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

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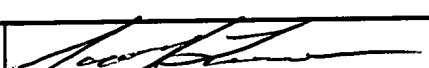
|---|---|---|---|---|---|
| Signature |  | Registration No. (Attorney/Agent) | 38,532 | Telephone | 610-738-6463 |
| Name (Print/Type) | Scott K. Larsen | | | Date | January 12, 2006 |

PTO/SB/05 (09-04)
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*Prior application information:* *Examiner* ____ *Art Unit:* ____

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| | | | | | |
|---|---|---|---|---|---|
| *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)
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|---|---|
| Application Number | UNKNOWN |
| Filing Date | January 12, 2006 |
| First Named Inventor | BRITTAIN |
| Examiner Name | UNKNOWN |
| Art Unit | UNKNOWN |
| Attorney Docket No. | CP391 |

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☒ 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)

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|---|---|---|---|---|---|---|---|
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| Application Type | Fee ($) | Fee($) | Fee($) | Fee($) | Fee($) | Fee($) | Fees Paid ($) |
| Utility | 300 | 150 | 500 | 250 | 200 | 100 | 1,000.00 |
| Design | 200 | 100 | 100 | 50 | 130 | 65 | ____ |
| Plant | 200 | 100 | 300 | 150 | 160 | 80 | ____ |
| Reissue | 300 | 150 | 500 | 250 | 600 | 300 | ____ |
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| | Fees Paid ($) |
|---|---|
| Non-English Specification, $130 fee (no small entity discount) | ____ |
| Other (e.g., late filing surcharge) : _____ | ____ |

## SUBMITTED BY

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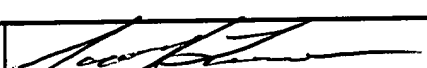
|---|---|---|---|---|---|
| Signature |  | Registration No. (Attorney/Agent) | 38,532 | Telephone | 610-738-6463 |
| Name (Print/Type) | Scott K. Larsen | | | Date | January 12, 2006 |

# 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.

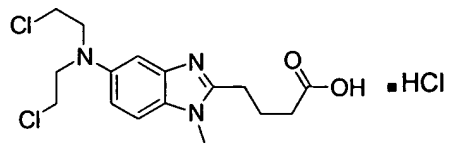
## BACKGROUND OF THE INVENTION

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.

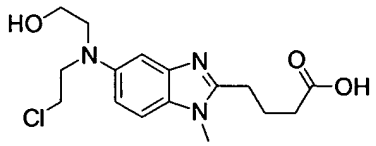
The lyophilized nitrogen mustard Ifosfamide is disclosed in International Publication No. WO 2003/066027; US Pat. Nos. 6,613,927; 5,750,131; 5,972,912; 5,227,373; and 5,204,335.

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.

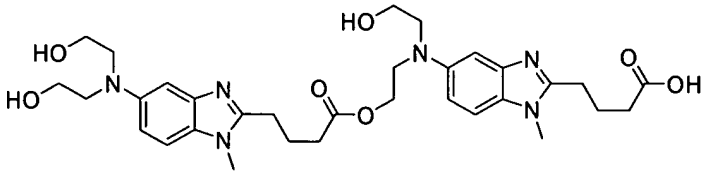
An embodiment of the invention is a pharmaceutical composition of bendamustine containing not more than about 0.5% to about 0.9% (area percent of bendamustine) HP1, as shown in Formula II,




Formula II

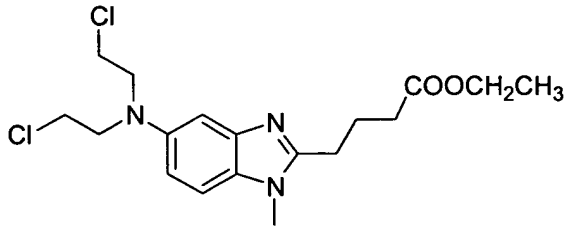
at the time of release or where the HP1 is the amount of HP1 present at time zero after reconstitution of a lyophilized pharmaceutical composition of bendamustine as described herein. In a preferred embodiment is a pharmaceutical composition of bendamustine containing not more than about 0.5% (area percent 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<br>25.5 mg/mL mannitol<br>Water, q.s. to desired volume | 14 mg/mL | 11 mg/mL | 17 mg/mL | 47 mg/mL |
| 30% (v/v) TBA<br>25.5 mg/mL mannitol<br>Water, q.s. to desired volume | 20 mg/mL | 18 mg/mL | 27 mg/mL | 65 mg/mL |

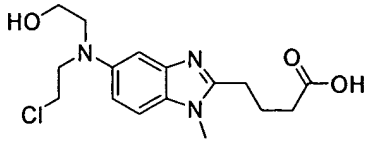
C. Stability

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).



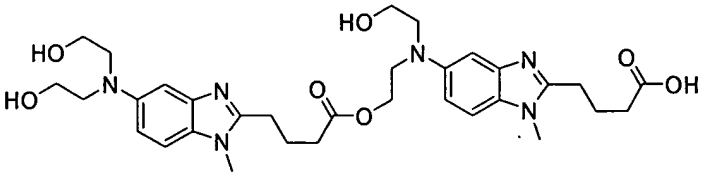

Monohydroxy bendamustine (HP1)

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 |

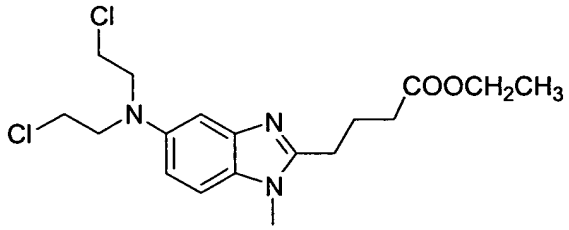
The other major degradant observed during this study and other long term stability studies was the dimer of bendamustine.



Bendamustine Dimer (BM1 Dimer)

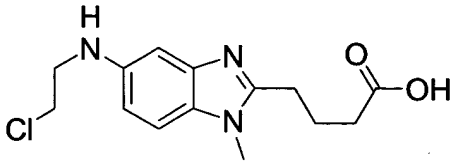
Formula III

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 $\mu$m 4.6 × 150 mm
Column temp.: 30°C
Sample temp.: 5°C
Injection Volume: 2 $\mu$L
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.

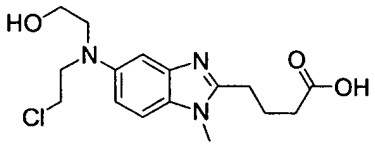
All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications are herein incorporated by reference to

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.

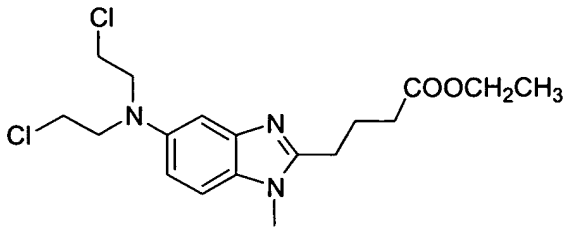
28. A process for manufacturing a lyophilized preparation of bendamustine which comprises controlling for the concentration of bendamustine degradants in the final product, such that, at release, the concentration of bendamustine degradants is less than 4.0 % (area percent of bendamustine) and the concentration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

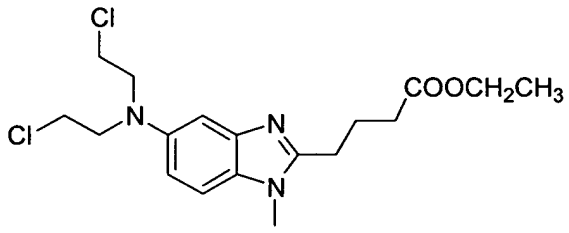
31. A lyophilized preparation of bendamustine wherein the concentration of bendamustine ethylester (as shown in Formula IV)




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.

