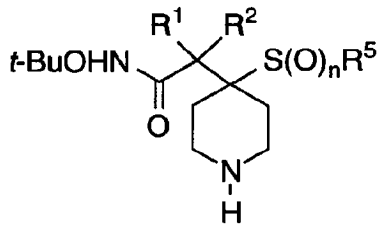


where n, R^1, R^2 and R^5 are as defined in the compounds of formula I, except that R^2 cannot be $-NR^6R^7$;

with a compound of the formula RX, where R is lower alkyl, cycloalkylalkyl, acyl, alkoxycarbonylalkyl, acetamido, picolyl, $-SO_2R^a$, where R^a is lower alkyl or NR^bR^c, where R^b and R^c are independently hydrogen or lower alkyl; and X is chloro, bromo or iodo.

9. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

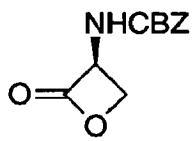


where n, R^1, R^2 and R^5 are as defined in the compounds of formula I, except that R^2 cannot be $-NR^6R^7$;

with acetone under hydrogen in the presence of a catalyst, for example, palladium on carbon, to give the *N*-isopropyl derivative.

10. Alternatively, a process for preparing compounds of Formula I comprises:

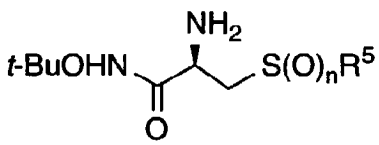
reacting a compound of the formula:



with an anion of a compound of the formula R^5 SH, where R^5 is as defined in the compounds of formula I.

11. Alternatively, a process for preparing compounds of Formula I comprises:

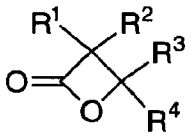
reacting a compound of the formula:



where R^5 is as defined in the compounds of formula I, with an acylating agent, for example CBZ-(*S*)-valine in the presence of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide and 1-hydroxybenzotriazole and a tertiary amine, or an alkylating agent, for example, methyl iodide in the presence of a base or a sulfamoyl halide, such as dimethylsulfamoyl chloride in the presence of a base.

12. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:

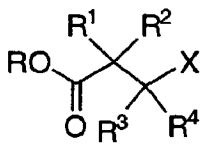


where R^1, R^2, R^3 and R^4 are as defined in the compounds of formula I, except that R^2 cannot be $-NR^6R^7$;

with a compound of the formula R^5 SH, where R^5 is as defined in the compounds of formula I, in the presence of a secondary base.

13. Alternatively, a process for preparing compounds of Formula I comprises:

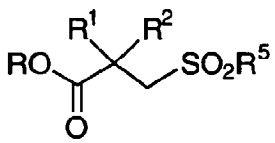
reacting a compound of the formula:



with an anion of a compound of the formula R^5SH, where R^5 is as defined in the compounds of formula I.

14. Alternatively, a process for preparing compounds of Formula I comprises:

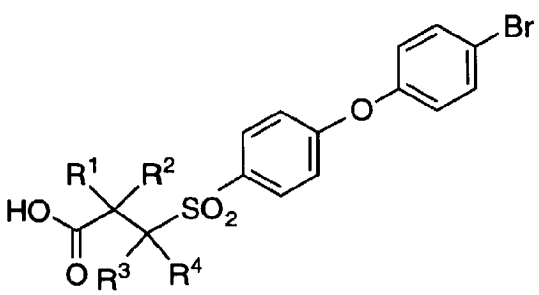
reacting a compound of the formula:



with an alkyl or aralkyl halide in the presence of a hindered base.

15. Alternatively, a process for preparing compounds of Formula I comprises:

reacting a compound of the formula:



with a compound of the formula $R^{11}B(OH)_2$ or $R^{11}SnMe_3$, where R^{11} is aryl or heteroaryl, in the presence of tetrakis(triphenylphosphine)-palladium(0).

The compounds of Formula I inhibit mammalian matrix metalloproteases, such as the stromelysins, gelatinases, matrilysin and collagenases, and are therefore useful as therapeutically active substances, especially for treating diseases associated with the MMP-induced excessive degradation of matrix and connective tissue within the mammal, for example, arthritic diseases (rheumatoid arthritis and osteoarthritis), multiple sclerosis, bone resorptive diseases (such as osteoporosis), the enhanced collagen destruction associated with diabetes, chronic obstructive pulmonary disease, cerebral hemorrhaging associated with stroke, periodontal disease, corneal ulceration, ulceration of the skin, tumor invasion and metastasis, and aberrant angiogenesis.

The compounds of Formula I substantially inhibit the release of tumor necrosis factor (TNF) from cells, and are therefore useful for the treatment of conditions mediated by TNF, for example inflammation, fever, cardiovascular effects, hemorrhage, coagulation and acute phase response, cachexia and anorexia, acute infections, shock states, restinosis, aneurysmal disease, graft versus host reactions and autoimmune disease.

The compounds of Formula I also inhibit the release of other biologically active molecules from cells, including soluble receptors (CD30 and receptors for TNF (p55 and p75), IL-6, IL-1 and TSH), adhesion molecules (*e.g.,* L-selection, ICAM-1, fibronectin) and other growth factors and cytokines, including Fas ligand, TGF-α, EGF, HB-EGF, SCF and M-CSF. Inhibition of the release or shedding of such proteins, and are therefore useful for treating a number of disease states, for example rheumatoid arthritis, multiple sclerosis, vascular disease, Type II diabetes, HIV, cachexia, psoriasis, allergy, hepatitis, inflammatory bowel disease, and cancer.

The ability of the compounds of Formula I to inhibit matrix metalloprotease activity, such as the activity of collagenase-1, -2 and -3, stromelysin-1, gelatinases A and B, and matrilysin may be demonstrated by a variety of *in vitro* assays known to those of ordinary skill in the art, such as the assay described in the MMP Enzymatic Assay described in *FEBS,* **296**, 263 (1992) or modifications thereof. The ability of the compounds of Formula I to inhibit MMP mediated processes *in vivo* may be tested using the interleukin-1 stimulated cartilage explant assay and cartilage plug implantation assay.

The ability of the compounds of Formula I to inhibit the release of TNF as shown in Examples 45 to 47.

The present invention also relates to a pharmaceutical composition comprising a pharmaceutically acceptable non-toxic excipient and a therapeutically effective amount of a compound of formula I.

Administration of the compounds of Formula I or their pharmaceutically acceptable salts, in pure form or in an appropriate pharmaceutical composition, can be carried out via any of the accepted modes of administration or agents for serving similar utilities. Thus, administration can be, for example, orally, nasally, parenterally, topically, transdermally, or rectally, in the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as for example, tablets, suppositories, pills, soft elastic and hard gelatin capsules, powders, solutions, suspensions, or aerosols, or the like, preferably in unit dosage forms suitable for simple administration of precise dosages. The compositions will include a conventional pharmaceutical carrier or excipient and a compound of Formula I as the/an active agent, and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, *etc.*

Generally, depending on the intended mode of administration, the pharmaceutically acceptable compositions will contain about 1% to about 99% by weight of a compound(s) of Formula I, or a pharmaceutically acceptable salt thereof, and 99% to 1% by weight of a suitable pharmaceutical excipient. Preferably, the composition will be about 5% to 75% by weight of a compound(s) of Formula I, or a pharmaceutically acceptable salt thereof, with the rest being suitable pharmaceutical excipients.

The preferred route of administration is oral, using a convenient daily dosage regimen which can be adjusted according to the degree of severity of the disease-state to be treated. For such oral administration, a pharmaceutically acceptable composition containing a compound(s) of Formula I, or a pharmaceutically acceptable salt thereof, is formed by the incorporation of any of the normally employed excipients, such as for example, pharmaceutical grades of mannitol, lactose, starch, pregelatinized starch, magnesium stearate, sodium saccharine, talcum, cellulose ether derivatives, glucose, gelatin, sucrose, citrate, propyl gallate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations, and the like.

Preferably such compositions will take the form of capsule, caplet or tablet and therefore will also contain a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant, such as croscarmellose sodium or derivatives thereof; a lubricant such as magnesium stearate and the like; and a binder such as a starch, gum acacia, polyvinylpyrrolidone, gelatin, cellulose ether derivatives, and the like.

The compounds of Formula I, or their pharmaceutically acceptable salts, may also be formulated into a suppository using, for example, about 0.5% to about 50% active ingredient disposed in a carrier that slowly dissolves within the body, *e.g.,* polyoxyethylene glycols and polyethylene glycols (PEG), *e.g.,* PEG 1000 (96%) and PEG 4000 (4%).

Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, *etc.,* a compound(s) of Formula I (about 0.5% to about 20%), or a pharmaceutically acceptable salt thereof, and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol and the like, to thereby form a solution or suspension.

If desired, a pharmaceutical composition of the invention may also contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, antioxidants, and the like, such as, for example, citric acid,

sorbitan monolaurate, triethanolamine oleate, butylated hydroxytoluene, *etc.*

Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences,* 18th Edition, Mack Publishing Company, Easton, Pennsylvania (1990). The composition to be administered will, in any event, contain a therapeutically effective amount of a compound of Formula I or a pharmaceutically acceptable salt thereof, for treatment of a disease-state alleviated by the inhibition of matrix metalloprotease activity in accordance with the teachings of this invention.

The compounds of Formula I or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount which will vary depending upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of the compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular disease-state, and the host undergoing therapy. Generally, a therapeutically effective daily dose is from about 0.014 mg to about 14.3 mg/kg of body weight per day of a compound of Formula I or a pharmaceutically acceptable salt thereof; preferably, from about 0.07 mg to about 5 mg/kg of body weight per day; and most preferably, from about 0.14 mg to about 1.4 mg/kg of body weight per day. For example, for administration to a 70 kg person, the dosage range would be from about 1 mg to about 1.0 gram per day of a compound of Formula I or a pharmaceutically acceptable salt thereof, preferably from about 5 mg to about 300 mg per day, and most preferably from about 10 mg to about 100 mg per day.

EXAMPLES

The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

EXAMPLE 1

Preparation of Compounds of Formula (1)

1A. Preparation of (1) where R^3 and R^4 when taken together with the Carbon to which they are attached represent *N*-CBZ-piperidine

1. A solution of benzyl chloroformate (35 ml, 247 mmol) in tetrahydrofuran (70 ml) was added to an ice-cold solution of 4-hydroxypiperidine (25 g, 247 mmol) and triethylamine (45 ml, 321 mmol) in tetrahydrofuran (350 ml). The mixture was stirred overnight at room temperature and the solvent removed under reduced pressure. The residue was partitioned between 5% hydrochloric acid and ethyl acetate, and the organic layer washed with brine, dried over magnesium sulfate, and the solvent removed under reduced pressure to give 4-hydroxy-*N*-CBZ-piperidine as a pale yellow oil.

2. Celite (66 g) was added to a solution of 4-hydroxy-*N*-CBZ-piperidine (18 g, 76.5 mmol) in methylene chloride (500 ml), followed by pyridinium chlorochromate (33 g, 153 mmol). The mixture was stirred overnight, and then isopropyl alcohol (12 ml) was added over a period of 3 hours. The reaction mixture was filtered through silica gel and the filter cake was repeatedly rinsed with methylene chloride and ethyl acetate. The combined filtrates were evaporated under reduced pressure. Silica gel chromatography using 50% ethyl acetate/hexane, gave 4-oxo-*N*-CBZ-piperidine as a yellow oil.

EXAMPLE 2

Preparation of Compounds of Formula (3)

2A. Preparation of (3) where R^2 is Hydrogen, and R^3 and R^4 when taken together with the Carbon to which they are attached represent *N*-CBZ-piperidine

tert-(Butoxycarbonylmethylene)triphenylphosphorane (28 g, 74.4 mmol) was added to 4-oxo-*N*-CBZ-piperidine (14.2 g, 61.3 mmol) in benzene (150 ml), and the solution was stirred at reflux overnight. The solution was concentrated, and the residue triturated with hexane (500 ml). Filtration and concentration of the filtrate gave 4-*tert*-butoxycarbonyl-methylene-*N*-CBZ-piperidine as a colorless oil.

2B. Preparation of (3), varying R^2 , R^3 , and R^4

Similarly, following the procedures of Example 2A above, but replacing 4-oxo-*N*-CBZ-piperidine with:

formaldehyde;
acetone;
propionaldehyde;
cyclopentanone;
cyclohexanone;
1,4-cyclohexanedione mono-ethylene ketal;
4-methylcyclohexanone;
phenylacetaldehyde;
4-(biphen-4-yl)butyraldehyde;
cyclopentylacetaldehyde;
tetrahydropyranone; and
tetrahydrothiopyran;

and optionally replacing *tert*-(butoxycarbonylmethylene)triphenylphosphorane with:

tert-butyl-3-phenylpropionate-2-triphenylphosphorane;
tert-butyl-propionate-2-triphenylphosphorane; and
tert-butyl-3-methylpropionate-2-triphenylphosphorane;

the following compounds of Formula (3) were prepared:

1-(*tert*-butoxycarbonyl)-1-benzylethene;
1-(*tert*-butoxycarbonyl)-2,2-dimethylethene;
1-(*tert*-butoxycarbonyl)-1-methyl-2-ethylethene;
tert-butoxycarbonylmethylenecyclopentane;
tert-butoxycarbonylmethylenecyclohexane;
tert-butoxycarbonylmethylene-4-methylcyclohexane;
1-(*tert*-butoxycarbonyl)-2-benzylethene;
1-(*tert*-butoxycarbonyl)-1-isopropyl-2-benzylethene;
1-(*tert*-butoxycarbonyl)-2-[3-(biphen-4-yl)]propylethene;
1-(*tert*-butoxycarbonyl)-2-cyclopentylmethylethene;
4-(*tert*-butoxycarbonylmethylene)-tetrahydropyran; and
4-(*tert*-butoxycarbonylmethylene)-tetrahydrothiopyran.

2C. Preparation of (3), varying R^2, R^3, and R^4

Similarly, following the procedures of Example 2A above, but optionally replacing 4-oxo-*N*-CBZ-piperidine with other compounds of Formula (1), and optionally replacing (*tert*-butoxycarbonylmethylene)triphenyl-phosphorane with other compounds of Formula (2), other compounds of Formula (3) are prepared.

EXAMPLE 3

Preparation of Compounds of Formula (4)

3A. Preparation of (4) where R^2 is Hydrogen, and R^3 and R^4 when taken together with the Carbon to which they are attached represent *N*-CBZ-piperidine, a Compound of Formula (4a)

Trifluoroacetic acid (10 ml) was added to 4-*tert*-butoxycarbonylmethylene-*N*-CBZ-piperidine (20 g, 60.3 mmol) in methylene chloride (30 ml) and the solution was stirred at room temperature for 1.5 hours. After evaporation of the solvent, the residue was triturated with diethyl ether to give 4-carboxymethylene-*N*-CBZ-piperidine as a crystalline white solid.

3B. Preparation of (4) where R^2 is Hydrogen, and R^3 and R^4 when taken together with the Carbon to which they are attached represent Tetrahydropyran, a Compound of Formula (4b)

Methanol (204 ml) was slowly added to a suspension of sodium hydride (5.48 g, 228.2 mmol) in tetrahydrofuran (204 ml) at 0°C. When addition was complete, trimethylphosphonoacetate (34.22 ml, 211.4 mmol) was added to the mixture at such a rate as to maintain the temperature below 12°C. Stirring was continued for a further 10 minutes. To this reaction mixture was added a solution of 2,3,5,6-tetrahydropyran-4-one (16.28 g, 163.0 mmol) in tetrahydrofuran

(20 ml), keeping the temperature below 30°C. After the addition was complete, stirring was continued for 30 minutes at room temperature, then methanol (100 ml) and 2M sodium hydroxide (326 ml) was added, and the mixture stirred overnight at room temperature. The resulting solution was concentrated to one half of the original volume, and acidified to pH 1.2 with 6M hydrochloric acid (108 ml). The reaction mixture was partitioned between ethyl acetate and water, the combined organic extracts dried over magnesium sulfate, and solvent removed under reduced pressure to give 4-(carboxymethylene)-2,3,5,6-tetrahydropyran (22.62 g), which was used with no further purification.

3C. Preparation of (4), varying R^2, R^3, and R^4

Similarly, following the procedures of Example 3A above, but replacing 4-(*tert*-butoxycarbonylmethylene)-*N*-CBZ-piperidine with other compounds of Formula (3), the following compounds of Formula (4) were prepared:

1-benzyl-1-carboxyethene;
1-carboxy-2,2-dimethylethene;
1-carboxy-2-ethyl-1-methylethene;
carboxymethylenecyclopentane;
carboxymethylenecyclohexane;
carboxymethylene-(4-methylcyclohexane);
4-carboxymethylenecyclohexanone mono-ethylene ketal;
2-benzyl-1-carboxyethene;
2-[3-(biphen-4-yl)propyl]-1-carboxyethene;
2-benzyl-1-carboxy-1-isopropylethene;
1-carboxy-2-cyclopentylmethylethene;
4-carboxymethylene-tetrahydrothiopyran; and
4-carboxymethylene-(tetrahydrothiopyran-1,1-dioxide).

3D. Preparation of (4), varying R^2, R^3, and R^4

Similarly, following the procedures of Example 3A above, but replacing 4-(*tert*-butoxycarbonylmethylene)-*N*-CBZ-piperidine with other compounds of Formula (3), other compounds of Formula (4) are prepared, or may be prepared by means well known to those skilled in the art. Alternatively, they are commercially available, for example, 1-cyclopentene carboxylic acid and 1-cyclohexene carboxylic acid are available from Lancaster Synthesis Inc.

EXAMPLE 4

Preparation of Compounds of Formula (5)

4A. Preparation of (5) where R^5 is 4-Phenoxyphenyl

A solution of sodium thiomethoxide (25 g) and 4-bromodiphenyl ether (25 g) in *N,N*-dimethylformamide (DMF) (150 ml) was refluxed overnight. The mixture was cooled and added to dilute aqueous sodium hydroxide. The water layer was washed with ether to remove by-products and acidified with hydrochloric acid. The product, 4-(phenoxy)thiophenol, was extracted with ether, and the ether layer dried and evaporated to give 4-(phenoxy)thio-phenol (19-20 g) as a red oil. This material can be used without further purification.

4B. Alternative Preparation of (5) where R^5 is 4-(4-Bromophenoxy)phenyl

A solution of 4-bromodiphenyl ether (50 g, 200.7 mmol) in methylene chloride (118 ml) was cooled to 0°C and chlorosulfonic acid (14.7 ml, 220.8 mmol) was added dropwise over a 20 minute period. The solution was stirred an additional 10 minutes, warmed to room temperature and stirred an additional 1 hour. To this mixture was added oxalyl chloride (23.6 ml, 270.9 mmol), followed by *N,N*-dimethylformamide (1.5 ml) as a catalyst, and the mixture refluxed for 2 hours. The mixture was cooled to room temperature, and additional oxalyl chloride (23.6 ml, 270.9 mmol) was added, the mixture refluxed for 3 hours, cooled to room temperature and stirred 12 hours more. The solution was concentrated to an oil, azeotroped several times using methylene chloride and put under high vacuum (1 torr) for several hours until the mixture had completely solidified. This mixture was immediately dissolved in methylene chloride (160 ml) which was added dropwise to a solution of triphenylphosphine (157.0 g, 602 mmol) in methylene chloride (160 ml) containing *N,N*-dimethylformamide (4 ml, 52.2 mmol). The mixture was stirred 2 hours, diluted with 1M aqueous hydrochloric acid (300 ml) and stirred for 1 hour. The aqueous layer was separated, extracted with methylene chloride (200 ml), and the organic layers were combined, washed with 200 ml of brine, dried ($MgSO_4$) and concentrated *in vacuo*. The resulting

solid was further purified through trituration with 750 ml of hexane. The solid was then dissolved in 750 ml of diethyl ether, extracted with 2M aqueous sodium hydroxide (2 x 350 ml), and the basic aqueous layer back extracted using diethyl ether (2 x 400 ml). The aqueous layer was adjusted to pH 2, extracted with diethyl ether (3 x 200 ml) and the combined organic layers dried ($MgSO_4$) and concentrated to afford 4-(4-bromophenoxy)thiophenol (45.6 g, 81%). ^{1}HNMR ($CDCl_3$) δ 3.43 (s, 1H), 6.86 (d, J = 8.9 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 7.28 (d, J = 8.6 Hz, 2H), 7.43 (d, J = 8.9 Hz, 2H).

The corresponding 4-chloro and 4-fluoro analogues were obtained in similar fashion from the corresponding commercially available 4-halodiphenylethers, respectively.

4-(4-chlorophenoxy)thiophenol: ^{1}HNMR ($CDCl_3$) δ 3.43 (s, 1H), 6.90 (m_c, 4H), 7.27 (m_c, 4H).
4-(4-fluorophenoxy)thiophenol: ^{1}HNMR ($CDCl_3$) δ 3.41 (s, 1H), 6.85 (d, J = 8.7 Hz, 2H), 7.00 (m_c, 4H), 7.26 (d, J = 8.7 Hz, 2H).
4-(4-pyridyloxy)thiophenol: ^{1}HNMR ($CDCl_3$) δ 7.05 (d, J =9.0 Hz, 2H), 7.29 (d, J = 7.3 Hz, 2H), 7.44 (d, J = 8.8 Hz, 2H), 8.70 (d, J = 7.3 Hz, 2H); EIMS (M^+): 203.
4-(5-chloro-2-pyridyloxy)thiophenol: ^{1}HNMR ($CDCl_3$) δ 6.87 (d, J =8.5 Hz, 1H), 7.01 (d, J = 8.7 Hz, 2H), 7.32 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.6 Hz, 1H), 8.15 (d, J = 2.8 Hz, 1H).

EXAMPLE 5

Preparation of Compounds of Formula (10)

5A. Preparation of a Compound of Formula (8) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, a Compound of Formula (8a)

A solution of 1.5M diisobutylaluminum hydride (DIBAL-H) (419 ml, 629 mmol) in toluene was added to a 3-L Morton flask equipped with a nitrogen gas inlet, mechanical stirrer, low temperature thermometer, 500 ml pressure equalizing funnel, and containing tetrahydropyran-4,4-dicarboxylic acid diethyl ester (70.78 g, 307.4 mmol) in toluene (600 ml) at -40°C, at a rate to maintain an internal temperature no higher than -25°C. The mixture was stirred an additional 10 minutes and anhydrous ethanol (595 ml) was added dropwise over 20 minutes maintaining an internal temperature no higher than -15°C. Solid sodium borohydride (11.6 g, 307.4 mmol) was added in three portions over a 15 minute period, the cooling bath was removed, the mixture allowed to warm to room temperature over 1 hour, and saturated aqueous sodium sulfate (325 ml) added over 15 minutes. The mixture was cooled to -15°C, ethyl acetate (250 ml) was added, and the flocculent white precipitate filtered over a pad of celite. The celite pad was washed with ethyl acetate (7 x 450 ml), the filtrate washed with brine (200 ml), dried over magnesium sulfate, and concentrated *in vacuo*. The residue was dissolved in the minimum amount of ethyl acetate, filtered through a sintered glass funnel containing silica gel (40 g), eluting with ethyl acetate, and the filtrate concentrated *in vacuo* to afford the hydroxyester, 4-(hydroxymethyl)tetrahydropyran-4-carboxylic acid ethyl ester, as a pale yellow oil (48.5 g, 84%).

5B. Alternative Preparation of a Compound of Formula (8) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran

1. To a solution of tetrahydropyran-4,4-dicarboxylic acid diethyl ester (400 mg, 1.74 mmol) in *N,N*-dimethylformamide (4 ml), was added lithium iodide (1.16 g, 8.66 mmol), followed by sodium cyanide (94 mg, 1.91 mmol). The mixture was heated at 130°C for 7 hours, 140°C for 25 hours, after which GC analysis indicated the reaction to be >95% complete. The mixture was partitioned between 33% diethyl ether/hexanes (100 ml) and brine (25 ml). The organic layer was washed with additional brine (25 ml), dried ($MgSO_4$) and concentrated *in vacuo* to afford the tetrahydropyran-4-carboxylic acid ethyl ester (253 mg, 92%). Note: Substitution of 2 equivalents of sodium acetate for 1.1 equivalents of sodium cyanide in this reaction and heating 12 hours longer provides identical results.

2. Lithium diisopropylamide was prepared by the addition of 2.5M *N*-butyl lithium (30.3 ml, 75.6 mmol) in hexanes to a solution of diisopropylamine (10.6 ml, 75.6 mmmol) in tetrahydrofuran (244 ml) at 0°C and stirring for 20 minutes. Then a solution of tetrahydropyran-4-carboxylic acid ethyl ester (10 g, 63.2 mmol) in tetrahydrofuran (50 ml) was added to the solution of lithium diisopropylamide over 15 minutes at -78°C. The resulting solution was stirred an additional 50 minutes, and solid paraformaldehyde (10 g) was added in one portion. The mixture was slowly allowed to warm to room temperature over 9 hours, diluted with 2M aqueous hydrochloric acid (100 ml), and filtered over a pad of celite pad which was washed with diethyl ether (2 x 200 ml). The aqueous layer of the filtrate was washed with additional portions of diethyl ether (2 x 200 ml). The combined organic layers were washed once with 2M aqueous hydrochloric acid (100 ml), saturated aqueous sodium bicarbonate (100 ml), dried over magnesium sulfate, and concentrated *in vacuo* to afford a slightly impure product 4-(hydroxymethyl)tetrahydropyran-4-carbox-

ylic acid ethyl ester (11.5 g, 97%), which was taken into the next reaction without further purification. IR (neat) 3433 (br), 1726 cm-^{1}; ^{1}HNMR ($CDCl_3$) δ 1.30 (t, *J* = 7.1 Hz, 3H), 1.57 (ddd, *J* = 13.8, 10.1, 4.4 Hz, 2H), 2.07 (dm, *J* = 13.8 Hz, 2H), 2.30-2.45 (br s, 1H), 3.56 (ddd, *J* = 11.9, 10.3, 2.7 Hz, 2H), 3.66 (s, 2H), 3.82 (dt, *J* = 11.9, 4.2 Hz, 2H), 4.24 (q, *J* = 7.2 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 14.25 (q), 30.54 (t), 46.63 (s), 61.04 (t), 64.79 (t), 69.02 (t), 175.24 (s); HRMS Calcd for $C_9H_{16}O_4$: 188.1049. Found: 188.1053.

5C. Preparation of a Compound of Formula (8) where R^1 and R^2 taken together with the Carbon to which they are attached represent Piperidine. a Compound of Formula (8)

Lithium diisopropylamide was prepared by the addition of 1.6M *N*-butyl lithium (29.1 ml, 46.6 mmol) in hexanes to a solution diisopropylamine (6.5 ml, 46.6 mmmol) in tetrahydrofuran (150 ml) at 0°C with stirring for 20 minutes at -78°C. Then a solution of neat *N*-(*tert*-butoxycarbonyl)-piperidine-4-carboxylic acid ethyl ester (10 g, 38.9 mmol) was added over 5 minutes, and the resulting solution was stirred an additional 50 minutes. Solid paraformaldehyde (13.5 g, 155.4 mmol) was added in one portion, and the mixture slowly allowed to warm to room temperature over 9 hours. The mixture was diluted with 2M aqueous hydrochloric acid (100 ml), filtered over a pad of celite, washed with diethyl ether (2 x 200 ml). The combined organic layers were washed once with 2M aqueous hydrochloric acid (100 ml), saturated aqueous sodium bicarbonate (100 ml), dried over magnesium sulfate, and concentrated *in vacuo*. Chromatography on silica gel, and eluting with 50% ethyl acetate/hexanes, yielded slightly impure *N*-(*tert*-butoxycarbonyl)-4-(hydroxymethyl)piperidine-4-carboxylic acid ethyl ester (10.57 g, 95%) as a pale yellow oil which was taken immediately into the hydrolysis reaction (LiOH): ^{1}H NMR ($CDCl_3$) δ 1.26 (t, *J* = 7.4 Hz, 3H), 1.40-1.53 (m, 2H), 1.46 (s, 9H), 2.00-2.12 (m, 2H), 3.05-3.16 (m, 2H), 3.65 (s, 2H), 3.70-3.83 (m, 2H), 4.23 (q, *J* = 7.2 Hz, 2H).

5D. Preparation of a Compound of Formula (9) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, a Compound of Formula (9a)

Lithium hydroxide monohydrate (16.7 g, 398.5 mmol) was added to a solution of 4-(hydroxymethyl)tetrahydropyran-4-carboxylic acid ethyl ester (25.0 g, 132.8 mmol) in 4.5:1 methanol/water (220 ml). The mixture was heated to reflux for 40 minutes and the methanol removed *in vacuo* by concentration using a bath temperature no higher than 45°C. The aqueous layer was then extracted into diethyl ether (4 x 100 ml) and the combined ether layers washed twice with 2M sodium hydroxide (15 ml). The combined aqueous base layers were cooled to 0°C, acidified to pH 3.0 with 8M aqueous hydrochloric acid, saturated with solid sodium chloride and extracted with ethyl acetate (8 x 250 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo*. The white fluffy powder residue was recrystallized from the minimum amount of methylene chloride/hexanes to afford pure 4-(hydroxymethyl)tetrahydropyran-4-carboxylic acid (17.05 g, 80%).

5E. Alternative Preparation of a Compound of Formula (9) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran

Lithium diisopropylamide was prepared by the addition of 2.45M *N*-butyl lithium (16.5 ml) in hexanes to a solution diisopropylamine (5.80 ml, 41.4 mmmol) in tetrahydrofuran (40 ml) at 0°C with stirring for 20 minutes. Then a solution of tetrahydropyran-4-carboxylic acid (2.5 g, 19.2 mmol) in tetrahydrofuran (10 ml) was added to the solution of lithium diisopropylamide over 15 minutes to form a slurry, followed by hexamethylphosphoramide (2 ml). The resulting solution was stirred for 25 minutes, then immediately warmed to room temperature after a stream of gaseous formaldehyde (prepared by heating 4 g of paraformaldehyde at 175-200°C over 5-10 minutes) was passed through the solution. The slurry was carefully concentrated at ambient temperature, acidified to pH 3 with 8M hydrochloric acid, saturated with solid sodium chloride, and extracted with ethyl acetate (8 x 100 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel (80 g), and eluting with 10% methanol/methylene chloride, yielded 4-(hydroxymethyl)tetrahydropyran-4-carboxylic acid as a white solid (1.80 g, 58%). mp 113.7-115°C; IR (KBr) 3420 (br), 1724 cm-^{1}, ^{1}HNMR (DMSO-d_6) δ 1.43 (ddd, *J* = 13.5, 11.0,4.4 Hz, 2H), 1.85 (dm, *J* = 13.4 Hz, 2H), 3.37 (td, *J* = 11.3, 3.0 Hz, 2H), 3.43 (s, 2H), 3.71 (dt, *J* = 11.6, 3.9 Hz, 2H), 4.81 (br, s, 1H); 12.24 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 30.42 (t), 46.38 (s), 64.35 (t), 68.15 (t), 69.02 (t), 176.08 (s); HRMS Calcd. for $C_7H_{12}O_4$: 160.0735. Found: 160.0731. Anal. Calcd. for $C_7H_{12}O_3$: C, 52.49; H, 7.55. Found: C, 52.50; H, 7.62.

5F. Preparation of a Compound of Formula (9) where R^1 and R^2 taken together with the Carbon to which they are attached represent Piperidine. a Compound of Formula (9b)

Lithium hydroxide monohydrate (6.95 g, 165.6 mmol) was added to solution of *N*-(*tert*-butoxycarbonyl)-4-(hydroxymethyl)piperidine-4-carboxylic acid ethyl ester (9.52 g, 33.1 mmol) in 2:1 methanol/water (100 ml). The mixture was heated to reflux for 30 minutes, the methanol removed *in vacuo* by concentration using a bath temperature no

higher than 45°C. The aqueous layer was cooled to 0°C, acidified to pH 3.0 using 6M aqueous hydrochloric acid, and extracted with ethyl acetate (4 x 75 ml). The combined organic layers were dried over magnesium sultate, and concentrated *in vacuo*, and recrystallized from dichloromethane/hexanes to afford *N*-(*tert*-butoxycarbonyl)-4-(hydroxymethyl)piperidine-4-carboxylic acid (8.59 g, 100%).

5G. Alternative Preparation of a Compound of Formula (9) where R^1 and R^2 taken together with the Carbon to which they are attached represent Piperidine

Lithium diisopropylamide was prepared by the addition of 2.45M *N*-butyllithium (69 ml, 168.8 mmol) in hexanes to a solution diisopropylamine (24 ml, 171.2 mmmol) in tetrahydrofuran (40 ml) at 0°C with stirring for 20 minutes. Then a solution of *N*-(*tert*-butoxycarbonyl)-piperidine-4-carboxylic acid (18 g, 78.5 mmol) in tetrahydrofuran (35 ml) was added to the solution of lithium diisopropylamide over 15 minutes to form a slurry, followed by hexamethylphosphoramide (2 ml). The resulting solution was stirred for 25 minutes, then stream of gaseous formaldehyde (prepared by heating paraformaldehyde (16.4 g, 189 mmol) at 175-200°C over 5-10 minutes) was passed through the solution, which was allowed to immediately warm to room temperature. The slurry was concentrated at ambient temperature, acidified to pH 4 with 6M hydrochloric acid, saturated with solid sodium chloride, and extracted with ethyl acetate (8 x 100 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 1% methanol/ methylene chloride, afforded *N*-(*tert*-butoxycarbonyl)-4-(hydroxymethyl)piperidine-4-carboxylic acid as a white solid (4 g, 20%). mp 156.6-157.3 °C; ^{1}HNMR (DMSO-d_6) δ 1.25-1.37 (m, 2H), 1.38 (s, 9H), 1.85 (dm, *J* = 13.7 Hz, 2H), 2.78-2.94 (br m, 2H), 3.41 (s, 1H), 3.70 (dm, *J* = 12.8 Hz, 2H), 4.87 (br s, 1H), 12.34 (s, 1H); Anal. Calcd. for $C_{12}H_{21}NO_5$: C, 55.58; H, 8.16; N, 5.40. Found: C, 55.72; H, 8.10; N, 5.53.

5H. Preparation of (10) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran a Compound of Formula (10a)

Trifluoromethanesulfonic anhydride (11.1 ml, 66.2 mmol), followed by triethylamine (17.8 ml, 127.4 mmol) was added to a slurry of 4-(hydroxymethyl)tetrahydropyran-4-carboxylic acid (10.20 g, 63.68 mmol) in anhydrous diethyl ether cooled to 0°C (115 ml). The biphasic solution was stirred for 20 hours, warmed to room temperature, stirred an additional 2 hours. The layers were separated by decantation, and the lower layer diluted with 2% aqueous sodium bicarbonate solution (50 ml) and extracted with methylene chloride (4 x 200 ml). The combined organic extracts were washed with additional 2% aqueous sodium bicarbonate (100 ml), dried over magnesium sulfate, and concentrated *in vacuo* to afford 2,7-dioxa-spiro[3.5]nonane-1-one as a pale yellow oil (10.8 g). IR (KBr) 1821 cm^{-1}; ^{1}HNMR (CD_3Cl_3) δ 1.92 (ddd, *J* = 13.4, 8.1, 4.0 Hz, 2H), 2.10 (dddd, *J* = 13.4, 6.1,3.4, 0.8 Hz, 2H), 3.70 (ddd, *J* = 11.8, 6.3, 3.9 Hz, 2H), 3.92 (ddd, *J* = 11.8, 7.9, 3.4 Hz, 2H), 4.15 (s, 2H); ^{13}CNMR (CD_3Cl_3) δ 30.78 (t), 55.78 (s), 64,46 (t), 71.50 (t), 173.42 (s), MS(EI) m/e=142. MS(CI) M+ =H m/e=143, M+ +HNH_4 m/e=160.

5I. Preparation of a Compound of Formula (10) where R^1 and R^2 taken together with the Carbon to which they are attached represent Piperidine, a Compound of Formula (10b)

Trifluoromethanesulfonic anhydride (2.60 ml, 15.39 mmol), followed by triethylamine (4.30 ml, 30.78 mmol) was added to a slurry of *N*-(*tert*-butoxycarbonyl)-4-hydroxymethylpiperidine-4-carboxylic acid (3.80 g, 14.65 mmol) in anhydrous diethyl ether (27 ml) cooled to 0°C. The biphasic solution was stirred for 23 hours, warmed to room temperature, stirred an addition 1 hour, and the upper diethyl ether layer separated by decantation. The lower was extracted with additional portions of diethyl ether (2 x 100 ml), and the combined organic extracts washed with aqueous sodium bicarbonate solution (2 x 50 ml), dried over magnesium sulfate, and concentrated *in vacuo* to afford 7-(butoxycarbonyl)-2-oxa-7-azaspiro[3.5]nonan-1-one as a pale yellow oil (2.88 g, 82%). ^{1}HNMR ($CDCl_3$) δ 1.48 (s, 9H), 1.79-1.89 (m, 2H), 2.02-2.10 (m, 2H), 3.48-3.66 (m, 4H), 4.13 (s, 2H).

EXAMPLE 6

Preparation of a Compound of Formula (13)

6A. Preparation of (13) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, and X is Iodo

Lithium diisopropylamide was prepared by the addition of 2.5M *N*-butyl lithium (5.6 ml, 13.9 mmol) in hexanes to a solution of diisopropylamine (1.95 ml, 13.9 mmmol) in tetrahydrofuran (30 ml) at 0°C with stirring for 20 minutes. Then a solution of tetrahydropyran-4-carboxylic acid ethyl ester (2 g, 12.7 mmol) in tetrahydrofuran (8 ml) was added to the solution of lithium diisopropylamide at a temperature of -78°C over 15 minutes. The resulting solution was stirred an

additional 50 minutes, and diiodomethane (1.14ml, 14.2 mmol) was added. The resulting mixture was stirred an additional 50 minutes, warmed to room temperature over 30 minutes, then recooled to 0°C. The mixture was diluted with 1M aqueous hydrochloric acid (25 ml), extracted with diethyl ether (2 x 100 ml), and washed with additional portions of diethyl ether (2 x 50 ml). The combined organic layers were washed once with 1M aqueous hydrochloric acid (100 ml), saturated aqueous sodium bisulfite (100 ml), saturated aqueous sodium bicarbonate (100 ml), and dried over magnesium sulfate, and concentrated *in vacuo*. The residue was filtered over a plug of silica gel, eluting successively with hexanes and ethyl acetate, removing excess alkylating agent with the hexane wash, to afford pure 4-(iodomethyl)tetrahydropyran-4-carboxylic acid ethyl ester as a pale yellow oil which was taken directly into the next reaction without further purification (3.20 g, 85%). IR (KBr) 1732 cm^{-1}; ^{1}HNMR ($CDCl_3$) 1.31 (q, J = 7.3 Hz, 3H), 1.56 (ddd, J = 14.6, 10.9, 4.5, 2H), 2.17 (ddd, J = 14.6, 5.7, 3.3, 2H), 3.31 (s, 2H), 3.51 (ddd, J = 11.7, 11.1, 2.5 Hz, 2H), 3.51 (td, J = 11.7, 4.3 Hz, 2H), 4.24 (q, J = 7.1 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 14.33 (q), 15.04 (t), 34.70 (t), 45.26 (s), 61.34 (t), 65.22 (t), 172.89 (s); EIHRMS Calcd. for $C_9H_{15}IO_3$ (M^+): 298.0066. Found: 298.0066. Anal. Calcd. for $C_9H_{15}IO_3$: C, 36.26; H, 5.07. Found: C, 36.56; H, 5.09.

6B. Preparation of (13) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, and Varying X

Similarly, replacing diiodomethane with dibromomethane or bromochloromethane, the following compounds of Formula (13) were prepared:

4-(bromomethyl)tetrahydropyran-4-carboxylic acid ethyl ester: IR (neat) 1732 cm^{-1}; ^{1}HNMR ($CDCl_3$) 1.30 (q, J = 7.1 Hz, 3H), 1.59 (ddd, J = 14.6, 10.9, 4.5, 2H), 2.17 (dm, J = 14.7, 2H), 3.48 (s, 2H), 3.53 (dt, J = 11.9, 4.5 Hz, 2H), 3.84 (dt, J = 11.9, 4.5 Hz, 2H), 4.23 (q, J = 7.1 Hz, 2H); ^{13}CNMR ($CDCl_3$)δ 14.27 (q), 33.17 (t), 40.16 (t), 46.05 (s), 61.29 (t), 64.97 (t), 172.91 (s); CIMS (M^+ + H): 251, (M^+ + NH_4+) 268.

4-(chloromethyl)tetrahydropyran-4-carboxylic acid ethyl ester: IR (neat) 1734 cm^{-1}; ^{1}HNMR ($CDCl_3$) 1.30 (q, J = 7.1 Hz, 3H), 1.59 (ddd, J = 14.6, 10.9, 4.5, 2H), 2.16 (dm, J = 14.7, 2H), 3.53 (dt, J = 11.9, 4.5 Hz, 2H), 3.61 (s, 2H), 3.84 (dt, J = 11.7, 4.3 Hz, 2H), 4.24 (q, J = 7.1 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 14.24 (q), 32.14 (t), 46.69 (s), 51.40 (t), 61.29 (t), 64.85 (t), 173.01 (s); CIMS (M^+ + H): 207. Anal. Calcd. for $C_9H_{15}ClO_3$: C, 52.31; H, 7.32. Found: C, 52.51; H, 7.30.

