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(54) Title: METHODS FOR CONTROLLING ANGIOGENESIS AND CELL PROLIFERATION

(57) Abstract: The present invention relates generally to methods of inhibiting angiogenesis in a patient by administering an effective angiogenesis-inhibiting amount of a thrombin inhibitor, and to the treatment of disease states that result from uncontrolled cell proliferation by administering a thrombin inhibitor alone or co-administering a thrombin inhibitor with an anticancer or cytotoxic agent. Specifically, the thrombin inhibitors used in the methods of the present invention are hirudins.

METHODS FOR CONTROLLING ANGIOGENESIS AND CELL PROLIFERATION

5

RELATED APPLICATION

The present invention claims priority of U.S. Provisional Application No. 60/561,741 filed April 12, 2004, which is incorporated herein in its entirety by this referenced.

BACKGROUND OF THE INVENTION

10 1. **Field of the Invention**

The present invention relates generally to methods of inhibiting angiogenesis in a patient by administering an effective angiogenesis-inhibiting amount of a thrombin inhibitor, and to the treatment of disease states that result from uncontrolled cell proliferation by administering a thrombin inhibitor alone or co-administering a 15 thrombin inhibitor with an anticancer or cytotoxic agent. Specifically, the thrombin inhibitors used in the methods of the present invention are hirudins.

2. **Discussion of the Related Art**

The circulatory system serves an important role in the transport of nutrients, proteins, hormones, and other vital molecules that are necessary to maintain life. 20 Blood vessels, which form an intricate network of pathways, represent an integral component of the circulatory system. In mammalian species, the internal surface of a blood vessel lumen is comprised of endothelial cells. These endothelial cells impart a smooth and low resistance quality to the luminal surface. Critical to the free flow and transport of blood and blood constituents, the smooth and nonadhesive internal 25 surface of the blood vessel increases the ease with which fluid flows. Without a smooth internal surface, blood vessels would become obstructed due to the formation of thrombi or other blockages at "sticky" locations on the internal walls. Complete or even partial blood vessel blockage would cause restriction of blood flow, thereby compromising the viability of living tissue served by the vessel. Thus, endothelial 30 cells represent an important structural component of blood vessels and also provide blood vessels with a smooth internal surface.

The formation of blood vessels in vivo takes place in response to stimuli, which are provided in the form of specialized growth factors. These growth factors induce mitosis in cells already present in blood vessels. The new cells may replace nearby damaged cells, or the new cells may arrange themselves such that new blood vessels are formed. The process of growing blood vessels from endothelial cells is termed "angiogenesis," which results in, among other characteristics, the vascularization of tissue.

Thrombin may play a role in metastasis and angiogenesis of tumors. Generally, for a tumor to grow larger than a few millimeters in diameter, vascular endothelium must proliferate and form vesicle walls to provide circulation and nutrients to the cells inside of the tumor mass. Thrombin likely potentiates this process by virtue of its ability to induce proliferation of endothelial cells. In addition, thrombin has been shown to disrupt the normal intercellular endothelial cell contacts important in preventing cells and plasma factors from escaping or entering the microvasculature. The present hypothesis that thrombin may increase metastasis by disrupting these contacts is supported by studies demonstrating a correlation between decreased levels of anti-thrombin III (which removes thrombin and other proteases from plasma) and increased tumor metastasis.

Cancerous cells derive from a single cell that has mutated in a way that permits it to escape from the biochemical controls that limit the multiplication of normal cells. Once that cell fails to respond normally to growth inhibitors, it starts to proliferate. When the growing tumor reaches a certain diameter, however, simple diffusion in and out of the tumor tissue no longer suffices to supply oxygen and nutrients and remove waste. Further growth depends on angiogenesis (i.e., the formation of new blood vessels from the existing vascular bed), and the small tumor must produce factors that stimulate the growth of blood vessels. Therefore, the inhibition of angiogenesis, in turn leads to the decrease of proliferation of malignant and neoplastic cells.

Carney, *et al.*, *Cell*, 15:1341 (1978) have postulated that high-affinity cell surface thrombin receptors may be involved in tumor metastasis and angiogenesis. For example, studies have indicated that thrombin receptors can serve as binding sites for tissue plasminogen activator, a molecule secreted from metastatic tumor cells.

Moreover, other studies demonstrate the involvement of tissue plasminogen activator in metastasis and angiogenesis. Thus, many of the effects of plasminogen activator may be mediated through its interaction with the cell surface thrombin receptor. Carney, *et al.*, have therefore proposed that stimulation of the thrombin receptor 5 serves to promote tumor metastases, while inhibition of the receptor will decrease metastatic activity.

Angiogenesis has become a central theme in promoting the understanding of how tissue grows. As indicated above, endothelial cell proliferation is not only desirable, but also necessary to carry out a number of physiological processes, for 10 example the *in utero* formation of tissues and organs. In other contexts, however, angiogenesis may be harmful to the overall health of an organism. For example, angiogenesis makes tumor growth and metastasis possible by vascularizing the tumor, thereby supplying the tumor with blood and nutrients that are necessary to sustain the tumor's growth, as well as providing routes by which tumor cells can migrate to 15 distant parts of the body. (Folkman *Cancer Res.* **46**(2):467-473 (1986). Clearly then, the prevention or reduction of angiogenesis may be a desirable goal in treating some disorders and diseases. Compounds have been tested for their ability to inhibit or reduce angiogenesis. Inhibitors of vascular endothelial growth factor (VEGF), a protein that selectively induces mitosis of vascular endothelial cells, have been 20 investigated. For example, U.S. Patent No. 6,284,751 to Aiello, *et al.*, describes using inhibitors of the beta isozyme of protein kinase C to counteract the effects of VEGF. Antibiotics such as minocycline have also been reported to inhibit angiogenesis. Some investigators have reported inhibition of tumor growth as well as reduction in 25 the number of metastatic tumors following administration of minocycline in combination with radiation or chemotherapy. See Tamargo, *et al. Cancer Res.*, **51**(2):672-675 (1991), and Teicher, *Cancer Res.*, **52**(23):6702-6704 (1992). U.S. Patent No. 5,843,925 to Backer, *et al.*, describes inhibition of angiogenesis upon administration of certain deoxytetracyclines. Many of these angiogenesis-inhibiting compounds, however, have only been tested *in vitro* for their antiproliferative activity.