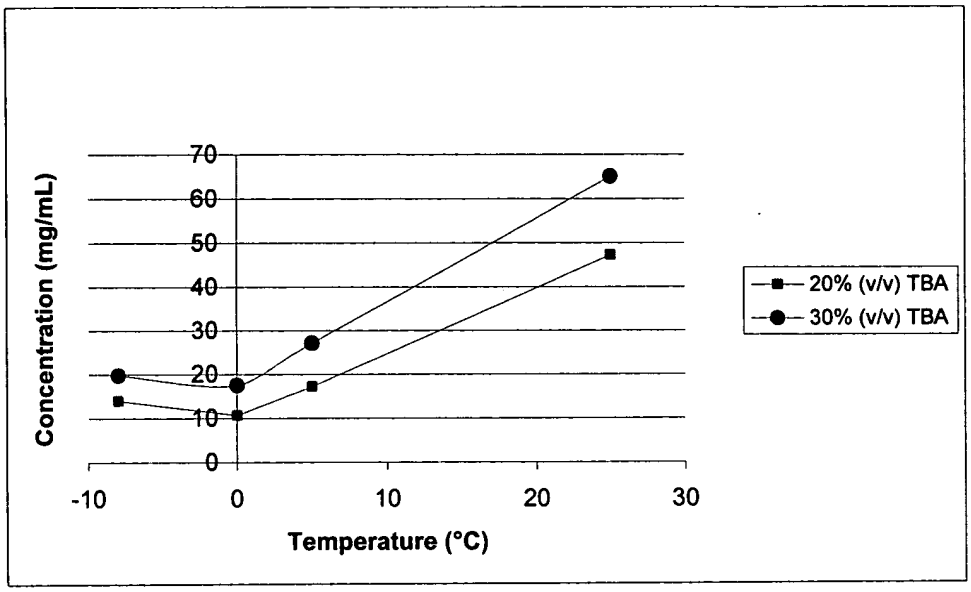



Fig. 1

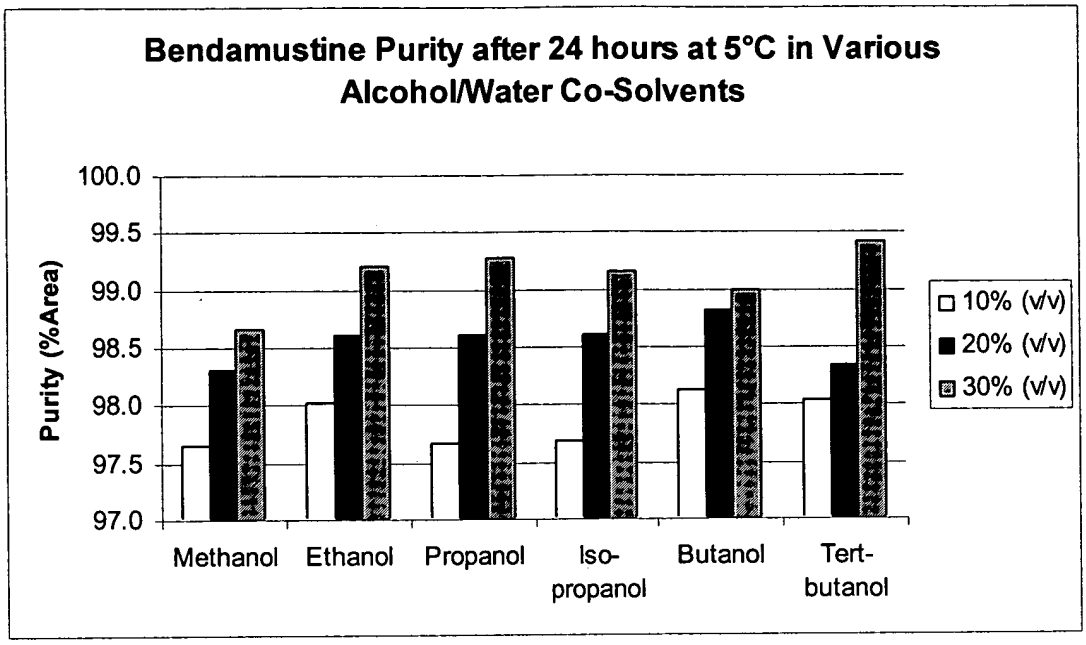



Fig 2

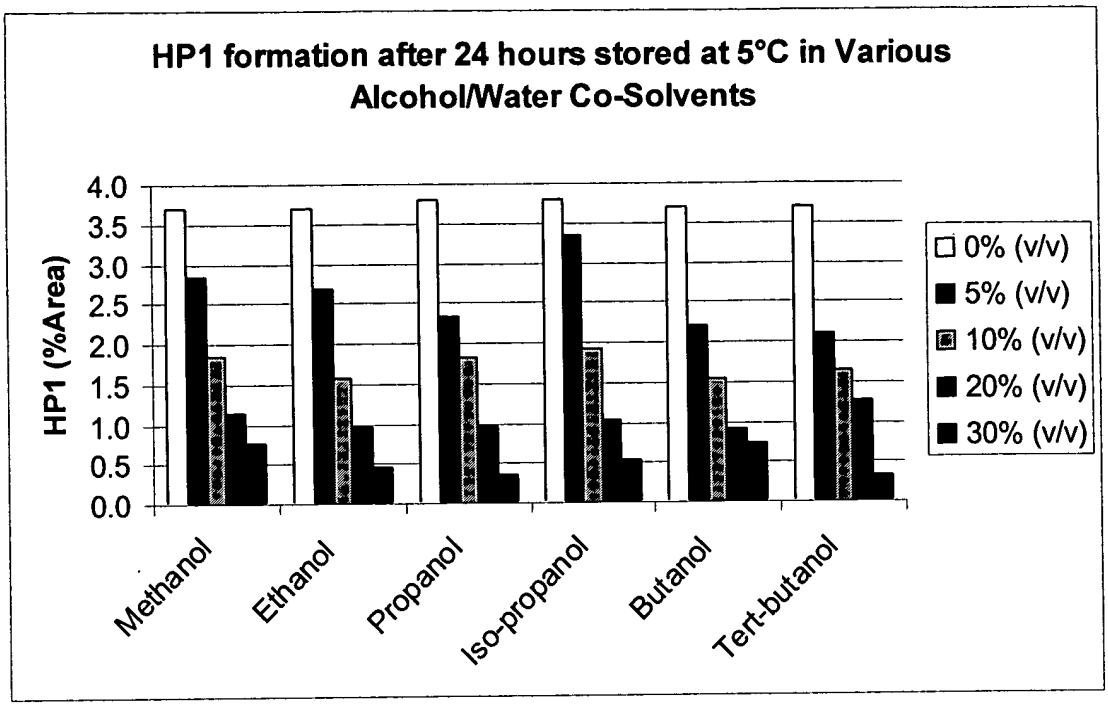
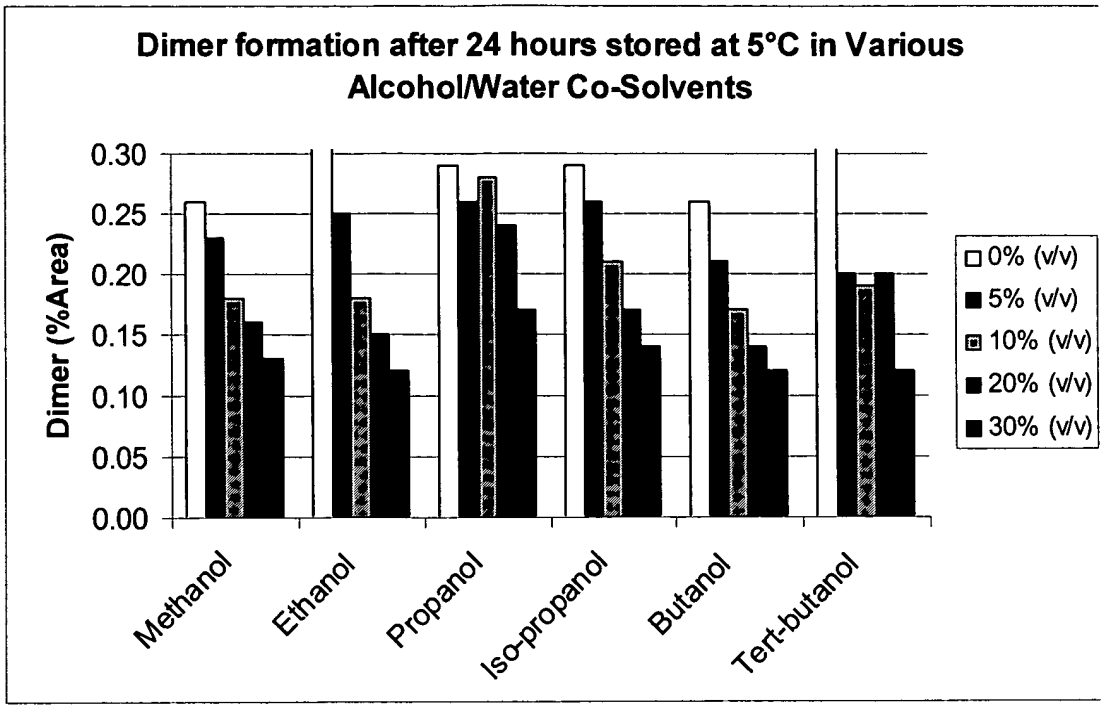



Fig 3

Figure 4.