6C. Alternative Preparation of a Compound of Formula (13) where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, and X is *p*-Tosyl

To a solution of tetrahydropyran-4-carboxylic acid ethyl ester (820 mg, 4.356 mmol) in pyridine (10 ml) at 0°C, was added *p*-toluenesulfonyl chloride (997 mg, 5.23 mmol), and the mixture allowed to warm to room temperature over 1 hour period. The mixture was stirred 36 hours and partitioned between methylene chloride (150 ml) and 3N aqueous hydrochloric acid (50 ml). The organic layer was washed with 25 ml of saturated aqueous sodium bicarbonate, dried ($MgSO_4$), concentrated and the residue chromatographed over 45 g of silica gel, eluting with 30% ethyl acetate/hexanes, to afford the tosylate as a white solid (1.03 g, 69%). mp 87.7-88.6 °C; IR (KBr) 1717 cm^{-1}; 1 NMR ($CDCl_3$) δ 1.21 (q, J = 17.1 Hz, 3H), 1.52 (ddd, J = 13.4, 10.6, 4.1 Hz, 2H), 2.00 (dm, J = 13.4 Hz, 2H), 2.46 (s, 3H), 3.49 (ddd, J = 11.7, 10.6, 2.5 Hz, 2H), 3.76 (dt, J = 11.9, 4.1 Hz, 2H), 4.03 (s, 2H), 4.13 (q, J = 7.1 Hz, 2H), 7.35; ^{13}C NMR ($CDCl_3$) δ 14.10 (q), 21.67 (q), 30.43 (t), 44.93 (s), 61.37 (t), 64.43 (t), 74.65 (t), 127.95 (d), 129.89 (d), 132.67 (s), 145.05 (s), 172.57 (s); HRMS Calcd for $C_{16}H_{22}O_6$: 343.1215. Found: 343.1217. Anal. Calcd. for $C_{16}H_{22}O_6$: C, 56.12; H, 6.48. Found: C, 56.22; H, 6.46.

EXAMPLE 7

Preparation of Compounds of Formula Ia

7A. Preparation of Ia where R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached represent Piperidine, and R^5 is Diphenylether, from a Compound of Formula (4)

1. 4-Phenoxythiophenol (7.4 g, 36.3 mmol), 4-carboxymethylene-*N*-CBZ-piperidine (10 g, 36.3 mmol) and piperidine (1.8 ml, 36.3 mmol) were stirred overnight at 100-110°C in a sealed flask. After cooling, the crude reaction mixture was partitioned between ethyl acetate and 1N hydrochloric acid, the organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated *in vacuo* to give a yellow solid. The solid was triturated in 1:1 (v/v) ethyl ether/hexane (500 ml) to give 2-[4-(4-phenoxyphenylthio)-*N*-CBZ-piperidin-4-yl]-acetic acid as a white solid.

2. A solution of 2-[4-(4-phenoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetic acid (150 mg, 0.29 mmole) in dry 1,2 dichloroethane (3 ml) under nitrogen was cooled to -10°C and saturated with hydrochloric acid gas for 15 minutes. The reaction vessel was then sealed and the solution stirred for two days at 25°C. The tube was cooled to -10°C prior to opening to release gaseous hydrochloric acid, and then allowed to warm to 25°C. The solvent was removed *in vacuo* and the product triturated with ethyl acetate to give 2-[4-(4-phenoxyphenylthio)-piperidin-4-yl)]-acetic acid hydrochloride as a white powder. ^{1}HNMR (CD_3OD): 7.93 (d,2H); 7.45 (t,2H); 7.27 (t,1H), 7.14 (t,4H); 3.52 (m,2H); 3.25 (m,2H); 2.70 (s,2H), 2.35 (m,4H).

7B. Preparation of Ia where R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached represent Cyclopentyl, and R^5 is Diphenylether, from a Compound of Formula (4)

A mixture of cyclopentylideneacetic acid (2 mmol) and *p*-(phenoxy)-thiophenol (2 mmol) was heated at 110°C under nitrogen in the presence of piperidine (100 μL) for 24 hours. The residue was dissolved in ethyl acetate and washed with dilute hydrochloric acid. The organic layer was separated, dried and evaporated under reduced pressure to give crude 2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetic acid, which can be used in the next reaction without further purification.

7C. Preparation of Ia where R^1, R^2 and R^3 are Hydrogen, R^4 is Benzyl, and R^5 is 4-Bromophenyl

A mixture of *E*-2-benzylacrylic acid (1 g) and *p*-bromothiophenol (1.12 g) were stirred overnight at 110°C in the presence of piperidine (300 μL). The residue was partitioned between ethyl acetate and dilute hydrochloric acid. The organic layer was separated, dried and evaporated under reduced pressure to give 3-benzyl-3-(4-bromophenylthio)-propionic acid (Iaa), which was used in the next reaction with no further purification.

7D. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl, from a Compound of Formula (10)

2,7-dioxa-spiro[3.5]nonane-1-one (10.8 g), obtained as described in Example 5H, was immediately dissolved in *N,N*-dimethylformamide (95 ml) and slowly added to a solution containing the sodium salt of 4-(4-chlorophenoxy)thiophenol (generated by the addition of sodium hydride powder (2.14 g, 89.2 mmol) to a solution of 4-(4-chlorophenoxy)thiophenol (15.83 g, 66.8 mmol) in *N,N*-dimethylformamide (19 ml) at 0°C and stirring for 30 minutes) over a 10-15 minute period, and then stirred an additional 15 minutes. The resulting slurry was heated to 40°C, stirred for 5 minutes, *tert*-butanol (2 ml) was added, and the mixture cooled to room temperature over 20 minutes. The majority of the *N,N*-dimethylformamide was removed *in vacuo*, the pH adjusted to 9.2, the resultant slurry diluted with 30% diethyl ether-hexanes (120 ml) and filtered. The filter cake was washed with additional portions of ether (3 x 70 ml), acidified to pH 3.5 with 2N aqueous hydrochloric acid, and extracted into methylene chloride (4 x 350 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo*. The solid residue was recrystallized from the minimum amount of methylene chloridehexanes to afford pure 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid as a white crystalline solid (19.50 g). mp 140.6-141.9°C; IR (KBr) 3429 (br), 1732 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.54 (ddd, *J* = 14.2, 10.0, 4.2 Hz, 2H), 1.95 (dm, *J* = 14.2 Hz, 2H), 3.19 (s, 2H), 3.56 (ddd, *J* = 11.8, 10.0, 4.2 Hz, 2H), 3.70 (dt, J = 11.8, 4.2 Hz, 2H), 6.98 (d, *J* = 8.8 Hz, 2H), 7.02 (d, J = 8.9 Hz, 2H), 7.02 (d, *J* = 8.9 Hz, 2H), 7.42 (d, *J* = 9.0 Hz, 4H), 12.66 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 33.06 (t), 43.56 (t), 45.03 (s), 64.13 (t), 119.43 (d), 120.11 (d), 110.43 (d), 127.35 (s), 129.80 (d), 131.09 (s), 131.59 (d), 154.90 (s), 155.50 (s), 175.25 (s); HRMS Cald. for $C_{19}H_{19}SO_4Cl$: 378.0693. Found: 378.0685. Anal. Calcd. for $C_{19}H_{19}SO_4Cl.O.25\ H_2O$: C,59.53; H, 513. Found: C, 59.53; H, 5.07.

Similarly, replacing 4-(4-chlorophenoxy)thiophenol with 4-(4-bromophenoxy)thiophenol and 4-(4-fluorophenoxy)thiophenol, the following compounds were prepared:

4-[4-(4-bromophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid: mp 143.7-144.5 °C; IR (KBr) 3434 (br), 1732 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.54 (ddd, *J* = 13.8, 10.1, 4.3 Hz, 2H), 1.94 (dm, *J* = 13.5 Hz, 2H), 3.19 (s, 2H), 3.37 (ddd, *J* = 11.8, 10.1, 2.5 Hz, 2H), 3.70 (dt, *J* = 11.8 Hz, 4.0 Hz, 2H), 6.96 (d, *J* = 9.2 Hz, 2H), 6.98 (d, *J* = 8.8 Hz, 2H), 7.41 (d, *J* = 8.8 Hz, 2H), 7.55 (d, *J* = 9.0 Hz, 2H), 12.68 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 33.04 (t),43.34 (t), 45.00 (s), 64.10 (t), 115.14 (s), 119.59 (d), 120.53 (d), 131.15 (s), 131.51 (d), 132.77 (s), 154.71 (s), 156.06 (s), 175.28 (s); EIMS (M^+): 424. Anal. Calcd. for $C_{19}H_{19}SO_4Br$: C, 53.91; H, 4.52. Found: C, 53.53; H, 4.54;

4-[4-(4-fluorophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid: mp 143.0-143.4 °C; IR (KBr) 3436 (br), 1721 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.54 (ddd, *J* = 13.5, 10.1, 4.0 Hz, 2H), 1.94 (dm, *J* = 13.5 Hz, 2H), 3.17 (s, 2H), 3.38 (td, *J* = 11.8, 2.5 Hz, 2H), 3.70 (dt, *J* = 11.8 Hz, 4.0 Hz, 2H), 6.93 (d, *J* = 8.8 Hz, 2H),7.05 (dd, *J* = 9.2. 4.6 Hz, 2H), 7.21 (dd, *J* = 9.1, 8.4 Hz, 2H), 7.40 (d, *J* = 8.8 Hz, 2H), 12.65 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 33.05 (t), 43.65 (t),

45.49 (s), 64.12 (t), 116.53 (dd, J_{C-F} = 23.2 Hz), 118.71 (d), 120.63 (dd, J_{C-F} = 8.5 Hz), 130.31 (s), 131.69 (d), 152.38 (s), 155.85 (s), 158.29 (d, J_{C-F} = 239.9 Hz), 175.28 (s); EIMS (M^+): 362. Anal. Calcd. for $C_{19}H_{19}SO_4F$: C, 62.97; H, 5.28. Found: C, 62.79; H, 5.26.

7E. Alternative Preparation of Ia where R^1 and R^2 are both Methyl. R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

Sodium hydride powder (0.86 g, 35.8 mmol) was added to a mixture of 4-(4-chlorophenoxy)thiophenol (3.55 g, 15 mmol) in *N,N*-dimethylformamide (12 ml) at 0°C. The mixture was warmed to room temperature over 5 minutes, stirred for an additional 20 minutes, and solid chloropivalic acid (1.64 g, 12.0 mmol) was added in one portion. This mixture was heated to 80°C for 18 hours, cooled to room temperature, and water (1 ml) added. The residue was partitioned between methylene chloride (50 ml) and 2N hydrochloric acid (25 ml). The aqueous layer was separated and washed with additional methylene chloride (2 x 25 ml). The combined organic extracts were dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 5% methanol/methylene chloride, gave slightly impure 3-[4-(4-chlorophenoxy)-phenylthio]-2,2-dimethyl propionic acid (4 g, 99%). This material was recrystallized from the minimum amount of diethyl ether/hexanes to afford analytically pure acid as a white solid (3.20 g, 80%). mp 84.4-84.9°C; IR (KBr) 3433 (br), 1732 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.19 (s, 6H), 3.14 (s, 2H), 6.97 (d, J = 8.7 Hz, 2H), 7.01 (d, J = 8.9, 2H), 7.40 (d, J = 8.8 Hz, 2H), 12.36 (br s, 1H). EIMS(M+): 378. Anal. Calcd. for $C_{17}H_{17}SO_3Cl$: C, 60.62; H, 5.09. Found: C, 60.31; H, 4.96.

7F. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent *N*-BOC-Piperidine. R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl, from a Compound of Formula (10b)

7-(*tert*-Butoxycarbonyl)-2-oxa-7-azaspiro[3.5]nonan-1-one obtained in Example 5I above, was immediately dissolved in *N,N*-dimethylformamide (4 ml), slowly added to a solution containing the sodium salt of 4-(4-chlorophenoxy)thiophenol (generated by the addition of sodium hydride power (340 mg, 14.17 mmol) to a solution of 4-(4-chlorophenoxy)thiophenol (3.00 g, 12.7 mmol) in *N,N*-dimethylformamide (19 ml), at 0°C and stirred for 30 minutes) over a 10-15 minute period, and was stirred an additional 15 minutes. The resulting slurry was heated to 80°C, stirred for 5 minutes, *tert*-butanol (2 ml) added, and the mixture cooled to room temperature over 20 minutes. The majority of the *N,N*-dimethylformamide was removed *in vacuo*, the pH adjusted to 3.5 using 2M aqueous hydrochloric acid and extracted into ethyl acetate (4 x 150 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo* and the residue chromatographed over silica gel, eluting with 1% to 10% methanol/methylene chloride, to afford the piperidine acid, 4-[4-(4-chlorophenoxy)phenylthiomethyl]-*N*-(*tert*-butoxycarbonyl)-piperidin-4-yl carboxylic acid as a pale yellow oil (5 g, 89%). ^{1}HNMR (OH not observed; $CDCl_3$) δ 1.37 (s, 9H), 1.55 (m_c, 2H), 2.10 (m_c, 2H), 3.05 (m_c, 2H), 3.06 (s, 2H), 3.72 (m_c, 2H), 6.81 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.9 Hz, 2H), 7.21 (d, J = 8.9 Hz, 2H), 7.30 (d, J = 8.7 Hz, 4H).

7G. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, R^5 is 4-(4-Chlorophenoxy)phenyl, from a Compound of Formula Ia where R is Ethyl

To a solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid ethyl ester (70 mg, 0.17 mmol) in ethanol (2 ml) containing two drops of water, was added potassium hydroxide (58.3 mg, 1.04 mmol). The mixture was refluxed for 13 hours, cooled to room temperature, acidified to pH 4, and extracted with ethyl acetate (4 x 50 ml). The combined organic layers were dried over magnesium sulfate, and concentrated to afford 4-[4-(4-chlorophenoxy)-phenylthiomethyl]-tetrahydropyran-4-carboxylic acid (66 mg, 100%), which is spectroscopically identical to that isolated from the prior procedure of Example 7D.

7H. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, R^5 is 4-(4-Bromophenoxy)phenyl, from a Compound of Formula Ia where R is Ethyl

Similarly, following the procedure of Example 7G above, 4-[4-(4-bromophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid and 4-[4-(4-fluorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid were prepared.

7I. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, R^5 is 4-(4-Chlorophenoxy)phenyl, and R is Methyl, from the Corresponding Carboxylic Acid

To a solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid (580 mg, 1.53 mmol) and *N, N*-dimethylformamide catalyst (22 μL) in methylene chloride (15 ml) at 0°C was added oxalyl chloride (0.33 ml, 3.83 mmol) dropwise over 10 minutes. The mixture was warmed to room temperature over 1 hour, the partial slurry stirred an additional 12 hours, and concentrated *in vacuo* until the theoretical mass of the acid chloride was obtained. The residue was suspended in tetrahydrofuran (7.5 ml), and methanol (0.19 ml, 4.59 mmol), followed by triethylamine (0.64 ml, 4.59 mmol) was added. The mixture was heated to reflux for 14 hours, concentrated, and the resulting residue partitioned between methylene chloride (150 ml) and 1M aqueous hydrochloric acid (50 ml). The aqueous layer was back extracted with additional portions of methylene chloride (2 x 30 ml), the combined extracts dried over magnesium sulfate, and concentrated to afford crude 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid methyl ester, which was taken directly into the next reaction without further purification. ^{1}HNMR ($CDCl_3$) δ 1.62 (m_c, 2H), 2.15 (dm, *J* = 13.6 Hz, 2H), 3.13 (s, 2H), 3.47 (td, *J* = 11.9, 2.4 Hz, 2H), 3.59 (s, 3H), 3.81 (dt, *J* = 12.0, 4.1 Hz, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), 7.29 (d, *J* = 8.8 Hz, 2H), 7.36 (d, *J* = 8.8 Hz, 2H).

7J. Preparation of Ia where R^1 and R^2 taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, R^5 is 4-4-Chlorophenoxy)phenyl, and R is Ethyl, from a Compound of Formula (13)

4-(Iodomethyl)tetrahydropyran-4-carboxylic acid ethyl ester (300 mg, 1 mmol) was added to a solution containing the sodium salt of 4-(4-chlorophenoxy)thiophenol (generated by the addition of sodium hydride powder (36 mg, 1.5 mmol) to a solution of 4-(4-chlorophenoxy)thiophenol (262 mg, 1.1 mmol) in *N,N*-dimethylformamide (2 ml) at 0°C and stirring for 30 minutes). The mixture was warmed to room temperature over 5 minutes, stirred for an additional 20 minutes, cooled to room temperature, and 1M aqueous hydrochloric acid (5 ml) added. The mixture was then partitioned between ethyl acetate (100 ml) and 2M hydrochloric acid (25 ml). The aqueous layer was separated and washed with additional ethyl acetate (2 x 50 ml). The organic extracts were combined, washed with 1M sodium hydroxide (2 x 30 ml), dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 20% ethylacetate/ hexanes, yielded pure 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid ethyl ester (370 mg, 91%), followed by impure 4-[4-(4-chlorophenoxy)-phenylthiomethyl]tetrahydropyran-4-carboxylic acid ethyl ester (40 mg). IR (KBr) 1728 cm^{-1}; ^{1}HNMR ($CDCl_3$) 1.23 (q, *J* = 7.1 Hz, 3H), 1.56 (ddd, *J* = 14.6, 10.9, 4.4, 2H), 1.63 (ddd, *J* = 14.6, 5.7, 3.3, 2H), 3.13 (s, 2H), 3.51 (ddd, *J* = 11.8, 11.1, 2.4 Hz, 2H), 3.80 (dt, *J* = 11.8, 4.1 Hz, 2H), 4.07 (q, *J* = 7.1 Hz, 2H), 6.91 (d, *J* = 8.9 Hz, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), 7.29 (d, *J* = 9.0 Hz, 2H), 7.39 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 14.20 (q), 33.72 (t), 45.72 (t), 46.07 (s), 60.92 (t), 65.06 (t), 119.29 (d), 120.20 (d), 128.43 (s), 129.85 (d), 130.57 (s), 133.05 (s), 155.40 (s), 156.21(s), 174.02 (s); EIHRMS Calcd. for $C_{21}H_{23}SO_4Cl$ (M^+): 406.1006. Found: 406.1008. Anal. Calcd. for $C_{21}H_{23}SO_4Cl$: C, 61.98; H, 5.70. Found: C, 61.86; H, 5.68.

7K. Preparation of Ia where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, R^5 is 4-(4-Bromophenoxy)phenyl, and R is Ethyl, from a Compound of Formula (13)

Similarly, replacing 4-(4-chlorophenoxy)thiophenol with 4-(4-bromophenoxy)thiophenol, and following the procedures of Example 7J above, 4-[4-(4-bromophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid ethyl ester was prepared (2.10 g, 93%). IR (KBr) 1728 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.22 (q, *J* = 7.1 Hz, 3H), 1.60 (ddd, *J* = 14.6, 10.9, 4.5, 2H), 2.14 (ddd, *J* = 14.6, 5.7, 3.3, 2H), 3.13 (s, 2H), 3.81 (ddd, *J* = 11.8, 11.1, 2.4 Hz, 2H), 4.07 (q, *J* = 7.1 Hz, 2H), 6.87 (d, *J* = 9.0 Hz, 2H), 6.92 (d, *J* = 8.8 Hz, 2H), 7.37 (d, *J* = 8.8 Hz, 2H), 7.43 (d, *J* = 9.0 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 14.20 (q), 33.71 (t), 45.69 (t), 46.05 (s), 60.92 (t), 65.05 (t), 116.06 (s), 119.40 (d), 120.59 (d), 130.69 (s), 132.81 (d), 133.03 (s), 156.04 (s), 156.16 (s), 174.01 (s); EIHRMS Calcd. for $C_{21}H_{23}SO_4Br$ (M^+): 450.0500. Found: 450.0505. Anal. Calcd. for $C_{21}H_{23}SO_4Cl$: C, 55.88; H, 5.14. Found: C, 55.52; H, 5.09.

Similar reactions were carried out, starting from compounds of Formula (13) where X is iodo, bromo, and chloro, and moderate to good yields were obtained in all cases.

7L. Preparation of Ia, varying R^1, R^2, R^3, R^4, and R^5

Similarly, optionally replacing 4-carboxymethylene-*N*-CBZ-piperidine with other *N*-protected compounds of Formula (4) and following the procedures of Example 7A (1) and (2) above, or optionally replacing cyclopentylideneacetic acid with other compounds of Formula (4) and following the procedures of Example 7B above, and optionally replacing p-phenoxythiophenol with other compounds of Formula (5), the following compounds of Formula Ia were prepared:

2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl-]-acetic acid;
2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-acetic acid;
2-benzyl-3-(3-methoxyphenylthio)-propionic acid;
2-benzyl-3-(4-methoxyphenylthio)-propionic acid;
3-benzyl-3-(4-methoxyphenylthio)-propionic acid;
3,3-dimethyl-3-[(4-chlorophenoxy)phenylthio]-propionic acid;
2-{4-[4-(4-fluorophenoxy)phenylthio]-piperidin-4-yl}-acetic acid;
2-{4-[4-(4-fluorophenoxy)phenylthio}-*N*-CBZ-piperidin-4-yl}-acetic acid;
3-benzyl-3-[(4-phenylthiophenyl)thio]-propionic acid;
3-benzyl-3-(phenylthio)-propionic acid;
3-benzyl-3-(4-phenoxphenylthio)-propionic acid;
3-benzyl-3-[(4-biphenyl)thio]-propionic acid;
3-benzyl-3-(2-naphthylthio)-propionic acid;
3-benzyl-3-(4-methoxystyrylphenylthio)-propionic acid;
3-cyclopentylmethyl-3-(4-methoxyphenylthio)-propionic acid;
3-cyclopentylmethyl-2-isopropyl-3-(4-methoxyphenylthio)-propionic acid;
3-ethyl-2-methyl-3-(4-methoxyphenylthio)-propionic acid;
3,3-dimethyl-(4-methoxyphenylthio)-propionic acid;
2-[1-(4-methoxyphenylthio)-cyclopent-1-yl]-acetic acid;
2-[4-(4-methoxyphenylthio)-cyclohexanone-4-yl]-acetic acid ethylene ketal;
2-[1-(4-methoxyphenylthio)-(4-methylcyclohex-1-yl]-acetic acid;
2-[1-(4-phenoxyphenylthio)-cyclohex-1-yl]-acetic acid;
2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetic acid;
[4-[4-(4-benzo[b]thiophen-2-yl-phenoxy)phenylthio)-tetrahydropyran-4-yl]-acetic acid;
2-{4-[4-(phenylmethyl)phenylthio]-tetrahydropyran-4-yl}-acetic acid;
2-{4-[4-(4-fluorophenoxy)phenylthio]-tetrahydropyran-4-yl}-acetic acid;
2-{4-[4-(4-chlorophenoxy)phenylthio]-tetrahydropyran-4-yl}-acetic acid: mp 138.5-138.8 °C; ^{1}HNMR ($CDCl_3$, OH not seen) δ 1.73 (d, J = 14.7, 2H), 1.91 (ddd, J = 14.7, 10.1, 4.3 Hz, 2H), 2.58 (s, 2H), 3.76 (dt, J = 11.8, 4.1 Hz, 2H), 4.02 (dt, J = 11.8, 2.6 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 7.33 (d, J = 8.9 Hz, 2H), 7.53 (d, J = 8.8 Hz, 4H); FABMS (M^+): 379.2. Anal. Calcd. for $C_{19}H_{19}SO_4Cl$: C, 60.23; H, 5.05. Found: C, 60.39; H, 5.01;
2-{4-[4-(4-chlorophenoxy)phenylthio]-tetrahydropyran-4-yl}-acetic acid;
2-{4-[4-(4-bromophenoxy)phenylthio]-tetrahydropyran-4-yl}-acetic acid;
2-[4-(4-phenoxyphenylthio)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetic acid;
trans-2-(4-methoxyphenylthio)-cyclopentanecarboxylic acid; and
2-(4-methoxyphenylthio)-cyclohexanecarboxylic acid.

7M. Preparation of Ia, varying R^1, R^2, R^3, R^4, and R^5

Similarly, optionally replacing 2,7-dioxa-spiro[3.5]nonane-1-one with other compounds of Formula (10) and following the procedures of Example 7D above, and optionally replacing 4-(4-chlorophenoxy)-thiophenol with other compounds of Formula (5), the following compounds of Formula Ia were prepared:

4-[4-(4-fluorophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid;
4-[4-(4-bromophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid;
3-(4-benzoylphenylthio)-2,2-dimethyl propionic acid;
3-[4-(4-chlorophenoxy)phenylthio]-2,2-dimethyl propionic acid;
4-[(4-phenoxypyrid-4-yl)thiomethyl]tetrahydropyran-4-carboxylic acid: ^{1}HNMR (OH not observed; $CDCl_3$) δ 1.65 (m_c, 2H), 2.16 (dm, J = 14.2 Hz, 2H), 3.20 (s, 2H), 3.57 (tm, J = 11.4 Hz, 2H), 3.84 (dm, J = 12.0 Hz, 2H), 6.87 (d, J =6.2 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 7.47 (d, J = 8.9 Hz, 2H), 8.43 (d, J = 6.0 Hz, 2H).

7N. Preparation of Ia, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 7 above, other compounds of Formula Ia are prepared.

EXAMPLE 8

Preparation of Compounds of Formula Iba

8A. Preparation of Iba where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

Oxalyl chloride (37.5 ml, 429.5 mmol) was added dropwise over 10 minutes to a suspension of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid (65.1 g, 171.8 mmol) and *N,N*-dimethylformamide catalyst (2 ml) in methylene chloride (1 litre) at 0°C. The mixture was warmed to room temperature over 1 hour and the resultant partial slurry stirred an additional 20 hours, concentrated under reduced pressure until the theoretical mass of the acid chloride was obtained. This mixture was dissolved in methylene chloride (600 ml), cooled to 0°C, and *N,O*-bis(trimethylsilyl)hydroxylamine (109.1 ml, 510.45 mmol) added dropwise over 10 minutes. The mixture was immediately warmed to room temperature, stirred 3 hours, and recooled to 0°C. Aqueous 2.4M hydrochloric acid solution (400 ml, 960 mmol) was added to the solution, causing precipitation of the hydroxamic acid product within several minutes after the addition. The slurry was stirred an additional 30 minutes and filtered. The filter cake was washed with water (3 x 30 ml) and 50% diethyl ether-hexanes (2 x 25 ml) and dried at 70°C to afford 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) (61.8 g, 92%). mp 146.6-148.0 °C; IR (KBr) 3426 (br), 1636 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.54 (ddd, J = 13.8, 10.2, 4.0 Hz, 2H), 2.00 (dm, J = 13.8 Hz, 2H), 3.16 (s, 2H), 3.39 (m, 2H), 3.66 (dt,J= 11.7,3.8 Hz, 2H), 6.98 (d,J = 8.8Hz, 2H), 7.02 (d,J=9.0Hz, 2H), 7.40 (d, J = 8.8 Hz, 2H), 7.41 (d, J = 8.9 Hz, 2H), 8.78 (s, 1H), 10.63 (s, 1H); ^{13}CNMR ($CDCl_3$) δ 32.79 (t), 43.60 (s), 43.70 (t), 63.93 (t), 119.56 (d), 120.07 (d), 127.19 (s), 129.85 (d), 131.24 (d), 131.34 (s), 154.62 (s), 155.59 (s), 169.69 (s); FABHRMS Calcd. for $C_{19}H_{21}NSO_4Cl$ (M^+ + H): 394.0880. Found: 378.0872. Anal. Calcd. for $C_{19}H_{20}NSO_4Cl$: C, 57.94; H, 5.12; N, 3.56. Found: C, 57.98; H, 5.04; N, 3.68.

8B. Alternative Preparation of Iba where R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

Oxalyl chloride (37.5 ml, 429.5 mmol) was added dropwise over 10 minutes to a solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid (65.1 g, 171.8 mmol) and *N,N*-dimethylformamide catalyst (2 ml) in methylene chloride (1 litre) at 0°C. The mixture was warmed to room temperature over 1 hour, and the resultant partial slurry stirred an additional 20 hours and concentrated *in vacuo* until the theoretical mass of the acid chloride was obtained. A solution of the acid chloride mixture (650 mg, 1.68 mmol) in methylene chloride (3.4 ml) was added dropwise over 2 minutes to a solution of 50% aqueous hydroxylamine (556 mg) in 2:1 tetrahydrofuran/*tert*-butanol (5.1 ml). The mixture was stirred 1.5 hours and concentrated until approximately 1 ml of aqueous solution was remaining. The slurry was filtered, washed with 1:1 diethyl ether-hexanes (3 X 15 ml) and the solid dried overnite at 70°C in a vacuum oven, to afford 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) (584 mg, 91%). mp 146.6-148.0 °C; IR (KBr) 3426 (br), 1636 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.54 (ddd, J = 13.8, 10.2, 4.0 Hz, 2H), 2.00 (dm, J = 13.8 Hz, 2H), 3.16 (s, 2H), 3.39 (m, 2H), 3.66 (dt, J = 11.7, 3.8 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 7.02 (d, J = 9.0 Hz, 2H), 7.40 (d, J = 8.8 Hz, 2H), 7.41 (d, J = 8.9 Hz, 2H), 8.78 (s, 1H), 10.63 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.79 (t), 43.60 (s), 43.70 (t), 63.93 (t), 119.56 (d), 120.07 (d), 127.19 (s), 129.85 (d), 131.24 (d), 131.34 (s), 154.62 (s), 155.59 (s), 169.69 (s); FABHRMS Calcd. for $C_{19}H_{21}NSO_4Cl$ (M^+ + H): 394.0880. Found: 378.0872. Anal. Calcd. for $C_{19}H_{20}NSO_4Cl$: C, 57.94; H, 5.12; N, 3.56. Found: C, 57.98; H, 5.04; N, 3.68.

8C. Preparation of Iba, varying R^1, R^2, R^3, R^4, and R^5

Similarly, replacing 4-[4-(4-chlorophenoxy)phenyl-thiomethyl]-tetrahydropyran-4-carboxylic acid with other compounds of Formula Ia and following the procedures of Example 8A above, the following compounds of Formula Iba were prepared:

4-[4-(4-fluorophenoxy)phenylthiomethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 146.2-146.5 °C; IR (KBr) 3431 (br), 1628 cm^{-1}; ^{1}HNMR ($CDCl_3$; NH and OH not observed) δ 1.35 (ddd, J = 13.8, 10.2, 4.0 Hz, 2H), 1.83 (dm, J = 13.8 Hz, 2H), 2.85 (s, 2H), 3.23 (m, 2H), 3.46 (dt, J = 11.9, 3.9 Hz, 2H), 6.58 (d, J = 8.8 Hz, 2H), 6.57 (d, J = 8.8 Hz, 2H), 6.65-6.78 (m, 4H),7.06 (d, J = 8.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 32.99 (t), 44.27 (s), 45.49 (t), 64.63 (t), 116.28 (dd, J_{C-F} = 23.2 Hz), 118.64 (d), 120.49 (dd, J_{C-F} = 8.5 Hz), 130.41 (s), 132.49 (d), 152.46 (s), 156.49 (s), 160.29 (d, J_{C-F} = 241.9 Hz), 170.23 (s); FABMS (M^+ + H): 378. Anal. Calcd. for $C_{19}H_{20}NSO_4F$: C, 60.46; H, 5.34; N, 3.71. Found: C, 60.08; H, 5.29; N, 3.65.

4-[4-(4-bromophenoxy)phenylthiomethyl]tetrahydropyran-4-*N*-hydroxycarboxamide: mp 153.1-154.0 °C; IR (KBr) 3434 (br), 1634 cm^{-1}; 1HNMR ($CDCl_3$; NH and OH not observed) δ 1.68 (ddd, J = 14.0, 10.0, 4.0 Hz, 2H), 2.13 (dm, J = 14.0 Hz, 2H), 3.15 (s, 2H), 3.55 (ddd, J = 12.0, 10.2, 2.5 Hz, 2H), 3.76 (dt, J = 12.0 Hz, 4.1 Hz, 2H), 6.87

(d, J = 9.0 Hz, 2H), 6.90 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.43 (d, J = 9.0 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 33.01 (t), 44.32 (s), 45.40 (t), 64.65 (t), 115.95 (s), 119.50 (d), 120.53 (d), 130.67 (s), 132.76 (d), 132.80 (d), 155.92 (s), 156.16 (s), 170.60 (s); FABMS (M^+ + H): 438. Anal. Calcd. for $C_{19}H_{20}NSO_4Br$: C, 52.06; H, 4.60; N, 3.20. Found: C, 51.84; H, 4.52; N, 3.54.

3-(4-benzoylphenylthio)-2,2-dimethyl-N-hydroxypropionamide;

3-[4-(4-chlorophenoxy)phenylthio]-2,2-dimethyl-N-hydroxypropionamide: mp 114.7-115.3 °C; ^{1}HNMR ($CDCl_3$) δ 1.30 (s, 6H), 3.14 (s, 2H), 6.90 (d, J = 8.8 Hz, 2H), 6.92 (d, J = 8.8 Hz, 2H), 7.29 (d, J = 8.9 Hz, 2H), 7.37 (d, J = 8.8 Hz, 1H); FABHRMS Calcd. for $C_{17}H_{18}NSO_3Cl$ (M^+ + H): 352.0772. Found: 352.0774. Anal. Calcd. for $C_{17}H_{18}NSO_3Cl$: C, 58.03; H, 5.16; N, 3.98. Found: C, 57.85; H, 5.10; N, 4.12.

3,3-dimethyl-3-[(4-chlorophenoxy)phenylthio]-N-hydroxypropionamide;

{4-[4-(4-benzo[b]thiophen-2-yl-phenoxy)phenylthio)-tetrahydropyran-4-yl]-N-hydroxyacetamide;

2-{4-[4-(phenylmethyl)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;

2-{4-[4-(4-chlorophenoxy)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide; and

2-{4-[4-(4-bromophenoxy)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide.

8D. Preparation of Iba, varying R^1, R^2, R^3, R^4, and R^5

Similarly, replacing 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid with other compounds of Formula Ia and following the procedures of Example 8A above, other compounds of Formula Iba are prepared, for example:

4-(4-phenoxyphenylthiomethyl)tetrahydropyran-4-(*N*-hydroxycarboxamide);

4-[4-(4-fluorophenoxy)phenylthiomethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]piperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]-1-methylpiperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]-1-(cyclopropyl-methyl)piperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]-1-acetylpiperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]-1-(3-pyridyl)-piperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylthiomethyl]-1-(3-pyridoyl)-piperidine-4-(*N*-hydroxycarboxamide);

2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl-]-*N*-hydroxyacetamide;

2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-*N*-hydroxyacetamide;

2-benzyl-3-(3-methoxyphenylthio)-*N*-hydroxypropionamide;

2-benzyl-3-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

3-benzyl-3-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

2-{4-[4-(4-fluorophenoxy)phenylthio]-piperidin-4-yl}-*N*-hydroxyacetamide;

2-{4-[4-(4-fluorophenoxy)phenylthio]-*N*-CBZ-piperidin-4-yl}-*N*-hydroxyacetamide;

3-benzyl-3-[(4-phenylthiophenyl)thio]-*N*-hydroxypropionamide;

3-benzyl-3-(phenylthio)-*N*-hydroxypropionamide;

3-benzyl-3-(4-phenoxphenylthio)-*N*-hydroxypropionamide;

3-benzyl-3-[(4-biphenyl)thio]-*N*-hydroxypropionamide;

3-benzyl-3-(2-naphthylthio)-*N*-hydroxypropionamide;

3-benzyl-3-(4-methoxystyrylphenylthio)-*N*-hydroxypropionamide;

3-cyclopentylmethyl-3-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

3-cyclopentylmethyl-2-isopropyl-3-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

3-ethyl-2-methyl-3-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

3,3-dimethyl-(4-methoxyphenylthio)-*N*-hydroxypropionamide;

2-[1-(4-methoxyphenylthio)-cyclopent-1-yl]-*N*-hydroxyacetamide;

2-[4-(4-methoxyphenylthio)-cyclohexanone-4-yl]-*N*-hydroxyacetamide ethylene ketal;

2-[1-(4-methoxyphenylthio)-(4-methylcyclohex-1-yl]-*N*-hydroxyacetamide;

2-[1-(4-phenoxyphenylthio)-cyclohex-1-yl]-*N*-hydroxyacetamide;

2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-*N*-hydroxyacetamide;

2-{4-[4-(4-fluorophenoxy)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;

2-[4-(4-phenoxyphenylthio)-tetrahydrothiopyran-1,1-dioxide-4-yl]-*N*-hydroxyacetamide;

trans-2-(4-methoxyphenylthio)-cyclopentanecarboxylic acid; and

2-(4-methoxyphenylthio)-cyclohexanecarboxylic acid.

EXAMPLE 9

Preparation of Compounds of Formula Ib

9A. Preparation of Ib where R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Cyclopentyl, and R^5 is 4-Phenoxyphenyl

The 2-[1-(4-phenoxypheny]thio)-cyclopent-1-yl]-acetic acid obtained in Example 5 was dissolved in methylene chloride (8 ml) and treated with 4-dimethylaminopyridine (180 mg), *O*-(*tert*-butyl)-hydroxylamine hydrochloride (360 mg), triethylamine (540 μL), pyridine (400 μL), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (750 mg). After stirring overnight the reaction mixture was partitioned between ethyl acetate and water, the organic layer separated, and the solvent removed under reduced pressure. Preparative TLC of the residue and elution with 2:1 hexane/ethyl acetate gave *N*-(*tert*-butoxy)-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide (270 mg) as a white foam, which can be used in the next reaction without further purification.

9B. Preparation of Ib where R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Tetrahydropyran, and R^5 is 4-Phenoxyphenyl

O-(*tert*-Butyl)hydroxylamine hydrochloride (9.57 g), 4-methylmorpholine (15.64 ml), hydroxybenzotriazole (6.87 g), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (19.5 g) was added to a solution of 2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetic acid (17.5 g) in methylene chloride (200 ml). After stirring for 3 hours at room temperature, 0.5 M hydrochloric acid (200 ml) was added to the mixture, and the mixture extracted with methylene chloride. The solvent was removed from the combined extracts under reduced pressure. Silica gel chromatography of the residue and elution with 35%-80% ethyl acetate/hexane gave *N*-*tert*-butoxy-2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetamide (15.3 g) as an oil, which can be used in the next reaction without further purification.

9C. Preparation of Ib where R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached are *N*-BOC-Piperidine, and R^5 is 4-(4-Chlorophenoxy)phenyl

4-Methylmorpholine (2.60 ml, 23.68 mmol) was added dropwise to a solution of 2-{4-[4-(4-chlorophenoxy)phenylthiomethyl]-*N*-BOC-piperidin-4-yl}-carboxylic acid obtained in Example 6 (2.83 g, 5.92 mmol), *O*-(*tert*-butyl)hydroxylamine hydrochloride (2.23 g, 17.76 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.27 g, 11.84 mmol) in anhydrous methylene chloride (25 ml) cooled to 0°C. After the resulting mixture was allowed to warm to room temperature over 1 hour and stirred for an additional 12 hours, the mixture was partitioned between diethyl ether/1 N aqueous hydrochloric acid (300 ml). The acid layer was back extracted using diethyl ether (2 x 100 ml), and the combined ether extracts dried over magnesium sulfate and concentrated. Chromatography over silica gel, and eluting with 25% ethyl acetate/hexanes, gave *N*-(*tert*-butoxy)-2-{4-[4-(4-chlorophenoxy)phenylthiomethyl]-*N*-BOC-piperidin-4-yl}-carboxamide (2.88 g, 89%). ^{1}HNMR ($CDCl_3$) δ 1.31 (s, 9H), 1.45 (s, 9H), 1.58 (m_c, 2H), 2.10 (br d, J = 14.2 Hz, 2H), 3.13 (s, 2H), 3.19 (m_c, 2H), 3.73 (m_c, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.95 (d, J = 8.9 Hz, 2H), 7.30 (d, J = 8.9 Hz, 2H), 7.38 (d, J = 8.7 Hz, 2H), 8.15 (br s, 1H).