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

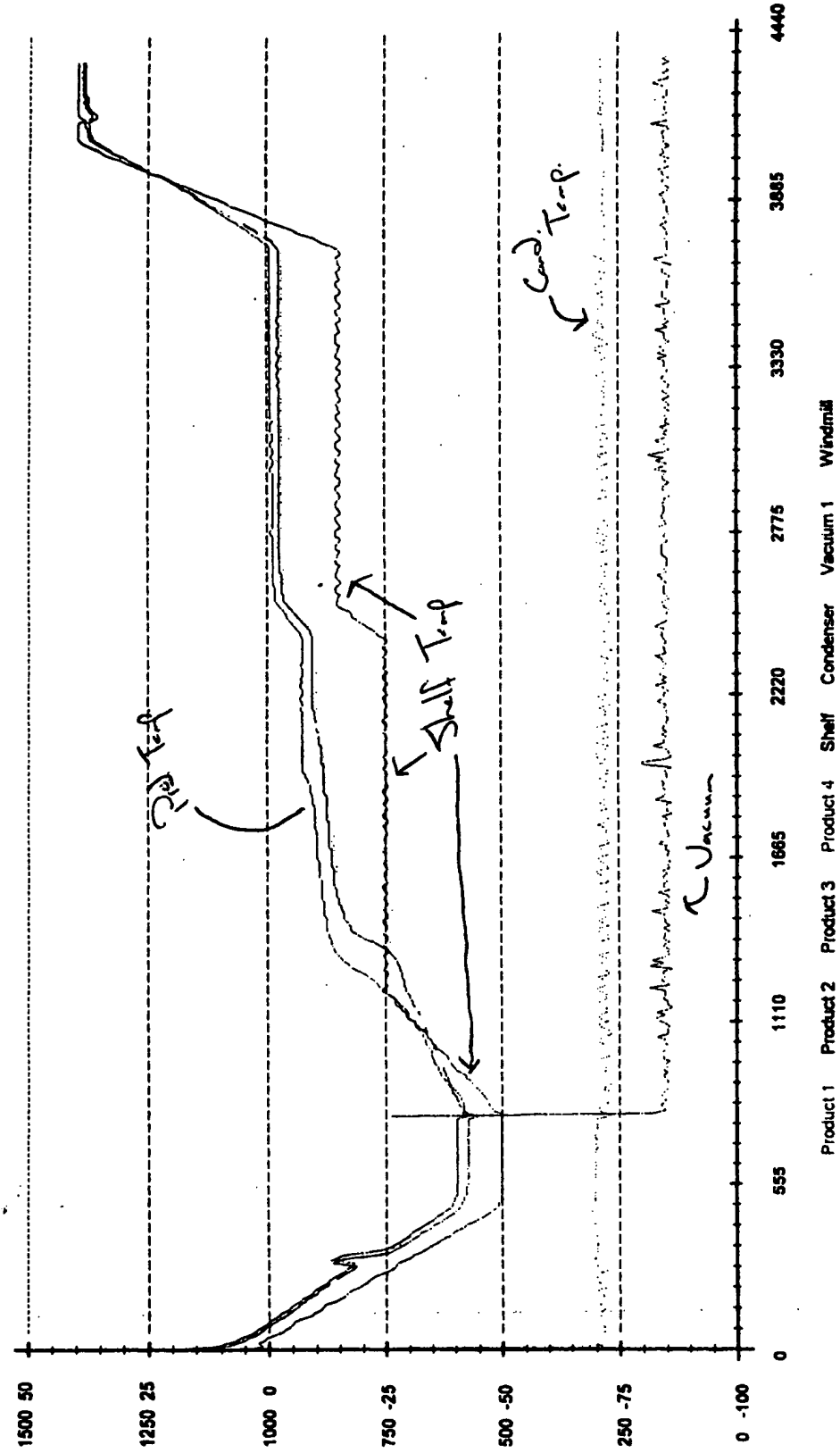



Figure 5

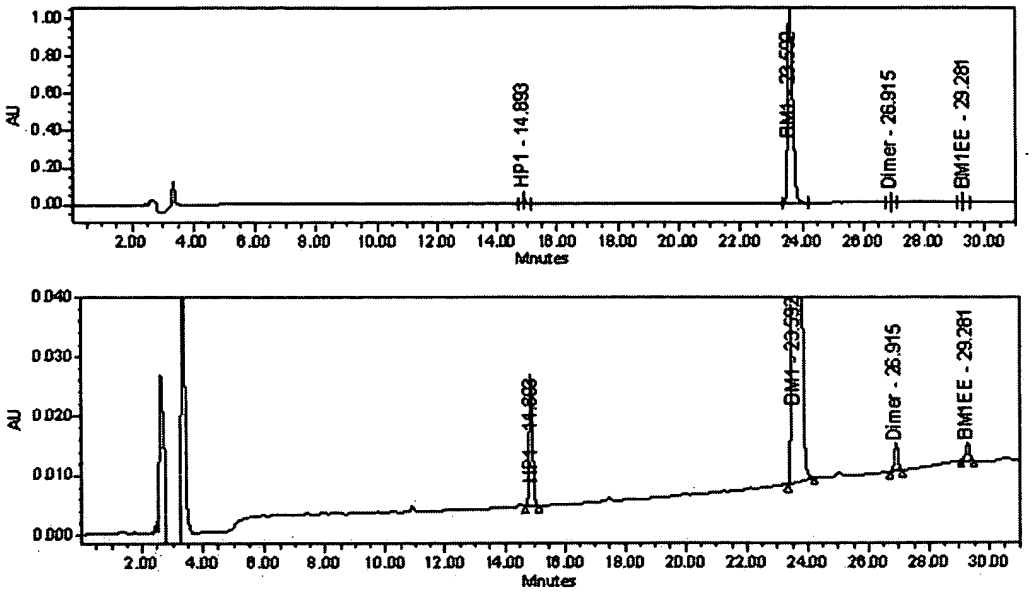



Fig. 6

**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.

| | |
|---|---|
| **Name: Jason Edward Brittain** | |
| **Mailing Address:**<br>1580 Chiswick Ct.<br>El Cajon, CA 92020 | _____<br>**Signature**<br><br>**Date of Signature:**_____ |
| **City/State of Actual Residence:**<br>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** | |
| **Mailing Address:**<br>11519 Kirby Place<br>San Diego, CA 92126 | _____<br>**Signature**<br><br>**Date of Signature:**_____ |
| **City/State of Actual Residence:**<br>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
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875 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.





*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

**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**

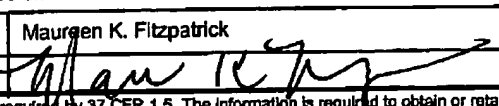
Remarks

**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
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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).

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**SUMMARY OF FEES DUE:**

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- **$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:**<br>1580 Chiswick Ct.<br>El Cajon, CA 92020 | _____<br>**Signature**<br><br>**Date of Signature:**_____ |
| **City/State of Actual Residence:**<br>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 | **Date of Signature:**_____ |
| **City/State of Actual Residence:** Tulsa, Oklahoma | **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:

| 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:**<br>11519 Kirby Place<br>San Diego, CA 92126 | _____<br>**Signature**<br><br>**Date of Signature:** _____ |
| **City/State of Actual Residence:**<br>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**

**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.***

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
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)*

| | |
|---|---|
| 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 |

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APR 04 2006

**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**

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**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
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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

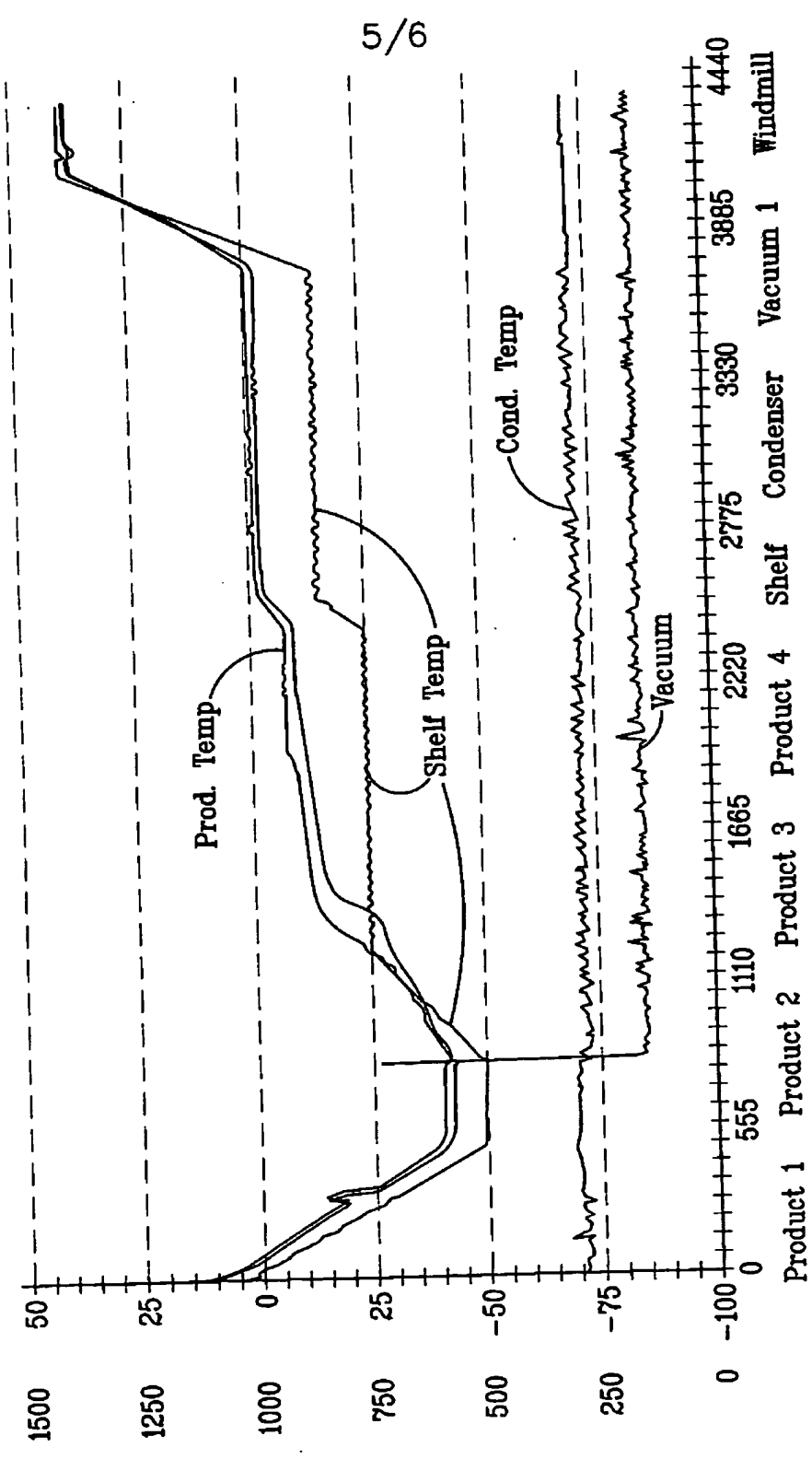

Prod. Temp
Shelf Temp
Cond. Temp
Vacuum
Product 1
Product 2
Product 3
Product 4
Shelf
Condenser
Vacuum 1
Windmill
555
1110
1665
2220
2775
3330
3885
4440
50
25
0
-50
-75
-100
1500
1250
1000
750
500
250
0

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 |

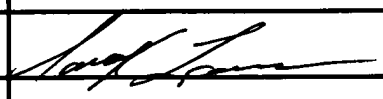
| | |
|---|---|
| Application Number | 11/330,868 |
| Filing Date | January 12, 2006 |
| First Named Inventor | Brittain |
| Art Unit | 1616 |
| Examiner Name | A. Soroush |
| Attorney Docket Number | CP391 |

**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**

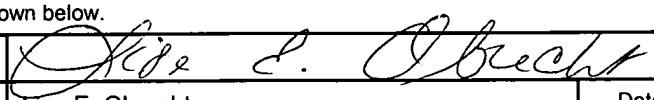
**Remarks**

**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

# 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 |
|---|---|---|---|---|---|---|
| | 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<br><br>**INFORMATION DISCLOSURE STATEMENT BY APPLICANT**<br><br>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<br><br>**INFORMATION DISCLOSURE STATEMENT BY APPLICANT**<br><br>List of Patent and Publications Cited by Applicant | Attorney Docket | CP391 |
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| | Application Number | 11/330,868 |
| | Filing Date | January 12, 2006 |
| | First Named Inventor | Brittain |
| | Group Art Unit | 1616 |
| | Examiner Name | A. Soroush |
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## (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.

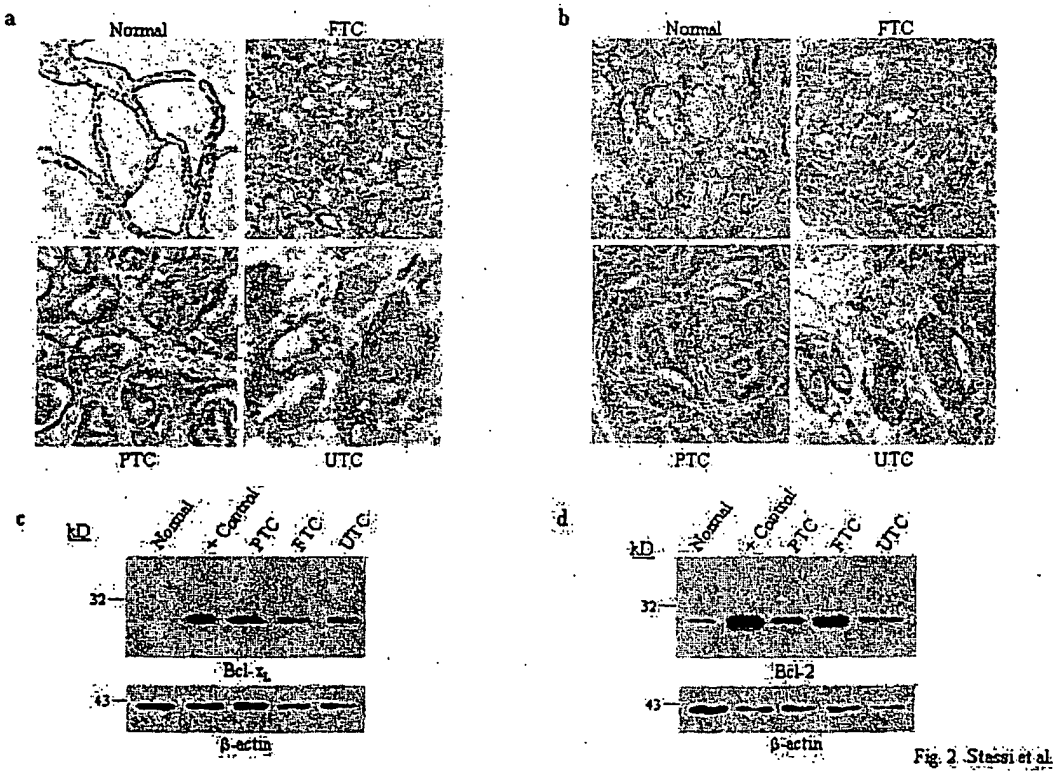


Fig. 2 Stassi et al.

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## 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, basaliom, 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.

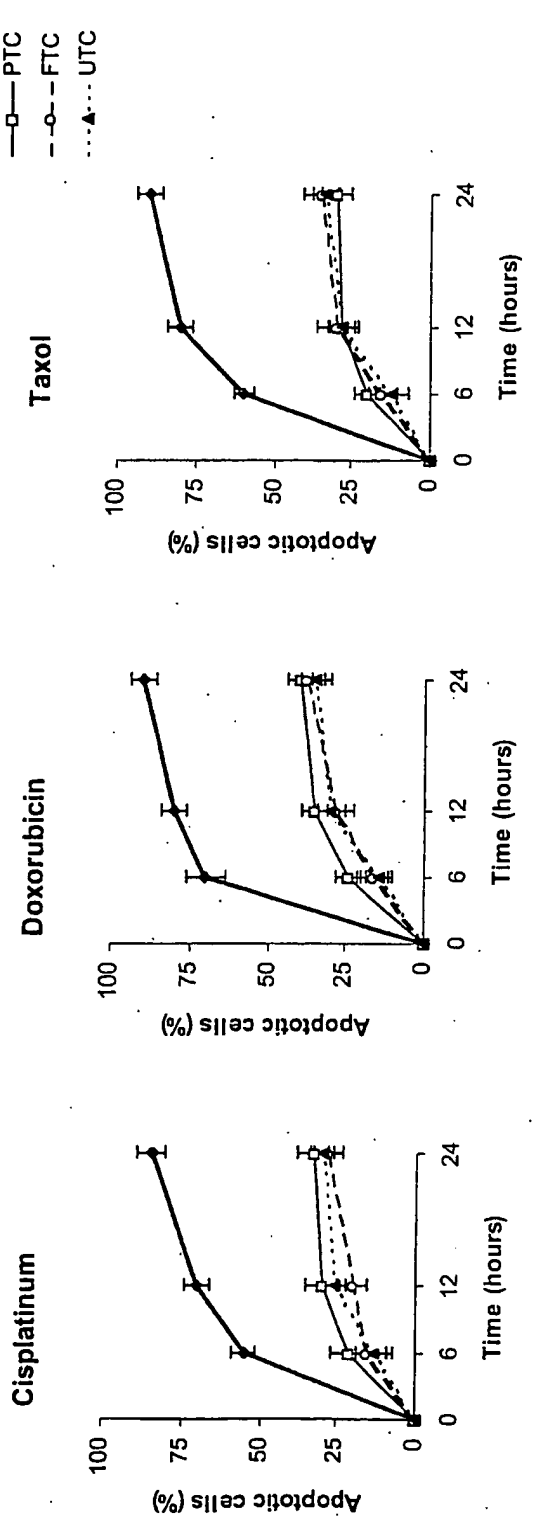


Fig. 1 Stassi et al.

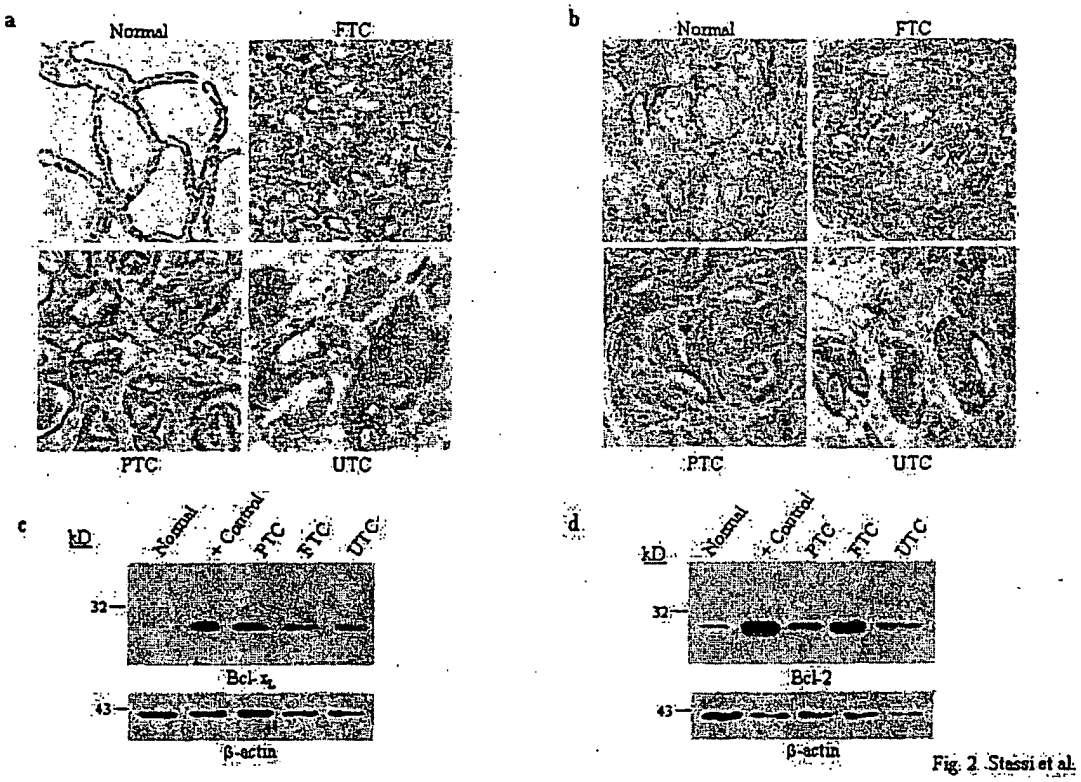


Fig. 2 Stassi et al.