9D. Preparation of Ib, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 9A above, but replacing 2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetic acid with other compounds of Formula Ia, the following compounds of Formula Ib were prepared:

N-*tert*-butoxy-2-[4-(4-phenoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-*tert*-butoxy-2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-*tert*-butoxy-2-{4-[4-(4-fluorophenoxy)phenylthio]-*N*-CBZ-piperidin-4-yl}-acetamide;
N-*tert*-butoxy-2-{4-[4-(4-fluorophenoxy)phenylthio]-piperidin-4-yl}-acetamide;
N-*tert*-butoxy-2-[4-(4-phenoxyphenylthio)-piperidin-4-yl)]-acetamide;
N-*tert*-butoxy-2-[4-(3-methoxyphenylthio)-piperidin-4-yl)]-acetamide;
N-*tert*-butoxy-2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-acetamide;
N-*tert*-butoxy-2-benzyl-3-(phenylthio)-propionamide;
N-*tert*-butoxy-3-benzyl-3-(phenylthio)-propionamide;
N-*tert*-butoxy-3-benzyl-3-(4-methoxyphenylthio)-propionamide;
N-*tert*-butoxy-3-benzyl-3-[(4-phenylthiophenyl)thio]-propionamide;
N-*tert*-butoxy-3-benzyl-3-(4-phenoxyphenylthio)-propionamide;
N-*tert*-butoxy-3-benzyl-3-[(4-biphenyl)thio]-propionamide;

N-tert-butoxy-3-benzyl-3-(2-naphthylthio)-propionamide;
N-tert-butoxy-3-benzyl-3-(4-methoxystyrylphenylthio)-propionamide;
N-tert-butoxy-3-cyclopentylmethyl-3-(4-methoxyphenylthio)-propionamide;
N-tert-butoxy-3-cyclopentylmethyl-2-isopropyl-3-(4-methoxyphenylthio)-propionamide;
N-tert-butoxy-3-ethyl-2-methyl-3-(4-methoxyphenylthio)-propionamide;
N-tert-butoxy-3,3-dimethyl-(4-methoxyphenylthio)-propionamide;
N-tert-butoxy-2-[1-(4-methoxyphenylthio)-cyclopent-1-yl]-acetamide;
N-tert-butoxy-2-[1-(4-methoxyphenylthio)-(4-methylcyclohex-1-yl]-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylthio)-cyclohexanone-4-yl]-acetamide ethylene ketal;
N-tert-butoxy-2-[1-(4-phenoxyphenylthio)-cyclohex-1-yl]-acetamide;
N-tert-butoxy-2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-tert-butoxy-2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-acetamide.
N-tert-butoxy-2-{4-[4-(4-fluorophenoxy)phenylthio]-tetrahydropyran-4-yl}-acetamide;
N-tert-butoxy-2-{4-[4-(4-chlorophenoxy)phenylthio]-tetrahydropyran-4-yl]-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylthio)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide;
N-tert-butoxy-4-[4-(4-pyridyloxy)phenylthiomethyl]-tetrahydropyran-carboxamide: ^{1}HNMR ($CDCl_3$) δ 1.31 (s, 9H), 1.70 (m_c, 2H), 2.14 (dm, J = 11.8 Hz, 2H), 3.21 (s, 2H), 3.63 (m_c, 2H), 3.82 (m_c, 2H), 6.84 (d, J =6.4 Hz, 2H), 7.03 (d, J = 8.6 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 8.20 (s, 1H), 8.48 (d, J = 5.8 Hz, 2H).
N-tert-butoxy-4-[4-(5-chloro-2-pyridyloxy)phenylthiomethyl]-tetrahydropyran-carboxamide: mp 100.5-102.7 °C; IR (KBr) 3438 (br), 1657 cm^{-1}; ^{1}HNMR (DMSO-d_6) 1.19 (s, 9H), 1.57 (ddd, J = 13.5, 10.1, 4.0 Hz, 2H), 2.05 (dm, J = 13.5 Hz, 2H), 3.34 (s, 2H), 3.42 (m_c, 2H), 3.65 (dm, J = 11.6 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.41 (d, J = 8.7 Hz, 2H), 7.95 (dd, J = 8.8, 2.7 Hz, 1H), 8.19 (d, J = 2.7 Hz, 1H), 10.37 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 26.66 (q), 33.03 (t), 43.20 (t), 44.25 (s), 64.10 (t), 80.78 (s), 113.00 (d), 121.88 (d), 124.88 (s), 130.43 (d), 132.67 (s), 139.93 (d), 145.51 (d), 151.89 (s), 161.58 (s), 171.64 (s); FABHRMS Calcd. for $C_{22}H_{28}N_2SO_4Cl$ (M^+ + H): 451.1458. Found: 451.1461. Anal. Calcd. for $C_{22}H_{27}N_2SO_4Cl$: C, 58.59; H, 6.03; N, 6.21. Found: C, 58.70; H, 6.05; N, 6.43.
N-tert-butoxy-3-[4-(5-chloro-2-pyridyloxy)phenylthio]-2,2-dimethyl-*N*-hydroxypropionamide: mp 90.8-91.9°C; IR (KBr) 3438 (br), 1651 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.18 (s, 9H), 1.21 (s, 6H), 3.20 (s, 2H), 7.08 (m_c, 3H), 7.40 (d, J = 8.7 Hz, 2H), 7.93 (dd, J = 8.7, 2.7 Hz, 1H), 8.17 (d, J = 2.7 Hz, 1H), 10.17 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.67 (q), 26.48 (q), 42.54 (s), 44.31 (t), 80.62 (s), 112.95 (d), 121.79 (d), 125.28 (s), 130.32 (d), 133.31 (s), 139.86 (d), 145.48 (d), 151.77 (s), 161.58 (s), 173.77 (s); FABHRMS Calcd. for $C_{20}H_{26}N_2SO_3Cl$ (M^+ + H): 409.1353. Found: 409.1354. Anal. Calcd. for $C_{20}H_{25}N_2SO_3Cl$: C, 58.74; H, 6.16; N, 6.85. Found: C, 58.91; H, 6.13; N, 7.07.
N-tert-butoxy-2-(4-methoxyphenylmercapto)-cyclohexane-carboxamide; and
N-tert-butoxy-*trans*-2-(4-methoxyphenylmercapto)-cyclopentanecarboxamide.

9E. Preparation of Ib, varying R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 9A above, but replacing 2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetic acid with other compounds of Formula Ia, other compounds of Formula Ib are prepared.

EXAMPLE 10

Preparation of Compounds of Formula Id

10A. Preparation of Id where n is 0, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Cyclopentyl, and R^5 is 4-Phenoxyphenyl

The *N-tert*-butoxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide was dissolved in trifluoroacetic acid (6 ml) and allowed to stand for 24 hours. The acid was evaporated off under reduced pressure and the product purified by preparative TLC, eluting with 6.5% methanol/methylene chloride gave *N*-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide (100 mg).

10B. Preparation of Id where n is 0, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 10A above, but replacing *N-tert*-butoxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide with other compounds of Formula Ib, the following compounds of Formula Id where n is 0 are prepared:

N-hydroxy-2-[4-(4-phenoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;

N-hydroxy-2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;
2-{4-[4-(4-fluorophenoxy)phenylthio]-*N*-CBZ-piperidin-4-yl}-*N*-hydroxy-acetamide;
2-{4-[4-(4-fluorophenoxy)phenylthio]-piperidin-4-yl}-*N*-hydroxy-acetamide;
3-benzyl-*N*-hydroxy-3-(3-methoxyphenylthio)-propionamide;
N-hydroxy-2-[4-(4-phenoxyphenylthio)-piperidin-4-yl)]-acetamide;
N-hydroxy-2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-acetamide;
2-benzyl-*N*-hydroxy-3-(phenylthio)-propionamide;
3-benzyl-*N*-hydroxy-3-(phenylthio)-propionamide;
3-benzyl-*N*-hydroxy-3-(4-methoxyphenylthio)-propionamide;
3-benzyl-*N*-hydroxy-3-[(4-phenylthiophenyl)thio]-propionamide;
3-benzyl-*N*-hydroxy-3-(4-phenoxyphenylthio)-propionamide;
3-benzyl-*N*-hydroxy-3-[(4-biphenyl)thio]-propionamide;
3-benzyl-*N*-hydroxy-3-(2-naphthylthio)-propionamide;
3-benzyl-*N*-hydroxy-3-(4-methoxystyrylphenylthio)-propionamide;
3-cyclopentylmethyl-*N*-hydroxy-3-(4-methoxyphenylthio)-propionamide;
3-cyclopentylmethyl-*N*-hydroxy-2-isopropyl-3-(4-methoxyphenylthio)-propionamide;
3-ethyl-*N*-hydroxy-2-methyl-3-(4-methoxyphenylthio)-propionamide;
3,3-dimethyl-*N*-hydroxy-(4-methoxyphenylthio)-propionamide;
N-hydroxy-2-[1-(4-methoxyphenylthio)-cyclopent-1-yl]-acetamide;
N-hydroxy-2-[1-(4-methoxyphenylthio)-(4-methylcyclohex-1-yl]-acetamide;
N-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclohex-1-yl]-acetamide;
N-hydroxy-2-[4-(4-methoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-hydroxy-2-[4-(4-methoxyphenylthio)-piperidin-4-yl)]-acetamide;
N-hydroxy-2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetamide; 2-{4-[4-(4-chlorophenoxy)-phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxy-acetamide;
2-{4-[4-(4-fluorophenoxy)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxy-acetamide, m.p. 50-55°C; and
N-hydroxy-2-[4-(4-phenoxyphenylthio)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide.

10C. Preparation of Id where n is 0, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 10A above, but replacing *N-tert*-butoxy-2-[1-(4-phenoxyphenylthio)cyclopent-1-yl]-acetamide with other compounds of Formula Ib, other compounds of Formula Id where n is 0 are prepared.

EXAMPLE 11

Preparation of Compounds of Formula Id

11A. Preparation of Id where n is 1, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Cyclopentyl, and R^5 is 4-Phenoxyphenyl

A solution of *N*-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide (45 mg) in acetone (4 ml) was treated with sodium periodate (260 mg) in water (2 ml). Over the course of 24 hours, two additional portions of sodium periodate (260 mg) were added. After complete disappearance of starting material the solution was diluted with methylene chloride, filtered, dried, and the solvent evaporated under reduced pressure. Preparative TLC on silica gel and elution with 10% methanol/methylene chloride gave *N*-hydroxy-2-[1-(4-phenoxyphenylsulfinyl)-cyclopent-1-yl]-acetamide (15 mg), ^{1}H NMR (CDCl3) 7.64 (d,2H), 7.44 (t,2H), 7.30-7.05 (m,5H), 2.97 (d,1H), 2.53 (d,1H), 2.15-1.65 (m,8H).

11B. Preparation of Id where n is 1, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Tetrahydropyran-4-yl, and R^5 is 4-(4-Fluorophenoxy)-phenyl

2-[4-[4-(4-Fluorophenoxy)phenylthio]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide (500 mg) was dissolved in methanol (25 ml). OXONE (400 mg) in water (5 ml) was added. After stirring for 30 minutes, the mixture was partitioned between methylene chloride and water. Preparative TLC on silica gel and elution with 10% methanol/methylene chloride gave 2-{4-[4-(4-fluorophenoxy)phenyl-sulfinyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide (402 mg, m.p. 120°C).

11C. Preparation of Id where n is 1, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 11A or 11B above, but replacing *N*-hydroxy-2-[1-(4-phenoxyphe-

nylthio)-cyclopent-1-yl]-acetamide with other compounds of Formula Id where n is 0, other compounds of Formula Id where n is 1 are prepared, for example;

N-hydroxy-2-[4-(4-phenoxyphenylsulfinyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-hydroxy-2-[4-(4-phenoxyphenylsulfinyl)-piperidin-4-yl)]-acetamide;
N-hydroxy-2-[4-(4-methoxyphenylsulfinyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;
2-{4-[4-(4-fluorophenoxy)phenylsulfinyl]-piperidin-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-[4-(4-methoxyphenylsulfinyl)-piperidin-4-yl)]-acetamide;
2-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfinyl)-propionamide;
3-benzyl-*N*-hydroxy-3-(3-methoxyphenylsulfinyl)-propionamide;
3-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfinyl)-propionamide;
3-benzyl-*N*-hydroxy-3-[(4-phenylthiophenyl)sulfinyl]-propionamide;
3-benzyl-*N*-hydroxy-3-(4-phenoxyphenylsulfinyl)-propionamide;
3-benzyl-*N*-hydroxy-3-[(4-biphenyl)sulfinyl]-propionamide;
3-benzyl-*N*-hydroxy-3-(2-naphthylsulfinyl)-propionamide;
3-benzyl-*N*-hydroxy-3-(4-methoxystyrylphenylsulfinyl)-propionamide;
3-cyclopentylmethyl-*N*-hydroxy-3-(4-methoxyphenylsulfinyl)-propionamide;
3-cyclopentylmethyl-*N*-hydroxy-2-isopropyl-3-(4-methoxyphenylsulfinyl)-propionamide;
3-ethyl-*N*-hydroxy-2-methyl-3-(4-methoxyphenylsulfinyl)-propionamide;
3,3-dimethyl-*N*-hydroxy-(4-methoxyphenylsulfinyl)-propionamide;
N-hydroxy-2[1-(4-methoxyphenylsulfinyl)-cyclopent-1-yl]-acetamide;
N-hydroxy-2-[1-(4-methoxyphenylsulfinyl)-(4-methylcyclohex-1-yl]-acetamide;
N-hydroxy-2-[1-(4-phenoxyphenylsulfinyl)-cyclohex-1-yl]-acetamide;
N-hydroxy-2-[4-(4-methoxyphenylsulfinyl)-*N*-CBZ-piperidin-4-yl)]-acetamide; and
N-hydroxy-2-[4-(4-methoxyphenylsulfinyl)-piperidin-4-yl)]-acetamide.
N-hydroxy-2-[4-(4-phenoxyphenylsulfinyl)-tetrahydropyran-4-yl]-acetamide;
4-[4-(4-chlorophenoxy)phenylsulfinylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 141.3-142.1 °C; IR (KBr) 3436 (br), 1649 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.67 (dm, J = 13.9 Hz, 1H), 1.79 (dm, J = 13.9 Hz, 1H), 1.97 (dm, J = 13.9 Hz, 1H), 2.24 (dm, J = 13.9 Hz, 1H), 2.97 (d, J = 13.7 Hz, 1H), 3.07 (d, J = 13.7 Hz, 1H), 3.33-3.54 (m_c, 2H), 3.69 (m_c, 2H), 7.12 (d, J = 8.9 Hz, 2H), 7.21 (d, J = 8.8 Hz, 2H), 7.48 (d, J = 8.9 Hz, 2H), 7.66 (d, J = 8.8 Hz, 2H), 8.87 (br s, 1H), 10.76 (s, 1H), ^{13}CNMR (DMSO-d_6) ∂32.43 (t), 33.71 (t), 42.69 (s), 63.65 (t), 67.12 (t), 118.90 (d), 121.07 (d), 126.11 (d), 128.19 (s), 130.07 (d), 139.51 (s), 154.62 (s), 158.72 (s), 169.68 (s); FABHRMS Calcd. for $C_{19}H_{21}NSO_5Cl$ ($M^+ + H$): 410.0829 Found: 426.0825. Anal. Calcd. for $C_{19}H_{20}NSO_5Cl$: C, 55.68; H, 4.92; N, 3.42. Found: C, 55.70; H, 4.93; N, 3.64.
2-{4-[4-(4-chlorophenoxy)-phenylsulfinyl]-tetrahydropyran-4-yl } -*N*-hydroxyacetamide; and
N-hydroxy-2-[4-(4-phenoxyphenylsulfinyl)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide.

EXAMPLE 12

Preparation of Compounds of Formula Id

12A. Preparation of Id where n is 2, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Cyclopentyl, and R^5 is 4-Phenoxyphenyl

A solution of *N*-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide (45 mg) in methanol (4 ml) was treated with a solution of OXONE (260 mg) in water (2 ml). The mixture was stirred for 1 hour, then partitioned between methylene chloride and water. The organic layer was separated, and the solvent removed under reduced pressure. Preparative TLC on silica gel and elution with 10% methanol/methylene chloride gave *N*-hydroxy-2-[1-(4-phenoxyphenylsulfonyl)cyclopent-1-yl]-acetamide (20 mg), m/e = 393 (MNH_4^+, CIMS).

12B. Preparation of Id where n is 2, R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a mechanically stirred suspension of 4-[4-(4-chlorophenoxy)-phenylthiomethyl]tetrahydropyran-4-(N-hydroxycarboxamide) (59.8 g, 151.8 mmol) in 20% tetrahydrofuran-methanol (1570 ml) cooled to 5°C was added dropwise a solution of OXONE (152 g, 247 mmol) in water (1 litre), maintaining an internal temperature of 15-20°C. The mixture was stirred for 5.5 hours, and the mixture then partitioned between 30% ethyl acetate/water (3 litres). The aqueous layer was washed with ethyl acetate (2 x 300 ml), the combined ethyl acetate layers dried over magnesium sulfate, concentrated under reduced pressure, and the residue crystallized from the minimum amount of methylene chloride/hexanes,

to afford analytically pure 4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) as a white powder (54.2 g, 84%). mp 147.7-148.9 °C; IR (KBr) 3429 (br), 1636 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.70 (dm, J = 13.9, 2H), 1.96 (dm, J = 13.9 Hz, 2H), 3.38-3.48 (m, 2H), 3.58-3.68 (m, 2H), 3.58-3.68 (m, 2H), 3.66 (s, 2H), 7.19 (d, J = 8.9 Hz, 2H), 7.19 (d, J = 8.9 Hz, 2H), 7.52 (d, J = 8.9 Hz, 2H), 7.85 (d, J = 8.9 Hz, 2H), 8.68 (d, J = 2.0 Hz, 1H), 10.54 (d, J = 2.0 Hz, 1H), ^{13}CNMR (DMSO-d_6) δ 32.83 (t), 41.70 (s), 61.02 (t), 63.19 (t), 118.01 (d), 121.71 (d), 128.73 (s), 130.08 (d), 130.19 (d), 135.20 (s), 153.83 (s), 160.86 (s), 168.96 (s); FABHRMS Calcd. for $C_{19}H_{20}NSO_6Cl$: 426.0778. Found: 426.0774. Anal. Calcd. for $C_{19}H_{20}NSO_6Cl$: C, 53.59; H, 4.73; N, 3.29. Found: C, 53.58; H, 4.70; N, 3.40.

12C. Preparation of Id where n is 2, R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 is hydrogen, R^4 is Benzyl, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a solution of 3-benzyl-4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid (316 mg, 0.63 mmol) and *N,N*-dimethylformamide catalyst (10 μL) in methylene chloride (6 ml) at 0°C was added oxalyl chloride (200 μL, 2.20 mmol) dropwise over 10 minutes. The mixture was warmed to room temperature over 1 hour, the partial slurry stirred an additional 8 hours, and concentrated *in vacuo* until the theoretical mass of the acid chloride was obtained. This mixture was dissolved in methylene chloride (8 ml), cooled to 0°C, and a neat solution of *N,O*-bis(trimethylsilyl)hydroxylamine (0.56 g, 3.15 mmol) added dropwise over 5 minutes. The mixture was immediately warmed to room temperature, stirred for 48 hours, and recooled to 0°C. To this solution was added aqueous 1M hydrochloric acid (5 ml, 150 mmol), and the solution stirred for an additional 30 minutes, partitioned between ethyl acetae (150 ml) and brine (50 ml). The organic layer was dried over magnesium sulfate, concentrated *in vacuo*, chromatographed over silica gel, eluted with 4% methanol/methylene chloride) to afford 280 mg (86%) of 3-benzyl-4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarbamide) hydroxamic acid. mp 108-113°C; IR (KBr) 3422 (br), 1653 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.76-1.86 (m, 1H), 2.08-2.27 (m, 2H), 2.34 (dm, J = 13.8 Hz, 1H), 2.91 (dd, J = 16.5, 7.2 Hz, 1H), 3.17 (dd, J = 16.4, 4.0 Hz, 1H), 3.19-3.23 (tm, J = 9.0 Hz, 1H), 3.43 (td, J = 11.9, 2.4 Hz, 2H), 6.65-6.72 (m, 2H), 6.76 (d, J = 8.9 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 6.98-7.04 (m, 3H), 7.30 (d, J = 8.9 Hz, 2H), 7.49 (d, J = 8.8 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 31.76 (t), 34.23 (t), 47.30 (s), 64.07 (t), 64.66 (t), 72.68 (d), 117.50 (d), 121.64 (d), 126.47 (d), 127.96 (d), 128.53 (d), 130.31 (d), 130.69 (d), 132.91 (s), 137.83 (s), 153.34 (s), 162.12 (s), 171.30 (s); FABMS (M^+ +H): 516; Anal. Calcd. for $C_{26}H_{26}NSO_6Cl$: C, 60.52; H, 5.08; N, 2.71. Found: C, 60.45; H, 5.10; N, 2.55.

12D. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 12C above, but replacing 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) with other compounds of Formula Iba, the following compounds of Formula Id where n is 2 were prepared:

4-[4-(4-fluorophenoxy)phenylsulfonylmethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 153.1-153.9 °C; IR (KBr) 3434 (br), 1636 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.87 (ddd, J = 13.6, 8.8, 4.0 Hz, 2H), 2.22 (dm, J = 13.6 Hz, 2H), 3.52-3.78 (m, 4H), 7.00-7.16 (m, 6H), 7.84 (d, J = 8.9 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 33.12 (t), 42.19 (s), 62.52 (t), 63.96 (t), 116.88 (dd, J_{C-F} = 21.3 Hz), 117.30 (d), 121.97 (dd, J_{C-F} = 8.4 Hz), 130.18 (s), 134.21 (d), 150.66 (d, J_{C-F} = 2.6 Hz), 159.73 (d, J_{C-F} = 243.8 Hz), 162.61 (s), 169.73 (s); FABMS (M^+ + H): 410. Anal. Calcd. for $C_{19}H_{20}NSO_6F$: C, 55.74; H, 4.92; N, 3.42. Found: C, 55.45; H, 4.91; N, 3.38.

4-[4-(4-bromophenoxy)phenylsulfonylmethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 150.1-151.0 °C; IR (KBr) 3432 (br), 1636 cm^{-1}; ^{1}HNMR ($CDCl_3$; NH and OH not observed) ∂ 1.87 (ddd, J = 13.6, 8.7, 3.9 Hz, 2H), 2.12 (dm, J = 13.6 Hz, 2H), 3.52 (s, 2H), 3.62-3.80 (m, 4H), 6.97 (d, J = 8.8 Hz, 2H), 7.06 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 7.85 (d, J = 8.8 Hz, 2H); ^{13}CNMR ($CDCl_3$) δ 33.10 (t), 42.16 (s), 62.49 (t), 63.93 (t), 117.66 (s), 117.83 (d), 121.93 (d), 130.20 (d), 133.17 (d), 134.61(s), 154.13 (s), 161.79 (s), 169.53 (s); FABHRMS Calcd. for $C_{19}H_{20}NSO_6Br$ (M^+ + H): 470.0273. Found: 470.0268. Anal. Calcd. for $C_{19}H_{20}NSO_6Br$: C, 48.51; H, 4.28; N, 2.98. Found: C, 48.29; H, 4.02; N, 2.94.

3-(4-benzoylphenylsulfonyl)-2,2-dimethyl-N-hydroxypropionamide;

3-[4-(4-chlorophenoxy)phenylsulfonyl]-2,2-dimethyl-*N*-hydroxypropionamide: mp 154.9-156.1 °C; ^{1}HNMR ($CDCl_3$) δ 1.45 (s, 6H), 3.48 (s, 2H), 7.02 (d, J = 8.9 Hz, 2H), 7.04 (d, J = 8.9 Hz, 2H), 7.38 (d, J = 8.9 Hz, 2H), 7.85 (d, J = 8.9 Hz, 2H); FABMS (M^+ +H): 384.0. Anal. Calcd. for $C_{17}H_{18}NSO_5Cl$: C, 53.19; H, 4.73; N, 3.65. Found: C, 52.98; H, 4.69; N, 3.73.

4-(4-phenoxyphenylsulfonylmethyl)-tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 141.8-142.9 °C; IR (KBr) 3432 (br), 1636 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.74 (ddd, J = 13.8, 10.0, 3.9 Hz, 2H), 1.98 (dm, J = 13.8 Hz, 2H), 3.45 (m_c, 2H), 3.64 (m_c, 2H), 3.65 (s, 2H), 7.15 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 7.5 Hz, 2H), 7.47 (t, J = 7.5 Hz, 1H), 7.85 (d, J = 8.8 Hz, 2H), 8.68 (s, 1H), 10.52 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 32.87 (t), 41.76 (s), 61.19 (t), 63.28 (t), 117.71 (d), 119.99 (d), 124.91 (d), 130.04 (d), 130.34 (d), 134.85 (s), 154.85 (s), 161.39 (s), 168.97 (s); FABHRMS Calcd. for $C_{19}H_{22}NSO_6$ (M^+ + H): 392.1168. Found: 392.1162. Anal. Calcd. for $C_{19}H_{21}NSO_6.0.5H_2O$: C,

56.99; H, 5.54; N, 3.50. Found: C, 57.06; H, 5.35; N, 3.93.

4-[4-(4-thiophen-2-yl)phenoxyphenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 172.2-176.5 °C; IR (KBr) 3428 (br), 1636 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.72 (dm, *J* = 14.5 Hz, 2H), 1.99 (dm, *J* = 14.5 Hz, 2H), 3.46 (m_c, 2H), 3.65 (m_c, 2H), 3.66 (s, 2H), 7.14 (dd, *J* = 4.9, 3.6 Hz,1H), 7.19 (d, *J* = 8.7 Hz, 2H), 7.20 (d, *J* = 8.9 Hz, 2H), 7.48 (dd, *J* = 3.6, 1.2 Hz, 1H), 7.52 (dd, *J* = 4.9, 1.2 Hz, 1H), 7.73 (d, *J* = 8.8 Hz, 2H), 7.86 (d, *J* = 8.8 Hz, 2H), 8.68 (s, 1H), 12.58 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 32.89 (t), 41.78 (s), 61.20 (t), 63.28 (t), 117.88 (d), 120.55 (d), 123.66 (d), 125.56 (d), 127.34 (d), 128.45 (d), 130.07 (d), 130.62 (s), 135.04 (s), 142.45 (s), 154.30 (s), 161.16 (s), 169.03 (s); FABHRMS Calcd. for $C_{23}H_{24}NS_2O_6$ (M^+ + H): 474.1045. Found: 474.1050. Anal. Calcd. for $C_{23}H_{23}NS_2O_6$: C, 58.33; H, 4.90; N, 3.00. Found: C, 58.18; H, 4.84; N, 3.19.

4-[4-(4-thiophen-3-yl)phenoxyphenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 183.5-184.4 °C; IR (KBr) 3432 (br), 1636 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.72 (m_c, 2H), 1.98 (m_c, 2H), 3.48 (m_c, 2H), 3.65 (m_c, 4H), 7.18 (m_c, 4H), 7.55 (dd, *J* = 5.1 Hz, 1H), 7.62 (d, *J* = 4.9, 3.7 Hz, 2H), 7.80 (d, *J* = 8.6 Hz, 2H), 7.86 (m_c, 3H), 8.69 (s, 1H), 10.58 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 32.88 (t), 41.79 (s), 61.19 (t), 63.28 (t), 117.71 (d), 120.42 (d), 120.81 (d), 126.09 (d), 127.10 (d), 127.97 (d), 130.06 (d), 132.10 (s), 134.89 (s), 140.54 (s), 153.86 (s), 168.85 (s); FAB-HRMS Calcd. for $C_{23}H_{24}NS_2O_6$ (M^+ + H): 474.1045. Found: 474.1049. Anal. Calcd. for $C_{23}H_{23}NS_2O_6.0.75H_2O$: C, 56.72; H, 5.07; N, 2.88. Found: C, 56.74; H, 4.78; N, 3.22.

3,3-dimethyl-3-[(4-chlorophenoxy)phenylsulfonyl]-*N*-hydroxypropionamide;

{4-[4-(4-benzo[b]thiophen-2-yl-phenoxy)phenylsulfonyl)-tetrahydropyran-4-yl]-*N*-hydroxyacetamide;

2-{4-[4-(phenylmethyl)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;

2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]tetrahydropyran-4-yl}-*N*-hydroxyacetamide; and

2-{4-[4-(4-bromophenoxy)phenylsulfonyl]tetrahydropyran-4-yl}-*N*-hydroxyacetamide.

12E. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 12A or 12B above, but replacing *N*-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide with other compounds of Formula Id where n is 0, the following compounds of Formula Id where n is 2 are prepared, for example;

4-(4-phenoxyphenylsulfonylmethyl)tetrahydropyran-4-(*N*-hydroxycarboxamide);

4-[4-(4-fluorophenoxy)phenylsulfonylmethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]piperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-methylpiperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-cyclopropylmethylpiperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-acetylpiperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-(3-pyridyl)-piperidine-4-(*N*-hydroxycarboxamide);

4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-(3-pyridoyl)-piperidine-4-(*N*-hydroxycarboxamide);

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;

N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;

2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-*N*-CBZ-piperidin-4-yl}-*N*-hydroxyacetamide;

2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;

N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide;

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide;

2-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide;

3-benzyl-*N*-hydroxy-3-(3-methoxyphenylsulfonyl)-propionamide;

3-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide;

3-benzyl-*N*-hydroxy-3-[(4-phenylthiophenyl)sulfonyl]-propionamide;

3-benzyl-*N*-hydroxy-3-(phenylsulfonyl)-propionamide;

3-benzyl-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide;

3-benzyl-3-[(4-biphenyl)sulfonyl]-*N*-hydroxypropionamide;

3-benzyl-*N*-hydroxy-3-(2-naphthylsulfonyl)-propionamide;

3-benzyl-*N*-hydroxy-3-(4-methoxystyrylphenylsulfonyl)-propionamide;

3-(cyclopentylmethyl)-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide;

3-(cyclopentylmethyl)-*N*-hydroxy-2-isopropyl-3-(4-methoxyphenyl-sulfonyl)-propionamide;

3-ethyl-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-2-methylpropionamide;

3,3-dimethyl-*N*-hydroxy-(4-methoxyphenylsulfonyl)-propionamide;

N-hydroxy-2-[1-(4-methoxyphenylsulfonyl)-cyclopent-1-yl]-acetamide;

N-hydroxy-2-[1-(4-methoxyphenylsulfonyl)-(4-methylcyclohex-1-yl]-acetamide;

N-hydroxy-2-[1-(4-phenoxyphenylsulfonyl)-cyclohex-1-yl]-acetamide;

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide;

2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-N-hydroxyacetamide;

2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide; and

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide.

12F. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 12A above, but replacing *N*-hydroxy-2-[1-(4-phenoxyphenylthio)-cyclopent-1-yl]-acetamide with other compounds of Formula Id where n is 0, other compounds of Formula Id where n is 2 are prepared.

EXAMPLE 13

Preparation of Compounds of Formula I where Y is *tert*-BuONH-

13A. Preparation of Ic where n is 2, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Tetrahydropyran, and R^5 is 4-Phenoxyphenyl

To a cooled solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetamide (14.1 g, 33.9 mmol) in methanol (340 ml) was added a solution of OXONE (33.9 g) in water (170 ml). The reaction mixture was stirred for 5 hours at room temperature, concentrated to half the original volume under reduced pressure, and the residue then partitioned between ethyl acetate and water. The solvent was removed from the ethyl acetate extracts under reduced pressure. The residue chromatographed on silica gel, eluting with 10% methanol/methylene chloride, to give *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide as a white foam.

13B. Preparation of Ic where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached are *N*-BOC-Piperidine, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylthiomethyl)-*N*-BOC-piperidin-4-yl]-carboxamide (4.96 g, 9.03 mmol) in anhydrous methylene chloride (70 ml) cooled to 0°C, was added 60% 3-chloroperoxybenzoic acid (4.96 g). After the resulting mixture was allowed to warm to room temperature over 30 minutes and stirred for 5 minutes, 13.6M aqueous methyl sulfide (1 ml, 13.62 mmol) was added in one portion. The mixture was stirred 10 minutes, partitioned with saturated aqueous sodium bicarbonate (2 x 50 ml), dried over magnesium sulfate, and concentrated *in vacuo*. Chromatography over silica gel, and eluting with 25% ethyl acetate/hexanes, gave *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-*N*-BOC-piperidin-4-yl]-carboxamide as a white foam (4.70 g, 90%). ^{1}HNMR ($CDCl_3$) δ 1.31 (s, 9H), 1.46 (s, 9H), 1.59 (m_c, 2H), 2.18 (m_c, 2H), 3.42 (m_c, 2H), 3.45 (s, 2H), 3.62 (m_c, 2H), 7.01 (d, J = 8.9 Hz, 2H), 7.04 (d, J = 8.8 Hz, 2H), 7.38 (d, J = 8.8 Hz, 2H), 7.84 (d, J = 8.8 Hz, 2H), 8.44 (br s, 1H).

13C. Preparation of Ic where n is 2 and Y is *tert*-BuONH-, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 13B above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylthiomethyl)-*N*-BOC-piperidin-4-yl]-carboxamide with other compounds of Formula Ib, the following compound of Formula Ic where n is 2 and Y is *tert*-BuONH- was prepared:

N-tert-butoxy-4-[4-(4-pyridyloxy)phenylsulfonylmethyl]-tetrahydropyran-carboxamide: IR (KBr) 3434, 1684 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.33 (s, 9H), 2.01 (m_c, 2H), 2.24 (m_c, 2H), 3.55 (s, 2H), 3.79 (m_c, 4H), 6.93 (d, J =6.3 Hz, 2H), 7.22 (d, J = 8.8 Hz, 2H), 7.96 (d, J = 8.8 Hz, 2H), 8.38 (s, 1H), 8.57 (d, J = 6.3 Hz, 2H); FABHRMS Calcd. for $C_{22}H_{28}N_2SO_6$ (M^+ + H) 449.1746. Found: 449.1757.

N-tert-butoxy-4-[4-(5-chloro-2-pyridyloxy)phenylsulfonylmethyl]-tetrahydropyran-carboxamide: mp (broad) 100.8-135.8 °C; IR (KBr) 3436 (br), 1684 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.20 (s, 9H), 1.72 (m_c, 2H), 2.03 (m_c, 2H), 3.48 (m_c, 2H), 3.67 (m_c, 2H), 3.76 (s, 2H), 7.23 (dd, J = 8.8, 0.5 Hz, 1H), 7.41 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.8 Hz, 2H), 8.03 (dd, J = 8.8, 2.7 Hz, 1H), 8.25 (dd, J = 2.7, 0.5 Hz, 1H), 8.30 (s, 1H), 10.32 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 26.66 (q), 33.09 (t), 42.37 (s), 61.03 (t), 63.36 (t), 80.64 (s), 113.89 (d), 121.38 (d), 126.33 (s), 129.53 (d), 137.00 (s), 140.34 (d), 145.74 (d), 157.87 (s), 160.66 (s), 171.25 (s); FABHRMS Calcd. for $C_{22}H_{28}N_2SO_6Cl$ (M^+ + H): 483.1357. Found: 483.1354. Anal. Calcd. for $C_{22}H_{27}N_2SO_6Cl$: C, 54.71; H, 5.63; N, 5.80. Found: C, 54.46; H, 5.60; N, 5.98.

N-tert-butoxy-3-[4-(5-chloro-2-pyridyloxy)phenylsulfonyl]-2,2-dimethyl-propionamide: mp (broad) 64.5-70.5 °C; ^{1}HNMR (DMSO-d_6) δ 1.19 (s, 9H), 1.29 (s, 6H), 3.65 (s, 2H), 7.24 (d, J = 8.7 Hz, 1H), 7.41 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.8 Hz, 2H), 8.04 (dd, J = 8.8, 2.7 Hz, 1H), 8.26 (d, J = 2.7 Hz, 1H), 10.17 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 25.01 (q), 26.47 (q), 40.74 (s), 63.03 (t), 80.79 (s), 113.91 (d), 121.38 (d), 126.32 (s), 129.35 (d), 130.66 (s), 140.36

(d), 145.75 (d), 157.72 (s), 160.68 (s), 173.14 (s); FABHRMS Calcd. for $C_{20}H_{26}N_2SO_5Cl$ (M^+ + H): 441.1251. Found: 441.1248. Anal. Calcd. for $C_{20}H_{25}N_2SO_5Cl$: C, 54.48; H, 5.71; N, 6.35. Found: C, 54.37; H, 5.69; N, 6.57.

13D. Preparation of Ic where n is 2 and Y is *tert*-BuONH-, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 13A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylthio)-tetrahydropyran-4-yl]-acetamide with other compounds of Formula Ib, the following compounds of Formula Ic where n is 2 and Y is *tert*-BuONH-were prepared;

N-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-tert-butoxy-2-[4-(4-methoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide;
N-tert-butoxy-2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-piperidin-4-yl}-acetamide;
N-tert-butoxy-2-[4-(4-methoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide;
2-benzyl-*N-tert*-butoxy-3-(4-methoxyphenylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-(3-methoxyphenylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-(4-methoxyphenylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-[(4-phenylthiophenyl)sulfonyl]-propionamide;
3-benzyl-*N-tert*-butoxy-3-(phenylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-[(4-biphenyl)sulfonyl]-propionamide;
3-benzyl-*N-tert*-butoxy-3-(2-naphthylsulfonyl)-propionamide;
3-benzyl-*N-tert*-butoxy-3-(4-methoxystyrylphenylsulfonyl)-propionamide;
N-tert-butoxy-3-(cyclopentylmethyl)-3-(4-methoxyphenylsulfonyl)-propionamide;
N-tert-butoxy-3-(cyclopentylmethyl)-2-isopropyl-3-(4-methoxyphenylsulfonyl)-propionamide;
N-tert-butoxy-3-ethyl-2-methyl-3-(4-methoxyphenylsulfonyl)-propionamide;
N-tert-butoxy-3,3-dimethyl-(4-methoxyphenylsulfonyl)-propionamide;
N-tert-butoxy-2-[1-(4-methoxyphenylsulfonyl)-cyclopent-1-yl]-acetamide;
N-tert-butoxy-2-[1-(4-methoxyphenylsulfonyl)-(4-methylcyclohex-1-yl]-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-cyclohexanone-4-yl]-acetamide ethylene ketal;
N-tert-butoxy-2-[1-(4-phenoxyphenylsulfonyl)-cyclohex-1-yl]-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide;
N-tert-butoxy-2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-acetamide;
N-tert-butoxy-2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-acetamide;
N-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide;
N-tert-butoxy-2-(4-methoxyphenylsulfonyl)-cyclohexanecarboxamide; and
N-tert-butoxy-*trans*-2-(4-methoxyphenylsulfonyl)-cyclopentanecarboxamide.

13E. Preparation of Ic where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 13A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylthio)-*N*-CBZ-piperidin-4-yl)]-acetamide with other compounds of Formula Ib, other compounds of Formula Ic where n is 2 and Y is *tert*-BuONH- are prepared.

EXAMPLE 14

Preparation of Compounds of Formula Ic where Y is *tert*-BuONH-

14A. Preparation of Ic where n is 2, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Piperidine and R^5 is 4-Phenoxyphenyl

To a solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide (1.2 g, 2.1 mmol) in ethanol (21 ml) was added 10% palladium on carbon (1 g) and ammonium formate (6.7 g), and the mixture refluxed for 1 hour. The mixture was filtered through Celite, the filter cake washed with ethanol (150 ml) followed by 10% methanol in methylene chloride (150 ml). Solvent was removed from the filtrate under reduced pressure and the residue was dissolved in hot ethyl acetate. Filtration, concentration of the filtrate, followed by silica gel chromatography and elution with 10% methanol/methylene chloride gave *N*-tert-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide as a colorless oil.

14B. Preparation of Ic where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 14A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide with other *N*-CBZ protected compounds of Formula I, other compounds of Formula I where n is 2 and Y is *tert*-BuONH- are prepared.

EXAMPLE 15

Preparation of Compounds of Formula Id where Y is HONH-

15A. Preparation of Id where n is 2, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Piperidine, and R^5 is 4-Phenoxyphenyl

A solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperid-4-yl)]-acetamide (27 mg, 0.05 mmol) in dichloroethane (2 ml) was cooled to -20°C, and saturated with hydrochloric acid gas for 30 minutes. The reaction vessel was then sealed and the solution stirred for two days at 25°C. Solvent was removed from the reaction mixture under reduced pressure, and the residue dissolved in 50% methanol in methylene chloride. Addition of hexane precipitated *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide, m/e = 391 (MH^+, FAB).