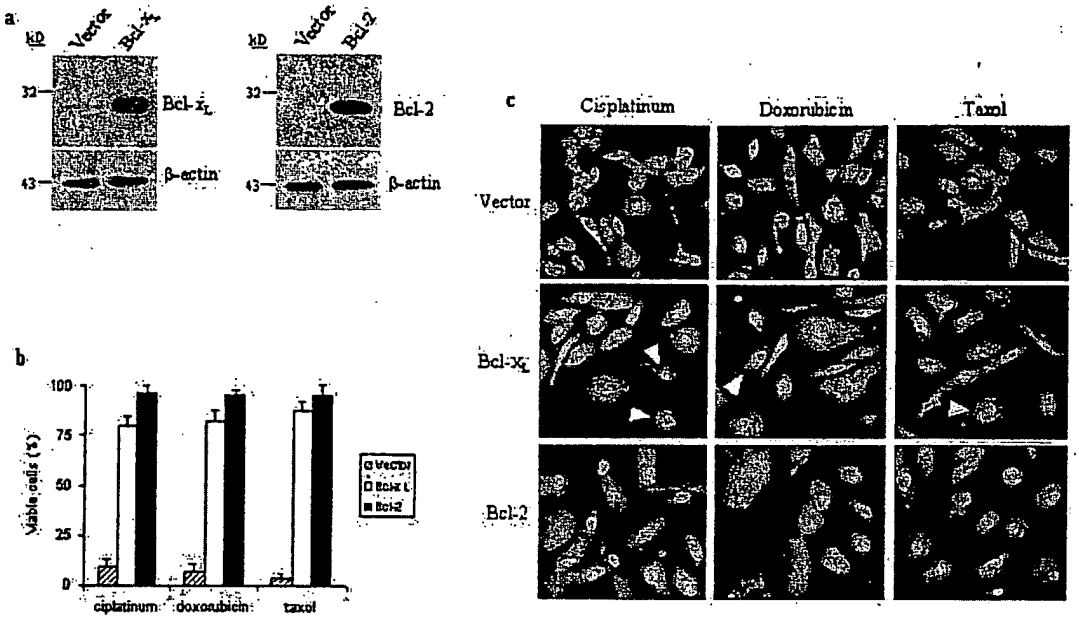


Fig. 3 Stassi et al.

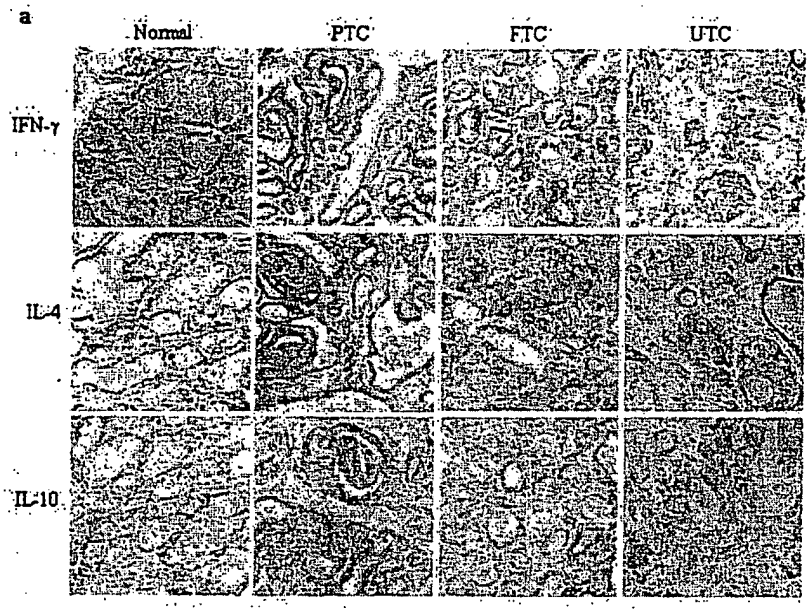


Fig. 4: Stassi et al.

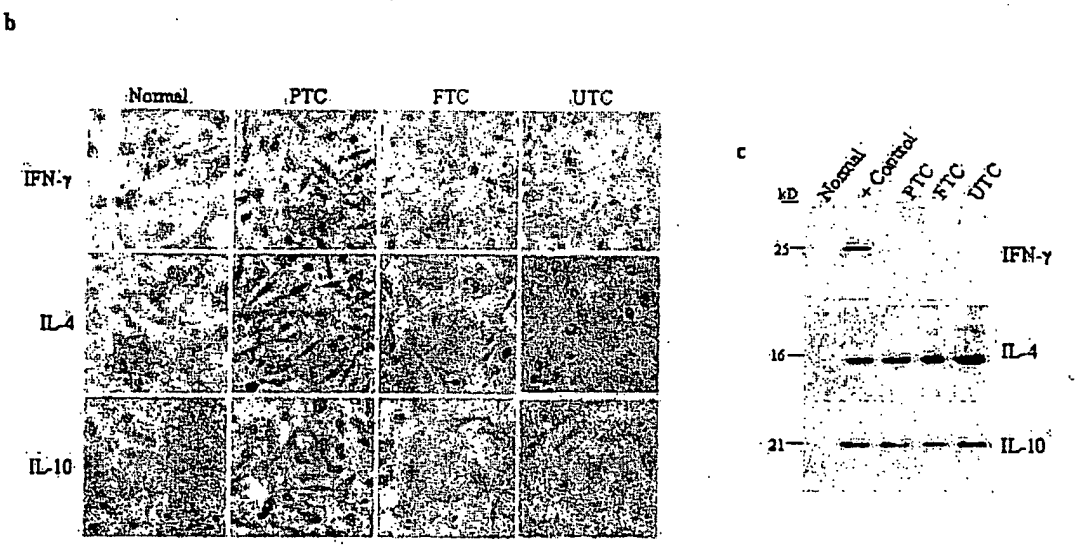


Fig. 4 Stassi et al.

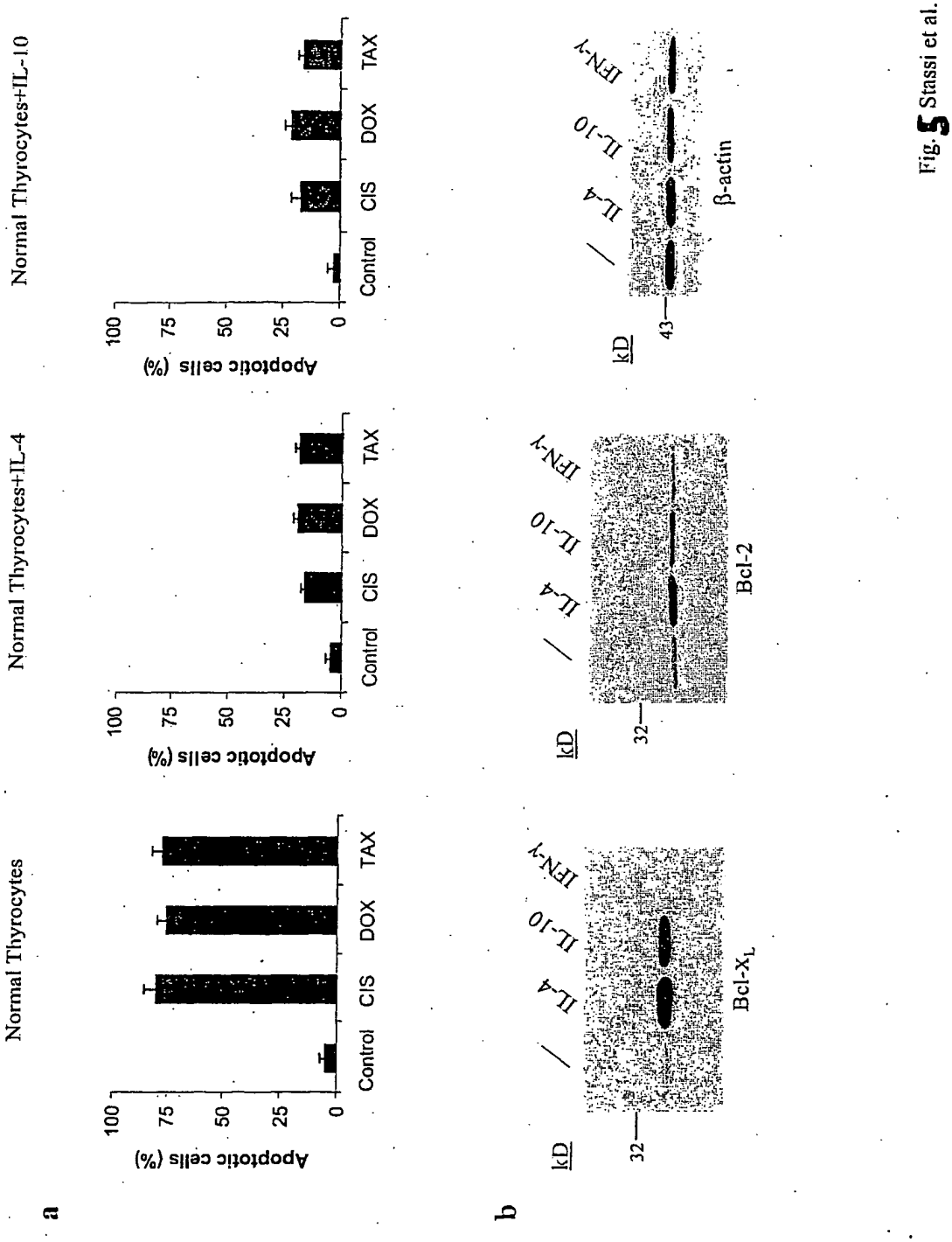


Fig. 5 Stassi et al.