15B. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 15A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide with other compounds of Formula Ic where Y is *tert*-BuONH-, the following compounds of Formula Id where n is 2 and Y is HONH- were prepared:

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide, m/e = 525 (MH^+);
N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-*N*-CBZ-piperidin-4-yl)]-acetamide, m/e = 463 (MH^+, FAB);
2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide, m.p. 196-197°C;
2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide, m.p. 200-201°C;
2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide: mp 135.7-136.1 °C; ^{1}HNMR ($CDCl_3$) δ 1.60 (m_c, 2H), 1.83 (m_c, 2H), 3.00 (s, 2H), 3.66 (m_c, 2H), 3.88 (m_c, 2H), 7.06 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 8.9 Hz, 2H), 7.79 (d, J = 8.9 Hz, 2H), 7.25 (s, 1H), 9.49 (s, 1H); FABHRMS Calcd. for $C_{19}H_{20}NSO_6Cl$ (M^+ + H): 426.0778. Found: 426.0775. Anal. Calcd. for $C_{19}H_{20}NSO_6Cl$: C, 53.59; H, 4.73; N, 3.29. Found: C, 53.30; H, 4.67; N, 3.35.
2-[4-(4-cyclohexyloxyphenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide: m.p. 77-78°C;
N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide, m/e = 329 (MH^+);
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide, m/e = 391 (MH^+);
2-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide, m/e = 350.2 (MH^+);
3-benzyl-*N*-hydroxy-3-(3-methoxyphenylsulfonyl)-propionamide, m/e = 350.2 (MH^+);
3-benzyl-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide, m/e = 350.2 (MH^+);
3-benzyl-*N*-hydroxy-3-[(4-phenylthiophenyl)sulfonyl]-propionamide, m/e = 427 (MH^+);
3-benzyl-*N*-hydroxy-3-(phenylsulfonyl)-propionamide, m/e = 320 (MH^+);
3-benzyl-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide, m/e = 412.2 (MH^+);
3-benzyl-*N*-hydroxy-3-[(4-biphenyl)sulfonyl]-propionamide; m/e = 395 (MH^+);
3-benzyl-*N*-hydroxy-3-(2-naphthylsulfonyl)-propionamide, m/e = 370.1 (MH^+);
3-benzyl-*N*-hydroxy-3-[(4-methoxystyrylphenylsulfonyl]-propionamide, m/e = 452.2 (MH^+);
3-(cyclopentylmethyl)-*N*-hydroxy-3-(4-methoxyphenylsulfonyl)-propionamide, m/e = 342 (MH^+);
3-(cyclopentylmethyl)-*N*-hydroxy-2-isopropyl-3-(4-methoxyphenylsulfonyl)-propionamide;
3-ethyl-*N*-hydroxy-2-methyl-3-(4-methoxyphenylsulfonyl)-propionamide, m/e = 301 (MH^+);
3,3-dimethyl-3-(4-methoxyphenylsulfonyl)-*N*-hydroxypropionamide, elemental analysis: C_1H_1N;
N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-cyclopent-1-yl]-acetamide, m/e = 313 (MH^+);
N-hydroxy-2-[4-(4-methoxyphenylsulfonyl)-(4-methylcyclohex-1-yl]-acetamide, m/e = 341 (MH^+);
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)cyclohex-1-yl]-acetamide, m/e = 389 (MH^+);
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide, m.p. 88.5-90°C, m/e = 391 (MH^+);
2-{ 4-[4-(4-chlorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide;
2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide, m.p. 91-95°C;
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)tetrahydrothiopyran-1,1-dioxide-4-yl]-acetamide, m/e = 440.1 (MH^+);
N-hydroxy-*trans*-2-(4-methoxyphenylsulfonyl)-cyclopentanecarboxamide, m/e = 313 (MH^+);
N-hydroxy-*trans*-2-(4-methoxyphenylsulfonyl)-cyclohexanecarboxamide, m/e = 327 (MH^+); and

2-benzyl-*N*-hydroxy-*trans*-2-(4-methoxyphenylsulfonyl)-cyclopentane-carboxamide, m/e = 390 (MH^+, FABMS).

15C. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 15A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide with other compounds of Formula Ic where Y is *tert*-BuONH-, other compounds of Formula Id where n is 2 and Y is HONH-are prepared, for example:

2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-*N*-CBZ-piperidin-4-yl]-*N*-hydroxyacetamide;
2-{1-methyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide;
N-hydroxy-2-{1-methyl-4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-acetamide; and
2-{4-[4-(4-bromophenoxy)-phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide.

15D. Preparation of Id where n is 2, R^1 and R^2 are Hydrogen, R^3 and R^4 when taken together with the Carbon to which they are attached are Cyclohexanone, and R^5 is 4-Phenoxyphenyl

Following the procedure outlined in Example 15A, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-cyclohexanone-4-yl]-acetamide ethylene ketal (400 mg) was prepared from the corresponding *N-tert*-butoxy precursor. The above product was dissolved in a 1:1 mixture of acetone and 1M hydrochloric acid (40 ml) and stirred at room temperature for 18 hours. The reaction was concentrated under reduced pressure and extracted with ethyl acetate. Silica gel chromatography using 10% methanol/methylene chloride gave 2-[4-(4-phenoxyphenylsulfonyl)cyclohexanone-4-yl]-*N*-hydroxyacetamide as a white solid: m.p. 106°C (dec), m/e = 404 (MH^+, FABMS).

15E. Preparation of Id where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached are Piperidine, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a sealed tube containing the free base *N-tert*-butoxy-2-{4-[4-(4-phenoxy)phenylsulfonylmethyl]-piperidin-4-yl}-carboxamide (780 mg, 1.62 mmol) in 1,2-dichloroethane (35 ml) at -30°C, was bubbled in gaseous hydrochloric acid until the saturation point was reached. The reaction vessel was then sealed and the solution stirred for two days. After the vessel was recooled to -30°C and opened, a stream of nitrogen gas bubbled through the solution, which was then warmed to room temperature. The mixture was concentrated to afford 2-{4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-piperidin-4-yl}-*N*-hydroxycarboxamide (747 mg, 100%). mp 166.7-176.2°C; ^{1}HNMR (CD_3OD) δ 2.39 (m_c, 2H), 3.12 (m_c, 2H), 3.36 (m_c, 2H), 3.63 (s, 2H), 7.12 (d, *J* = 8.9 Hz, 2H), 7.15 (d, *J* = 8.9 Hz, 2H), 7.44 (d, *J* = 9.0 Hz, 2H), 7.89 (d, *J* = 8.9 Hz, 2H); FABMS (M^+ +H): 425.0; Anal. Calcd. for $C_{19}H_{21}N_2SO_5Cl.HCl.1.5\,H_2O$: C, 46.73; H, 4.33; N, 5.74. Found: C, 46.83; H, 4.66; N, 5.71.

15F. Preparation of Id where n is 2, varying R^1, R^2, R^3, R^4, and R^5

Similarly, following the procedures of Example 15E above, but replacing *N-tert*-butoxy-2-{4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-piperidin-4-yl)}-carboxamide with other compounds of Formula Ic where Y is *tert*-BuONH-, other compounds of Formula Id where n is 2 and Y is HONH- were prepared, for example:

2-{4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-1-(cyclopropylmethyl)piperidin-4-yl}-*N*-hydroxycarboxamide hydrochloride (1.30 g, 84%). mp 120.5-124.0 °C; IR (KBr) 3429 (br), 1582 cm^{-1}; ^{1}HNMR (CD_3OD) δ 0.40-0.50 (m, 2H), 0.73-0.81 (m, 2H), 1.12 (m_c, 1H), 2.18 (m_c, 2H), 2.41 (d, *J* = 14.8 Hz, 2H), 2.63 (d, *J* = 14.3 Hz, 2H), 3.03 (m_c, 2H), 3.10 (m_c, 2H), 3.60 (m_c, 3H), 7.13 (m_c, 4H), 7.43 (d, *J* = 8.7 Hz, 2H), 7.89 (d, *J* = 8.8 Hz, 2H), 7.93 (d, *J* = 8.8 Hz, 2H); FABMS (M^+ +H): 479.1. Anal. Calcd. for $C_{23}H_{27}N_2SO_5Cl.HCl.H_2O$: C, 51.77; H, 5.09; N, 5.25. Found: C, 51.90; H, 5.53; N, 5.26.

2-{4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-*N*-hydroxy-1-nicotinoylmethylpiperidin-4-yl}-carboxamide hydrochloride (590 mg, 89%). mp 160.5 °C (effervescence); IR (KBr) 3426 (br), 1638 cm^{-1}; ^{1}HNMR (CD_3OD) δ 1.97 (m_c, 2H), 2.25 (m_c, 2H), 3.55 (m_c, 4H), 3.64 (s, 2H), 7.10 (d, *J* = 8.9 Hz, 2H), 7.13 (d, *J* = 8.7 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 2H), 8.12 (m_c, 1H), 8.61 (d, *J* = 7.9 Hz, 2H), 8.92 (d, *J* = 5.5 Hz, 2H), 8.98 (br s, 1H); FABMS (M^+ +H): 530.0. Anal. Calcd. for $C_{25}H_{29}N_3SO_6Cl.HCl.0.5H_2O$: C, 51.38; H, 4.14; N, 7.19. Found: C, 51.80; H, 4.46; N, 7.25.

2-{4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-*N*-hydroxy-1-methansulfonylpiperidin-4-yl}-carboxamide hydrochloride (682 mg, 69%). mp 107.3-112.3 °C; ^{1}HNMR ($CDCl_3$) δ 1.95 (m_c, 2H), 2.40 (m_c, 2H), 2.79 (s, 3H), 3.12 (m_c, 2H), 3.42 (s, 2H), 3.51 (m_c, 2H), 7.01 (d, *J* = 8.9 Hz, 2H), 7.07 (d, *J* = 8.9 Hz, 2H), 7.39 (d, *J* = 8.9 Hz, 2H), 7.83 (d, *J* = 8.9 Hz, 2H); FABMS (M^+ +H): 503.2. Anal. Calcd. for $C_{20}H_{23}N_2S_2O_7Cl$: C, 47.76; H, 4.61; N, 5.57. Found: C, 47.32; H, 4.56; N, 5.52.

4-[4-(4-pyridyloxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) hydrochloride: mp 188-

197°C; IR (KBr) 3431, 1638 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.73 (m_c, 2H), 2.01 (dm, J =14.7 Hz, 2H), 3.43 (m_c, 2H), 3.65 (m_c, 2H), 3.78 (s, 2H), 7.56 (m_c, 4H), 8.02 (d, J = 8.7 Hz, 2H), 8.82 (d, J = 6.6 Hz, 2H), 10.64 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 33.01 (t), 39.78 (t), 61.13 (s), 63.26 (t), 114.48 (d), 121.81 (d), 130.87 (d), 138.41 (s), 144.92 (d), 156.14 (s), 168.4 (s), 168.8 (s); Anal. Calcd. for $C_{18}H_{21}N_2SO_6Cl.HCl.0.6\ H_2O$: C, 49.17; H, 5.09; N, 6.37. Found: C, 49.16; H, 5.03; N, 6.27.

4-[4-(5-chloro-2-pyridyloxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide): mp 141.9-142.7°C; IR (KBr) 3432, 1636 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.73 (m_c, 2H), 2.01 (dm, J = 14.7 Hz, 2H), 3.33 (s, 2H), 3.46 (m_c, 2H), 3.64 (m_c, 2H), 7.23 (dd, J = 8.7, 0.4 Hz, 2H), 7.40 (d, J = 8.8 Hz, 2H), 7.92 (d, J = 8.8 Hz, 2H), 8.03 (d, J = 8.7, 2.7 Hz, 2H), 8.26 (dd, J = 2.7, 0.4 Hz, 1H), 8.69 (s, 1H), 10.62 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 32.89 (t), 41.81 (s), 60.96 (t), 63.26 (t), 113.88 (d), 121.32 (d), 126.31 (s), 129.58 (d), 136.93 (s), 140.33 (s), 145.74 (d), 157.82 (s), 160.69 (s), 169.02 (s); FABHRMS Calcd. for $C_{18}H_{19}N_2SO_6Cl$ (M^+ + H): 427.0731. Found: 427.0726. Anal. Calcd. for $C_{18}H_{19}N_2SO_6C1.0.5H_2O$: C, 49.49; H, 4.61; N, 6.41. Found: C, 49.54; H, 4.35; N, 6.47.

3-[4-(5-chloro-2-pyridyloxy)phenylsulfonyl]-2,2-dimethyl-*N*-hydroxypropionamide: mp 115.8-116.6 °C; IR (KBr) 3412 (br), 1644 cm^{-1}; ^{1}HNMR (CD_3OD) δ 1.38 (s, 6H), 3.58 (s, 2H), 7.13 (d, J = 8.7 Hz, 1H), 7.34 (d, J = 8.8 Hz, 2H), 7.89 (dd, J = 8.7, 2.7 Hz, 2H), 7.95 (d, J = 8.8 Hz, 1H), 8.15 (d, J = 2.5 Hz, 1H); ^{13}C NMR (CD_3OD) δ 25.55 (q), 41.76 (s), 65.06 (t), 114.91 (d), 122.35 (d), 128.40 (s), 130.98 (d), 138.21 (s), 141.44 (d), 146.88 (d), 159.89 (s), 162.32 (s), 174.51 (s); FABHRMS Calcd. for $C_{16}H_{18}N_2SO_5Cl$ (M^+ + H): 385.0625. Found: 383.0625. Anal. Calcd. for $C_{16}H_{17}N_2SO_5Cl$: C, 49.94; H, 4.48; N, 7.28. Found: C, 49.58; H, 4.42; N, 7.30.

15G. Preparation of Id where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached are 1-Picolylpiperidine, and R^5 is 4-(4-Chlorophenoxy)-phenyl

A solution containing *N-tert*-butoxy-2-{4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-1-picolylpiperidin-4-yl}-carboxamide (324 mg, 0.566 mmol) in trifluoroacetic acid (5 ml) was heated to 30°C for 1.5 hours, cooled to room temperature, and concentrated *in vacuo*. The residue was dissolved in ethyl acetate (100 ml), washed with saturated sodium bicarbonate (2 x 30 ml), dried over magnesium sulfate, and concentrated *in vacuo*. Chromatography over silica gel, eluting with 6% methanol/methylene chloride, yielded 2-{4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-1-picolylpiperidin-4-yl}-*N*- hydroxycarboxamide hydrochloride: mp 222.5-223.9°C; IR (KBr) 3436 (br), 1645 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 2.15 (m_c, 3H), 2.40 (m_c, 2H), 3.32 (m_c, 2H), 3.57 (m_c, 2H), 3.97 (m_c, 2H), 4.44 (m_c, 2H), 4.51 (m_c, 2H), 7.19 (m_c, 4H), 7.50 (d, J = 8.8 Hz, 2H), 7.87 (m_c, 3H), 8.49 (m_c, 1H), 8.85 (m_c, 1H), 8.99 (br s, 1H); FABMS (M^+ +H): 516.1. Anal. Calcd. for $C_{29}H_{34}N_3SO_5Cl.2HCl.0.5\ H_2O$: C, 50.22; H, 4.89; N, 7.03. Found: C, 50.17; H, 4.65; N, 7.00.

EXAMPLE 16

Preparation of Compounds of Formula Ih

16A. Preparation of Ie where R^1 , R^2 and R^3 are Hydrogen, and R^4 is Benzyl

To a cooled solution of 3-benzyl-3-(4-bromophenylthio)-propionic acid in methanol (50 ml) was added a solution of OXONE (8 g) in water (50 ml). The reaction mixture was stirred for 2 hours at room temperature, and then partitioned between methylene chloride and water. The solvent was removed from the organic layer under reduced pressure, to give 3-benzyl-3-(4-bromophenylsulfonyl)-propionic acid, as a crystalline solid.

16B. Preparation of If where R^1 , R^2 and R^3 are Hydrogen, and R^4 is Benzyl

1. A solution of 3-(4-bromophenyl)sulfonyl-4-benzylpropionic acid (200 mg, 0.52 mmol), phenylboronic acid (127 mg, 1.04 mmol), and tetrakis(triphenylphospine)palladium(0) (24 mg, 0.021 mmol) in a 1:1 mixture of ethanol and benzene (5 ml) was heated to reflux temperature with stirring. A solution of 2M sodium carbonate (1 ml) was added to the reaction mixture, and stirring continued at reflux for approximately 2 hours. The mixture was cooled and then partitioned between ethyl acetate and water. The solvent layer was washed with brine, dried over magnesium sulfate, filtered, and solvent removed under reduced pressure. The residue was chromatographed, eluting with 7% methanol/methylene chloride, to yield 3-(4-biphenyl)-sulfonyl-4-benzylpropionic acid. ^{1}HNMR ($CDCl_3$): 7.75 ppm (m, 14H); 3.42 ppm (dd, 1H); 2.82 ppm (dd, 1H); 2.77 ppm (dd, 1H); 2.51 ppm (dd, 1H).

16C. Preparation of Ih where R^1, R^2, and R^3 are Hydrogen and R^4 is Benzyl

The 3-(4-biphenyl)sulfonyl-4-benzylpropionic acid, prepared as shown above, was then converted to 3-(4-biphenyl)sulfonyl-4-benzyl-*N*-hydroxypropionamide, m.p. 65°C (shrinks with decomposition) as described in Examples 10A.

16D. Preparation of Ifb where R^1 and R^2 Together with the Carbon to which they are attached represent Tetrahydropyran-4-yl, R^3 and R^4 are Hydrogen, R^5 is 4-(Thiophen-2-yl)phenoxyphenyl

1. To a mechanically stirred suspension of 4-[4-(4-bromophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid (5.50 g, 13.0 mmol) in 20% tetrahydrofuran/methanol (135 ml) cooled to 15°C, was added a solution of OXONE (13.0 g, 21.2 mmol) in water (86 ml) dropwise, maintaining an internal temperature of 15-20°C. The mixture was stirred for 12 hours and dissolved in 40% ethyl acetate/water (1200 ml). The layers were partitioned, and the water layer back extracted using ethyl acetate (2 x 300 ml). The combined ethyl acetate layers were dried ($MgSO_4$), concentrated, and the residue crystallized from the minimum amount of methylene chloride/hexanes to afford 4-[4-(4-bromophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid as a white powder, which was used without further purification (5.00 g, 84%).

2. To a solution of 4-[4-(4-bromophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid (1.10 g, 2.42 mmol) of in *N, N*-dimethylformamide (15 ml) was added tetrakis(triphenylphosphine)-palladium(0) (108 mg), 2-thiophene boronic acid (857 mg, 6.70 mmol), followed by 2M aqueous sodium carbonate (2.7 ml, 5.4 mmol). The reaction was heated to reflux for 10 hours, cooled to room temperature, and the mixture partitioned between methylene chloride (100 ml) and 1N aqueous hydrochloric acid (20 ml). The aqueous layer was back extracted with methylene chloride (100 ml), and the combined organic layers dried ($MgSO_4$), the residue chromatographed over 100 g of silica gel (eluted with methylene chloride to 10% methanol/methylene chloride), and the resulting foam crystallized from the minimum amount of methylene chloride/hexanes to afford 4-[4-(4-(thiophen-2-yl)phenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid (1.04 g, 94%). mp 181.2-193.3°C; IR (KBr) 3432 (br), 1718.9 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.67 (ddd, J = 13.8, 9.4, 4.0 Hz, 2H), 1.95 (dm, J = 13.8 Hz, 2H), 3.47 (m_c, 2H), 3.67 (m_c, 2H), 3.68 (s, 2H), 7.14 (dd, J = 4.9, 3.6 Hz, 1H), 7.20 (d, J = 8.8 Hz, 2H), 7.22 (d, J = 8.9 Hz, 2H), 7.50 (dd, J = 3.6, 1.2 Hz, 1H), 7.54 (dd, J = 4.9, 1.2 Hz, 1H), 7.74 (d, J = 8.8 Hz, 2H), 7.87 (d, J = 8.8 Hz, 2H), 12.80 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 32.92 (t), 42.25 (s), 61.73 (t), 63.26 (t), 117.82 (d), 123.75 (d), 125.66 (d), 127.39 (d), 128.50 (d), 130.08 (d), 130.74 (s), 134.90 (s), 142.42 (s), 154.13 (s), 161.33 (s), 174.39 (s); FABHRMS Calcd. for $C_{23}H_{24}S_2O_6$ (M^+ + H): 459.0936. Found: 459.0936. Anal. Calcd. for $C_{23}H_{23}S_2O_6$: C, 60.24; H, 4.83. Found: C, 60.57; H, 4.90.

16E. Preparation of Ifb where R^1 and R^2 Together with the Carbon to which they are attached represent Tetrahydropyran-4-yl, R^3 and R^4 are Hydrogen, R^5 is 4-(Thiophen-3-yl)phenoxyphenyl

Similarly, following the above procedure, other compounds of Formula Ifb, were prepared, for example replacing 2-thiophene boronic acid with 3-thiophene boronic acid, 4-[4-(4-(thiophen-3-yl)phenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid was prepared: mp 206.6-212.4 °C; IR (KBr) 3430 (br), 1719 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.67 (m_c, 2H), 1.95 (m_c, 2H), 3.47 (m_c, 2H), 3.66 (m_c, 2H), 3.67 (s, 2H), 7.20 (m_c, 4H), 7.56 (dd, J = 5.0, 1.4 Hz, 1H), 7.64 (d, J = 5.0, 2.9 Hz, 2H), 7.81 (d, J = 8.7 Hz, 2H), 7.87 (m_c, 2H), 7.96 (s, 1H), 12.77 (s, 1H); ^{13}CNMR (DMSO-d_6) δ 32.92 (t), 40.38 (s), 61.19 (t), 63.26 (t), 117.66 (d), 120.54 (d), 120.87 (d), 126.04 (d), 127.07 (d), 127.96 (d), 130.02 (d), 132.00 (s), 134.66 (s), 140.45 (s), 160.80 (s), 174.32 (s); FABHRMS Calcd. for $C_{23}H_{23}S_2O_6$ (M^+ + H): 459.0936. Found: 459.0934. Anal. Calcd. for $C_{23}H_{22}S_2O_6.0.5H_2O$: C, 59.08; H, 4.96. Found: C, 58.82; H, 4.69.

16F. Catalytic Reduction of 4-[4-(4-bromophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid

A solution of 660 mg (1.45 mmol) of 4-[4-(4-bromophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid in 80% ethanol/tetrahydropyran (40 ml) was hydrogenated at atmospheric pressure for 14 hours using palladium on carbon catalyst, filtered over a celite pad washing with methylene chloride and concentrated *in vacuo* to afford 4-[4-phenoxyphenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid as a light orange solid (546 mg, 100%), which was taken directly into the next reaction without further purification: mp 162.5-165.3°C; IR (KBr) 3431 (br), 1727 cm^{-1}; ^{1}HNMR (DMSO-d_6) δ 1.67 (ddd, J = 14.1, 10.0, 4.0 Hz, 2H), 1.95 (dm, J = 14.1 Hz, 2H), 3.47 (m_c, 2H), 3.65 (m_c, 2H), 3.66 (s, 2H), 7.15 (d, J = 8.8 Hz, 2H), 7.27 (t, J = 7.4 Hz, 1H), 7.45 (t, J = 7.5 Hz, 2H), 7.86 (d, J = 7.9 Hz, 2H), 12.74 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 32.88 (t), 42.26 (s), 61.75 (t), 63.26 (t), 117.64 (d), 120.11 (d), 125.03 (d), 130.04 (d), 130.39 (s), 134.69 (s), 154.69 (s), 161.53 (s), 174.39 (s); FABHRMS Calcd for $C_{19}H_{21}SO_6$ (M^+ + H): 377.1059. Found: 378.1064. Anal. Calcd. for $C_{19}H_{20}SO_6.0.75H_2O$: C, 58.52; H, 5.56. Found: C, 58.54; H, 5.19.

EXAMPLE 17

Preparation of Compounds of Formula Ij

17A. Preparation of Ii where R^1, R^2 and R^3 are Hydrogen, and R^4 is Benzyl

Thiophenol (80 mg) was stirred for 45 min with potassium hydride (40 mg) in *N,N*-dimethylformamide (1 ml) to produce a homogeneous solution of potassium thiophenolate. To this mixture was added 3-benzyl-3-(4-bromophenylsulfonyl)-propionic acid (100 mg) dissolved in *N,N*-dimethylformamide (1 ml) at room temperature. After stirring for 16 hours at 75°C the mixture was partitioned between aqueous citric acid and water, giving a product which was purified by preparative TLC to afford 3-benzyl-3-(4-phenylthiophenylsulfonyl)-propionic acid (30 mg).

17B. Preparation of Ij where R^1, R^2 and R^3 are Hydrogen, and R^4 is Benzyl

The 3-benzyl-3-(4-phenylthiophenylsulfonyl)-propionic acid, prepared as shown above, was then converted to 3-benzyl-3-(4-phenylthiophenylsulfonyl)-*N*-hydroxypropionamide as descibed in Example 10A.

EXAMPLE 18

Preparation of Compounds of Formula Ik

18A. Preparation of Ik where R^1, R^2 and R^3 are Hydrogen, and R^4 is Benzyl

A mixture of 3-benzyl-3-(4-bromophenylsulfonyl)-propionic acid (250 mg), *p*-methoxystyrene (0.1 ml), diisopropylethylemine (0.25 ml), palladium acetate (5 mg) and tri(o-methylphenyl)phosphine (16 mg) was stirred overnight at 80°C. The reaction mixture was dissolved in methylene chloride and washed with aqueous citric acid. Solvent was removed from the methylene chloride solution, and the residue chromatographed on silica gel (preparative TLC, eluting with 10% methanol/methylene chloride), to afford 3-benzyl-3-(4-styrylphenylsulfonyl)-propionic acid (21 mg).

18B. Preparation of Ik where R^1, R^2 and R^3 are Hydrogen, and R^4 is Benzyl

The 3-benzyl-3-(4-styrylphenylsulfonyl)-propionic acid, prepared as shown above, was then converted to 3-benzyl-3-(4-styrylphenylsulfonyl)-*N*-hydroxypropionamide, LSIMS m/e=452.2 $(M+H)^+$, as descibed in Example 10A.

EXAMPLE 19

Preparation of Compounds of Formula Il

Preparation of Il where n is 2, R^1 and R^2 together with the Carbon to which they are attached are Piperidine, R^2 and R^3 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

Trifluoroacetic acid (4 ml) was added to a solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-*N*-BOC-piperidin-4-yl]-carboxamide (2 g, 3.64 mmol) dissolved in methylene chloride (4 ml). The reaction mixture was stirred for 1.3 hours and concentrated *in vacuo*. The crude salt residue was dissolved in ethyl acetate (150 ml), washed with saturated aqueous sodium bicarbonate (2 x 50 ml), dried over magnesium sulfate, concentrated *in vacuo*, to afford the free base, *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-piperidin-4-yl]-carboxamide (1.57 g, 90%). ^{1}HNMR ($CDCl_3$) δ 1.28 (s, 9H), 2.23 (m_c, 2H), 2.56 (m_c, 2H), 3.30 (m_c, 2H), 3.44 (m_c, 2H), 3.53 (m_c, 2H), 7.00 (d, J = 8.9 Hz, 2H), 7.05 (d, J = 8.8 Hz, 2H), 7.38 (d, J = 8.8 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 8.25 (br s, 1H), 8.48 (br s, 1H).

EXAMPLE 20

Preparation of Compounds of Formula Im

20A. Preparation of Im where n is 2, R is Ethoxycarbonylmethyl, R^1 and R^2 are Hydrogen, and R^5 is 4-Phenoxyphenyl

A solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide (750 mg) in *N, N*-dimethylformamide (10 ml) was treated with ethyl bromoacetate (0.2 ml) and potassium carbonate (600 mg). The mixture was stirred overnight at room temperature, and then partitioned between ethyl acetate and water. After drying, solvent was removed from the organic layer under reduced pressure to yield *N-tert*-butoxy-2- [4-(4-phenoxyphenyl-sulfonyl)-1-

(ethoxycarbonylmethyl)piperidin-4-yl]-acetamide, which was used in the next step without further purification.

20B. Preparation of Im where n is 2, R is Isopropyl, R^1 and R^2 are Hydrogen, and R^5 is 4-Phenoxyphenyl

To a solution of *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide (500 mg) in acetone (20 ml) was added 10% palladium on carbon (100 mg), and the mixture stirred under hydrogen for three days. The catalyst was filtered off, and solvent removed from the filtrate under reduced pressure. The residue was chromatographed on silica gel, eluting with 10% methanol/methylene chloride, to give *N-t*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(isopropyl)piperidin-4-yl)]-acetamide (300 mg).

20C. Preparation of Im where n is 2, varying R

Similarly, following the procedures of Example 20A above, but replacing ethyl bromoacetate with 3-picolyl chloride, *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(3-picolyl)piperidin-4-yl]-acetamide was prepared.

Similarly, following the procedures of Example 20A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)piperidin-4-yl)]-acetamide with *N-tert*-butoxy-2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-acetamide, and replacing ethyl bromoacetate with cyclopropylmethyl bromide, *N-tert*-butoxy-2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-1-(cyclopropylmethyl)-piperidin-4-yl}-acetamide was prepared.

Similarly, *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(acetamidocarbonylmethyl)piperidin-4-yl]-acetamide was prepared.

20D. Preparation of Im where n is 2, varying R

Similarly, following the procedures of Example 20A above, but optionally replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperid-4-yl)]-acetamide with other compounds of Formula Iy, and optionally replacing ethyl bromoacetate with other compounds of formula RX, where R is lower alkyl, cycloalkylalkyl, acyl, alkoxycarbonylalkyl, picoline, $-SO_2R^a$, where R^a is lower alkyl or $-NR^bR^c$, where R^b and R^c are independently hydrogen or lower alkyl; and the like, and X is chloro, bromo or iodo, other compounds of Formula Im were prepared:

N-tert-butoxy-2-[1-ethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide;
N-tert-butoxy-2-[1-methyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide, m.p. 152-155°C;
N-tert-butoxy-2-[1-(2-methylpropyl)-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide;
N-tert-butoxy-2-[1-cyclopropylmethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide;
N-tert-butoxy-2-[1-cyclopropylmethyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl]-acetamide; and
N-tert-butoxy-2-[1-acetyl-4-[4-(4-fluorophenoxy)phenylsulfonyl]-piperidin-4-yl]-acetamide.

20E. Preparation of Ic where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached is 1-CyclopropylmethylPiperidine, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a solution of the free base *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-piperidin-4-yl]-carboxamide (1.28 g, 2.66 mmol) dissolved in *N,N*-dimethylformamide (17 ml), was added cyclopropylmethyl bromide (0.26 ml, 2.66 mmol), followed by potassium carbonate (1.84 g, 13.3 mmol). After the reaction mixture was stirred for 20 hours, water was added (100 ml), and the aqueous solution extracted with ethyl acetate (3 x 100 ml). The combined organic extracts were washed with brine (2 x 50 ml), dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 25% ethyl acetate/hexanes, gave *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-1-(cyclopropyl)piperidin-4-yl]-carboxamide (1.30 g, 92%). ^{1}HNMR ($CDCl_3$) δ 0.10 (ddd, *J* = 5.6, 4.7, 4.6 Hz, 2H), 0.53 (ddd, *J* =8.7, 4.7, 4.5 Hz, 2H), 0.85 (m_c, 1H), 1.31 (s, 3H), 1.64 (m_c, 2H), 2.06 (m_c, 2H), 2.24 (m_c, 2H), 2.28 (d, *J* = 6.5 Hz, 2H), 2.67 (m_c, 4H), 3.50 (m_c, 2H), 7.01 (d, *J* = 8.8 Hz, 2H), 7.04 (d, *J* = 8.8 Hz, 2H), 7.37 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 8.8 Hz, 2H), 8.33 (br s, 2H); FABMS (M^+ +H): 535.2.

20F. Preparation of Ic where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached is 1-(3-Picolyl)piperidine, and R^5 is 4-(4-Chlorophenoxy)-phenyl

Similarly, following the procedures of Example 20E above, but replacing cyclopropylmethyl bromide with 1.25 equivalents of 3-picolyol chloride hydrochloride, *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-1-(3-picolyl)piperidin-4-yl]-carboxamide was prepared: mp 83.3-93.8°C; IR (KBr) 3436, 1661 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.31 (s, 9H), 2.00 (m_c, 2H), 2.24 (m_c, 2H), 2.55 (m_c, 4H), 3.48 (s, 2H), 3.53 (s, 2H), 7.01 (d, *J* = 8.9 Hz, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 7.25 (dd, *J* = 7.6, 4.6 Hz, 2H), 7.38 (d, *J* = 8.8 Hz, 2H), 7.64 (brd, *J* = 7.8 Hz, 2H), 7.85 (d, *J* = 8.9 Hz, 2H), 8.36 (br s, 1H), 8.52 (m, 2H); FABMS (M^+ +H): 572.0. Anal. Calcd. for $C_{29}H_{34}N_3SO_5Cl.0.5\ H_2O$: C, 59.03; H, 5.81; N, 7.12. Found: C,

59.37; H, 6.15; N, 7.98.

20G. Preparation of Ic where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached is 1-(Nicotinoyl)Piperidine, and R^5 is 4-(4-Chlorophenoxy)-phenyl

To a solution of the free base *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-piperidin-4-yl]-carboxamide (491 mg, 1.02 mmol) and *N,N*-diisopropylethylamine (444 mg, 2.55 mmol) in methylene chloride (2 ml) cooled to 0°C, was added nicotinyl chloride hydrochloride (219 mg, 1.27 mmol) in one portion. After the reaction mixture was stirred for 3 hours, water (30 ml) was added, and the aqueous solution extracted with ethyl acetate (2 x 60 ml). The combined organic extracts were washed with brine (2 x 50 ml), dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 6% methanol/methylene chloride, afforded *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-1-(nicotinoyl)piperidin-4-yl]-carboxamide (233 mg, 39%). ^{1}HNMR ($CDCl_3$) δ 1.33 (s, 9H), 1.95 (m_c, 2H), 2.35 (m_c, 2H), 3.45 (m_c, 2H), 3.49 (s, 2H), 3.55 (m_c, 4H), 7.01 (d, J = 8.8 Hz, 2H), 7.06 (d, J = 8.8 Hz, 2H), 7.39 (d, J = 8.8 Hz, 2H), 7.41 (m_c, 2H), 7.79 (m_c, 2H), 7.83 (d, J = 8.8 Hz, 2H), 8.69 (br s, 1H), 8.52 (m_c, 2H).

20H. Preparation of Ic where n is 2, R^3 and R^4 are Hydrogen, R^1 and R^2 when taken together with the Carbon to which they are attached is 1-(Methanesulfonyl)Piperidine, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a solution of the free base *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-piperidin-4-yl]-carboxamide (1.57 g, 3.26 mmol) in 67% methylene chloride/pyridine (16.5 ml) cooled to -78°C, was added a solution of methanesulfonyl chloride (0.51 ml, 6.53 mmol) in methylene chloride (2 ml). After the reaction mixture was stirred for 4 hours, 3N aqueous hydrochloric acid (25 ml) was added, and the aqueous solution extracted with ethyl acetate (2 x 60 ml). The combined organic extracts were washed with brine (2 x 50 ml), dried over magnesium sulfate, concentrated *in vacuo*. Chromatography over silica gel, and eluting with 45% ethyl acetate/hexanes, afforded *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonylmethyl)-1-(methanesulfonyl)piperidin-4-yl]-carboxamide (1.16 g, 64%). ^{1}HNMR ($CDCl_3$) δ 1.33 (s, 9H), 2.05 (m_c, 2H), 2.37 (m_c, 2H), 2.79 (s, 3H), 3.23 (m_c, 2H), 3.43 (s, 2H), 3.47 (m_c, 2H), 7.01 (d, J = 8.9 Hz, 2H), 7.06 (d, J = 8.9 Hz, 2H), 7.39 (d, J = 8.9 Hz, 2H), 7.85 (d, J = 8.9 Hz, 2H); FABMS (M^+ +H): 559.1.

EXAMPLE 21

Preparation of Compounds of Formula In

21A. Preparation of In where n is 2, R is Ethoxycarbonylmethyl, R^1 and R^2 are Hydrogen, and R^5 is 4-Phenoxyphenyl

The product from Example 20A, *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(ethoxycarbonylmethyl)piperidin-4-yl]-acetamide, was dissolved in dichloroethane (10 ml), cooled to 0°C, and saturated with hydrochloric acid gas. The reaction vessel was then sealed and the solution stirred for two days at 25°C. Solvent was removed from the reaction mixture under reduced pressure, and the residue purified by preparative TLC, eluting with 10% methanol/ methylene chloride, to give *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(ethoxycarbonylmethyl)piperidin-4-yl]-acetamide (420 mg), m/e = 477.1 (MH^+, FABMS).

21B. Preparation of In where n is 2, R is Isopropyl, R^1 and R^2 are Hydrogen, and R^5 is 4-Phenoxyphenyl

The product from Example 20B, *N-t*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(isopropyl)piperidin-4-yl)]acetamide, was reacted with hydrochloric acid gas as described above, to yield *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(isopropyl)piperidin-4-yl)]-acetamide (155 mg), m.p. 128°C, m/e = 432 (MH^+,EIMS).

21C. Preparation of In where n is 2, varying R

Similarly, following the procedures of Example 21A above, but replacing ethyl bromoacetate with 3-picolyl chloride, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-1-(3-picolyl)piperidin-4-yl]-acetamide was prepared, m.p. 185-192°C (dec).

Similarly, following the procedures of Example 19A above, but replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide with *N-tert*-butoxy-2-[4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl]-acetamide, and replacing ethyl bromoacetate with cyclopropylmethyl bromide, *N*-hydroxy-2-[4-[4-(4-fluorophenoxy)phenylsulfonyl]-1-cyclopropylmethylpiperidin-4-yl]-acetamide was prepared, m.p. 104-105°C.

Similarly, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-1-acetamidocarbonylmethylpiperidin-4-yl]-acetamide was prepared.

21D. Preparation of In where n is 2, varying R

Similarly, following the procedures of Example 21A above, but optionally replacing *N-tert*-butoxy-2-[4-(4-phenoxyphenylsulfonyl)-piperid-4-yl)]-acetamide with other compounds of Formula Iy, and optionally replacing ethyl bromoacetate with other compounds of formula RX, where R is lower alkyl, cycloalkylalkyl, acyl, alkoxycarbonylalkyl, picoline, $-SO_2R^a$, where R^a is lower alkyl or $-NR^bR^c$, where R^b and R^c are independently hydrogen or lower alkyl; and the like, and X is chloro, bromo or iodo, other compounds of Formula In were prepared:

2-[1-ethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-*N*-hydroxyacetamide, m.p. 182-183°C;
N-hydroxy-2-[1-methyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide, m.p. 152-155°C;
N-hydroxy-2-[1-(2-methylpropyl)-4-(4-phenoxyphenylsulfonyl)-piperid-4-yl]-acetamide, m.p. 226-227°C;
2-[1-cyclopropylmethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide, m.p. 210-211°C;
2-[1-cyclopropylmethyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]-piperidin-4-yl]-*N*-hydroxyacetamide, m.p. 110-112°C; and
2-[1-acetyl-4-[4-(4-fluorophenoxy)phenylsulfonyl]-piperidin-4-yl]-*N*-hydroxyacetamide, m/e = 450 (MH^+).

EXAMPLE 22

Preparation of Compounds of Formula Iab

Preparation of Iab where R^5 is 4-phenoxyphenyl

4-Phenoxythiophenol (4.8 g) was stirred for 45 min with potassium hydride (0.98 g) in *N, N*-dimethylformamide (100 ml) to produce a homogeneous solution of potassium 4-phenoxythiophenolate. The lactone, (S)-3-carbobenzyloxyamino-2-oxetanone (5.3 g) (Arnold, L.D. *et al., J. Am. Chem. Soc.,* **107,** 7105 (1985)), dissolved in *N, N*-dimethylformamide (50 ml) was then added at room temperature. After stirring for 30 minutes the mixture was poured into water and extracted with ethyl acetate. The combined extracts were dried over magnesium sulfate, and solvent removed under reduced pressure to give (*R*)-2-(benzyloxycarbonylamino)-3-(4-phenoxyphenylthio)-propionic acid (9.2 g). It can be used directly in the next step.

EXAMPLE 23

Preparation of Compounds of Formula Io

Preparation of Io where R^5 is 4-phenoxyphenyl

The above-prepared (*R*)-2-(benzyloxycarbonylamino)-3-(4-phenoxyphenylthio)-propionic acid was dissolved in methylene chloride (175 ml), cooled to 0°C, and treated with *O-(tert*-butyl)hydroxylamine hydrochloride (7.7 g), 4-methylmorpholine (9.4 ml), 1-hydroxybenzotriazole (2.8 g), and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (7.9 g). The mixture was allowed to warm to room temperature, stirred for 1.5 hours, then partitioned between methylene chloride and water. Solvent was removed from the organic phase under reduced pressure, and the residue purified by flash chromatography on silica gel, eluting with 0 to 50% ethyl acetate/hexane, to provide (*R*)-2-(benzyloxycarbonylamino)-*N-tert*-butoxy-3-(4-phenoxyphenylthio)-propionamide (7.4 g) as a white foam.

EXAMPLE 24

Preparation of Compounds of Formula Ip

Preparation of Ip where n is 2 and R^5 is 4-phenoxyphenyl

(*R*)-*N-tert*-butoxy-2-(benzyloxycarbonylamino)-3-(4-phenoxyphenylthio)-propionamide (1.5 mmol) was dissolved in methanol (140 ml), and a solution of OXONE (15 g) in water (50 ml) was added with vigorous stirring. The oxidation is usually complete within 2 hours. The mixture is then partitioned between methylene chloride and water. Solvent was removed from the dried organic phase under reduced pressure, to afford (*R*)-2-(benzyloxycarbonylamino)-*N-tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (8.3 g) in near-quantitative yield.

EXAMPLE 25

Preparation of Compounds of Formula Iq

Preparation of Iq where n is 2, R^1 is Hydrogen, R^2 is $-NR^6R^7$, in which R^6 is Hydrogen and R^7 is Benzyloxycarbonylamino, and R^5 is 4-phenoxyphenyl

A solution of (*R*)-2-(benzyloxycarbonylamino)-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (1.2 g) obtained from Example 16 in methylene chloride (5 ml) was diluted with trifluoroacetic acid (30 ml). The solution was allowed to stand overnight, and solvent was removed under reduced pressure. This residue was chromatographed on silica gel, eluting with 10% methanol/methylene chloride to give (*R*)-2-(benzyloxycarbonylamino)-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide (400 mg), m.p. 195-202°C.

EXAMPLE 26

Preparation of Compounds of Formula Ir

Preparation of Ir where n is 2 and R^5 is 4-phenoxyphenyl

(*R*)-2-(benzyloxycarbonylamino)-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (6.0 g) obtained from Example 17 was dissolved in ethanol (100 ml) and hydrogenated at 1 atmosphere in the presence of 10% palladium on carbon (6 g) for a period of 18 hours. The catalyst was filtered off and the solvent removed from the filtrate under reduced pressure to give (*R*)-2-amino-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide as a glass.