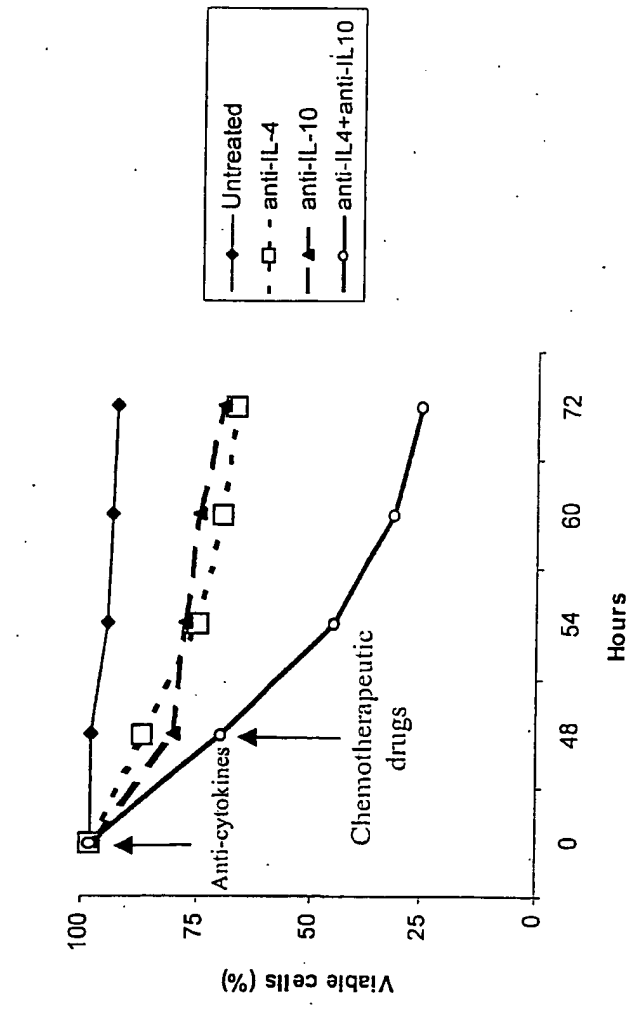


Fig. 6 Stassi et al.

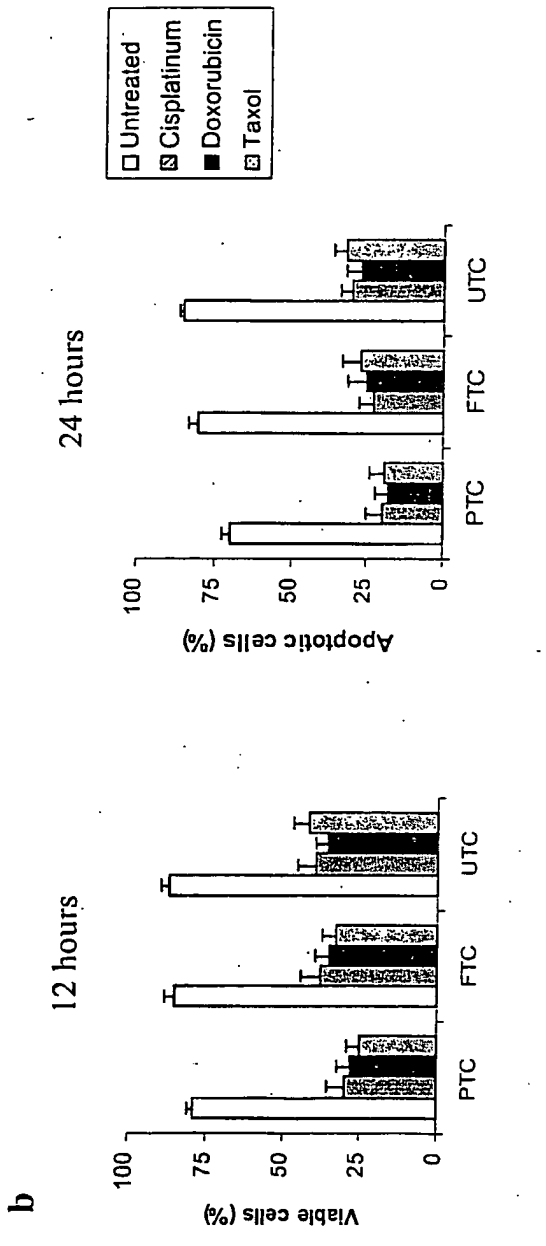


Fig. 6 Stassi et al.

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.

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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

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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

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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.

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# 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)

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# 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
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16-07-2003

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| 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

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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.

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## 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, pernicious 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) |
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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) |
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EPO FORM 1503 03.82 (P04C10)

European Patent Office

# PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 02 00 8199

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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

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EPO FORM P0459

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

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**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

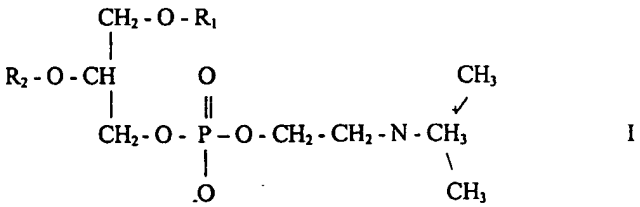
nach an sich bekannten Verfahren zur Herstellung pharmazeutischer Formulierungen, die für eine orale, paranterale (i.v., s.c., i.m.), rektale, nasale und inhalatorische Applikation geeignet sind.

**[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.

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**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

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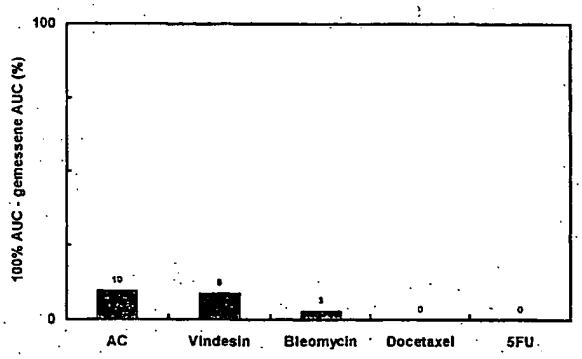
**Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen**

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

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## 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