EXAMPLE 27

Preparation of Compounds of Formula Is

Preparation of Is where n is 2, R^1 is Hydrogen, R^2 is $-NR^6R^7$, in which R^6 and R^7 are both Hydrogen, and R^5 is 4-phenoxyphenyl

Similarly as in Example 25, (*R*)-2-amino-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (6.0 g) was dissolved in 1,2-dichloroethane (5 ml) and cooled to -20°C and bubbled for 20 minutes with hydrochloric acid gas in a pressure tube. The flask was then sealed and the mixture stirred overnight. The tube was cooled, vented, and allowed to warm. The solution was rinsed with methanol, the solvent removed from the filtrate under reduced pressure, triturated with 1:1 hexane/ethyl acetate (4 ml). The residue was filtered and dried to give (*R*)-2-amino-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide hydrochloride, m.p. 178-180°C (dec).

EXAMPLE 28

Preparation of Compounds of Formula It

Preparation of It where n is 2, R^1 is Hydrogen, R^2 is $-NR^6R^7$, in which R^6 is Hydrogen and R^7 is CBZ-(*S*)-Valinamido, and R^5 is 4-phenoxyphenyl

To a solution of (*R*)-2-amino-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (1.9 g) in methylene chloride (30 ml) was added CBZ-(*S*)-valine (1.6 g), 1-hydroxybenzotriazole (0.9 g), triethylamine (1 ml), and *N*'-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide (1.3 g). After stirring overnight at room temperature, the solution was partitioned between methylene chloride and water, and after the organic layer was dried over magnesium sulfate, solvent was removed under reduced pressure to give (*R*)-*N*-*tert*-butoxy-2-(CBZ-valinamido)-3-(4-phenoxyphenylsulfonyl)-propionamide, which was used without further purification.

EXAMPLE 29

Preparation of Compounds of Formula Iu

Preparation of Iu where n is 2, R^1 is Hydrogen, R^2 is $-NR^6R^7$, in which R^6 is Hydrogen and R^7 is (*S*)-Valinamido, and R^5 is 4-phenoxyphenyl

A solution of (*R*)-*N*-*tert*-butoxy-2-(CBZ-valinamido)-3-(4-phenoxyphenylsulfonyl)-propionamide (prepared above) in a mixture of methanol (300 ml) and ethanol (100 ml) was stirred under hydrogen at 1 atmosphere with palladium on carbon catalyst (10% Pd, 4 g) for 3 hours. The mixture was filtered, and the filtrate evaporated under reduced pressure. The residue was chromatographed on silica gel, eluting with 0-3% methanol in methylene chloride, to give (*R*)-*N*-*tert*-butoxy-2-valinamido-3-(4-phenoxyphenylsulfonyl)-propionamide (1.6 g).

EXAMPLE 30

Preparation of Compounds of Formula Iv

Preparation of Iv where n is 2, R^1 is Hydrogen, R^2 is $-NR^6R^7$, in which R^6 is Hydrogen and R^7 is (*S*)-Valinamido, and R^5 is 4-phenoxyphenyl

A solution of (*R*)-*N*-*tert*-butoxy-2-valinamido-3-(4-phenoxyphenylsulfonyl)-propionamide (1.6 g) in 1,2-dichloroethane (50 ml) was cooled to -20°C and bubbled for 15-20 minutes with hydrochloric acid gas in a pressure tube. The flask was then sealed and the mixture stirred for 24 hours. After cooling the tube was cautiously vented and its contents evaporated to yield a gum, which upon trituration with ethyl acetate gave a crude product as a white powder. This product was stirred overnight with 10% methanol/methylene chloride (20 ml) and filtered to remove impurities. This was repeated three times to give (*R*)-*N*-hydroxy-2-valinamido-3-(4-phenoxyphenylsulfonyl)-propionamide hydrochloride (760 mg), m.p. 214-217°C.

EXAMPLE 31

Preparation of Compounds of Formula Iw

Preparation of Iw where n is 2, Y is hydroxy or lower alkoxy, R^1 and R^2 when taken together with the carbon to which they are attached are Tetrahydropyan-4-yl, R^3 is hydrogen, and R^4 is Benzyl, and R^5 is 4-(4-Chlorophenoxy)phenyl

1. To a solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-carboxylic acid methyl ester in 20% tetrahydrofuran-methanol (9.5 ml) was added dropwise a solution of OXONE (1.53 g, 2.49 mmol) in water (8 ml) while maintaining an internal temperature of 15-20°C. The mixture was stirred 2 hours and the mixture dissolved in 40% ethyl acetate/water (200 ml). The layers were partitioned, and the water layer back extracted using ethyl acetate (2 x 50 ml). The combined organic layers were dried over magnesium sulfate, concentrated, and the residue purified by preparative chromatography (20 x 40-1000 um plates), eluting with 50% ethyl acetate/hexanes) to afford 4-[4-(4-chlorophenoxy)phenyl-sulfonylmethyl]-tetrahydropyran-4-carboxylic acid methyl ester (460 mg, 71%). ^{1}HNMR ($CDCl_3$) δ 1.71-1.82 (m, 2H), 2.23 (dm, J = 13.6 Hz, 2H), 3.47 (s, 2H), 3.58-3.67 (m, 2H), 3.59 (s, 3H), 3.73-3.81 (m, 2H), 6.97-7.10 (m, 4H), 7.39 (d, J = 8.7 Hz, 2H), 7.84 (d, J = 8.7 Hz, 2H).

2. Lithium diisopropylamide was prepared by the addition of 2.5M *N*-butyl lithium (610 µL, 1.53 mmol) in hexanes to a solution of diisopropylamine (200 µL, 1.53 mmol) in tetrahydrofuran (3 ml) at 0°C and stirring for 20 minutes. Then a solution of 4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid methyl ester (540 mg, 1.27 mmol) in tetrahydrofuran (1 ml) was added to the solution of lithium diisopropylamide at -78°C, and stirred for an additional 60 minutes. Benzyl bromide (181 µL, 1.53 mmol) of was added to the mixture, stirred for an 50 minutes, warmed to room temperature over 30 minutes, and stirred for an additional 3 hours. The mixture was then diluted with 0.1M aqueous hydrochloric acid (25 ml) and extracted with methylene chloride (2 x 50 ml). The combined organic layers were dried over magnesium sulfate, concentrated *in vacuo*, chromatographed over silica gel, eluted with 20% ethyl acetate/hexanes, to afford 3-benzyl-4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid methyl ester (440 mg, 67%). IR (KBr) 1736 cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 1.78 (dm, J = 13.5 Hz, 1H), 2.02-2.17 (m, 2H), 2.39 (dm, J = 13.5 Hz, 1H), 3.19-3.23 (m, 2H), 3.37-3.45 (td, J = 11.9, 2.4 Hz, 2H), 3.77-3.85 (m, 1H), 3.84 (s, 3H), 3.88-3.98 (m, 2H), 4.07-4.17 (m, 2H), 6.83-6.90 (m, 4H), 6.94 (d, J = 8.7 Hz, 2H), 7.08-7.15 (m, 3H), 7.37 (d, J = 8.7 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H); FABMS (M^+ +H): 515.

EXAMPLE 32

Preparation of Compounds of Formula Ix

Preparation of Ix where n is 2, Y is hydroxy, R^1 and R^2 when taken together with the carbon to which they are attached are Tetrahydropyan-4-yl, R^3 is hydrogen, and R^4 is Benzyl, and R^5 is 4-(4-Chlorophenoxy)phenyl

To a solution of 3-benzyl-4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid methyl ester (410 mg, 0.80 mmol) in *N,N*-dimethylformamide (4 ml) was added lithium iodide (1.06 g, 7.96 mmol), followed by sodium cyanide (78 mg, 1.59 mmol). The mixture was heated to 120°C for 8 hours, cooled to room temperature, the *N,N*-dimethylformamide solvent removed by heating under reduced pressure, and the residue partitioned between ethyl acetate (150 ml) and saturated aqueous sodium bisulfite (50 ml). The ethyl acetate layer was dried over magnesium sulfate, concentrated *in vacuo*, purified by preparative chromatography (20 x 40-1000 um plates), eluted with 8% methanol/methylene chloride) to afford 317 mg (80%) of 3-benzyl-4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-carboxylic acid ^{1}HNMR (*N,N*-dimethylformamide contaminant, $CDCl_3$) δ 1.74 (dm, J = 13.5 Hz, 1H), 2.05-2.18 (m, 2H), 2.42 (dm, J = 13.5 Hz, 1H), 3.22-3.26 (m, 2H), 3.48-3.58 (m, 2H), 3.78-4.18 (m, 5H), 6.83-6.88 (m, 4H), 6.93 (d, J = 8.5 Hz, 2H), 7.08-7.13 (m, 3H), 7.36 (d, J = 8.7 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H); CIMS (NH_3, $M^+ + NH_4+$): 518.

EXAMPLE 33

Preparation of Compounds of Formula I

Preparation of I where n is 2, R^2 is -NR^6R^7, in which R^6 and R^7 are both Methyl, and R^5 is 4-phenoxyphenyl

To a solution of (*R*)-2-amino-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (1.6 g) in *N,N*-dimethylformamide (5 ml) was added potassium carbonate (0.5 g) and methyl iodide (550 µl). After stirring for 2.5 hours, the mixture was partitioned between ethyl acetate and water, and after the organic layer was dried over magnesium sulfate, solvent was removed under reduced pressure. The residue was chromatographed on silica gel, eluting with 50% ethyl acetate/hexane to give (*R*)-*N*-*tert*-butoxy-2-dimethylamino-3-(4-phenoxyphenylsulfonyl)-propionamide (0.6 g).

This compound, (*R*)-*N*-*tert*-butoxy-2-dimethylamino-3-(4-phenoxyphenylsulfonyl)-propionamide, was dissolved in 1,2-dichloroethane (50 ml), cooled to -30°C and bubbled for 15-20 minutes with hydrochloric acid gas in a pressure tube. The flask was then sealed and the mixture stirred overnight. After cooling the tube was cautiously vented and its contents evaporated, to yield a gum, which upon trituration with 2:1 hexane/ethyl acetate gave a white powder, (*R*)-2-dimethylamino-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide hydrochloride (0.43 g), m.p. 65-70°C.

EXAMPLE 34

Preparation of Compounds of Formula I

Preparation of I where n is 2, R^2 is -NR^6R^7, in which R^6 is Hydrogen and R^7 is Dimethylaminosulfonyl, and R^5 is 4-phenoxyphenyl

To a solution of (*R*)-2-amino-*N*-*tert*-butoxy-3-(4-phenoxyphenylsulfonyl)-propionamide (1.5 g) in methylene chloride (20 ml) and pyridine (1.2 ml) was added dimethylsulfamoyl chloride (1 ml), and the mixture stirred overnight at room temperature. The mixture was partitioned between methylene chloride and water, and after the organic layer was dried over magnesium sulfate, solvent was removed under reduced pressure. The residue was chromatographed on silica gel, eluting with 0-45% ethyl acetate/hexane, to give (*R*)-*N*-*tert*-butoxy-2-dimethylaminosulfonamido-3-(4-phenoxyphenylsulfonyl)-propionamide (1.6 g).

This compound, (*R*)-*N*-*tert*-butoxy-2-dimethylaminosulfonamido-3-(4-phenoxyphenylsulfonyl)-propionamide, was dissolved in trifluoroacetic acid (30 ml) and the mixture stirred overnight at room temperature. The trifluoroacetic acid was removed under reduced pressure, and the residue chromatographed on silica gel, eluting with 10% methanol/methylene chloride, to give (*R*)-2-dimethylaminosulfonamido-3-(4-phenoxyphenylsulfonyl)-*N*-hydroxypropionamide hydrochloride (550 mg). ^{1}H NMR (d6-DMSO) 7.90 (d,2H), 7.47 (d,2H), 7.25 (t,1H), 7.13 (m,4H), 3.95 (m,1H), 3.55 (m,2H), 2.6 (s,6H).

EXAMPLE 35

Example of Preparation of Compounds of Formula I on a Large Scale

Preparation of I where n is 2, R^1 and R^2 when taken together with the Carbon to which they are attached represent Tetrahydropyran, R^3 and R^4 are Hydrogen, and R^5 is 4-(4-Chlorophenoxy)phenyl

1. Preparation of a Compound of Formula (7a)

To a mixture of *N,N*-dimethylformamide (56 Kg) and diethyl malonate (22 Kg) was added a 21% solution of sodium ethoxide in ethanol (45 Kg), followed by 2-chloroethyl ether (19 Kg). The mixture was heated to 85°C, causing ethanol to distil from the mixture. The temperature was raised to 120°C until all the ethanol formed was removed (3 hours), and then the mixture was allowed to cool to 25°C. The mixture was then rewarmed to 120°C and a further 45 Kg of a 21% solution of sodium ethoxide in ethanol added at such a rate as to cause the ethanol formed to distil off. When the distillation was complete, the mixture was cooled to 100°C, and after it was determined that the reaction was complete then cooled to 25°C. The mixture was partitioned between toluene (80 Kg) and water (216 Kg) and solvent removed from the organic layer by distillation. The product was used in the next step with no further purification.

2. Preparation of a Compound of Formula (8a) where R^1 and R^2 when taken together with the Carbon Atom to which they are attached represent Tetrahydropyran

A solution of diethyl tetrahydro-4H-pyran-4,4-dicarboxylate, the compound of Formula (7a), (12 Kg) in toluene (104 Kg) was cooled to between -30°C to -35°C, and diisobutylaluminum hydride (69 Kg) was added at such a rate so as to maintain a reaction temperature of -25°C. After the addition was complete, the temperature was raised to 15°C over 3 hours, and the reaction stirred until all starting material was consumed. The mixture was then recooled to -15°C and allowed to stand overnight. The product was partitioned between ethyl acetate (54 Kg), ethanol (48 Kg), and saturated sodium sulfate solution (60 litres), and the mixture stirred overnight at 25°C. The precipitated salts were filtered off, washed with tetrahydrofuran, and the filtrate washed with brine and separated. The organic layer was dried over magnesium sulfate and solvent removed under reduced pressure, to give ethyl 4-hydroxymethyltetrahydropyran-4-carboxylate (3.8 Kg), the compound of Formula (8a).

3. Preparation of a Compound of Formula (9a) where R^1 and R^2 when taken together with the Carbon Atom to which they are attached represent Tetrahydropyran

To a solution of lithium hydroxide monohydrate (4.46 Kg) in methanol (44 litres) and water (11 Kg) was added ethyl 4-hydroxymethyl-tetrahydropyran-4-carboxylate (8.0 Kg). The mixture was refluxed for 30 minutes, then solvent removed under reduced pressure. The mixture was cooled to 20°C, methyl *tert*-butyl ether (14.8 Kg) added, stirred for 10 minutes, and allowed to settle. The top organic layer was separated. This was repeated twice more, then the remaining mixture cooled to -10°C, and a solution of 31% hydrochloric acid (13 Kg) in water (3 Kg) added, maintaining the temperature below 5°C. The mixture was extracted several times with tetrahydrofuran, and the combined organic phases dried over magnesium sulfate. Approximately 90% of the tetrahydrofuran was removed, and the remaining solution added to a mixture of hexane (64.5 Kg) and methyl *tert*-butylether (23.7 Kg) with stirring. The precipitated solid material was filtered off and dried under reduced pressure at 60°C, to give 4-hydroxymethyl-tetrahydropyran-4-carboxylic acid (3.7 Kg), the compound of Formula (9a).

4. Preparation of a Compound of Formula Ia where R^1 and R^2 when taken together with the Carbon Atom to which they are attached represent Tetrahydropyran

To a mixture of 4-hydroxymethyl-tetrahydropyran-4-carboxylic acid (3.84 Kg), 4-dimethylaminopyridine (0.6 Kg) in dichloromethane (32 litres) was added triethylamine (4.88 Kg). The mixture was cooled to -20°C, and a solution of benzenesulfonyl chloride (4.66 Kg) in dichloromethane (5 litres) was added over a period of 35 minutes, maintaining the temperature below -10°C. The mixture was stirred at -10°C for 30 minutes, then 3N hydrochloric acid (10 litres) and water (10 litres) were added with stirring, then the layers allowed to separate. The organic layer was separated, the aqueous layer washed with dichloromethane (16 litres), the combined organics washed with aqueous 5% sodium bicarbonate solution (12 litres), then with water (12 litres), and solvent removed under reduced pressure, to give 2,7-dioxaspiro[3,5]nonane-1-one, a compound of Formula (10a)

To a mixture of 60% sodium hydride (0.92 Kg) in tetrahydrofran (26 litres) at 0°C was added a solution of 4-(4-chlorophenoxy)thiophenol (4.37 Kg) in tetrahydrofuran (15 litres), maintaining the temperature below 10°C. The mixture was allowed to warm to room temperature for 30 minutes, then recooled to 0°C. The concentrated solution of 2,7-dioxas-

piro[3,5]nonane-1-one obtained above was then added slowly to this mixture, maintaining the temperature below 10°C. The mixture was allowed to warm to room temperature, and stirred for 30 minutes. The mixture was then treated with 3N hydrochloric acid (16 litres) and dichloromethane (30 litres). The organic layer was separated and the aqueous layer extracted twice with dichloromethane (20 litres). The combined organics were washed with water (20 litres), filtered, and 100 litres of solvent removed under atmospheric pressure. To the remaining reaction product was added acetonitrile (60 litres) and after a further 60 litres of solvent were removed by distillation, acetonitrile (40 litres) was added and the total volume of the remainder reduced to 30 litres by distillation. This mixture was then heated to mild reflux (80°C), and then slowly cooled to 0°C. The product was filtered off, washed with hexane, and dried to about 60°C under reduced pressure, to yield 4-[4-(4-chlorophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid (5.61 Kg).

5. Preparation of a Compound of Formula Iba where R^1 and R^2 when taken together with the Carbon Atom to which they are attached represent Tetrahydropyran

A solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]tetrahydropyran-4-carboxylic acid (5.5 Kg) and *N,N*-dimethylformamide (27 ml) in dichloromethane (27.5 litres) was cooled to 5°C, and oxalyl chloride (1.4 litres) added slowly with stirring. After addition was complete, the mixture was allowed to warm to room temperature and stirred for 2 hours, thus forming a compound of Formula (12). The solution was then recooled to 10°C, and a mixture of 50% aqueous hydroxylamine (5.4 litres), *tert*-butanol (12.1 litres) and tetrahydrofuran (30.5 litres) was added slowly, maintaining the temperature below 21°C. The mixture was then allowed to warm to room temperature until the reaction was complete. The solvent was then evaporated under reduced pressure until 90% had been removed, at which point acetonitrile (42.5 litres) was added and the remaining dichloromethane removed by distillation under reduced pressure. The remaining solution was heated under reflux, and water (126 Kg) added at such a rate so as to maintain reflux. The solution was then cooled to 5°C for 12 hours, and the solid thus obtained filtered off. This product was washed with water and dried under vacuum at 50°C to yield 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) (5.06 Kg), a compound of Formula Iba.

6. Preparation of a Compound of Formula Id where R^1 and R^2 when taken together with the Carbon Atom to which they are attached represent Tetrahydropyran

To a solution of 4-[4-(4-chlorophenoxy)phenylthiomethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) (5.06 Kg) in tetrahydrofuran (28 litres) and methanol (112 litres) at 15°C was added a solution of OXONE (14.23 Kg) in water (72 litres) with stirring, ensuring that the temperature did not exceed 16°C. After the addition was complete, the temperature was raised to 20°C and the mixture stirred for 3 hours, then poured into a cold mixture (5°C) of toluene (60 litres) and ethyl acetate (98 litres) with stirring. The resultant mixture was filtered, the organic and aqueous layers thus obtained separated, and the aqueous layer washed with a mixture of ethyl acetate (25 litres) and toluene (10 litres). This wash was repeated twice more. The combined extracts and organic layer was washed twice with water (25 litres), and solvent removed under reduced pressure to a volume of 30 litres. The solution was cooled to 5°C, and the solid filtered off, washed with ethyl acetate/water and dried under vacuum at 50°C, to yield 4-[4-(4-chlorophenoxy)phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide) (4.3 Kg).

7. Similarly other Compounds of Formula I may be prepared.

EXAMPLE 36

This example illustrates the preparation of representative pharmaceutical compositions for oral administration containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide:

A.	
Ingredients	% wt./wt.
Compound of Formula I	20.0%
Lactose	79.5%
Magnesium stearate	0.5%

The above ingredients are mixed and dispensed into hard-shell gelatin capsules containing 100 mg each, one capsule would approximate a total daily dosage.

B.	
Ingredients% wt./wt.	
Compound of Formula I	20.0%
Magnesium stearate	0.9%
Starch	8.6%
Lactose	79.6%
PVP (polyvinylpyrrolidine)	0.9%

The above ingredients with the exception of the magnesium stearate are combined and granulated using water as a granulating liquid. The formulation is then dried, mixed with the magnesium stearate and formed into tablets with an appropriate tablet machine.

C.	
Ingredients	
Compound of Formula I	0.1 g
Propylene glycol	20.0 g
Polyethylene glycol 400	20.0 g
Polysorbate 80	1.0 g
Water	q.s. 100 ml

The compound of Formula I is dissolved in propylene glycol, polyethylene glycol 400 and polysorbate 80. A sufficient quantity of water is then added with stirring to provide 100 ml of the solution which is filtered and bottled.

D.	
Ingredients	% wt./wt.
Compound of Formula I	20.0%
Peanut Oil	78.0%
Span 60	2.0%

The above ingredients are melted, mixed and filled into soft elastic capsules.

EXAMPLE 37

This example illustrates the preparation of a representative pharmaceutical formulation for parenteral administration containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide:

Ingredients	
Compound of Formula I	0.02 g
Propylene glycol	20.0 g
Polyethylene glycol 400	20.0 g
Polysorbate 80	1.0 g
0.9% Saline solution	q.s. 100 ml

The compound of Formula I is dissolved in propylene glycol, polyethylene glycol 400 and polysorbate 80. A sufficient quantity of 0.9% saline solution is then added with stirring to provide 100 ml of the I.V. solution which is filtered through a 0.2 μ membrane filter and packaged under sterile conditions.

EXAMPLE 38

This example illustrates the preparation of a representative pharmaceutical composition in suppository form containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide:

Ingredients	% wt./wt.
Compound of Formula I	1.0%
Polyethylene glycol 1000	74.5%
Polyethylene glycol 4000	24.5%

The ingredients are melted together and mixed on a steam bath, and poured into molds containing 2.5 g total weight.

EXAMPLE 39

This example illustrates the preparation of a representative pharmaceutical formulation for insufflation containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide

Ingredients	% wt./wt.
Micronized compound of Formula I	1.0%
Micronized lactose	99.0%

The ingredients are milled, mixed, and packaged in an insufflator equipped with a dosing pump.

EXAMPLE 40

This example illustrates the preparation of a representative pharmaceutical formulation in nebulized form containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide:

Ingredients	% wt./wt.
Compound of Formula I	0.005%
Water	89.995%
Ethanol	10.000%

The compound of Formula I is dissolved in ethanol and blended with water. The formulation is then packaged in a nebulizer equipped with a dosing pump.

EXAMPLE 41

This example illustrates the preparation of a representative pharmaceutical formulation in aerosol form containing a compound of Formula I, or a pharmaceutically acceptable salt thereof, *e.g.*, *N*-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl)]-acetamide:

Ingredients	% wt./wt.
Compound of Formula I	0.10%
Propellant 11/12	98.90%
Oleic acid	1.00%

The compound of Formula I is dispersed in oleic acid and the propellants. The resulting mixture is then poured into an aerosol container fitted with a metering valve.

EXAMPLE 42

In Vitro Assay

42A. Isolation of MMPs for Assays

The catalytic domain of human collagenase-1 was expressed as a fusion protein with ubiquitin in *E. Coli* (Gehring, E.R. *et at., J. Biol. Chem.*, **270**, 22507, (1995)). After purification of the fusion protein, the fibroblast collagenase-1 catalytic domain was released by treatment with 1mM of aminophenylmercuric acetate (APMA) for 1 hour at 37°C and purified by zinc chelate chromatography.

Human collagenase-2 and gelatinase B were isolated in active form from buffy coats (Mookhtiar, K.A. *et at., Biochemistry,* **29**, 10620, (1990)).

The propeptide and catalytic domain portion of human collagenase-3 was expressed in *E. Coli* as an *N*-terminal fusion protein with ubiquitin. After purification, the catalytic domain was obtained by treatment with 1 mM APMA for 1 hour at 37°C, and purified by zinc chelate chromatography.

Rat collagenase-3 was purified in active form from the culture media of uterine smooth muscle cells (Roswit, W.T. *et al., Arch. Biochem. Biophys.*, **225**, 285-295 (1983)).

The catalytic and fibronectin-like portion of human progelatinase A was expressed as a fusion protein with ubiquitin in *E. Coli.* Assays were carried out on autolytically activated material. Rat progelatinase A was purified from the culture media of interleukin-1 stimulated keratinocytes and activated by treatment with 1 mM APMA for 1 hour at 37°C, and subsequently dialyzed to remove excess APMA.

Human prostromelysin-1 was purified from the culture medium of synovial fibroblasts by affinity chromatography using an immobilized monoclonal antibody. The zymogen was activated by treatment with trypsin (1.5 μg/ml) for 1 hour at 23°C to give a mixture of 45 and 28 kD species. The catalytic domain of human stromelysin was prepared by expression and purification of prostromelysin-1 from *E. Coli* and activated with 1 mM APMA for 1 hour at 37°C, followed by dialysis. Rat prostromelysin-1 was expressed in Chinese Hampster Ovary cells and purified from the culture media. It was activated by 1 mM APMA for 1 hour at 37°C, followed by dialysis.

Human promatrilysin was expressed and purified from Chinese Hampster Ovary cells (Barnett, *J. et al., Prot.*

Expres. Pur., **5**, 27, (1994)). The zymogen was activated by treatment with 1 mM APMA for 1 hour at 37°C, and purified by zinc chelate chromatography.

Compounds of Formula I exhibited the ability to inhibit the collagenases when tested in this assay.

42B. In Vitro Assay Procedure

Assays were performed in assay buffer (50 mM Tricine pH 7.5, 200 mM sodium chloride, 10 mM calcium chloride, 0.005% Brij-35) containing 2.5% methyl sulfoxide (DMSO) once the substrate and inhibitor were diluted into it. Stock solutions of inhibitors were prepared in 100% DMSO. Stock solutions of the substrate were prepared in 100% DMSO at a concentration of 2 mM.

The assay method was based on the hydrolysis of MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 (Bachem, Inc.) at 37°C (Knight, C.G. *et al., FEBS,* **296**, 263-266 (1992)). The fluorescence changes were monitored with a Perkin-Elmer LS-50B fluorimeter using an excitation wavelength of 328 nm and an emission wavelength of 393 nm. The substrate concentration used in the assays was 10 μmole. The inhibitor was diluted into the assays from a solution in 100% DMSO, and controls substituted an equal volume of DMSO so that the final DMSO concentration from inhibitor and substrate dilutions in all assays was 2.5%. The inhibition results are expressed as the inhibitor concentration that produced 50% inhibition (IC_{50}) of the activity in the control (non-inhibited) reaction.

EXAMPLE 43

In Vitro Assay

This assay determines the ability of the compounds of Formula I to inhibit the degradation of the collagen matrix (as judged by release of hydroxyproline), and proteoglycan (as judged by the release of ^{35}S-labelled glycosaminoglycans) from cartilage explants.

Small cartilage explants (3 mm diameter) were prepared from freshly sacrificed bovine knee joints and labeled with $^{35}SO_4$. ^{35}S-labelled glycosaminoglycans (GAG's) and collagen fragments are released into the culture medium in response to the addition of rhIL-1-alpha, which induces the expression of chondrocyte matrix metalloproteases (MMP's), including stromelysin and collagenase. The percent inhibition of hydroxyproline and GAG's released was corrected for spontaneous release in the absence of rhIL-1-alpha.

Compounds of Formula I, when tested in this assay, displayed the ability to inhibit the release of both collagen fragments and ^{35}S-labelled GAG's from cartilage explants.

EXAMPLE 44

In Vivo Assay

The cartilage plug implantation assay measures the destruction of the collagen matrix of a cartilage plug implanted in a rat (Bishop, J. *et al., J. Pharm. Tox. Methods,* **30**, 19, (1993)).

Previously frozen bovine nasal cartilage plugs weighing approximately 20 mg were embedded in polyvinyl sponges impregnated with *Mycobacterium tuberculosis* and implanted subcutaneously in female Lewis rats. Dosing was begun 9 days after implantation and the plugs were harvested about one week later. The plugs were weighed, hydrolyzed, and the hydroxyproline content measured. Efficaciousness was determined by the comparison of the compound-treated groups with vehicle treated controls.

The compounds of Formula I exhibited the ability to inhibit the degradation of the cartilage plugs in this assay.

EXAMPLE 45

In Vivo Assay Procedure

45A. Determination of TNF Production Following LPS Stimulation

Female Balb/c mice, 6-8 weeks old (Jackson Labs or Harlan) were used. For each treatment group, 6-8 mice were used. Mice were injected I.P. with LPS (Sigma, 13129, 10-20 μg/mouse) after treatment with a compound of Formula I. The compound of Formula I or vehicle was administered subcutaneously (S.C.) once, 30-60 minutes prior to LPS challenge. Control animals received CMC vehicle alone or CMC + 2-5% DMSO. Animals were bled 1.5 hours after LPS injection under anesthesia with metofane from the retro-orbital plexus, using a Pasteur pipette. Blood was collected in a microtainer serum separator tube (Becton Dickinson #5960). The sera were separated and either tested the next day or they were kept at -20°C until ready to test for TNF-α.

45B. ELISA Assay for Murine TNF-α

The Endogen (EM-TNFA kit) mouse tumor necrosis factor alpha (mTNF-α) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of mouse TNF-α (ordering code: EM-TNFA; Endogen, 30 Commerce Way, Woburn, MA 01801-1059, USA). Standards (lyophilized recombinant *E. coli*-derived mouse TNF-a) or serum samples (50 µl each) were added in duplicate to each well of the precoated anti-mTNF-α plate. Biotinylated antibody (50 µl) was added, the plates were incubated for 2-3 hours at room temperature. The wells were washed five times with wash buffer and 100 µl of diluted strepavidin HRP were added to each well and then were incubated at room temperature for 30 minutes. After washing (5X), 100 µl premixed TMB substrate solution were added to each well and plates were developed at room temperature in the dark for 30 minutes. The reaction was stopped by adding 100 µl of the stop solution. Absorbance at 450-575 nm was measured in a plate reader (ThermoMax, Molecular Devices). Results are calculated at pg/ml TNF-α by comparison to the standard curve, using Immunofit Beckman software. They are expressed as mean pg/ml of TNF-α, and as percentage of inhibition compared to controls (animals injected with LPS alone), considered 100% of TNF-α: production.

The compounds of Formula I, when tested in this assay, exhibited the ability to inhibit TNF-α production.

EXAMPLE 46

TNF Conjugate Immunoassay

Human Monomac 6 cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum to a density of 1 X 10^5 cells/mL. All subsequent incubations were performed at 37°C. 230 µl of these cells were placed in each well of a 96-well tissue culture plate and the cells incubated for 15 minutes. 10 µl of desired concentration of compounds of Formula I in the above mentioned medium were added to the appropriate wells and incubated for an additional 15 minutes. To each well was added 10 µl of an LPS/PMA mixture which brings the final concentration of LPS to 10 ng/mL and the final PMA concentration to 30 ng/mL. The cells were then incubated for 2 hours after which the plate was centrifuged and the medium removed and analyzed for TNF content. The analysis was performed using an R & D Systems TNF Quantikine Immunoassay and following the manufacturer's protocol (R & D. Systems, 614 Mckinley Place N.E., Minneapolis, MN 55413, USA; Catalog No. DTA50). The IC_{50} was calculated from the percent inhibition of TNF released into the medium.

The compounds of Formula I, when tested in this assay, exhibited the ability to inhibit TNF production.

EXAMPLE 47

TNFR Shedding Immunoassay

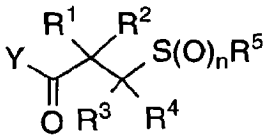
Human Monomac 6 cells are cultured to a density of 1 X 10^6 cells/mL at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. All subsequent incubations are performed at 37°C. 230 µl of these cells are placed in each well of a 96-well tissue culture plate and the cells are incubated for 15 minutes. 10 µl of desired concentration of compounds of Formula I in the above mentioned medium are added to the appropriate wells and incubated for an additional 15 minutes. To each well is added 10 µl of PMA at a final concentration of 30 ng/mL. The cells are then incubated for 16 hours after which the plate is centrifuged and the medium is removed and analyzed for TNF receptor content. The analysis is performed using the R & D Systems TNF receptor Quantikine Immunoassay following the manufacturer's protocol. Measurements of each TNF receptor (receptor I and receptor II) are performed in this way. The IC_{50} is calculated from the percent inhibition of TNF released into the medium.

The compounds of Formula I, when tested in this assay, exhibited the ability to selectively inhibit TNF production.

While the present invention has been described with respect to specific embodiments thereof, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto.

Claims

1. A compound of the formula:



I

wherein:

n is 0, 1 or 2;
Y is hydroxy or XONH-, where X is hydrogen or lower alkyl;
R^1 is hydrogen or lower alkyl;
R^2 is hydrogen, lower alkyl, heteroalkyl, aryl, aralkyl, arylheteroalkyl, cycloalkyl, cycloalkylalkyl, heteroaryl, heteroaralkyl, heteroarylheteroalkyl, heterocyclo, heterocylo-lower alkyl, heterocyclo-lower heteroalkyl or $-NR^6R^7$, wherein:

R^6 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl, aryl, heteroaryl and heteroaralkyl;
R^7 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, $-C(O)R^8$, $-C(O)NR^8R^9$, $-SO_2NR^8R^9$, $-SO_2R^{10}$, aryloxycarbonyl, or alkoxycarbonyl; or
R^6 and R^7 together with the nitrogen atom to which they are attached represent a heterocyclo group; wherein

R^8 and R^9 are independently hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl or heteroalkyl; and
R^{10} is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, heteroalkyl or heterocyclo; or

R^1 and R^2 together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group;
R^3 is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, heteroaralkyl, heteroalkyl or lower alkoxy;
R^4 is hydrogen, lower alkyl, cycloalkyl or cycloalkylalkyl; or
R^2 and R^3 together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or
R^3 and R^4 together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and
R^5 is lower alkyl, cycloalkyl, cycloalkylalkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl;

or a pharmaceutically acceptable salt or ester thereof.

2. The compound of Claim 1, wherein R^2 is $-NR^6R^7$.

3. The compound of Claim 1, wherein n is 2 and Y is XONH- in which X is hydrogen.

4. The compound of Claim 3, wherein R^1 is hydrogen and R^5 is aryl or heteroaryl.

5. The compound of Claim 4, wherein R^2 is hydrogen and R^3 is aralkyl and R^4 is hydrogen.

6. The compound of Claim 5, wherein R^3 is benzyl and R^5 is optionally substituted phenyl or naphthyl.

7. The compound of Claim 6, wherein R^5 is phenyl, 4-methoxyphenyl, 1-(4-methoxyphenyl)-2-phenylethene, phenylthiophenyl, phenoxyphenyl, or biphenyl.

8. The compound of Claim 7, wherein R^5 is 4-phenylthiophenyl, 4-phenoxyphenyl, or 4-biphenyl.

9. The compound of Claim 4, wherein R^3 and R^4 together with the carbon to which they are attached form a cycloalkyl group.

10. The compound of Claim 9, wherein R^5 is 4-methoxyphenyl or 4-phenoxyphenyl and the cycloalkyl group is cyclopentyl, cyclohexyl, or 4-methylcyclohexyl.

11. The compound of Claim 4, wherein R^3 and R^4 together with the carbon to which they are attached form a heterocyclo group.

12. The compound of Claim 11, wherein the heterocyclo group is optionally substituted piperidine or tetrahydropyranyl.

13. The compound of Claim 12, wherein the heterocyclo group is piperidin-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

14. The compound of Claim 12, wherein the heterocyclo group is 1-methylpiperidin-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

15. The compound of Claim 12, wherein the heterocyclo group is 1-(cyclopropylmethyl)piperidin-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

16. The compound of Claim 12, wherein the heterocyclo group is tetrahydropyran-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

17. The compound of Claim 3, wherein R^2 and R^3 together with the carbons to which they are attached form a cycloalkyl group and R^5 is aryl.

18. The compound of Claim 17, wherein the cycloalkyl group is cyclopentyl or cyclohexyl, R^4 is hydrogen, and R^5 is 4-methoxyphenyl.

19. The compound of Claim 3, wherein R^2 is $-NR^6R^7$, R^1, R^3 and R^4 are hydrogen, and R^5 is aryl.

20. The compound of Claim 19, wherein R^5 is 4-phenoxyphenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

21. The compound of Claim 3, wherein R^1 and R^2 together with the carbon to which they are attached form a heterocyclo group.

22. The compound of Claim 21, wherein R^3 and R^4 are both hydrogen and the heterocyclo group is optionally substituted piperidine or tetrahydropyranyl.

23. The compound of Claim 22, wherein the heterocyclo group is piperidin-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

24. The compound of Claim 22, wherein the heterocyclo group is tetrahydropyran-4-yl and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, 4-(4-fluorophenoxy)phenyl, 4-(thiophen-2-yl)phenoxyphenyl, 4-(thiophen-3-yl)phenoxyphenyl, 4-(2-pyridyloxy)phenyl, 4-(5-chloro-2-pyridyloxy)phenyl.

25. The compound of Claim 3, wherein R^1 and R^2 are both alkyl, R^3 and R^4 are hydrogen, and R^5 is 4-phenoxyphenyl, 4-(4-bromophenoxy)phenyl, 4-(4-chlorophenoxy)phenyl, or 4-(4-fluorophenoxy)phenyl.

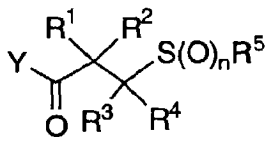
26. A compound of the group comprising

N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-tetrahydropyran-4-yl]-acetamide,
2-{4-[4-(4-chlorophenoxy)phenylsulfonyl]tetrahydropyran-4-yl}-*N*-hydroxyacetamide,
2-{4-[4-(4-fluorophenoxy)phenylsulfonyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide,
N-hydroxy-2-[4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide,
2-{4-[4-(4-chlorophenoxy)-phenylsulfonyl]piperidin-4-yl}-*N*-hydroxyacetamide,
2-{4-[4-(4-fluorophenoxy)-phenylsulfonyl]-piperidin-4-yl}-*N*-hydroxyacetamide,
N-hydroxy-2-[1-methyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-acetamide,
2-[1-cyclopropylmethyl-4-(4-phenoxyphenylsulfonyl)-piperidin-4-yl]-*N*-hydroxyacetamide,
2-{ 1-cyclopropylmethyl-4-[4-(4-chlorophenoxy)-phenylsulfonyl]piperidin-4-yl}-*N*-hydroxyacetamide,
2-{1-cyclopropylmethyl-4-[4-(4-fluorophenoxy)-phenylsulfonyl]piperidin-4-yl}-*N*-hydroxyacetamide
2-{4-[4-(4-fluorophenoxy)-phenylsulflnyl]-tetrahydropyran-4-yl}-*N*-hydroxyacetamide,
(*R*)-2-(CBZ-valinamido)-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide,
(*R*)-*N*-hydroxy-2-valinamido-3-(4-phenoxyphenylsulfonyl)propionamide,

(*R*)-2-dimethylamino-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)propionamide,
(*R*)-2-dimethylaminosulfonamido-*N*-hydroxy-3-(4-phenoxyphenylsulfonyl)-propionamide,
2-{4-[-(4-fluorophenoxy)-phenylthio]-tetrahydropyran-4-yl}-N-hydroxyacetamide,
4-[4-(4-chlorophenoxy)-phenylsulfonylmethyl]-tetrahydropyran-4-(*N*-hydroxycarboxamide),
4-[4-(4-thiophen-2-yl)phenoxyphenyl-sulfonylmethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide),
3-[4-(4-chlorophenoxy)-phenylsulfonyl]-2,2-dimethyl-N-hydroxypropionamide,
4-[4-(4-(thiophen-3-yl)-phenoxy)phenylsulfonylmethyl]tetrahydropyran-4-(*N*-hydroxycarboxamide)

and pharmaceutically acceptable salts thereof.

27. A process for preparing a compound of the Formula:

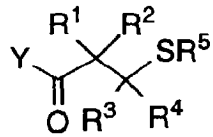


I

wherein:

n	is 1 or 2;
Y	is hydroxy or XONH-, where X is hydrogen or lower alkyl;
R^1	is hydrogen or lower alkyl;
R^2	is hydrogen, lower alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, or heterocyclo; or
R^1 and R^2	together with the carbon atom to which they are attached represent a cycloalkyl or heterocyclo group;
R^3	is hydrogen, lower alkyl, cycloalkyl, cycloalkylalkyl, aralkyl, heteroaralkyl, or lower alkoxy;
R^4	is hydrogen or lower alkyl; or
R^2 and R^3	together with the carbons to which they are attached represent a cycloalkyl or heterocyclo group; or
R^3 and R^4	together with the carbon to which they are attached represent a cycloalkyl or heterocyclo group; and
R^5	is lower alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl;

comprising contacting a compound of the Formula:



wherein R^1, R^2, R^3, R^4 and R^5 are as defined before,
with an oxidizing agent.

28. A pharmaceutical composition comprising a pharmaceutically acceptable non-toxic excipient and a therapeutically effective amount of a compound according to any one of claims 1-26.

29. Compounds according to any one of claims 1-26 for use as a therapeutically active substance.

30. Compounds according to any one of claims 1-16 for use in the treatment of a disease-state which is alleviated by treatment with a matrix metalloprotease inhibitor, especially wherein the disease state is rheumatoid arthritis, osteoarthritis, osteoporosis, periodontal disease, aberrant angiogenesis, multiple sclerosis, tumor metastasis, or corneal ulceration.

31. Compounds according to any one of claims 1-26 for use in the treatment of a disease state which is mediated by tumor necrosis factor, especially wherein the disease state is inflammation, hemorrhage, graft versus host reaction or an autoimmune disease.

32. The use of a compound according to any one of claims 1-26 in the treatment of of a disease-state which is alleviated by treatment with a matrix metalloprotease inhibitor, especially wherein the disease state is rheumatoid arthritis, osteoarthritis, osteoporosis, periodontal disease, aberrant angiogenesis, multiple sclerosis, tumor metastasis, or corneal ulceration.

33. The use of a compound according to any one of claims 1-26 in the treatment of a disease state which is mediated by tumor necrosis factor, especially wherein the disease state is inflammation, hemorrhage, graft versus host reaction or an autoimmune disease.

34. The use of a compound according to any one of claims 1-26 in the preparation of a medicament for the treatment of a disease-state which is alleviated by treatment with a matrix metalloprotease inhibitor, especially wherein the disease state is rheumatoid arthritis, osteoarthritis, osteoporosis, periodontal disease, aberrant angiogenesis, multiple sclerosis, tumor metastasis, or corneal ulceration or wherein the disease-state is mediated by tumor necrosis factor, especially wherein the disease state is inflammation, hemorrhage, graft versus host reaction or an autoimmune disease.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X,P	WO 96 15096 A (BAYER AG ;KLUENDER HAROLD CLINTON EUGENE (US); BENZ GUENTER H H H) 23 May 1996 * examples 196-198 * ---	1	C07D309/08 C07D211/54 C07D213/64 C07D401/06 C07D407/12 C07D413/12 C07D405/12 A61K31/35 A61K31/445
X	WO 90 05719 A (BRITISH BIO TECHNOLOGY) 31 May 1990 * examples1-3,8-18,20-24 * ---	1,28,29	
X	WO 95 09841 A (BRITISH BIOTECH PHARM ;CRIMMIN MICHAEL JOHN (GB); BECKETT PAUL RAY) 13 April 1995 * examples 1-7 * ---	1,28,29	
X,P	WO 96 06074 A (BRITISH BIOTECH PHARM ;BECKETT RAYMOND PAUL (GB); MILLER ANDREW (G) 29 February 1996 * example 13 * ---	1	
X	WO 93 20047 A (BRITISH BIO-TECHNOLOGY LTD) * examples 1-9,11-14 * --- -/--	1,28,29	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C07D
A61K
C07C
C25D

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
BERLIN	10 April 1997	Frelon, D

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	US 4 394 520 A (KALOPISSIS GREGOIRE) 19 July 1983 * examples 4,5,18; table I * ---	1	
X	US 4 268 516 A (LOMBARDINO JOSEPH G ET AL) 19 May 1981 * example 4 * ---	1	
X	FR 1 580 899 A (BRISTOL-MYERS COMPANY) 12 September 1969 * examples 4-7 * ---	1,28,29	
X	FR 2 355 095 A (M & T CHEMICALS INC) 13 January 1978 * compound page 21 * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
X	CAS REGISTRY HANDBOOK: , AMERICAN CHEMICAL SOCIETY , 1965-1971 XP002028919 * RN: 331-89-5;331-90-8;331-93-1;405-23-2;780-95-0;5463-52-5;5464-62-0;5445-04-5;5445-05-6;5460-58-2;6803-08-3;6534-37-8;21056-72-4 * ---	1	
X	TETRAHEDRON, vol. 49, no. 4, 1993, OXFORD GB, pages 939-946, XP002028918 S.E. CLAYTON ET AL.: * compound (8), page 940 * --- -/--	1	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X,P	CHEMICAL ABSTRACTS, vol. 125, no. 13, 23 September 1996 Columbus, Ohio, US; abstract no. 167779x, XP002028920 * abstract * & JP 08 127 581 A (SANKYO CO.) 21 May 1996 * CAS Registry (STN database), RN: 13739-36-1 * ---	1	
X	CHEMICAL ABSTRACTS, vol. 93, no. 19, 10 November 1980 Columbus, Ohio, US; abstract no. 186144y, XP002028921 * abstract * & JP 55 027 116 A (HOKKO CHEMICAL INDUSTRY CO.) * CAS Registry (STN database), RN:13739-35-0 * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
X	CHEMICAL ABSTRACTS, vol. 77, no. 13, 25 September 1972 Columbus, Ohio, US; abstract no. 88024j, XP002028922 * abstract * & TETRAHEDRON LETTERS, vol. 19, 1972, OXFORD GB, pages 1937-1940, R.H. RYNBRANDT ET AL.: * CAS Registry (STN database), RN: 36603-44-8;36603-37-9;36603-36-8;35930-65 -5 * --- -/--	1	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	CHEMICAL ABSTRACTS, vol. 76, no. 21, 22 May 1972 Columbus, Ohio, US; abstract no. 126719d, XP002028923 * abstract * & AUST. J. CHEM., vol. 25, no. 3, 1972, pages 647-653, G.F. KATEKAR ET AL.: * compounds I * ---	1	
X	CHEMICAL ABSTRACTS, vol. 74, no. 3, 18 January 1971 Columbus, Ohio, US; abstract no. 13116v, XP002028924 * abstract * & HELV. CHIM. ACTA, vol. 53, no. 7, 1970, pages 1813-1827, P. DOSTERT ET AL.: * CAS Registry (STN database), RN: 30013-62-8;30037-18-4;30037-19-5 * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
X	CHEMICAL ABSTRACTS, vol. 69, no. 19, 4 November 1968 Columbus, Ohio, US; abstract no. 76817s, XP002028925 * abstract * & ANN. UNIV. MARIAE CURIE-SKLODOWSKA, SECT. AA. 1966, vol. 21, 1967, pages 49-64, M. JANCZEWSKI ET AL.: * CAS Registry (STN database), RN: 20025-59-6 * ---	1	
	-/--		

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	CHEMICAL ABSTRACTS, vol. 69, no. 19, 4 November 1968 Columbus, Ohio, US; abstract no. 75267a, XP002028926 * abstract * & BIOCHEM. J. , vol. 109, no. 1, 1968, pages 143-147, B. GILLHAM ET AL.: * CAS Registry (STN database), RN: 21462-48-6 * ---	1	
X	CHEMICAL ABSTRACTS, vol. 67, no. 15, 9 October 1967 Columbus, Ohio, US; abstract no. 73477d, XP002028927 * abstract * & J. INDIAN CHEM. SOC., vol. 43, no. 7, 1966, pages 521-525, A.B. SEN ET AL.: * CAS Registry (STN database), RN: 15773-92-9 * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
X	CHEMICAL ABSTRACTS, vol. 67, no. 9, 28 August 1967 Columbus, Ohio, US; abstract no. 43615e, XP002028928 * abstract * & BULL. SOC. CHIM. FR., vol. 11, 1966, pages 3674-3682, P. CAGNIANT ET AL.: * CAS Registry (STN database), RN: 6112-45-9 * ---	1	
	-/--		

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	CHEMICAL ABSTRACTS, vol. 66, no. 11, 13 March 1967 Columbus, Ohio, US; abstract no. 46292n, XP002028929 * abstract * & BOLL. SCI. FAC. CHIM. IND. BOLOGNA, vol. 24, no. 2-3, 1966, pages 75-91, I. DEGANI ET AL.: * CAS Registry (STN database), RN:13735-04-1;13735-02-9 * ---	1	
Y	WO 95 33731 A (HOFFMANN LA ROCHE ;BROADHURST MICHAEL JOHN (GB); BROWN PAUL ANTHON) 14 December 1995 * the whole document * ---	1,28,29	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
Y	WO 95 29892 A (DU PONT MERCK PHARMA) 9 November 1995 * the whole document * ---	1,28,29	
Y	EP 0 606 046 A (CIBA GEIGY AG) 13 July 1994 * the whole document * ---	1,28,29	
Y	CHEMICAL ABSTRACTS, vol. 93, no. 7, 18 August 1980 Columbus, Ohio, US; abstract no. 61046m, XP002028930 * abstract * & ARZNEIM.-FORSCH., vol. 30, no. 4A, 1980, pages 695-702, R.G. CHILD ET AL.: --- -/--	1,28,29	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 96 11 9780

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y,D	J. ENZYME INHIBITION, vol. 2, 1987, pages 1-22, XP000197047 W.H. JOHNSON ET AL.: * compound (V), page 7; table III, page 9 * -----	1,28,29	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

EPO FORM 1503 03.82 (P04C10)

European Patent Office

EP 96119780 - C -

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extend that is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely:
Claims searched incompletely: all
Claims not searched:

Reason for the limitation of the search:

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of all claims.
Reason:

The huge number of theoretically conceivable compounds resulting from the combinations of all the substituent definitions claimed in claim 1 prevents the search from being carried out comprehensively. Additionally such an uncertainty on the claimed scope may introduce contradictions and render unity questionable. Guided by the description, the search has been limited to the scope (IPC sub-divisions) which is illustrated by the compounds explicitely mentioned in the application. It is noted nevertheless that many individual compounds fall within the searched scope and therefore it is not possible to cite all of the documents found which are prejudicial to the novelty of the claimed invention. The documents cited as X-documents in the present search report are only a selection thereof.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07D 501/00, A61K 31/545 — A1

(11) International Publication Number: **WO 97/08174**

(43) International Publication Date: 6 March 1997 (06.03.97)

(21) International Application Number: PCT/US96/13967

(22) International Filing Date: 29 August 1996 (29.08.96)

(30) Priority Data:
60/003,082 31 August 1995 (31.08.95) US

(71) Applicants *(for all designated States except US)*: SMITHKLINE BEECHAM CORPORATION [US/US]; Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US). SMITHKLINE BEECHAM PLC [GB/GB]; Three New Horizons Court, Great West Road, Brentford, Middlesex TW8 9EP (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LEVY, Mark, Alan [US/US]; 115 reveille Road, Wayne, PA 19087 (US). LEE, Dennis [CA/US]; 205 Haverford Avenue, Swarthmore, PA 19081 (US). GLEASON, John, Gerald [CA/US]; 8 Heron Hill Drive, Downington, PA 19335 (US). TAYLOR, Andrew, William [GB/GB]; 64 Mazoe Road, Bishops Stortford CM23 3JT (GB). CORBERTT, David, Francis [GB/GB]; 12 Wilmots Close, Reigate, Surrey RH2 0NP (GB).

(74) Agents: VENETIANER, Stephen et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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

(54) Title: INTERLEUKIN CONVERTING ENZYME AND APOPTOSIS

(57) Abstract

The present invention is to the novel compounds of Formula (I), their pharmaceutical compositions, and to the novel inhibition of ICE and ICE-like proteins for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

Interleukin Converting Enzyme and Apoptosis

FIELD OF THE INVENTION

The present invention is to the discovery of a new method to block excessive or inappropriate apoptosis in a mammal.

BACKGROUND

It has been recognized for over a century that there are different forms of cell death. One form of cell death, necrosis, is usually the result of severe trauma and is a process that involves loss of membrane integrity and uncontrolled release of cellular contents, often giving rise to inflammatory responses. In contrast, apoptosis is a more physiological process that occurs in a controlled manner and is generally non-inflammatory in nature. For this reason apoptosis is often referred to as programmed cell death. The name itself (apoptosis: Greek for "dropping off", for example leaves from trees) implies a cell death that is part of a normal physiological process (Kerr et al., Br. J. Cancer, 26: 239-257 (1972)).

Apoptosis appears to be a carefully controlled series of cellular events which ultimately leads to death of the cell. This process for elimination of unwanted cells is active and requires expenditure of cellular energy. The morphological characteristics of apoptosis include cell shrinkage and loss of cell-cell contact, condensation of nuclear chromatin followed by fragmentation, the appearance of membrane ruffling, membrane blebbing and apoptotic bodies. At the end of the process, neighboring cells and macrophages phagocytose the fragments from the apoptotic cell. The process can be very fast, occurring in as little as a few hours (Bright et al., Biosci. Rep., 14: 67-82 (1994)).

The best defined biochemical event of apoptosis involves the orderly destruction of nuclear DNA. Signals for apoptosis promote the activation of specific calcium- and magnesium-dependent endonucleoases that cleave the double stranded DNA at linker regions between nucleosomes. This results in production of DNA fragments that are multiples of 180-200 base pair fragments (Bergamaschi et al., Haematologica, 79: 86-93 (1994); Stewart, JNCI, 86: 1286-1296 (1994)). When examined by agarose gel electrophoresis, these multiple fragments form a ladder pattern that is characteristic for most cells undergoing apoptosis.

There are numerous stimuli that can signal cells to initiate or promote cellular apoptosis, and these can be different in different cells. These stimuli can include glucocorticoids, TNFa, growth factor deprivation, some viral proteins, radiation and anticancer drugs. Some of these stimuli can induce their signals through a variety of cell surface receptors, such as the TNF / nerve growth factor family of receptors, which include CD40 and Fas/Apo-1 (Bright et al., supra). Given this diversity in stimuli that cause apoptosis it has been difficult to map out the signal transduction pathways and molecular factors involved in apoptosis. However, there is evidence for specific molecules being involved in apoptosis.

The best evidence for specific molecules that are essential for apoptosis comes from the study of the nematode C. elegans. In this system, genes that appear to be required for induction of apoptosis are Ced-3 and Ced-4. These genes must function in the dying cells and, if either gene is inactivated by mutation, cell death fails to occur (Yuan et al., Devel. Biol., 138: 33-41 (1990)). In mammals, genes that have been linked with induction of apoptosis include the proto-oncogene c-myc and the tumor suppresser gene p53 (Bright et al., supra; Symonds et al., Cell, 78: 703-711 (1994)).

In this critical determination of whether or not to undergo apoptosis, it is not surprising that these are genes that program for proteins that inhibit apoptosis. An example in C. elegans is Ced-9. When it is abnormally activated, cells survive that would normally die and, conversely, when Ced-9 is inactivated cells die that would normally live (Stewart, B.W., supra). A mammalian counterpart is bcl-2, which had been identified as a cancer-causing oncogene. This gene inhibits apoptosis when its product is overexpressed in a variety of mammalian cells, rendering them less sensitive to radiation, cytotoxic drugs and apoptotic signals such as c-myc (Bright et al., supra). Some viral proteins have taken advantage of this ability of specific proteins to block apoptosis by producing homologous viral proteins with analogous functions. An example of such a situation is a protein produced by the Epstein Barr virus that is similar to bcl-2, which prevents cell death and thus enhances viral production (Wells et al., J. Reprod. Fertil., 101: 385-391 (1994)). In contrast, some proteins may bind to and inhibit the function of bcl-2 protein, an example being the protein bax (Stewart, B.W., supra). The overall picture that has developed is that entry into apoptosis is regulated by a careful balancing act between specific gene products that promote or inhibit apoptosis (Barinaga, Science, 263: 754-756 (1994).

Apoptosis is an important part of normal physiology. The two most often cited examples of this are fetal development and immune cell development. In development of the fetal nervous system, over half of the neurons that exist in the early fetus are lost by apoptosis during development to form the mature brain (Bergamaschi et al., Haematologica, 79: 86-93 (1994)). In the production of immune competent T cells (and

to a lesser extent evidence exists for B cells), a selection process occurs that eliminates cells that recognize and react against self. This selection process is thought to occur in an apoptotic manner within areas of immune cell maturation (Williams, G. T., J. Pathol., 173: 1-4 (1994); Krammer et al., Curr. Opin. Immunol., 6: 279-289 (1994)).

Dysregulation of apoptosis can play an important role in disease states, and diseases can be caused by both excessive or too little apoptosis occurring. An example of diseases associated with too little apoptosis would be certain cancers. There is a follicular B-cell lymphoma associated with an aberrant expression of functional bcl-2 and an inhibition of apoptosis in that cell (Bergamaschi et al., supra). There are numerous reports that associate deletion or mutation of p53 with the inhibition of apoptosis and the production of cancerous cells (Kerr et al., Cancer, 73: 2013-2026 (1994); Ashwell et al., Immunol. Today, 15: 147-151, (1994)). In contrast, one example of excessive or inappropriate apoptosis is the loss of neuronal cells that occurs in Alzheimer disease, possible induced by b-amyloid peptides (Barr et al., BioTechnology, 12: 487-493 (1994)). Other examples include excessive apoptosis of $CD4^+$ T cells that occurs in HIV infection, of cardiac myocytes during infarction / reperfusion and of neuronal cells during ischemia (Bergamaschi et al., supra); Barr et al., supra).

Some pharmacological agents attempt to counteract the lack of apoptosis that is observed in cancers. Examples include topoisomerase II inhibitors, such as the epipodophyllotoxins, and antimetabolites, such as ara-c, which have been reported to enhance apoptosis in cancer cells (Ashwell et al., supra). In many cases with these anti-cancer drugs, the exact mechanism for the induction of apoptosis remains to be elucidated.

In the last few years, evidence has built that ICE and proteins homologous to ICE play a key role in apoptosis. This area of research has been spurred by the observation of homology between the protein coded by Ced-3, a gene known to be critical for C. Elegans apoptosis, and ICE. These two proteins share 29% amino acid identity, and complete identity in the 5 amino acid portion thought to be responsible for protease activity (QACRG) (Yuan et al., Cell, 75: 641-652 (1993)). Additional homologies are observed between ICE and the product of the nedd-2 gene in mice, a gene suspected of involvement in apoptosis in the developing brain (Kumar et al., Genes Dev., 8: 1613-1626 (1994)) and Ich-1 and CPP32 (ICE and Ced-3 homolog-1), human counterparts of nedd-2 isolated from human brain cDNA libraries (Wang et al., Cell, 78: 739-750 (1994); Fernandes-Alnemiri et al., J. Biol. Chem., 269: 30761-30764 (1994)).

Further proof for the role of these proteins in apoptosis comes from transfection studies. Over expression of murine ICE caused fibroblasts to undergo programmed cell

death in a transient transfection assay (Miura et al., Cell, 75: 653-660 (1993)). Cell death could be prevented by point mutations in the transfected gene in the region of greatest homology between ICE and Ced-3. As very strong support for the role of ICE in apoptosis, the authors showed that ICE transfection-induced apoptosis could be antagonized by overexpression of bcl-2, the mammalian oncogene that can prevent programmed cell death (Miura et al., supra). Additional experiments were performed using the crmA gene. This gene of the cowpox virus encodes a serpin protein, a family of proteins that are inhibitors of proteases (Ray et al., Cell, 69: 597-604 (1992)). Specifically, the protein of crmA has been shown to inhibit processing of pro-interleukin -1b by ICE. (Gagliardini et al. Science, 263: 826-828 (1994)) showed that microinjection of the crmA gene into dorsal root ganglion neurons prevented cell death induced by nerve growth factor deprivation. This result shows that ICE is involved in neuronal cell apoptosis. A more direct demonstration of ICE involvement comes from experiments in which ICE transfection is coupled with the co-expression of crmA, demonstrating a crmA-induced suppression of the ICE-induced apoptosis response (Miura et al., supra; Wang et al., supra).

In addition to ICE, researchers have examined the ability of ICE-like genes to promote apoptosis. (Kumar et al. supra) demonstrated that over expression of nedd-2 in fibroblasts and neuroblastoma cells resulted in cell death by apoptosis and that this apoptosis could also be suppressed by expression of the bcl-2 gene. Most recently, Wang et al., (Wang et al. , supra) examined the over expression of Ich-1 in a number of mammalian cells. Expression resulted in cell apoptosis, which could be antagonized by bcl-2 co-expression. Mutation of a cysteine residue, contained within the QACRG motif and presumed to be critical for protease function, to serine abolished apoptotic activity.

Further evidence for a role of a cysteine protease in apoptosis comes from a recent report by Lazebnik et al. (Nature, 371: 346-347 (1994)). These authors have used a cell-free system to mimic and study apoptosis. In their system there is a protease activity that cleaves the enzyme poly(ADP-ribose) polymerase at a site identical to a cleavage site in pre-interleukin-1b. However, this yet to be isolated protease and ICE appear to be different and to act on different substrate proteins. Blockade of protease activity in the system, using non-selective cysteine protease inhibitors, resulted in inhibition of apoptosis.

Taken together, the above evidence provides striking involvement of ICE and ICE-like proteins in the induction of apoptosis in mammalian cells. Brain interleukin-1 has been reported to be elevated in Alzheimer disease and Down syndrome (Griffin et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7611-7615 (1989)). There are also reports that interleukin-1 can increase the mRNA and production of b-amyloid protein, a major

component of senile plaques in Alzheimer disease as well as in brains of people with Down syndrome and with aging (Forloni et al., Mol. Brain Res., 16: 128-134 (1992); Buxbaum et al., Proc. Natl. Acad. Sci. U. S. A., 89: 10075-10078 (1992); Goldgaber et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7606-7610 (1989)). These reports can be viewed as additional evidence for the involvement of ICE in these diseases and the need for use of a novel therapeutic agent and therapy thereby.

To date, no useful therapeutic strategies have blocked excessive or inappropriate apoptosis. In one patent application, EPO 0 533 226 a novel peptide structure is diclosed which is said to be useful for determining the activity of ICE, and therefore useful in the diagnoses and monitoring of IL-1 mediated diseases. Therefore, a need exists to find better therapeutic agents which have non-toxic pharmacological and toxicological profiles for use in mammals. These compounds should block excessive or inappropriate apoptosis cells, and hence provide treatment for diseases and conditions in which this condition appears.

SUMMARY OF THE INVENTION

The present invention is to the novel compounds of Formula (I), their pharmaceutical compositions, and to the novel inhibition of ICE and ICE-like proteins for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death.

Another aspect of the present invention is to a pharmaceutical composition comprising a compound of Formula (I), or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier or diluent.

Another aspect of the present invention is to a method for the treatment of diseases or disorders associated with excessive IL-1b convertase activity, in a mammal in need thereof, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

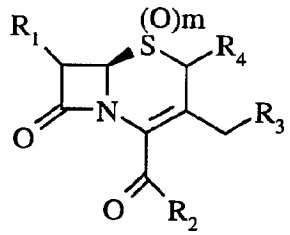
Another aspect of the present invention is to a method of preventing, or reducing apoptosis (i.e.blocking excess or inappropriate apoptosis) in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

Another aspect of the present invention is to a method of blocking or decresing the production of IL-1b and/or TNF, in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

DETAILED DESCRIPTION OF THE INVENTION

The compounds of the present invention may contain one or more asymmetric carbon atoms, in particular positions 6 and 7, and may exist in racemic and optically active forms. All of these compounds are included within the scope of the present invention. Preferably the compound has a 6R, 7S configuration.

Preferably the compounds of Formula (I) are represented by the structure:



(I)

wherein

R_1 is hydrogen, an optionally substituted alkoxy, or halogen;

R_2 is OR_a;

R_a is C_{1-4}alkyl, or optionally substituted aryl C_{1-4}alkyl;

R_3 is hydrogen, $-OC(O)R_5$, $S(O)_n R_6$, or bromine; provided that when R_3 is hydrogen, R_4 is other than hydrogen;

R_4 is hydrogen;

R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, or optionally substituted arylalkyl;

R_6 is optionally substituted aryl, or optionally substituted heteroaryl;

m is an integer having a value of 1 or 2;

n is 0, or an integer having a value of 1 or 2;

or a pharmaceutically acceptable salt thereof.

Suitably, for compounds of Formula (I), R_1 is hydrogen, an optionally substituted C_{1-4} alkoxy or halogen. When R_1 is alkoxy, the carbon chain may be optionally substituted, one or more times, suitably one to three times, independently by hydroxy, halogen, alkoxy, C(O)H, $C(O)_2R_c$, or $C(O)CH_3$ moieties; wherein R_c is hydrogen, C_{1-6} alkyl, aryl, or arylC_{1-4}alkyl. Preferably R_1 is methoxy.

Suitably, for compounds of Formula (I), R_2 is OR_a; wherein R_a is C_{1-4}alkyl, or an optionally substituted arylC_{1-4}alkyl, preferably benzyl. It is recognized that the alkyl group in the arylalkyl moiety may be branched or straightsuch as a methylene or substituted methylene group , i.e., $-CH(CH_3)$ - aryl.

When R_a is an optionally substituted arylC_{1-4}alkyl, the aryl ring may be substituted one or more times independently by hydroxy, halogen, alkyl or alkoxy. When R_a is an alkyl, it is preferably methyl or t-butyl.

Suitably, for compounds of Formula (I), m is 1 or 2. Preferably m is 2.

Suitably, for compounds of Formula (I), R_3 is hydrogen, -OC(O)R_5, S(O)n-R_6, or bromo; provided that when R_3 is hydrogen, then R_4 is other than hydrogen. When R_3 is -OC(O)R_5, the R_5 group is suitably C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, or optionally substituted arylalkyl; preferably R_5 is C_{1-6} alkyl, more preferably methyl.

When R_3 is S(O)n R_6, R_6 is suitably an optionally substituted aryl, or an optionally substituted aryl heteroaryl; and n is 0, or an integer having a value of 1 or 2. When R_6 is heteroaryl, as defined below, it is preferably a triazole, oxadiazole, or tetrazole moiety. When R_6 is aryl, as also defined below, it is preferably a phenyl; the n value is preferably 1 or 2. When R_6 is a heteroaryl, n is preferably 0. The heteroaryl or aryl ring may be optionally substituted one or more times independently by hydroxy, halogen, alkyl or alkoxy, preferably alkyl, more preferably methyl.

Compounds exemplified by Formula (I) include, but are not limited to:
3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethoxy)-3-cephem-4-carboxylate-1,1-dioxide
3,4- and 2,3-Dimethylbenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
4-Nitrobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl (1RS,6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate 1-oxide
3,4-Dichlorobenzyl-(6R,7R)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
4-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3-Iodo-4-methylbenzyl -(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl -(6R,7S)-7-[2-hydroxyethoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl -(6R,7S)-7-[n-butoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl -(6R,7S)-7-ethoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl-(6R,7S)-3-bromomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl-(6R,7S)-3-phenylsulfonylmethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl-(6R,7S)-3-[5-methyl-(1,3,4-oxadiazol)-2-thiomethyl]-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl-(6R,7S)-3-[(1-methyltetrazole)-5-thio]methyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl-(6R,7S)-3-[(1,2,3-triazole)-4-thiomethyl] -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Compounds of Formula (I) for use in the methods of the present invention include those noted above and:
tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7R)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
Benzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

The term "excessive IL-1b convertase activity" is used herein to mean an excessive expression of the protein, or activation of the enzyme.

The term "C_{1-6} alkyl" or "alkyl" is used herein to mean both straight and branched chain radicals of 1 to 6 carbon atoms, unless the chain length is otherwise specified, including, but not limited to, methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *sec*-butyl, *iso*-butyl, *tert*-butyl, and the like.

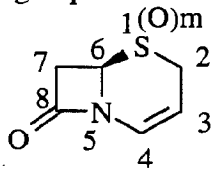
The term "heteroaryl" (on its own or in any combination, such as "heteroaryloxy", or "heteroaryl alkyl") is used herein to mean a 5-10 membered aromatic ring system in which one or more rings contain one or more heteroatoms selected from the group consisting of N, O or S, such as, but not limited, to pyrrole, pyrazole, furan, thiophene, quinoline, isoquinoline, quinazolinyl, pyridine, pyrimidine, oxazole, oxadiazole, tetrazole, thiazole, thiadiazole, triazole, imidazole, or benzimidazole.

The term "aryl" (on its own or in any combination, such as "aryloxy", or "arylalkyl") is used herein to mean a phenyl and naphthyl ring.

The term "cycloalkyl" is used herein to mean cyclic radicals, preferably of 3 to 7 carbons, including but not limited to cyclopropyl, cyclopentyl, cyclohexyl, and the like.

The term"halo" or "halogens", is used herein to include, unless otherwise specified, chloro, fluoro, bromo and iodo.

For purposes herein the "core" group for Formula (I) is numbered as follows:



The present invention is to the inhibition of ICE and ICE-like proteases by compounds of Formula (I). What is meant by the term "ICE-like proteases" are fragment, homologs, analogs and derivatives of the polypeptides Interleukin-1 b converting enzyme (or convertase). These analogs are structurally related to the ICE family. They generally encode a protein (s) which exhibits high homology to the human ICE over the entire sequence. Preferably, the pentapeptide QACRG is conserved. The ICE like proteases , which may include many natural allelic variants (such as substitutions, deletion or addition of nucleotides) does not substantially alter the function of the encoded polypeptide. That is they retain essentially the same biological function or activity as the ICE protease, although it is recognized that the biological function may be enhanced or reduced activity. The suitable activity is not IL-1b convertase activity, but the ability to induce apoptosis or involved in programmed cell death in some manner. Suitable ICE like proteases encompasses within this invention are those described in PCT US94/07127 filed 23 June 1994, Attorney Docket No.: 325800-184; and in USSN 08/334,251, filed 1 November 1994, Attorney Docket No.: 325800-249 whose disclosures are incorporated herein by reference in their entirety.

The term "blocking or inhibiting, or decreasing the production of IL-1b and/or TNF" as used herein refers to:

a) a decrease of excessive levels, or a down regulation, of the cytokine in a human to normal or sub-normal levels by inhibition of the *in vivo* release of the cytokine; or

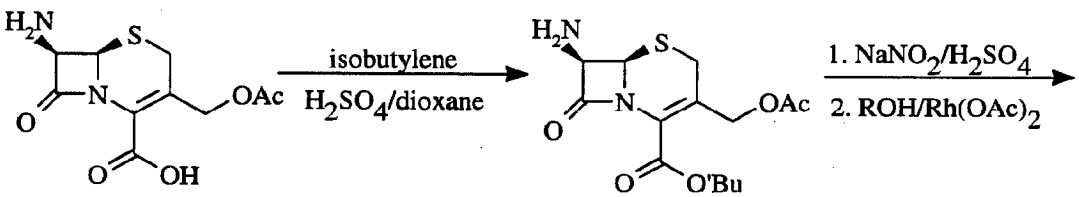
b) a down regulation, at the genomic level, of excessive *in vivo* levels of the cytokine (IL-1 or TNF) in a human to normal or sub-normal levels; or

c) a down regulation, by inhibition of the direct synthesis of the cytokine (IL-1, or TNF) as a postranslational event; or

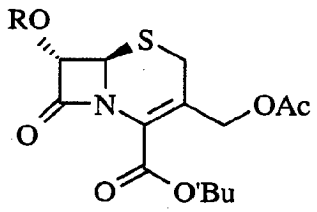
d) a down regulation, at the translational level, of excessive *in vivo* levels of the cytokine (IL-1, or TNF) in a human to normal or sub-normal levels.

The blocking or inhibiting, or decreasing the production of IL-1b and/or TNF is a discovery that the compounds of Formula (I) are inhibitors of the cytokines, IL-1 and TNF is based upon the effects of the compounds of Formulas (I) on the production of the IL-1 and TNF in *in vitro* and *in vivo* assays which are well known and recognized in the art, some of which are described herein.

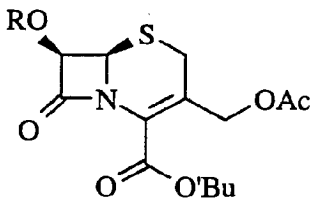
Compound of the present invention may be synthesized by methods well known in the art, such as those described by the procedures of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513 whose disclosure is incorporated herein by reference. Alternatively, compounds of Formula (I) may be made in accordance with the schemes illustrated below.



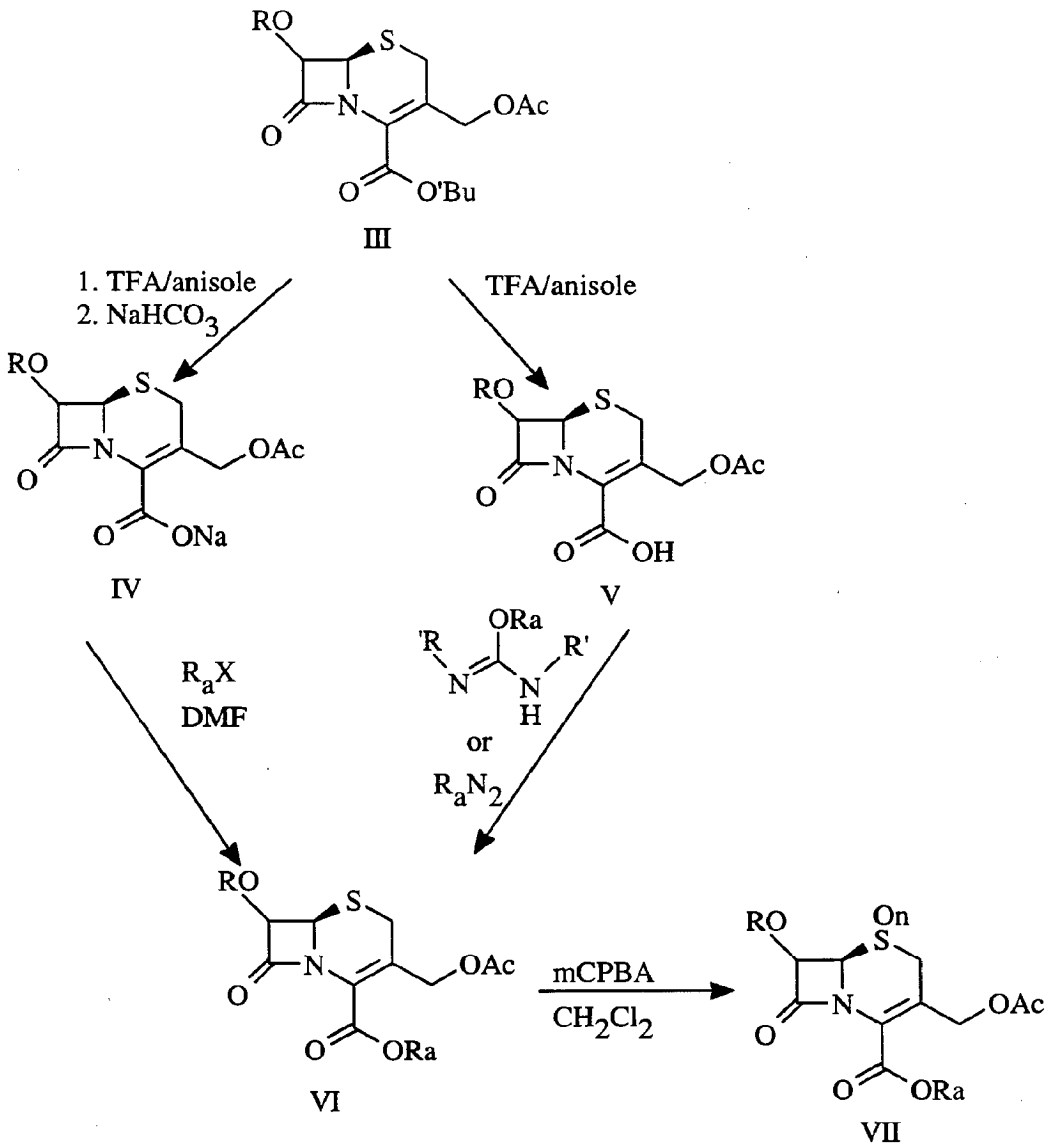
I II



IIIA



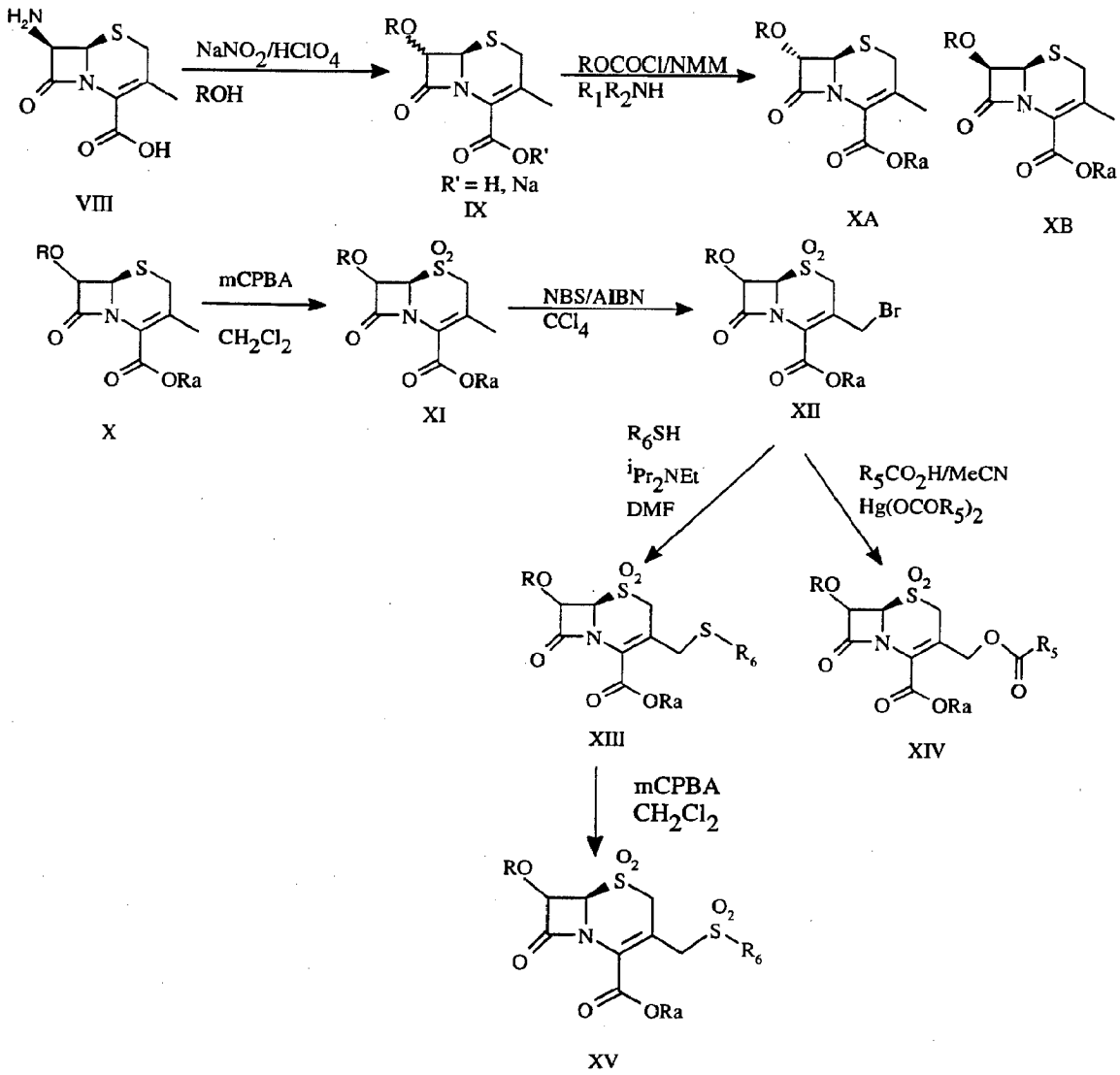
IIIB



Scheme 1

The t-Butyl ester, 2-Scheme I, is synthesized by treating commercially available 7-aminocephalosporonic acid (1-Scheme I) with isobutylene and sulfuric acid in dioxane. Following the procedure of Doherty et al. (*J. Med. Chem.* 1990, **33**, 2513-2521, which is incorporated herein by reference), 7-alkoxy substituted 3a-Scheme I and 3b-Scheme I are produced as a separable mixture. Deprotection of 3-Scheme I with trifluoroacetic acid/anisole at 0°C gives the free acid 5-Scheme I or the sodium salt 4-Scheme I upon titration with aqueous sodium bicarbonate. Benzyl halide alkylations of IV in DMF give esters 6-Scheme I. Treatment of 5-Scheme I with diazo derivatives (Braun et al. *J. Am. Chem. Soc.* 1958, **80**, 359-363,which is incorporated herein by reference) or with alkoxyisoureas (Schmidt et al. *Justus Liebigs Ann. Chem.* 1965, **685**,

161-166,which is incorporated herein by reference) yields various alkylester derivatives (6-Scheme I). Finally, sulfone or sulfoxides 7-Scheme I are obtained by m-chloroperoxybenzoic acid or oxone oxidation of 6-Scheme I.



VIII IX XA XB

X XI XII

XIII XIV

XV

Scheme 2

Alkoxy derivative 9-Scheme 2 is obtained in one step from 8-Scheme 2 by treatment with $NaNO_2$ and the alcohol in perchloric acid (Alpegiani et al. US 5,254,680, which is incorporated herein by reference). Ester 10-Scheme I is formed by esterification of 9-Scheme 2 by procedures described for 6-Scheme 1; m-chloroperoxybenzoic acid or oxone oxidation of 10-Scheme I yields 11-Scheme 2. The following derivatives can be synthesized according to procedures outlined by Alpegiani et al. *J. Med. Chem.* 1994, **37**, 4003-4019, which is incorporated herein by reference:

exposure of 11-Scheme 2 to N-bromosuccinimide under radical conditions gives the 3-bromomethyl derivative 12-Scheme 2; 13-Scheme 2 and 14-Scheme 2 are accessible through displacement of the bromide by aromatic thiols and mercuric acetate derivatives. Sulfones 15-Scheme 2 are obtained by oxidation of their corresponding thioethers (13-Scheme 2).