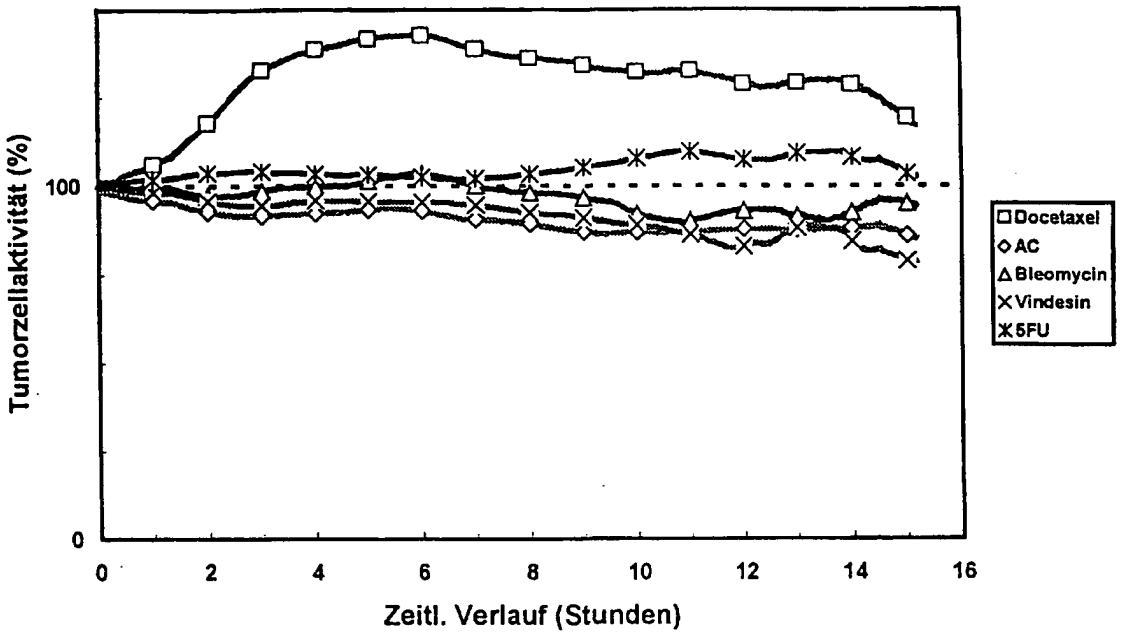


Fig. 1

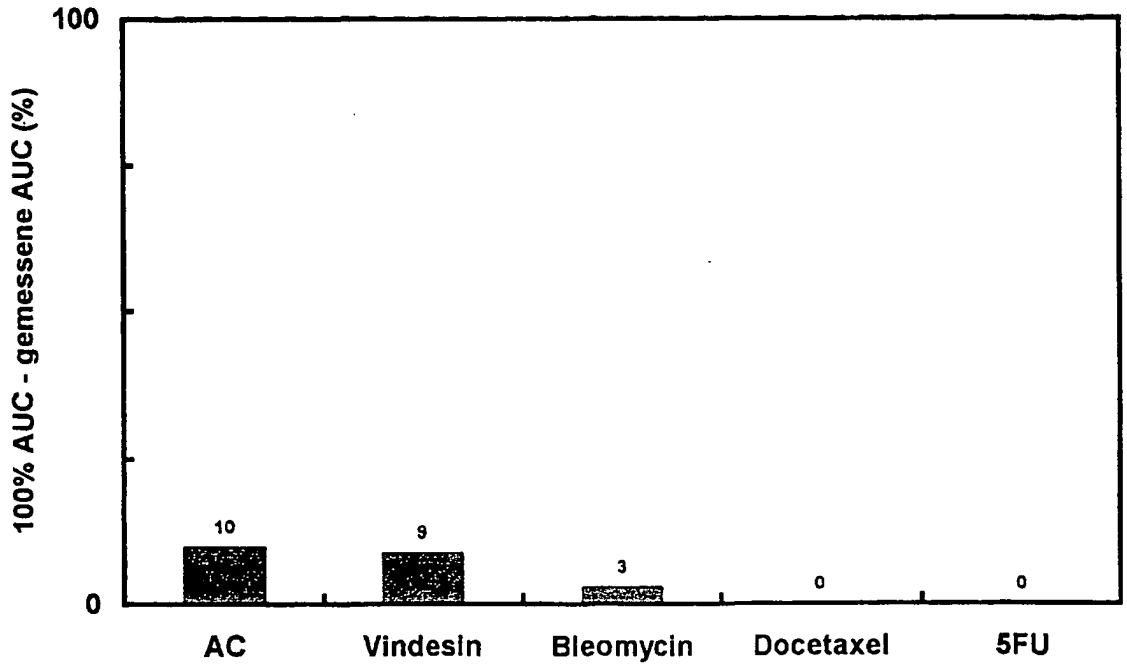


Fig. 2

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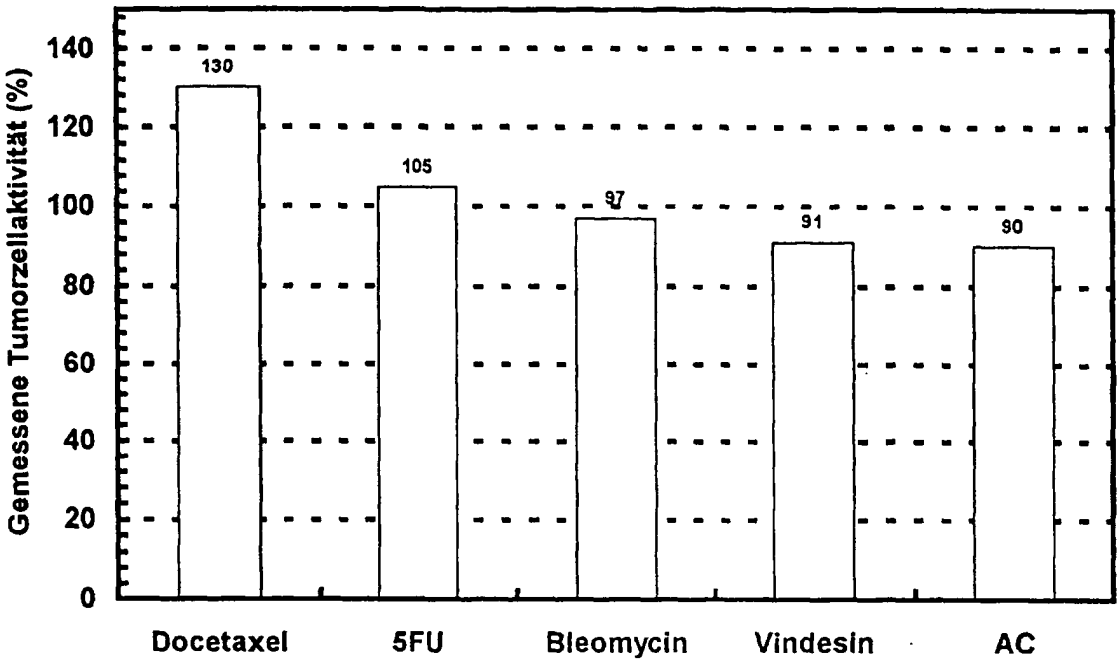


Fig. 3

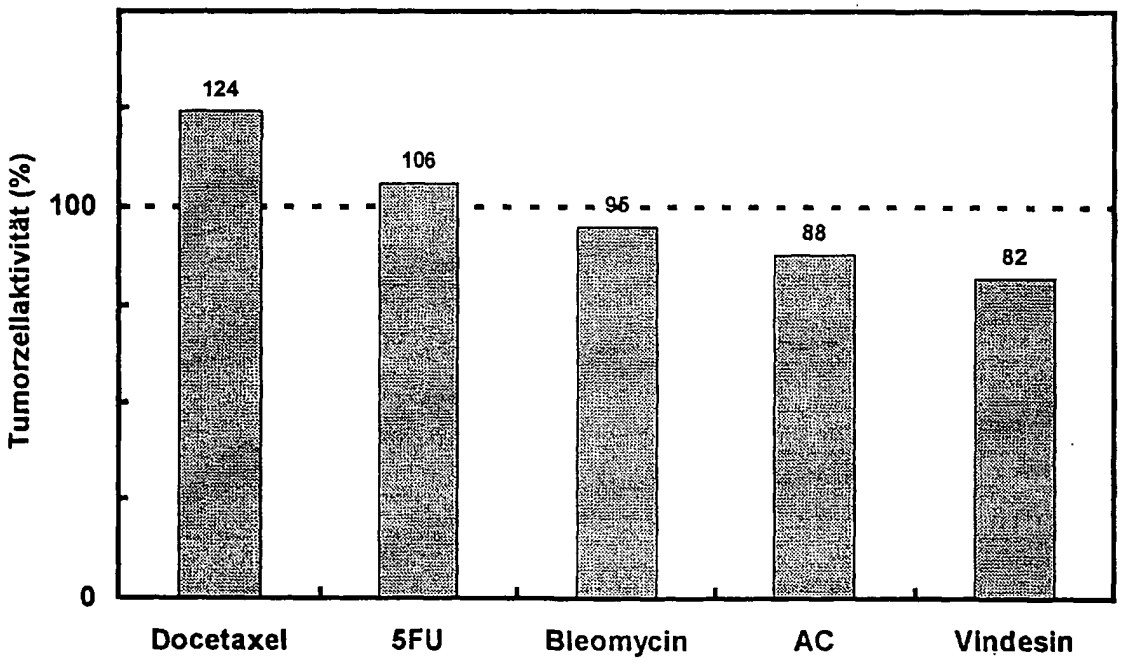


Fig. 4

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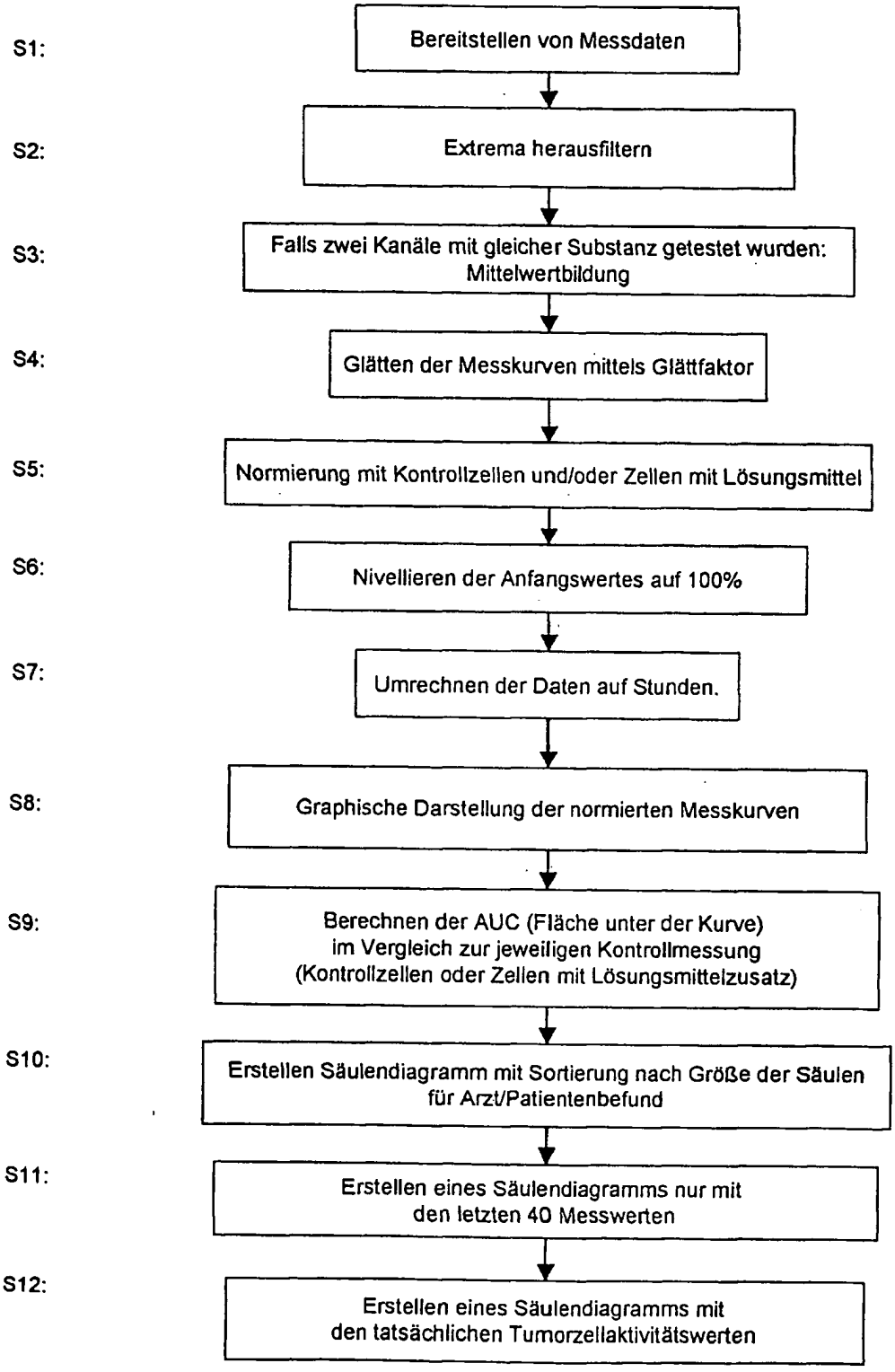