SYNTHETIC CHEMISTRY

Without further elaboration, it is believed that one skilled in the art can, using the preceding descriptions, utilize the present invention to its fullest extent. The following examples further illustrate the synthesis of compounds of this invention. The following examples are, therefore, to be construed as merely illustrative and not a limitation of the scope of the present invention in any way.

Temperatures are recorded in degrees centigrade unless otherwise noted.

Example 1

tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

The title compound was prepared according to the procedure of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513.

Example 2

tert-Butyl (6R,7R)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513, the title compound is isolated as a minor component of the final mixture.

Example 3

3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

a) 3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate

To tert-Butyl (6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate (prepared by the procedure of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513) (1.0 g, 2.9 mmol) and anisole (3.2 mL, 29 mmol) was added trifluoroacetic acid (16 mL) at 0_C under Ar. The solution was stirred for 30 min, and concentrated *in vacuo*.

The residue was dissolved in methylene chloride (50 mL), washed with water, washed with brine, dried ($MgSO_4$), filtered and concentrated *in vacuo* to an oil. The residue was dissolved in ethyl acetate (30 mL), and water (30 mL) was added. A solution of saturated sodium bicarbonate was dropped in until the aqueous layer reached pH 7. The aqueous layer was separated, and the procedure was repeated with

another 30 mL of water. The aqueous layers were combined and freeze-dried to afford a yellow solid (870 mg).

To the sodium salt (187 mg) in dimethylformamide (6 mL) was added 3,4-dichlorobenzyl chloride (168 uL) under Ar and the solution was stirred for 22 h. To the solution was added ether, the mixture was washed with water, dried ($MgSO_4$) and concentrated *in vacuo*. The oil was purified by flash chromatography (silica gel, 25-45% ethyl acetate/hexanes) to yield a 3:2 mixture of the title compound and the $\AE^2$ regioisomer (85 mg, 30% overall yield). ^1H NMR(400 MHz, $CDCl_3$) d 7.1-7.6 (m, 3H), 6.46, 4.5-5.3 (m, 6H), 3.3-3.7 (m, 5H), 3.55 (m, 3H).

b) 3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

To the ester (83 mg, 186 umol) of Example 2(a) in methylene chloride (3 mL) was added 85% m-chloroperoxybenzoic acid (114 mg, 558 umol) and the solution was stirred for 4 h. To the solution was added 20% sodium metabisulfite, followed by saturated sodium bicarbonate and the mixture was extracted with methylene chloride. The organic extract was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (silica gel, 40-50% ethyl acetate/hexanes) to yield the title compound (75 mg, 84%). MS(ES^+) m/e 478 $[M+H]^+$.

Example 4

tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethoxy)-3-cephem-4-carboxylate-1,1-dioxide

a) tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethoxy)-3-cephem-4-carboxylate

Following the procedure of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513, except substituting ethylene glycol for methanol, the title compound was prepared. ^1H NMR(400 MHz, $CDCl_3$) d 4.93 (d, J=13.7 Hz, 1H), 4.73 (d, J=13.7 Hz, 1H), 4.70 (s, 1H), 4.61 (s, 1H), 3.81 (br s, 4H), 3.58 (d, J=18.4 Hz, 1H), 2.07 (s, 3H), 1.54 (s, 9H).

b) tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethyloxy)-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3(b), except substituting the title compound of Example 4(a) for the ester of Example 3(a), the title compound was prepared. MS(ES^-) m/e 404 $[M-H]^-$.

Example 5

Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

a) (6R,7S)-3-Acetoxymethyl-7-methoxy-3-cephem-4-carboxylic acid

The intermediate sodium salt (85 mg) from Example 3(a) was purified by flash chromatography (0.5% acetic acid/10% methanol/methylene chloride) to yield the free acid (50 mg). ^{1}H NMR(400 MHz, 2:1 $CDCl_3/CD_3OD$) d 4.87 (d, J=12.6 Hz, 1H), 4.73 (d, J=12.6 Hz, 1H), 4.61 (s, 1H), 4.40 (d, J=1.7 Hz, 1H), 3.49 (d, J=17.8 Hz, 1H), 3.44 (s, 3H), 3.17 (d, J=17.8 Hz, 1H), 2.09 (s, 3H).

b) Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate

To the acid of Example 5(a) (48 mg, 167 umol) in tetrahydrofuran (3 mL) was added a 0.1 M ethereal solution of diazomethane (10 mL) at 0°C. The solution was stirred for 15 min, and quenched with an excess of acetic acid. The mixture was diluted with methylene chloride, washed with saturated sodium bicarbonate, concentrated in vacuo and purified by flash chromatography (silica gel, 15-25% ethyl acetate/hexanes) to yield the title compound (25 mg, 52%). ^{1}H NMR(400 MHz, $CDCl_3$) d 4.97 (d, J=13.2 Hz, 1H), 4.76 (d, J=13.2 Hz, 1H), 4.69 (s, 1H), 4.51 (s, 1H), 3.89 (s, 3H), 3.58 (d, J=18.3 Hz, 1H), 3.55 (s, 3H), 3.32 (d, J=18.3 Hz, 1H), 2.07 (s, 3H).

c) Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3(b), except substituting the title compound of Example 5(b) for the ester of Example 3(a), the title compound was prepared. MS(ES^-) m/e 332 $[M-H]^-$.

Example 6

Benzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting benzyl bromide for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^+) m/e 410 $[M+H]^+$.

Example 7

3,4- and 2,3-Dimethylbenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting 70% 3,4-dimethylbenzyl chloride (30% 2,3-dimethylbenzyl chloride) for 3,4-dichlorobenzyl chloride, the title compound was prepared as a 1:1 mixture with 2,3-dimethylbenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide. MS(ES^+) m/e 438 $[M+H]^+$.

Example 8

4-Nitrobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting 4-nitrobenzyl bromide for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^-) m/e 453 $[M-H]^-$.

Example 9

3,4-Dichlorobenzyl (1RS,6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate 1-oxide

Following the procedure of Example 3,.except one equivalent of m-chloroperoxybenzoic acid is used. MS(ES^-) m/e 460 $[M-H]^-$.

Example 10

3,4-Dichlorobenzyl-(6R,7R)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting tert-Butyl (6R,7R)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide for tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide, the title compound was prepared. MS(ES^-) m/e 478 $[M-H]^-$.

Example 11

4-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting 4-iodobenzyl chloride for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^+) m/e 536 $[M+H]^+$.

Example 12

3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting 3-iodobenzyl chloride for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^-) m/c 534 $[M-H]^-$.

Example 13

3-Iodo-4-methylbenzyl -(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting 3-iodo-4-methylbenzyl chloride for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^+) m/e 550 $[M+H]^+$.

Example 14

3,4-Dichlorobenzyl -(6R,7S)-7-[2-hydroxyethoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3, except substituting the title compound of Example 4(a) for tert-butyl (6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate. MS(ES^-) m/e 506 $[M-H]^-$.

Example 15

3,4-Dichlorobenzyl -(6R,7S)-7-[n-butoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 16, except substituting n-butanol for ethylene glycol, the title compound was prepared. MS(ES^-) m/e 518 $[M-H]^-$.

Example 16

3,4-Dichlorobenzyl -(6R,7S)-7-ethoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 16, except substituting ethanol for 3,4-dichlorobenzyl chloride, the title compound was prepared. MS(ES^-) m/e 490 $[M-H]^-$.

Example 17

3,4-Dichlorobenzyl-(6R,7S)-3-bromomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

a) 3,4-Dichlorobenzyl-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate

To a solution of (6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylic acid (1 g) in ethyl acetate (30 mL) is added water (30 mL). A solution of saturated sodium bicarbonate is dropped in until a pH of 7 in the aqueous layer is obtained. The aqueous layer is separated and lyophylized to afford sodium-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate (670 mg).

To sodium-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate (312 mg) in dimethylformamide (2 mL) is added 3,4-dichlorobenzyl chloride (500 uL), and the solution was stirred for 24 h. To the solution was added water and the solution was extracted with ether. The organic extract was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (silica gel, 15-25% ethyl acetate/hexanes) to yield 3,4-dichlorobenzyl-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate (148 mg). ^1H NMR(250 MHz, $CDCl_3$) d 7.2-7.6 (m, 3H), 5.23 (s, 2H), 4.67 (s, 1H), 4.50 (s, 1H), 3.4-3.6 (m, 4H), 3.19 (d, J=18.4 Hz, 1H), 2.10 (s, 3H).

b) 3,4-Dichlorobenzyl-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3b, except substituting 3,4-dichlorobenzyl-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate for the ester of example 2a, the sulfone was prepared. ^1H NMR(250 MHz, $CDCl_3$) d 7.2-7.6 (m, 3H), 5.21 (s, 2H), 5.13 (s, 1H), 4.62 (s, 1H), 3.88 (d, J=18.4 Hz, 1H), 3.66 (d, J=18.4 Hz, 1H), 3.56 (s, 3H), 2.10 (s, 3H).

c) 3,4-Dichlorobenzyl-(6R,7S)-3-bromomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide.

To the sulfone of Example 17b (91 mg) in carbon tetrchloride (4 mL) is added AIBN (5 mg) and N-bromosuccinimide (44 mg), and solution was reluxed under argon for 3 h. the reaction mixture was cooled, saturated sodium bicarbonate was added, and the mixture was extracted with methylene chloride. The organic extract was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by flash chromatography (silica gel, 15-35% ethyl acetate/hexanes) to yield the title compound (45 mg). MS(ES^-) m/e 496 $[M-H]^-$.

Example 18

3,4-Dichlorobenzyl-(6R,7S)-3-phenylsulfonylmethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

a) 3,4-Dichlorobenzyl-(6R,7S)-3-phenylthiomethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

To the bromide of Example 17 (110 mg) in dimethylformamide (15 mL) at 0°C was added thiophenol (25 uL) and N,N-diisopropyl-N-ethylamine (42 uL). The solution was stirred until the disappearance of starting material. Water was added, and the solution was extracted with ether. The organic extract concentrated *in vacuo*, and the residue was purified by flash chromatography (silica gel, ethyl acetate/hexanes) to yield the title compound. MS(ES$^+$) m/e 528 $[M+H]^+$.

b) 3,4-Dichlorobenzyl-(6R,7S)-3-phenylsulfonylmethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 3b, except substituting 3,4-dichlorobenzyl-(6R,7S)-7-methoxy-3-methyl-3-cephem-4-carboxylate for the ester of example 2a, the sulfone was prepared. MS(ES$^+$) m/e 560 $[M+H]^+$.

Example 19

3,4-Dichlorobenzyl-(6R,7S)-3-[5-methyl-(1,3,4-oxadiazol)-2-thiomethyl]-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 18a, except substituting 5-methyl-(1,3,4-oxadiazol)-2-mercaptan for thiophenol, the title compound was prepared. MS(ES$^+$) m/e 534 $[M+H]^+$.

Example 20

3,4-Dichlorobenzyl-(6R,7S)-3-[(1-methyltetrazole)-5-thio]methyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 18a, except substituting (1-methyltetrazole)-5-mercaptan for thiophenol, the title compound was prepared. MS(ES$^+$) m/e 534 $[M+H]^+$.

Example 21

3,4-Dichlorobenzyl-(6R,7S)-3-[(1,2,3-triazole)-4-thiomethyl] -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

Following the procedure of Example 18a, except substituting (1,2,3-triazole)-4-mercaptan for thiophenol, the title compound was prepared. MS(ES$^+$) m/e 519 $[M+H]^+$.

BIOLOGICAL ASSESSMENTS:

Assay I - DNA Ladder:

The present invention utilizes a model that measures apoptosis, by measuring the production of DNA ladders visualized on agarose gels. The observation of DNA ladders has been a hallmark of the apoptosis response for many years. The model used in our studies is the production of apoptosis in human monocytic HL-60 cells by the anti-cancer ether lipid 1-O-octadecyl-2-O-methyl-*sn*-3-phosphocholine (ET-18-OCH_3) and tumor necrosis factor a (TNF). The production of DNA ladders by ET-18-OCH_3 was recently reported (Mollinedo et al. Biochem. Biophys. Res. Commun., 192: 603-609 (1993)) and confirmed in house. The general method is to treat HL-60 cells with 6 μM ET-18-OCH_3 or 10 units of TNF for 24 hours, followed by extraction of small molecular weight DNA and removal of protein and RNA. The DNA is separated on a agarose gel and visualized with ethidium bromide staining. An internal standard is added to the cells just prior to extraction and preparation of DNA. Drugs are provided to cells 10 minutes prior to the apoptotic insult. This method provides a qualitative assessment of the ability of compounds to inhibit apoptosis.

Cell Conditions

- HL-60 cells (American Type Cell Culture) were grown and kept at log phase in RPMI 1640 w/L-glutamine and 10 % heat inactivated Fetal Bovine Serum (RPMI complete).
- On the day of the experiment, the desired number of cells (for example, 5 x 10^6 cells/treatment group) were resuspend in RPMI complete to give a final cell concentration of approximately 0.5 x 10^6 cells/ml. For each treatment group, 10 mls of cell suspension were placed in a culture flask. Cells were incubated for 2 hours at 37°C.

Exposures:

- For typical expose to ET-18-OCH_3, a 100 mM ET-18-OCH_3 stock solution in $CHCl_3$ was prepared, then diluted in RPMI complete to 600 μM. Then 100 μl of 600 μM ET-18-OCH_3 was added into 10 ml treatment group yielding a final concentration of 6 μM. The cell suspensions are then incubated overnight (18 hours). For a typical exposure to TNF, 300 to 3000 units of TNF were added to 10 ml of cell suspension.
- Cells were pretreated with desired agents (ICE compounds, etc.) 10 minutes prior to ET-18-OCH_3 or TNF addition. ICE compounds stocks were in DMSO. 50 μl of compound or DMSO vehicle was added to the 10 ml treatment groups yielding the final concentration of compound and 0.5 % DMSO.

DNA Extraction:

- Cells were spun (400 x g, 5 min) and washed 2x in 10 mLs PBS.
- Cells were lysed by resuspending them into 200 µL of cold, sterile detergent buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) and transferring the approximately 250 µL volume to sterile, 1.5 mL eppendorf tubes on ice. Then, tubes were incubated for 30 min. at 4°C, with mild shaking.
- Tubes were spun in a Microfuge for 15 min., the supernatant collected, taking care to avoid the cellular debris.
- The supernatants were incubated with 75 µg/mL RNAseA for 1 hr at 37°C then incubated with 200 µg/mL ProteinaseK and 0.5 % SDS [final] for 1 hr at 37°C.
- Ten µl of a 300 bp DNA was added as an internal standard to observe extraction efficiency.
- Supernatants were extracted twice with equal volume (200-300 µL) of cold, buffer saturated phenol (add phenol, vortex 15 seconds, microfuge 2 min., collect the top aqueous layer, avoiding the organic waste in between the two phases), once with 200 µL Phenol/Chloroform/Isoamyl alcohol 25:24:1 (v/v) and once with 100 µL Chloroform (100µL/sample is retrieved).
- Add 10 µL of sterile 3M NaCl (300 mM [final]) to the 100 µL sample and 200 µL of cold ethanol, vortex well and let stand overnight at -20°C.
- Samples were spun (Microfuge) 15 min and all but 25 µl of the ethanol was carefully removed. The DNA pellets were dried and resuspended in 30 µL of sterile 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 10 µL of gel loading Buffer. Load 20 µl/well.
- A DNA standard (for example, Sigma # D 5042, 123 bp ladder) was run on each gel.
- Samples were run on 1-2 % agarose gel with TBE buffer (5X TBE = 54 g Tris Base, 27.5 g Boric acid, 20ml 0.5 M EDTA, pH 8.0) with ethidium bromide added, for example for 90 - 120 min at 100 V, 50 mA.
- The resulting gels were visualized under UV light and the results recorded in a captured image.

Results

In HL-60 cells, treatment with ET-18-OCH_3 or TNF induced an apoptotic response that was prominent after 24 hours. Pretreatment with 50 µM of the compound of Example 3 was found to completely block the apoptotic response to both ET-18-OCH_3 or TNF (Table 1). Pretreatment with 5 µM of the compound of Example 3 was not effective over the 24 hour experiment. Addition of IL-1b (10 nM) had no effect on the ability of the compound of Example 3 to block apoptosis, suggesting that its primary mechanism of action is not inhibition of IL-1 production. These data support

that the compound of Example 3 blocks apoptosis by a novel mechanism of action, i.e., by inhibiting the activity of ICE and ICE-like proteases.

Table 1. Effect of Compound on Apoptosis

Drug	Concentration	Apoptotic Signal	Concentration	Presence of Apoptosis
None		ET-18-OCH_3	6 μM	yes
Example 3	50 μM	ET-18-OCH_3	6 μM	no
Example 3	5 μM	ET-18-OCH_3	6 μM	yes
None		TNF	270 U/ml	yes
Example 3	50 μM	TNF	270 U/ml	no

Assay II: Inhibition of ICE

Source of Enzyme

Human ICE was cloned and expressed in *E. coli* as its inactive precursor (p45) bearing a hexa-His flag on its *amino*-terminal end. Following harvesting, the cells were lysed, centrifuged, and the pellet containing the p45 solubilized with phosphate buffered 7 M urea at pH 7.5. The flagged p45 was applied to a Ni-nitrilo-acetic acid column, washed, and eluted with 300 mM imidazole. This yielded a highly enriched proenzyme preparation (390% pure p45). Catalytic autoproteolytic activation to p10/p20 dimer was achieved by concentrating the p45 on a Centricon ultrafiltration membrane (Amicon) at 10 _C for several hours. The formation of the catalytic subunits (p10 and p20) in activated samples was demonstrated by correlating time-dependent generation of ICE activity with p10/p20 signals in Western blots and by reversed-phase HPLC. Formation of authentic p10 and p20 was also confirmed by *N*-terminal sequence and MALD-mass spectral analyses of samples purified by reversed-phase HPLC. The activated enzyme was stored frozen at –80 _C.

Assay Protocol

ICE was assayed at 25 _C using the fluorogenic tetrapeptide substrate *N*-acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartyl-7-amido-4-methylcoumarin (Ac-**YVAD**-AMC). The assays were conducted at pH 7.5 in a buffered system containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS, and 2 mM DTT. The concentration of substrate was fixed at 25 uM. Fluorescence of the liberated 7-amino-4-methylcoumarin was continuously monitored at 460 nm following excitation at 335 nm.

Compound Testing

Compounds of Formula (I) were tested at a single dose of 100 uM following a 30 to 60-min preincubation with enzyme. The assay was initiated by the addition of 25 uM substrate (Ac-**YVAD**-AMC) and activity was monitored as described above.

Representative compounds of Formula (I), as exemplified by Examples 1 to 7 and 9 demonstrated positive inhibitiory activity in this assay ranging from about 36% to about 96%.

Assay III: Inhibition of ICE

ICE was assayed at 25 _C in 96-well plates using the fluorogenic tetrapeptide substrate *N*-acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartyl-7-amido-4-methylcoumarin (Ac-**YVAD**-AMC). The assays were conducted at pH 7.5 in a buffered system containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS, and 20-50 uM DTT. The concentration of substrate was fixed at 20 uM. Fluorescence of the liberated 7-amino-4-methylcoumarin was continuously monitored at 460 nm following excitation at 360 nm.

Compound Testing

Compounds were tested at a single dose of 50 to 100 uM. Activity was monitored as described above over a 30 to 60-minute time period following the simultaneous addition of substrate and inhibitor to initiate the reaction. The progress curves thus generated were fit by computer to Eq. 1 in order to assess potency and time-dependency:

$$v = \frac{(V_o(1 - e^{-k_{obs}t})}{k_{obs}} \qquad (1)$$

Representative compounds of formula (I) have demonstrated positive inhibitory activity in the above noted assay:

3,4-Dichlorobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

tert-Butyl 7-alpha-methoxycephalosporanate sulfone

3,4-Dichlorobenzyl-(6R,7S)-3-(1-methyltetrazol-5-yl)thiomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-(phenylsulfonyl)methyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[2-methyl(1,3,4-oxadiazol-5-yl)-2-thiomethyl]-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl (6R,7S)-3-(1,2,3-triazol-5-yl)thiomethyl-7-methoxy-3-cephem-4-carboxylate 1,1-dioxide

3,4-Dichlorobenzyl-5,5-dioxo-7-alpha-[2-hydroxyethyloxy]-cephalosporanate
3,4-Dichlorobenzyl-(6R,7R)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
Benzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
[(3,4)- and (2,3)-]Dimethylbenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
4-Nitrobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide N-3,4-Dichlorobenzyl-N-methyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide
(6R,7S)-4-Iodobenzyl--7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3-Iodo-4-methylbenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

METHODS OF TREATMENT

For therapeutic use the compounds of the present invention will generally be administered in a standard pharmaceutical composition obtained by admixture with a pharmaceutical carrier or diluent selected with regard to the intended route of administration and standard pharmaceutical practice. For example, they may be administered orally in the form of tablets containing such excipients as starch or lactose, or in capsule, ovules or lozenges either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavouring or colouring agents. They may be injected parenterally, for example, intravenously, intramuscularly or subcutaneously. For parenteral administration, they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The choice of form for administration as well as effective dosages will vary depending, inter alia, on the condition being treated. The choice of mode of administration and dosage is within the skill of the art.

The compounds of the present invention, particularly those noted herein or their pharmaceutically acceptable salts which are active when given orally, can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerin, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. Preferably the composition is in unit dose form such as a tablet or capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilized and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises a compound or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

The pharmaceutically acceptable compounds of the invention will normally be administered to a subject in a daily dosage regimen. For a patient this may be, for example, from about .001 to about 100mg/kg, preferably from about 0.001 to about 10mg/kg animal body weight. A daily dose, for a larger mammal is preferably from about 1 mg to about 1000 mg, preferably between 1 mg and 500 mg or a pharmaceutically acceptable salt thereof, calculated as the free base, the compound being administered 1 to 4 times per day. Unit dosage forms may contain from aobut 25μg to about 500mg of the compound.

There are many diseases and conditions in which dysregulation of apoptosis plays an important role. All of these conditions involve undesired, deleterious loss of specific cells with resulting pathological consequences.

Bone remodeling involves the initial resorption by osteoclasts, followed by bone formation by osteoblasts. Recently, there have been a number of reports of apoptotic events occurring during this process. Apoptotic events have been observed in both the bone forming and bone resorbing cells *in vitro* and indeed at the sites of these remodeling units *in vivo.*

Apoptosis has been suggested as one of the possible mechanisms of osteoclast disappearance from reversal sites between resorption and formation. TGF-ß1 induces apoptosis (approx. 30%) in osteoclasts of murine bone marrow cultures grown for 6 days *in vitro* . (Hughes, et al., *J. Bone Min. Res.* **9** , S138 (1994)). The anti-resorptive

bisphosphonates (clodronate, pamidronate or residronate) promote apoptosis in mouse osteoclasts *in vitro* and *in vivo* . (Hughes, et al., supra at S347). M-CSF, which has previously been found to be essential for osteoclast formation can suppress apoptosis, suggesting not only that maintenance of osteoclast populations, but also that formation of these multinucleated cells may be determined by apoptosis events. (Fuller, et al., *J. Bone Min. Res.* **8** , S384 (1993); Perkins, et al., *J. Bone Min. Res.* **8** , S390 (1993)). Local injections of IL-1 over the calvaria of mice once daily for 3 days induces intense and aggressive remodeling. (Wright, et al., *J. Bone Min. Res.* **9** , S174 (1994)). In these studies, 1% of osteoclasts were apoptotic 1 day after treatment, which increased 3 days later to 10%. A high percentage (95%) of these apoptotic osteoclasts were at the reversal site. This data suggests that ICE or ICE-like homologues are functionally very important in osteoclast apoptosis.

Therefore, one aspect of the present invention is the promotion of apoptosis in osteoclasts as a novel therapy for inhibiting resorption in diseases of excessive bone loss, such as osteoporosis, using compounds of Formula (I) as defined herein.

Apoptosis can been induced by low serum in highly differentiated rat osteoblast-like (Ros 17/2.8) cells (Ihbe, et al., (1994) *J. Bone Min. Res.* **9**, S167)). This was associated with a temporal loss of osteoblast phenotype, suggesting that maintenance of lineage specific gene expression and apoptosis are physiologically linked. Fetal rat calvaria derived osteoblasts grown *in vitro* undergo apoptosis and this is localized to areas of nodule formation as indicated by *in situ* end-labeling of fragmented DNA. (Lynch, et al., (1994) *J. Bone Min. Res.* **9**, S352). It has been shown that the immediate early genes c-fos and c-jun are expressed prior to apoptosis; c-fos and c-jun-Lac Z transgenic mice show constitutive expression of these transcription factors in very few tissues, one of which is bone (Smeyne, et al., (1992) *Neuron.* **8**, 13-23; and Morgan, J. (1993) Apoptotic Cell Death: Functions and Mechanisms. Cold Spring Harbor 13-15th October). Apoptosis was observed in these animals in the epiphyseal growth plate and chondrogenic zones as the petula ligament calcifies. Chondrogenic apoptosis has also been observed in PTHRP-less mice and these transgenics exhibit abnormal endochondral bone formation (Lee, et al., (1994) *J. Bone Min. Res.* **9**, S159). A very recent paper examined a human osteosarcoma cell line which undergoes spontaneous apoptosis. Using this cell line, LAP-4, but not ICE, could be detected and *in vitro* apoptosis could be blocked by inhibition or depletion of LAP-4 (Nicholson, et al., (1995) *Nature* **376**, 37-43). Thus, apoptosis may play a role in loss of osteoblasts and chondrocytes and inhibition of apoptosis could provide a mechanism to enhance bone formation.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to enhance bone formation using compounds of Formula (I) as defined herein.

Osteoarthritits (OA) is a degenerative disease characterized by progressive erosion of articular cartilage. Chondrocytes are the single cell-type found in articular cartilage and perturbations in metabolism of these cells may be involved in the pathogenesis of OA. Injury to cartilage initiates a specific reparative response which involves an increase in the production of proteoglycan and collagen in an attempt to reestablish normal matrix homeostasis. However, with the progress of the disease, the 3-dimensional collagen network is disrupted and cell death of chondrocytes occurs in OA lesions (Malemud, et al.: Regulation of chondrocytes in osteoarthritis. In: Adolphe, M. ed. Biological Regulation of Chondrocytes. Boca Raton:CRC Press, 1992, 295-319). It has been shown that in OA, chondrocytes adjacent to cartilage defects express high levels of bcl-2 (Erlacher, et al., (1995) *J. of Rheumatology*, 926-931). This represents an attempt to protect chondrocytes from apoptosis induced by the disease process.

Protection of chondrocytes during early degenerative changes in cartilage by inhibition of apoptosis may provide a novel therapeutic approach to this common disease. Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat osteoarthritis, using compounds of Formula (I) as defined herein.

Recent evidence shows that chronic, degenerative conditions of the liver are linked to hepatocellular apoptosis. These conditions include chemical-, infectious- and immune/inflammatory-induced hepatocellular degeneration. Apoptosis of liver cells has been observed in liver degenerative states induced by a variety of chemical agents, including acetaminophen (Ray, et a l.,(1993) *FASEB. J.* **7**, 453-463), cocaine (Cascales, et al., (1994) *Hepatology* **20**, 992-1001) and ethanol (Baroni, etal., (1994) *J. Hepatol.* **20**, 508-513). Infectious agents and their chemical components that have been shown to induce apoptosis include hepatitis ((Hiramatsu, et al., (1994) *Hepatology* **19**, 1354-1359; Mita, et al., (1994) *Biochem. Biophys. Res. Commun.* **204**, 468-474)), tumor necrosis factor and endotoxin . (Leist, et al., (1995) *J. Immunol.* **154**, 1307-1316; and Decker, K. (1993) *Gastroenterology* **28(S4)**, 20-25). Stimulation of immune / inflammatory responses by mechanisms such as allograft transplantation and hypoxia followed by reperfusion have been shown to induce apoptosis of hepatocytes (Krams, et al., (1995) *Transplant. Proc.* **27**, 466-467). Together, this evidence supports that hepatocellular apoptosis is central to degenerative liver diseases.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat degenerative liver diseases., using compounds of Formula (I) as defined herein.

Apoptosis is recognized as a fundamental process within the immune system where cell death shapes the immune system and effects immune functions. Apoptosis also is implicated in viral diseases (e.g AIDS). Recent reports indicate that HIV infection may produce an excess of apoptosis, contributing to the loss of $CD4^+$ T cells. Of additional interest is the observation that APO-1/Fas shares sequence homology with HIV-1 gp120.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat viral diseases, using compounds of Formula (I) as defined herein.

Additional therapeutic directions and other indications in which inhibition of apoptotic cysteine proteases is of therapeutic utility, along with relevant citations in support of the involvement for apoptosis in each indication, are presented below in Table 1.

Table 1: Therapeutic Indications Related to Apoptosis

Indication	Citations
Ischemia / reperfusion	Barr et al., (1994) *BioTechnology* **12**, 487-493; Thompson, C. B. (1995) *Science* **267**, 1456-1462
Stroke	Barr et al supra; and Thompson, C., supra
Polycystic kidney disease	Barr et al., supra; and Mondain, et al., (1995) *ORL J. Otorhinolaryngol. Relat. Spec.* **57**, 28-32
Glomerulo-nephritis	Barr et al., supra
Osteoporosis	Lynch et al., (1994) *J. Bone Min. Res.* **9**, S352; Nicholson et al., (1995) *Nature* **376**, 37-43
Erythropoiesis / Aplastic anemia	Thompson, C., supra; Koury et al., (1990) *Science* **248**, 378-381

Chronic liver degeneration	Thompson, C., supra; Mountz et al., 1994) *Arthritis Rheum.* **37**, 1415-1420; Goldin et al., (1993) *Am. J. Pathol.* **171**, 73-76
T-cell death	Thompson, C., supra; Ameison et al., (1995) *Trends Cell Biol.* **5**, 27-32
Osteoarthritis - chondrocytes	Ishizaki et al., (1994) *J. Cell Biol.* **126**, 1069-1077; Blanco et al., (1995) *Am. J. Pathol.* **146**, 75-85
Male pattern baldness	Mondain et al., supra; Seiberg et al., (1995) *J. Invest. Dermatol.* **104**, 78-82; Tamada et al., (1994) *Br. J. Dermatol.* **131**, 521-524
Alzheimer's disease	Savill, J.,(1994) *Eur. J. Clin. Invest.* **24**, 715-723; Su et al., (1994) *Neuroreport* **5**, 2529-2533; Johnson, E., (1994) *Neurobiol. Aging* **15 Suppl. 2**, S187-S189
Parkinson's disease	Savill, J., supra; Thompson, C., supra
Type I diabetes	Barr et al., supra

The IL-1 and TNF inhibiting effects of compounds of the present invention are determined by the following *in vitro* assays:

Interleukin - 1 (IL-1)

Human peripheral blood monocytes are isolated and purified from either fresh blood preparations from volunteer donors, or from blood bank buffy coats, according to the procedure of Colotta *et al*, J Immunol, **132**, 936 (1984). These monocytes ($1x10^6$) are plated in 24-well plates at a concentration of 1-2 million/ml per well. The cells are allowed to adhere for 2 hours, after which time non-adherent cells are removed by gentle washing. Test compounds are then added to the cells for about 1hour before the addition of lipopolysaccharide (50 ng/ml), and the cultures are incubated at 37°C for an additional 24 hours. At the end of this period, culture super-natants are removed and clarified of cells and all debris. Culture supernatants are then immediately assayed for IL-1 biological activity, either by the method of Simon *et al.*, J. Immunol. Methods, **84**, 85, (1985) (based on ability

of IL-1 to stimulate a Interleukin 2 producing cell line (EL-4) to secrete IL-2, in concert with A23187 ionophore) or the method of Lee *et al.*, J. ImmunoTherapy, **6** (1), 1-12 (1990) (ELISA assay).

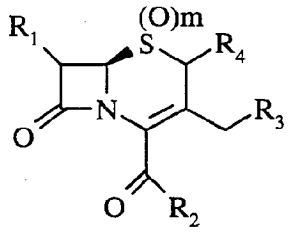
Tumour Necrosis Factor (TNF):

Human peripheral blood monocytes are isolated and purified from either blood bank buffy coats or platelet pheresis residues, according to the procedure of Colotta, R. *et al.*, J Immunol, **132**(2), 936 (1984). The monocytes are plated at a density of $1x10^6$ cells/ml medium/well in 24-well multi-dishes. The cells are allowed to adhere for 1 hour after which time the supernatant is aspirated and fresh medium (1ml, RPMI-1640, Whitaker Biomedical Products, Whitaker, CA) containing 1% fetal calf serum plus penicillin and streptomycin (10 units/ml) added. The cells are incubated for 45 minutes in the presence or absence of a test compound at 1nM-10mM dose ranges (compounds are solubilized in dimethyl sulfoxide/ethanol, such that the final solvent concentration in the culture medium is 0.5% dimethyl sulfoxide/0.5% ethanol). Bacterial lipopoly-saccharide (*E. coli* 055:B5 [LPS] from Sigma Chemicals Co.) is then added (100 ng/ml in 10 ml phosphate buffered saline) and cultures incubated for 16-18 hours at 37°C in a 5% CO_2 incubator. At the end of the incubation period, culture supernatants are removed from the cells, centrifuged at 3000 rpm to remove cell debris. The supernatant is then assayed for TNF activity using either a radio-immuno or an ELISA assay, as described in WO 92/10190 and by Becker *et al.*, J Immunol, 1991, **147**, 4307.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the are can, using the preceding description, utilize the present invention to its fullest extent. Therefore the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

What is claimed is:

1. A compound of the formula:



(I)

wherein

R_1 is hydrogen, an optionally substituted alkoxy or halogen;

R_2 is OR_a;

R_a is C_{1-4}alkyl, or optionally substituted aryl C_{1-4}alkyl;

R_3 is hydrogen, $-OC(O)R_5$, $S(O)_n R_6$, or bromine; provided that when R_3 is hydrogen, R_4 is other than hydrogen;

R_4 is hydrogen;

R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, optionally substituted arylalkyl;

R_6 is optionally substituted aryl, or optionally substituted heteroaryl;

m is an integer having a value of 1 or 2;

n is 0, or an integer having a value of 1 or 2;

or a pharmaceutically acceptable salt thereof; excluding the compounds tert-Butyl 7-alpha-methoxycephalosporanate sulfone; tert-Butyl 7-beta-methoxycephalosporanate sulfone; Methyl (6R,7S)-7-Methoxy-3-acetoxymethyl-3-cephem-4-carboxylic acid -1,1-dioxide; and Benzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide.

2. The compound according to Claim 1 wherein R_a is a benzyl moiety optionally substituted independently one or more times by hydroxy, halogen, alkyl, or alkoxy.

3. The compound according to Claim 1 wherein R_a is methyl or t-butyl.

4. The compound according to Claim 1 wherein the R_1 moiety is an optionally substituted alkoxy moiety.

5. The compound according to Claim 4 wherein the R_1 alkoxy is methoxy or 2-hydroxyethoxy.

6. The compound according to Claim 1 wherein m is 2.

7. The compound according to Claim 1 wherein R_3 is $S(O)_n R_6$.

8. The compound according to Claim 7 wherein R_6 is a heteroaryl which is an optionally substituted tetrazole, triazole, or oxadiazole.

9. The compound according to Claim 1 wherein R_3 is hydrogen.

10. The compound according to Claim 1 which is:
tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7R)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethoxy)-3-cephem-4-carboxylate-1,1-dioxide
Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
Benzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4- and 2,3-Dimethylbenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
4-Nitrobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl (1RS,6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate 1-oxide
3,4-Dichlorobenzyl-(6R,7R)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
4-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3-Iodo-4-methylbenzyl -(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl -(6R,7S)-7-[2-hydroxyethoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl -(6R,7S)-7-[n-butoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl -(6R,7S)-7-ethoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-bromomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-phenylsulfonylmethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[5-methyl-(1,3,4-oxadiazol)-2-thiomethyl]-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

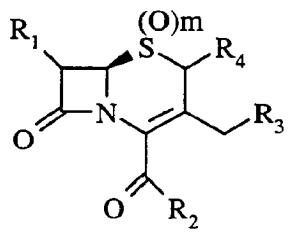
3,4-Dichlorobenzyl-(6R,7S)-3-[(1-methyltetrazole)-5-thio]methyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[(1,2,3-triazole)-4-thiomethyl] -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

11. A pharmaceutical composition comprising a compound according to Claim 1 and a pharmaceutically acceptable carrier or diluent.

12. A pharmaceutical composition comprising a compound according to Claim 10 and a pharmaceutically acceptable carrier or diluent.

13. A method of blocking excess or inappropriate apoptosis in a mammal in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of the formula:



(Ia)

wherein

R_1 is hydrogen, an optionally substituted alkoxy or halogen;

R_2 is OR_a;

R_a is C_{1-4}alkyl, or optionally substituted aryl C_{1-4}alkyl;

R_3 is hydrogen, $-OC(O)R_5$, $S(O)_n R_6$, or bromine; provided that when R_3 is hydrogen, R_4 is other than hydrogen, and that only one of R_3 and R_4 can be bromine;

R_4 is hydrogen;

R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, optionally substituted arylalkyl;

R_6 is optionally substituted aryl, or optionally substituted heteroaryl;

m is an integer having a value of 1 or 2;
n is 0, or an integer having a value of 1 or 2;
or a pharmaceutically acceptable salt thereof.

14. The method according to Claim 13 wherein the excessive or inappropriate apoptosis occurs in Alzheimer disease.

15. The method according to Claim 13 wherein the excessive or inappropriate apoptosis occurs in viral infections.

16. The method according to Claim 13 wherein the excessive or inappropriate apoptosis occurs during infarction or reperfusion injury.

17. The method according to Claim 13 wherein the excessive or inappropriate apoptosis occurs during ischemia.

18. The method according to Claim 13 wherein the excessive or inappropriate apoptosis results in excessive bone loss.

19. The method according to Claim 13 wherein the excessive or inappropriate apoptosis results in the disease of osteoarthritis.

20. The method according to Claim 13 wherein the excessive or inappropriate apoptosis results in hepatocellular degeneration.

21. The method according to Claim 13 wherein the compound is:
tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7R)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4-Dichlorobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
tert-Butyl (6R,7S)-3-acetoxymethyl-7-(2-hydroxyethoxy)-3-cephem-4-carboxylate-1,1-dioxide
Methyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
Benzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
3,4- and 2,3-Dimethylbenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide
4-Nitrobenzyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl (1RS,6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate 1-oxide

3,4-Dichlorobenzyl-(6R,7R)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

4-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3-Iodo-4-methylbenzyl -(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl -(6R,7S)-7-[2-hydroxyethoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl -(6R,7S)-7-[n-butoxy]-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl -(6R,7S)-7-ethoxy-3-acetoxymethyl-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-bromomethyl-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

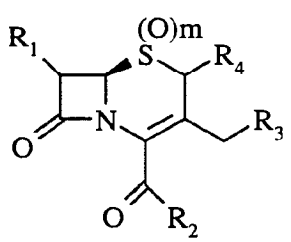
3,4-Dichlorobenzyl-(6R,7S)-3-phenylsulfonylmethyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[5-methyl-(1,3,4-oxadiazol)-2-thiomethyl]-7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[(1-methyltetrazole)-5-thio]methyl -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

3,4-Dichlorobenzyl-(6R,7S)-3-[(1,2,3-triazole)-4-thiomethyl] -7-methoxy-3-cephem-4-carboxylate-1,1-dioxide

22. A method for the treatment of diseases or disorders associated with excessive IL-1b convertase activity, in a mammal in need thereof, which method comprises administering to said mammal an effective amount of the formula:



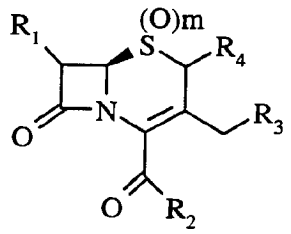
(Ia)

wherein

R_1 is hydrogen, an optionally substituted alkoxy or halogen;

R_2 is OR_a;
R_a is C_{1-4}alkyl, or optionally substituted aryl C_{1-4}alkyl;
R_3 is hydrogen, $-OC(O)R_5$, $S(O)_n R_6$, or bromine; provided that when R_3 is hydrogen, R_4 is other than hydrogen, and provided that only one of R_3 and R_4 can be bromine;
R_4 is hydrogen;
R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, optionally substituted arylalkyl;
R_6 is optionally substituted aryl, or optionally substituted heteroaryl;
m is an integer having a value of 1 or 2;
n is 0, or an integer having a value of 1 or 2;
or a pharmaceutically acceptable salt thereof.