Fig. 5

(19)
**Bundesrepublik Deutschland**

Deutsche Demokratische Republik

Amt für Erfindungs- und Patentwesen

# PATENTSCHRIFT 80 967

Wirtschaftspatent

Erteilt gemäß § 5 Absatz 1 des Änderungsgesetzes zum Patentgesetz

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:

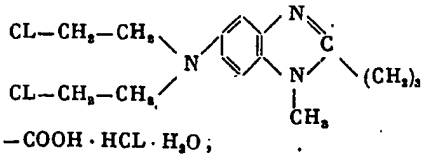
Prof. Dr. habil. Joachim Richter
Udo Reichenberger

## 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



$$CL-CH_2-CH_2 \quad CL-CH_2-CH_2 \quad N \quad C \quad N \quad (CH_2)_3 \quad CH_3 \quad -COOH \cdot HCL \cdot H_2O$$

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

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Zur PS Nr. 80 967

ist eine Zweitschrift erschienen.

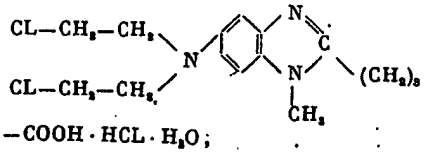
(Teilweise aufgehoben gem. § 6 Abs. 1 d. Änd. Ges. z. Pat. Ges.)

## 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.

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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.

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**Patentanspruch:**

Verfahren zur Herstellung eines stabilen injizierbaren Stickstofflostderiwat-Präparates, dadurch gekennzeichnet,

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daß Stickstofflostderivate, insbesondere γ-[1-Methyl-5-bis-(β-chloräthyl)-aminobenzimidazolyl-(2)]-buttersäurehydrochlorid, mit Askorbinsäure gemischt werden.

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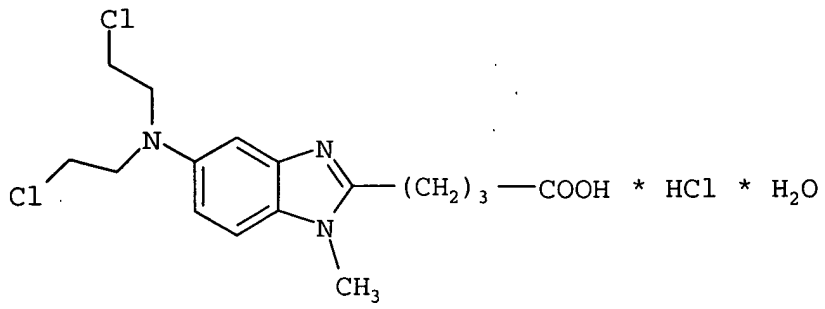
GDR Patent 80967

**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.

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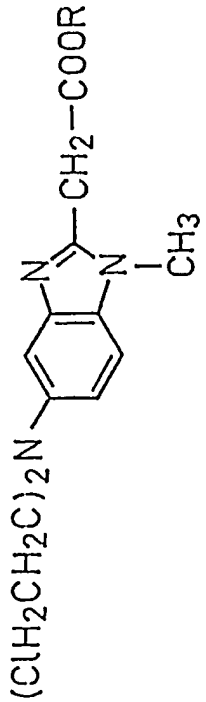
(54) Verfahren zur Herstellung von {5-[Bis(2-chlorethyl )amino]-1-methyl-benzimidazolyl(2)}ethansäurealkylestern

(55)
{5-[Bis(2-chlorethyl)amino]-1-methyl-benzimidazolyl-(2)}ethansäurealkylester; chemische Synthese; N¹-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 N¹-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

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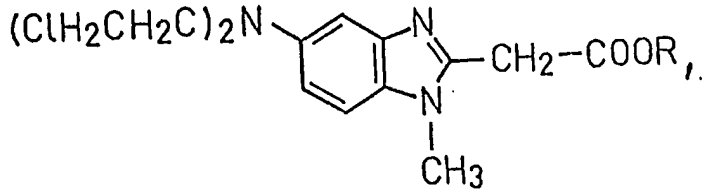
5 Seiten

**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-1 H-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 Stickstoffiostgruppe 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**

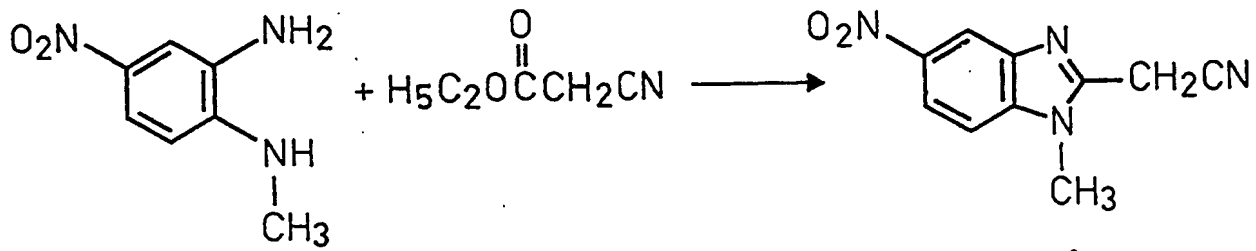
- Methode und Geräte
  Die Schmelzpunkte wurden auf dem Mikroheiztisch nach Boetius (korr. Werte) bestimmt. Die massenspektrometrischen Aufnahmen erfolgten mit dem doppelfokussierenden Gerät der Firma JEOL, Typ JMS-D 100. Zur Dünnschichtchromatographie wurden Glasplatten mit Merck-Kieselgel GF254 (0,5 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; $C_{11}H_{13}H_3O_2$ (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




A

Abb. 2



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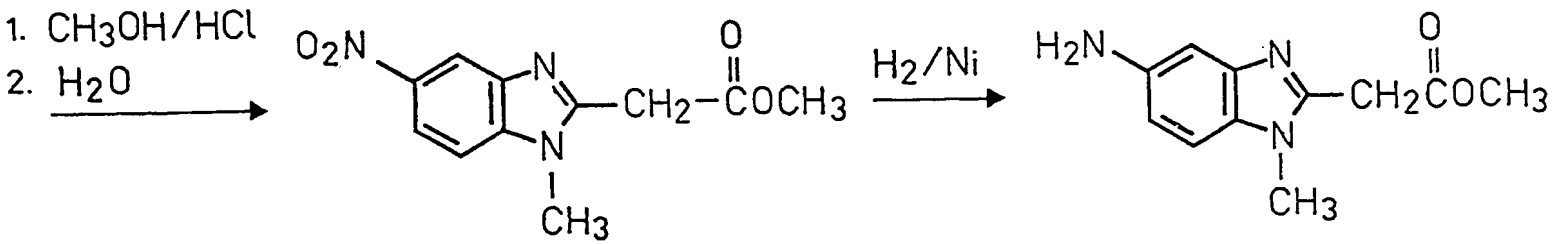

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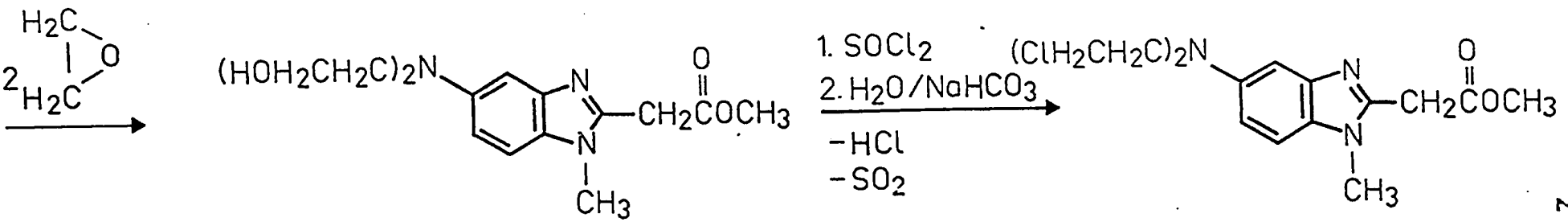

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## (54) VERFAHREN ZUR HERSTELLUNG VON 4-[1-METHYL-5-BIS (2-CHLORAETHYL)AMINOBENZIMIDAZOLYL-2]-BUTTERSAEURE

(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 Dünnschichtchromatographie 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 auoh 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,