23. A method of blocking or decresing the production of IL-1b and/or TNF, in a mammal in need of such treatment, which method comprises administering to said mammal an effective amount of a compound of the formula:



(Ia)

wherein
R_1 is hydrogen, an optionally substituted alkoxy or halogen;
R_2 is OR_a;
R_a is C_{1-4}alkyl, or optionally substituted aryl C_{1-4}alkyl;
R_3 is hydrogen, $-OC(O)R_5$, $S(O)_n R_6$, or bromine; provided that when R_3 is hydrogen, R_4 is other than hydrogen, and that only one of R_3 and R_4 can be bromine;
R_4 is hydrogen;
R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, optionally substituted arylalkyl;
R_6 is optionally substituted aryl, or optionally substituted heteroaryl;
m is an integer having a value of 1 or 2;
n is 0, or an integer having a value of 1 or 2;
or a pharmaceutically acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13967

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07D 501/00; A61K31/545
US CL :Please See Extra Sheet.
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)

U.S. : 540/215, 226, 229, 230;
514/204, 208, 209, 200

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 practicable, search terms used)

STN-CAS-on line
structure search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A,5,446,037 A(MAITI et al) 29 August 1995, column 3 and claim 1 when R4, here, is other than hydrogen.	1-23 1-23
X -P Y	Chemical Abstract, vol. 124, no.5. 29 January 1996(Columbus,OH,USA)page 1203,column 1,the abstract no.55675h. Alpengiani et al. WIPO document no. 94/28003, 08 December 1994.	1-23
X -- Y	Chemical Abstract, vol. 122,no.3.16 January 1995(Columbus OH. USA)page 905,column 1,the abstract no 31161n, Alpegiani et al., "Cephem Sulfones as Inactivators of Human Leukocyte Elastase", J. Med. Chem. (1994), vol. 37(23), pages 4003-19.	1-23 1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 12 DECEMBER 1996	Date of mailing of the international search report 03 FEB 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN M. FORD aco Telephone No. (703) 308-1235

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13967

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

540/215, 226, 229, 230;
514/204, 208, 209, 200

Electronic Acknowledgement Receipt

EFS ID:	14287044
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/Viantinna Campana Bordas
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	21-NOV-2012
Filing Date:	12-JAN-2006
Time Stamp:	11:41:14
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	Transmittal Letter	CEPH-4391_SIDS_Trans_11-21-12.PDF	103831 e1a6d3dfc23324ab026dacc52baf5daa46caa7a2	no	3
Warnings:					
Information:					

2	Information Disclosure Statement (IDS) Form (SB08)	CEPH-4391_SIDS_1449_11-21-12.PDF	121564 eca834bff97f85ad8420df394c4d3db7b7e5a5eb	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	EP_0780386.PDF	4879857 f206388169ee852d734596624362ead5bde06ea3	no	86
Warnings:					
Information:					
4	Foreign Reference	WO_97-08174.PDF	1928731 314ad9926d94be44d9567d2029bb6d5d53ba5721	no	40
Warnings:					
Information:					
5	Non Patent Literature	DHHS_FDA_ICH_GuidanceOnImpurities-ResidualSolvents_FederalRegister_1997_67377-67388.PDF	161538 76ab07042edb5580fd0fffde78b76895d07a213b	no	12
Warnings:					
Information:					
		Total Files Size (in bytes):	7195521		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

DOCKET NO.: CEPH-4391 / CP391 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Jason Edward Brittain **Confirmation No.: 9998**

Application No.: 11/330,868 **Group Art Unit: 1617**

Filing Date: January 12, 2006 **Examiner: Soroush, Ali**

For: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

Filed Via EFS

INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 CFR § 1.56 and in accordance with 37 CFR §§ 1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as an admission that this information is material as that term is defined in 37 CFR § 1.56(b).

☒ **IDS Filed Under 37 CFR 1.97(b)**

In accordance with § 1.97(b), since this Information Disclosure Statement is being filed either within three months of the filing date of the above-identified application, within three months of the date of entry into the national stage of the above identified application as set forth in § 1.491, before the mailing date of a first Office Action on the merits of the above-identified application, or before the mailing date of a first Office Action after the filing of request for continued examination under § 1.114, no additional fee is required.

☐ **IDS filed Under 37 CFR 1.97(c)**

In accordance with § 1.97(c), this Information Disclosure Statement is being filed after the period set forth in § 1.97(b) above but before the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311, or before an action that otherwise closes prosecution in the application, therefore:

☐ Certification in Accordance with § 1.97(e) is attached; or

☐ The fee of **$180.00** as set forth in § 1.17(p) is attached.

☐ **IDS filed Under 37 CFR 1.97(d)**

In accordance with § 1.97(d), this Information Disclosure Statement is being filed after the mailing date of either a Final Action under § 1.113 or a Notice of Allowance under § 1.311 but before, or simultaneously with, the payment of the Issue Fee, therefore included are: Certification in Accordance with § 1.97(e); and the submission fee of **$180.00** as set forth in § 1.17(p).

CONTENT OF IDS PURSUANT TO 37 CFR 1.98

☐ Copies of reference numbers listed on the attached Form 1449/PTO or Substitute for Form 1449/PTO are not required to be submitted pursuant to 37 CFR § 1.98(a)(2)(iii).

☒ Copies of reference numbers 95-97 listed on the attached Form 1449/PTO or Substitute for Form 1449/PTO are enclosed herewith.

☐ Copies of reference numbers are not being submitted because they were previously cited by or submitted to the U.S. Patent and Trademark Office in patent application number , filed for which a claim for priority under 35 U.S.C. § 120 has been made in the instant application.

☐ The month of publication for reference numbers is not available. However, the year of publication for these references is sufficiently earlier than the effective US filing date and any foreign priority date so that the particular month of publication is not in issue pursuant to 37 CFR § 1.98(b).

REFERENCES IN A LANGUAGE OTHER THAN ENGLISH

☐ The following documents are not in the English language. Accordingly, a concise explanation of the relevance of the document was incorporated in the specification passages identified below, the document was identified in a foreign communication as identified below or an English language counterpart application has been provided as indicated below.

Foreign Language Document	Cite No.	Pages of Reference in Specification or Relevance of Document

Foreign Language Document	Cite No.	English Language Counterpart	Cite No.

☐ **CERTIFICATION IN ACCORDANCE WITH § 1.97(e)**

I hereby certify that:

☐ Each item of information contained in this 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 this information disclosure statement.

☐ No item of information contained in this 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 this information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of this information disclosure statement.

Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

Date: November 21, 2012

/Stephanie A. Barbosa/
Stephanie A. Barbosa
Registration No. 51,430

WOODCOCK WASHBURN LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

46347 7590 02/04/2013
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

EXAMINER
SOROUSH, ALI

ART UNIT	PAPER NUMBER
1617	

DATE MAILED: 02/04/2013

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

TITLE OF INVENTION: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1770	$300	$0	$2070	05/06/2013

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

46347 7590 02/04/2013
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

TITLE OF INVENTION: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1770	$300	$0	$2070	05/06/2013

EXAMINER	ART UNIT	CLASS-SUBCLASS
SOROUSH, ALI	1617	548-304700

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☐ Issue Fee

☐ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

46347 7590 02/04/2013

WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

EXAMINER	
SOROUSH, ALI	
ART UNIT	PAPER NUMBER
1617	

DATE MAILED: 02/04/2013

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 802 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 802 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	11/330,868	BRITTAIN ET AL.	
	Examiner	Art Unit	
	ALI SOROUSH	1617	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the IDS submissions of 11/15/2012 and 11/21/2012*.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *83-91*.
4. ☐ 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 the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 11152012, 11212012
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/ALI SOROUSH/
Primary Examiner, Art Unit 1617

DETAILED ACTION

Claim Status

Claims 83-91 are pending.

Claims 31, 32, and 78-82 are cancelled and 1-30 and 33-77 were previously cancelled.

Claims 83-91 have been examined.

Claims 83-91 are rejected.

Priority

Priority to application 60/644,354 filed on 01/14/2005 is acknowledged.

Information Disclosure Statement

The information disclosure statements (IDSs) submitted on 11/15/2012 and 11/21/2012 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statements have been considered by the examiner.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance: the prior art teaches a formulation of bendamustine and mannitol to be lyophilized. The prior art also teach a combination of mannitol, tertiary-butyl alcohol, water, and an anti-neoplastic agent can be lyophilized. The prior art suggests using a combination of mannitol and tertiary-butyl alcohol with bendamustine to produce a formulation to be lyophilized. However, Applicant has unexpectedly found that the addition of tertiary-butyl alcohol stabilizes the formulation such that bendamustine degradation is negiligable (no more than 0.5% formation of bendamustine ethyl ester). Therefore, claims 83-91 are allowed.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Claims 83-91 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALI SOROUSH whose telephone number is (571)272-9925. The examiner can normally be reached on M-F (9am-6pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Fereydoun G. Sajjadi can be reached on (571)272-3311. 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.

/ALI SOROUSH/
Primary Examiner, Art Unit 1617

January 27, 2013

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	Art Unit
	ALI SOROUSH	1616

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner
34	284	08/20/2012	AS
548	304.7	08/20/2012	AS

SEARCH NOTES		
Search Notes	Date	Examiner
see search history printouts	08/20/2012	AS
Inventor/Assignee search EAST/PALM (Jason Edward Brittain, Joe Craig Franklin, Cephalon, Inc.)	08/20/2012	AS

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
34	284	08/20/2012	AS
548	304.7	08/20/2012	AS

/ALI SOROUSH/ Primary Examiner.Art Unit 1617	

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 9998

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/330,868	01/12/2006 RULE	548	1617	CP391

APPLICANTS

Jason Edward Brittain, El Cajon, CA;
Joe Craig Franklin, Tulsa, OK;

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

This appln claims benefit of 60/644,354 01/14/2005

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

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

02/27/2006

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /ALI SOROUSH/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	6	78	21

ADDRESS

WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891
UNITED STATES

TITLE

BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

FILING FEE RECEIVED		
0.00	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	2	treanda	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
S2	0	bendamustine same (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:41
S3	10	bendamustine and (lyophilize lyphilized)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
S4	46	bendamustine and (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
S5	3	bendamustine same (lyophilize lyphilized freeze$1dried)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:42
S6	88851	lyophilize lyophilization freeze$dry freeze$dried free$1drying	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:56
S7	22	S6 same (alkylating adj agent)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 19:57
S8	2	bendamustine same (aqueous adj	US-PGPUB;	OR	ON	2010/08/14

		solution) same unstable	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			20:03
S9	0	"cephalon.in"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:04
S10	563	cephalon.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
S11	11	S10 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:05
S12	4	bendamustine same (aqueous adj solution)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
S13	458	bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
S14	30	bendamustine adj hydrochloride	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:06
S15	58	bendamustine same injection	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:07
S16	18	bendamustine same solid	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2010/08/14 20:12

			IBM_TDB			
S17	2	bendamustine same unstable	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:13
S18	2	"0656211"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/14 20:29
S19	0	"0656211"	EPO	OR	ON	2010/08/14 20:29
S20	610	ku.in.	EPO	OR	ON	2010/08/14 20:29
S21	1	S20 and thiotepa	EPO	OR	ON	2010/08/14 20:30
S22	0	"5330835".pn.	EPO	OR	ON	2010/08/17 12:07
S23	2	"5330835".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/17 12:08
S24	3	"4145400".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/17 12:10
S25	3	"4145440".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/17 12:10
S26	1	10/417631.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2010/08/24 13:13
S27	0	benadmustine with mannitol with alcohol	EPO	OR	ON	2011/04/22 20:07
S28	0	benadmustine	EPO	OR	ON	2011/04/22 20:07
S29	11	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl)amino]-1-	EPO	OR	ON	2011/04/22 20:20

		methylbenzimidazol-2-yl]butanoic acid" "16506-27-7"				
S30	775	bendamustine ribomustin treanda "SDX-105" bendamustin Cytostasan "IMET 3393" "Zimet 3393" "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" "16506-27-7"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:20
S31	10	S30 with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S32	13	S30 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S33	13	S30 with alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:21
S34	22	S30 same alcohol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:22
S35	23	S30 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:24
S36	345	S30 and mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:36
S37	52	S36 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:38
S38	108	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) with (t-Butanol 2-Methyl-	US-PGPUB; USPAT; USOCR;	OR	ON	2011/04/22 20:44

		2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	FPRS; EPO; JPO; DERWENT; IBM_TDB			
S39	31	S38 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:44
S40	2	"5362718".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:52
S41	1	S30 same (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55
S42	15	S30 and (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:55
S43	18	S30 with rapamycin	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 20:56
S44	23	S30 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
S45	6	S30 same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:01
S46	132	S30 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2011/04/22 21:01

		propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	JPO; DERWENT; IBM_TDB			
S47	299	(mannitol "(2R,3R,4R,5R)-Hexane-1,2,3,4,5,6-hexol" Osmitrol Osmofundin) same (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02
S48	7	S47 and S30	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:02
S49	65	cyclophosphamide with mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07
S50	17	S49 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:07
S51	0	S50 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:12
S52	17166	(nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S53	113050	S52 sme (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S54	6	S52 same (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR;	OR	ON	2011/04/22 21:14

			FPRS; EPO; JPO; DERWENT; IBM_TDB			
S55	2335	S52 and (lyophilization lyophilize freeze$1dry freeze$1drying)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:14
S56	4	S35 and (t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:15
S57	3	S30 same tablet	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:18
S58	60242	(t-Butanol 2-Methyl-2-propanol ((t-Butyl tert-Butyl tertiary-Butyl) adj alcohol) 1,1-Dimethylethanol Dimethylethanol tert-Butanol 2-Methyl-propan-2-ol (Trimethyl adj carbinol) Trimethylcarbinol 2-metilpropan-2-ol Trimethylmethanol "2-Propanol, 2-methyl-")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
S59	81388	lyophilization lyophilize freeze$1dry freeze$1drying	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
S60	477	S58 same S59	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:22
S61	52	S60 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:23
S62	7	chlorambucil same lyophilization	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT;	OR	ON	2011/04/22 21:41

			IBM_TDB			
S63	49972	freeze$1dry freez$1drying lyophilisation lyophilization cryodesiccation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
S64	82	S63 and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:45
S65	6	S38 and S64	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:46
S66	13	S30 with water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:48
S67	10	fishman.in. and K4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50
S68	0	fishman.in. and S30	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:50
S69	2	"20020102215"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 21:53
S70	986	brittain.in. franklin.in. and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/22 22:53
S71	2	(brittain.in. franklin.in.) and bendamustine	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2011/04/22 22:54

			JPO; DERWENT; IBM_TDB			
S72	0	"4670262".pn.	EPO	OR	ON	2011/04/25 11:15
S73	2	"4670262".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:15
S74	626	jenapharm.as. ribosepharm.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:43
S75	0	S74 and (freeze$1dry freez$1drying lypholization lyophilize)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:44
S76	28	S74 and (powder)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 11:44
S77	396	GlOlA.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 15:35
S78	0	S77 and dinitroalanine	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 15:35
S79	4	S77 and dinitroaniline	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2011/04/25 15:35
S80	12	bendamustine "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105"	EPO	OR	ON	2012/08/20 17:07
S81	1158	bendamustine "4-[5-[Bis(2-	US-PGPUB;	OR	ON	2012/08/20

		chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105"	USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			17:09
S82	17	S81 near5 water	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:09
S83	15	S81 near5 (mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:10
S84	19	S81 with (mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:10
S85	203678	"tert-Butanol" "2-methyl-2-propanol" "tertiary-butyl alcohol" "2-Methylpropan-2-ol" "Dimethylethanol" "1,1-Dimethylethanol" ""tert-butyl alcohol" "t-butyl alcohol" ""1,1-Dimethyl ethanol" "trimethyl carbinol" "t-butyl hydroxide" "trimethyl methanol" "dimethyl ethanol" "methyl-2-propanol"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:15
S86	165922	(mannitol "(2R,3R,4R,5R)-Hexan-1,2,3,4,5,6-hexol")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:15
S87	24	S85 near5 S86	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
S88	107	S85 with S86	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
S89	2	S88 and S81	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2012/08/20 17:16

			JPO; DERWENT; IBM_TDB			
S90	364	S85 same S86	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
S91	7	S90 and S81	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
S92	7	S81 near5 S85	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:16
S93	8	S81 with S85	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:17
S94	183540	Freeze$1drying lyophilisation lyophilization cryodesiccation lyophilized lyophilize	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:22
S95	516	S94 and S81	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:23
S96	22	S94 same S81	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:23
S97	93	Mundipharma.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:29
S98	0	Mundipharma.as. and S81	US-PGPUB; USPAT;	OR	ON	2012/08/20 17:30

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S99	34	S81 same mannitol	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:36
S100	1160	bendamustine "4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid" Treakisym Ribomustin Treanda "SDX-105" "IMET 3393"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 17:53
S101	273	34/284.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
S102	0	34/284.ccls. and S81	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
S103	273	34/284.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:32
S104	2	"5977129".pn.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 18:39
S105	904	548/304.4.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:00
S106	11	S105 and (nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:01

S107	593	548/304.7.ccls.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:06
S108	14	S107 and (nitrogen adj mustard)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:06
S109	9	(brittain.in. franklin.in. cephalon.as.) and bendamustine.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/08/20 19:08

1/ 27/ 2013 9:18:13 PM
C:\ Users\ asoroush\ Documents\ EAST\ Workspaces\ 11330868.wsp

Substitute for 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(use as many sheets as necessary)*				***Complete if Known***	
				Application Number	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1616
				Examiner Name	Ali Soroush
Sheet	1	of	1	**Attorney Docket Number**	CEPH-4391 (CP391US)

U. S. PUBLICATION AND PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number Number – Kind Code (if known)	Publication or Grant Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document
/A.S./	**1**	5,192,743	03-09-1993	Hsu et al.
/A.S./	**2**	5,183,746	02-02-1993	Shaked et al.

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
/A.S./	**3**	WO 2006/065392	06-22-2006	Cephalon, Inc.	

Examiner Signature	/Ali Soroush/	**Date Considered**	01/27/2013

Substitute for 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(use as many sheets as necessary)*				***Complete if Known***	
				Application Number	11/330,868
				Filing Date	January 12, 2006
				First Named Inventor	Jason Edward Brittain
				Art Unit	1617
				Examiner Name	Soroush, Ali
Sheet	1	of	1	**Attorney Docket Number**	CEPH-4391 / CP391

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Foreign Patent Document Country Code- Number -Kind Code (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	T
/A.S./	**95**	EP 0780386	06-25-1997	F. Hoffmann-La Roche AG	
/A.S./	**96**	WO 97/08174	03-06-1997	Smithkline Beecham Corporation	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.	Include name of the author, 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
/A.S./	**97**	Department of Health and Human Services, Food and Drug Administration, “International Conference on Harmonisation; Guidance on Impurities: Residual Solvents,” Federal Register, December 24, 1997, 62(247), 67377-67388	

Examiner Signature	/Ali Soroush/	**Date Considered**	01/27/2013

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	**Art Unit**
	ALI SOROUSH	1617

CPC			
Symbol		**Type**	**Version**
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		
	/		

CPC Combination Sets				
Symbol	**Type**	**Set**	**Ranking**	**Version**
/				
/				

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
548	304.7

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
34	284				

INTERNATIONAL CLASSIFICATION

CLAIMED					NON-CLAIMED				
C	0	7	D	235 / 04 (2006.01.01)					

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 9	
/ALI SOROUSH/ Primary Examiner.Art Unit 1617 (Primary Examiner)	01/27/2013 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	**Art Unit**
	ALI SOROUSH	1617

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 9	
/ALI SOROUSH/ Primary Examiner.Art Unit 1617 (Primary Examiner)	01/27/2013 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11330868	BRITTAIN ET AL.
	Examiner	**Art Unit**
	ALI SOROUSH	1617

☒ **Claims renumbered in the same order as presented by applicant** ☐ **CPA** ☐ **T.D.** ☐ **R.1.47**

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	83														
2	84														
3	85														
4	86														
5	87														
6	88														
7	89														
8	90														
9	91														

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 9	
/ALI SOROUSH/ Primary Examiner.Art Unit 1617 (Primary Examiner)	01/27/2013 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure none

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

46347 7590 02/04/2013
WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	01/12/2006	Jason Edward Brittain	CP391	9998

TITLE OF INVENTION: BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1770	$300	$0	$2070	05/06/2013

EXAMINER	ART UNIT	CLASS-SUBCLASS
SOROUSH, ALI	1617	548-304700

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _____
2 _____
3 Woodcock Washburn LLP

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Cephalon, Inc.
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Frazer, PA

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 233050 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Stephanie A. Barbosa/ Date April 5, 2013

Typed or printed name Stephanie A. Barbosa Registration No. 51,430

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Electronic Patent Application Fee Transmittal

Application Number:	11330868
Filing Date:	12-Jan-2006
Title of Invention:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
First Named Inventor/Applicant Name:	Jason Edward Brittain
Filer:	Stephanie A. Barbosa/Ann Trevisani
Attorney Docket Number:	CP391

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:				
Utility Appl Issue Fee	1501	1	1780	1780
Publ. Fee- Early, Voluntary, or Normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**2080**

Electronic Acknowledgement Receipt

EFS ID:	15445344
Application Number:	11330868
International Application Number:	
Confirmation Number:	9998
Title of Invention:	BENDAMUSTINE PHARMACEUTICAL COMPOSITIONS
First Named Inventor/Applicant Name:	Jason Edward Brittain
Customer Number:	46347
Filer:	Stephanie A. Barbosa/Ann Trevisani
Filer Authorized By:	Stephanie A. Barbosa
Attorney Docket Number:	CP391
Receipt Date:	05-APR-2013
Filing Date:	12-JAN-2006
Time Stamp:	14:41:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2080
RAM confirmation Number	1008
Deposit Account	233050
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 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance 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	Issue Fee Payment (PTO-85B)	Issue_Fee_Transmittal_CP391_US.PDF	1396391 44ddff871ce138ec506000c60267ec1ac137ea70	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	32091 81dd640a75a8bd6cb31296f1a80d4a9246dbafb3	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1428482		

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.

<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

FORM PTO-1449 **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** List of Patent and Publications Cited by Applicant	Attorney Docket	CP391
	Application Number	11/330,868
	Filing Date	January 12, 2006
	First Named Inventor	Brittain
	Group Art Unit	1616
	Examiner Name	A. Soroush
	Sheet	2 of 5

FOREIGN PATENT DOCUMENTS

Examiner Initials	Cite No.	Document No.	Date	Country	Translation YES	Translation NO
/A.S./	B1	DD 159289	Jun. 1, 1981	Germany	x	
	B2	DD 159877	Apr. 13, 1983	Germany	x	
	B3	DD 293808	Sep. 12, 1991	Germany		x
	B4	DE 80967	Jun. 1, 1970	Germany	x	
	B5	DE 10016077	Dec. 13, 2001	Germany		x
	B6	DE 10304403	Aug. 5, 2004	Germany	A27	
	B7	DE 10306724	Sep. 18, 2003	Germany		x
	B8	EP 1354952	Oct. 22, 2003	Germany		
	B9	EP 1444989	Aug. 11, 2004	Italy		
	B10	WO 96/28148	~~Mar. 13, 1998~~	~~Australia~~		
	B11	WO 03/066027 A1	Feb. 7, 2003	PCT		
	B12	WO 03/081238	Oct. 2, 2003	PCT		
	B13	WO 03/086470 A3	May 6, 2004	PCT		
	B14	WO 03/094990	Mar. 11, 2003	PCT	A26	

Change(s) applied to document, /N.W.S./ 10/2/2012

1996-09-19

OTHER DOCUMENTS Non-Patent Literature Documents

Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), Title of Article, Title of Journal (book, magazine, catalog, etc.) Date, Pertinent Pages, Volume-Issue Number, publisher, city and/or country where published.
/A.S./	C1	AIVADO, MANUEL et al., *Bendamustine in the treatment of chronic lymphocytic leukemia: Results and future perspectives*, Seminars in Oncology, **2002**, pp. 19-22, Vol. 29 No. 4, Suppl. 13.
/A.S./	C2	BARMAN BALFOUR, JULIA A. et al., *Bendamustine*, Drugs, **2001**, pp. 631-638, Vol. 61(5), Auckland, New Zealand
/A.S./	C3	BREMER, KARL, *High rates of long-lasting remissions after 5-day bendamustine chemotherapy cycles in pre-treated low-grade non-hodgkin's-lymphomas*, Journal of Cancer Research and Clinical Oncology, **2002**, pp.603-609, Vol. 128(11).

Examiner's Signature	/Ali Soroush/	**Date:**	08/14/2009

FORM PTO-1449

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

List of Patent and Publications Cited by Applicant

Attorney Docket	CP391
Application Number	11/330,868
Filing Date	January 12, 2006
First Named Inventor	Brittain
Group Art Unit	1616
Examiner Name	A. Soroush
Sheet	1 of 5

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number	Name	Date of Publication	Class	Subclass	
/A.S./	A1	US-5204335	Sauerbier et al.	Apr. 20, 1993			
	A2	US-5227373	Alexander et al.	Jul. 13, 1993			
	A3	US-5750131	Wichert et al.	May 12, 1998			
	A4	US-5770230	Teagarden et al.	Jun. 23, 1998			
	A5	US-5776456	Anderson et al.	Jul. 7, 1998			
	A6	US-5955504	Wechter et al.	Sep. 21, 1999			
	A7	US-5972912	Marek et al.	Oct. 26, 1999			
	A8	US-6034256	~~Masferrer~~	Mar. 7, 2000			Carter, et al.
	A9	US-6077850	~~Masferrer~~	Jun. 20, 2000			Carter, et al.
	A10	US-6090365	Kaminski et al.	Jul. 18, 2000			
	A11	US-6271253 B1	~~Masferrer~~	Aug. 7, 2001			Carter, et al.
	A12	US-6380210	Desimone et al.	Apr. 30, 2002			
	A13	US-6492390 B2	~~Masferrer~~	Dec. 12, 2002			Carter, et al.
	A14	US-6545034 B1	Carson et al.	Apr. 8, 2003			
	A15	US-6569402	Cheesman et al.	May 27, 2003			
	A16	US-6573292 B1	Nardella	Jun. 3, 2003			
	A17	US-6613927 B1	Kwok	Sep. 2, 2003			
	A18	US-2003/0232874	Nardella	Dec 18, 2003			
	A19	US-2004/0053972	Nara	Mar. 18, 2004			
	A20	US-2004/0058956	Akiyama et al.	Mar. 25, 2004			
	A21	US-2004072889	Masjerrer	Apr. 15, 2004			
	A22	US-2004/0096436 A1	Carson et al.	May 20, 2004			
	A23	US-2004152672	Carson et al.	Aug. 5, 2004			
	A24	US-2004/0247600	Leoni	Dec. 9, 2004			
	A25	US-2005/0060028 A1	Horres et al.	Mar. 17, 2005			
	A26	US-2005/0176678	Horres et al.	Aug 11, 2005			
	A27	US-2006/0051412	Petereit et al.	Mar 9, 2006			

Change(s) applied to document, /N.W.S./ 10/2/2012

Examiner's Signature	/Ali Soroush/	Date:	08/14/2009

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/330,868	05/07/2013	8436190	CP391	9998

46347 7590 04/17/2013

WOODCOCK WASHBURN LLP
CIRA CENTRE, 12TH FLOOR
2929 ARCH STRET
PHILADELPHIA, PA 19104-2891

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 1748 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Jason Edward Brittain, El Cajon, CA;
Joe Craig Franklin, Tulsa, OK;

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/26/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	GLENMARK PHARMACEUTICALS LTD., GLENMARK GENERICS LTD., GLENMARK GENERICS S.A. and GLENMARK GENERICS INC., USA,

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	CEPHALON, INC.
2	8,436,190	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/26/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	HOSPIRA, INC.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	CEPHALON, INC.
2	US 8,436,190 B2	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	**Mail Stop 8** **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	**REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK**

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/26/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	SUN PHARMA GLOBAL FZE, SUN PHARMACEUTICAL INDUSTRIES LTD., and SUN PHARMACEUTICAL INDUSTRIES, INC.,

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	CEPHALON, INC.
2	US 8,436,190 B2	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/31/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
Cephalon, Inc.	Sandoz Inc.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	Cephalon, Inc.
2	8,436,190	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/20/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
Cephalon, Inc.	Dr. Reddy's Laboratories, Ltd. and Dr. Reddy's Laboratories, Inc.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	Cephalon, Inc.
2	8,436,190	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/20/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
Cephalon, Inc.	Innopharma, Inc.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	Cephalon, Inc.
2	8,436,190	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/20/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
Cephalon, Inc.	Agila Specialties Inc. f/k/a/ Strides, Inc. and Onco Therapies Limited

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	Cephalon, Inc.
2	8,436,190	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	1/31/2014	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	ACTAVIS LLC, f/k/a ACTAVIS INC. AND ACTAVIS ELIZABETH LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
13-2095-GMS	12/26/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	ACCORD HEALTHCARE, INC. and INTAS PHARMACEUTICALS LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	CEPHALON, INC.
2	8,436,190	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY
4/9/2014	☑ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,609,863	12/17/2013	CEPHALON, INC.
2			
3			
4			
5			

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	4/30/2014	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	UMAN PHARMA INC.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
2	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	5/27/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	BRECKENRIDGE PHARMACEUTICAL, INC. and NATCO PHARMA LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	9/2/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	SAGENT PHARMACEUTICALS, INC. and SAGENT AGILA LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	9/2/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	NANG KUANG PHARMACEUTICAL CO., LTD. and CANDA NK-1, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
13-2095-GMS	9/18/2014	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	ACCORD HEALTHCARE, INC. and INTAS PHARMACEUTICALS LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	CEPHALON, INC.
2	8,436,190	5/7/2013	CEPHALON, INC.
3	8,609,863	12/17/2013	CEPHALON, INC.
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☑ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading		
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,791,270	7/29/2014	CEPHALON, INC.
2			
3			
4			
5			

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	9/2/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	NANG KUANG PHARMACEUTICAL CO., LTD. and CANDA NK-1, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed Voluntarily — See Attached

CLERK	(BY) DEPUTY CLERK	DATE
~~John A Cerino, Clerk~~ United States District Court 844 N. King Street, Unit 18 ~~Wilmington, DE 19801~~		10/3/14

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	10/21/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	WOCKHARDT BIO LTD., WOCKHARDT LTD., and WOCKHARDT USA, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/26/2013	for the District of Delaware
PLAINTIFF		DEFENDANT
CEPHALON, INC.		SUN PHARMA GLOBAL FZE, SUN PHARMACEUTICAL INDUSTRIES LTD., and SUN PHARMACEUTICAL INDUSTRIES, INC.,

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	CEPHALON, INC.
2	US 8,436,190 B2	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed - See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A Cerino, Clerk United States District Court 844 N. King Street, Unit 18 Wilmington, DE 19801		3/26/15

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO: Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	**REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK**

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	3/14/2014	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	SUN PHARMA GLOBAL FZE, SUN PHARMACEUTICAL INDUSTRIES LTD., and SUN PHARMACEUTICAL INDUSTRIES, INC.

PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1 US 8,609,863 B2	12/17/2013	Cephalon, Inc.
2		
3		
4		
5		

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	**DATE OF PATENT OR TRADEMARK**	**HOLDER OF PATENT OR TRADEMARK**
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed – See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A Cerino, Clerk United States [illegible] Court 844 N. K[illegible] [illegible] 18 Wilmington, [illegible] 19801	[signature]	3/26/15

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	9/26/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	SUN PHARMA GLOBAL FZE and SUN PHARMACEUTICAL INDUSTRIES LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
2			
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☑ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed – See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A Cerino, Clerk United States District Court 844 N. King Street, Unit 18 Wilmington, DE 19801		3/24/15

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

IN RE BENDAMUSTINE CONSOLIDATED CASES	C.A. No. 13-2046-GMS **(Lead Case)**
CEPHALON, INC. *Plaintiff,* v. SUN PHARMA GLOBAL FZE and SUN PHARMACEUTICAL INDUSTRIES LTD., *Defendants.*	C.A. No. 13-2096-GMS C.A. No. 14-0333-GMS C.A. No. 14-1243-GMS (Consolidated Case)
CEPHALON, INC., *Plaintiff,* v. DR. REDDY'S LABORATORIES, LTD., *et al.*, *Defendants.*	C.A. No. 15-179-GMS (Consolidated Case)

STIPULATION OF DISMISSAL

Pursuant to Federal Rules of Civil Procedure 41(a)(1)(A)(ii) and 41(c), and an Agreement between Plaintiff Cephalon, Inc. ("Cephalon") and Defendants Sun Pharma Global FZE and Sun Pharmaceutical Industries Ltd. ("Defendants," collectively with Cephalon, the "Parties") (the "Agreement"), the Parties hereby stipulate and agree that all claims, counterclaims and affirmative defenses asserted by the Parties against one another in the above-captioned action solely with respect to Defendants (the "Action") are hereby dismissed without prejudice, and

without costs, disbursements, or attorneys' fees to any party. It is further stipulated that the U.S. District Court for the District of Delaware retains jurisdiction to enforce and resolve any disputes arising under the Agreement.

SO STIPULATED:

Dated: March 24, 2015

SHAW KELLER LLP

/s/Karen E. Keller
John W. Shaw (No. 3362)
Karen E. Keller (No. 4489)
300 Delaware Ave., Suite 1120
Wilmington, DE 19801
(302) 298-0700
jshaw@shawkeller.com
kkeller@shawkeller.com

Of Counsel:
David M. Hashmall
Calvin E. Wingfield Jr.
GOODWIN PROCTER LLP
The New York Times Building
620 Eighth Avenue
New York, NY 10018-1405
Tel.: (212) 813-8800

Daryl L. Wiesen
Emily L. Rapalino
Nicholas K. Mitrokostas
GOODWIN PROCTER LLP
Exchange Place
Boston, MA 02109
Tel.: (617) 570-1000

Counsel for Plaintiff Cephalon, Inc.

PHILLIPS, GOLDMAN & SPENCE, P.A.

/s/John C. Phillips, Jr.
John C. Phillips, Jr. (No. 110)
Megan C. Haney (No. 5016)
1200 North Broom Street
Wilmington, DE 19806
(302) 655-4200
jcp@pgslaw.com
mch@pgslaw.com

Of Counsel:
William A. Rakoczy
Paul J. Molino
John D. Polivick
RAKOCZY MOLINO MAZZOCHI SIWIK LLP
6 Hubbard Street, Suite 500
Chicago, IL 60654
(312) 527-2157

Counsel for Defendants Sun Pharma Global FZE and Sun Pharmaceutical Industries Ltd.

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/26/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	GLENMARK PHARMACEUTICALS LTD., GLENMARK GENERICS LTD., GLENMARK GENERICS S.A. and GLENMARK GENERICS INC., USA,

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	CEPHALON, INC.
2	8,436,190	5/7/2013	CEPHALON, INC.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed - See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A Cerino, Clerk United States District Court 844 N. King Street, Unit 18 Wilmington, DE 19801		4/15/15

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

IN RE BENDAMUSTINE CONSOLIDATED CASES	C.A. No. 13-2046-GMS LEAD CASE
CEPHALON, INC., Plaintiff, v. GLENMARK PHARMACEUTICALS LTD., *et al.*, Defendants.	C.A. No. 13-2093-GMS (Consolidated Case)
CEPHALON, INC., Plaintiff, v. SANDOZ INC., *et al.*, Defendants.	C.A. No. 15-178-GMS (Consolidated Case)

STIPULATION OF DISMISSAL

Pursuant to Federal Rules of Civil Procedure 41(a)(1)(A)(ii) and 41(c), and an Agreement between Plaintiff Cephalon, Inc. ("Cephalon") and Glenmark Pharmaceuticals Ltd., Glenmark Generics Ltd., Glenmark Generics S.A. and Glenmark Generics Inc., USA ("Defendants," collectively with Cephalon, the "Parties") dated April 13, 2015 (the "Agreement", the Parties hereby stipulate and agree that all claims, counterclaims and affirmative defenses asserted by the Parties against one another in the above-captioned action solely with respect to Defendants (the "Action") are hereby dismissed without prejudice, and without costs, disbursement, or attorneys'

fees to any party. It is further stipulated that the U.S. District Court for the District of Delaware retains jurisdiction to enforce and resolve any disputes arising under the Agreement.

April 13, 2015

BAYARD, P.A.

/s/ Stephen B. Brauerman
Stephen B. Brauerman (sb4952)
Vanessa R. Tiradentes (vt5398)
Sara E. Bussiere (sb5725)
222 Delaware Avenue, Suite 900
Wilmington, DE 19801
(302) 655-5000
sbrauerman@bayardlaw.com
vtiradentes@bayardlaw.com
sbussiere@bayardlaw.com

OF COUNSEL:
David M. Hashmall
Calvin E. Wingfield Jr.
GOODWIN PROCTER LLP
The New York Times Building
620 Eighth Avenue
New York, NY 10018
(212) 813-8800

Daryl L. Wiesen Emily L. Rapalino
Nicholas K Mitrokostas
GOODWIN PROCTER LLP
Exchange Place Boston, MA 02109
(617) 570-1000

Attorneys for Plaintiff Cephalon, Inc.

CONNOLLY GALLAGHER LLP

/s/ Ryan P. Newell
Arthur G. Connolly III (#2667)
Ryan P. Newell (#4744)
Mary I. Akhimien (#5448)
1000 West Street, Ste. 1400
Wilmington, DE 19801
(302) 757-7300
aconnolly@connollygallagher.com
mewell@connollygallagher.com
makhimien@connollygallagher.com

Sailesh K. Patel A.
Taylor Corbitt
SCHIFF HARDIN LLP
233 S. Wacker Drive, Ste. 6600 Chicago, IL
60606 (312) 258-5500

Gina M. Bassi
SCHIFF HARDIN LLP
666 Fifth Avenue, Ste. 1700 New York, NY
10103 (212) 753-5000

Attorneys for Glenmark Pharmaceuticals Ltd., Glenmark Generics Ltd., Glenmark Generics S.A. and Glenmark Generics Inc. USA

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	10/21/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	WOCKHARDT BIO LTD., WOCKHARDT LTD., and WOCKHARDT USA, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4	US 8,791,270 B2	7/29/2014	Cephalon, Inc.
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Consent Judgment - See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A. Cerino	/s/ Mark Buckson	5/18/2015

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 **Director of the U.S. Patent and Trademark Office** **P.O. Box 1450** **Alexandria, VA 22313-1450**	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Northern District of Texas on the following

☑ Trademarks or ☐ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
4:15-CV-404-A	5/26/2015	Northern District of Texas

PLAINTIFF	DEFENDANT
Mockingbird Dental Group, P.C.	Sabrina Canegie fka Iulianna Rychkova

PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1 4556482		Mockingbird Dental Group, P.C.
2		
3		
4		
5		

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Karen Mitchell	T. Schroeder	5/27/2015

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	5/27/2014	District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	BRECKENRIDGE PHARMACEUTICAL, INC. and NATCO PHARMA LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,445,524 B2	5/21/2013	Cephalon, Inc.
2	US 8,436,190 B2	5/7/2013	Cephalon, Inc.
3	US 8,609,863 B2	12/17/2013	Cephalon, Inc.
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed - See attached

CLERK	(BY) DEPUTY CLERK	DATE
John A. Cerino	/s/ Mark Buckson	5/27/2015

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	9/25/2014	for the District of Delaware

PLAINTIFF	DEFENDANT
CEPHALON, INC.	SANDOZ INC.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,791,270	7/29/2014	CEPHALON, INC.
2			
3			
4			
5			

In the above entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed - See Attached

CLERK	(BY) DEPUTY CLERK	DATE
John A Cerino, Clerk United States District Court 844 N. King Street, Unit 18 Wilmington, DE 19801		7/10/15

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/31/2013	for the District of Delaware

PLAINTIFF	DEFENDANT
Cephalon, Inc.	Sandoz Inc.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	8,445,524	5/21/2013	Cephalon, Inc.
2	8,436,190	5/7/2013	Cephalon, Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Dismissed - See Attached

CLERK	(BY) DEPUTY CLERK	DATE
~~John A Cerino, Clerk~~ United States District Court 844 N. King Street, Unit 18 ~~Wilmington, DE 19801~~		7/10/15

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 08/10)

TO: Mail Stop 8 Director of the U.S. Patent and Trademark Office P. O. Box 1450 Alexandria, VA 22313-1450	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK

In Compliance with 35 § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Eastern District of Louisiana on the following

X Trademarks ☐ Patents. (☐ the patent action involves 35 U.S.C § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
13-6560 c/w 14-810, 14-837 Section H(1)	12/3/13	Eastern District of Louisiana, 500 POYDRAS St., Rm C-151, New Orleans, LA 70130

PLAINTIFF	DEFENDANT
Uptown Grill, LLC	Michael Louis Shwartz et al

	PATENT OR	DATE OF PATENT	HOLDER OF PATENT OR TRADEMARK
1	1446870	7/7/87	Camellia Grill Holdings, Inc.
2	1471728	1/5/88	Camellia Grill Holdings, Inc.
3	1471729	1/5/88	Camellia Grill Holdings, Inc.
4	1440249	5/19/87	Camellia Grill Holdings, Inc.
5			

In the above—entitled case, the following patent(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgment issued:

DECISION/JUDGMENT
JUDGMENT in favor of Plaintiff Uptown Grill, LLC and against Defendants Michael Shwartz, Camellia Grill Holdings, Inc., and Camellia Grill, Inc.: Pursuant to the Bill of Sale, Uptown Grill, LLC is the owner of all "Camellia Grill" trademarks, particularly including those on file with the United States Patent and Trademark Office, including, but not limited to, serial numbers 73561921, 73503693, 735603694, and 73503696, now registration numbers 1440249, 1471729, 1471728, and 1446870.

CLERK	(BY) DEPUTY CLERK	DATE
William W. Blevins	E McNamara	7/10/2015

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**