30 Thus, there is a need to identify additional compounds as inhibitors of angiogenesis.

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SUMMARY OF THE INVENTION

The present invention provides methods and compositions for treating cancer by administering to a patient a composition comprising co-administering desulphohirudin with a cytotoxic or anticancer agent. The present invention is particularly useful for 5 treating or for repressing the growth of tumors. For example, administration of desulphohirudin to a human or animal with prevascularized metastasized tumors prevents the growth or expansion of those tumors.

According to one embodiment of the invention, there is provided a method of 10 effecting regression of tumor mass and size in a metastasized tumor in a subject, the method comprising administering to the subject a pharmaceutical formulation comprising an effective amount of a desulphohirudin variant selected from the group consisting of

a) SEQ ID NO:1 wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) Xaa at 66 not present,

b) SEQ ID NO:1 wherein Xaa at 27 is Ile or Glu and Xaa at 36, 47, 51 and 62-66 are as defined in a),

c) SEQ ID NO:1 wherein Xaa at 36 is Ile or Glu and Xaa at 27, 47, 51 and 62-66 are as defined in a),

d) SEQ ID NO:1 wherein Xaa at 47 is Ile or Glu and Xaa at 27, 36, 51 and 62-66 are as defined in a), and

e) SEQ ID NO:1 wherein Xaa at 51 is Leu or Asp and Xaa at 27, 36, 47 and 62-66 are as defined in a),

to effect regression in the subject of a metastasized tumor selected from the group consisting of adrenocortical cancer, cervical cancer, colon cancer, endometrial cancer, 25 esophageal cancer, eye cancer, gallbladder cancer, gastric cancer, head and neck cancer, laryngeal cancer, liver cancer, myeloproliferative disorders, neck cancer, nonmelanoma skin cancer, ovarian cancer and testicular cancer.

In another embodiment of the invention, there is provided a method of effecting regression of tumor mass and size in a metastasized pancreatic tumor in a subject, the 30 method comprising administering to the subject a pharmaceutical formulation comprising an effective amount of a desulphohirudin variant selected from the group consisting of

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a) SEQ ID NO:1 wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) Xaa at 66 not present,

5 b) SEQ ID NO:1 wherein Xaa at 27 is Ile or Glu and Xaa at 36, 47, 51 and 62-66 are as defined in a),

c) SEQ ID NO:1 wherein Xaa at 36 is Ile or Glu and Xaa at 27, 47, 51 and 62-66 are as defined in a),

d) SEQ ID NO:1 wherein Xaa at 47 is Ile or Glu and Xaa at 27, 36, 51 and 62-66 are as defined in a), and

10 e) SEQ ID NO:1 wherein Xaa at 51 is Leu or Asp and Xaa at 27, 36, 47 and 62-66 are as defined in a),

to effect regression in the subject of a metastasized pancreatic tumor.

The present invention further provides pharmaceutical formulations to treat a patient in need of anticancer therapy, wherein the formulation is comprised of a pharmaceutically acceptable solvent, a pharmaceutically acceptable carrier, and desulphohirudin in an effective quantity. The solvent may be aqueous or nonaqueous and may also be organic or inorganic. The solvent may be packaged in a separate container and be added to the desulphohirudin prior to administration. The desulphohirudin may be formulated as a lyophilized dry product. In another embodiment, the formulation further comprises potassium phosphate. In yet another embodiment, the formulation further comprises a sugar. In yet another embodiment, the formulation further comprises a divalent or trivalent metal ion.

DETAILED DESCRIPTION OF THE INVENTION

25 The present invention provides methods and compositions for treating diseases and processes mediated by undesired and uncontrolled angiogenesis by administering to a patient with the undesired angiogenesis a pharmaceutical composition comprising a thrombin inhibitor in a dosage sufficient to inhibit angiogenesis. In one embodiment, the angiogenesis is associated with a cellular proliferative disease. In particular, the invention provides a method of treating angiogenesis-dependent cancer in patients, comprising administering desulphohirudin or desirudin along or as an adjuvant to chemotherapy.

The term "cellular proliferative disease" is intended to refer to any condition characterized by the undesired propagation of cells. Included are conditions such as neoplasms, cancers, and tumors. "Cellular proliferative diseases" also include non-cancerous conditions such as benign melanomas, benign prostatic hyperplasia, psoriasis, and other cellular growths occurring within the epidermal layers.

"Cancer" means angiogenesis-dependent cancers and tumors, *i.e.*, tumors that require for their growth (expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying them with blood.

The terms "treating" and "treatment" as used herein refer to reduction in

severity and/or frequency of symptoms, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms and/or their underlying cause, and improvement or remediation of damage. Thus, for example, “treating” a patient involves prevention of a particular disorder or adverse physiological event in a

5 susceptible individual as well as treatment of a clinically symptomatic individual by inhibiting or causing regression of a disorder or disease. Thus, for example, the present method of “treating” a patient in need of anti-angiogenic therapy encompasses both prevention of a condition, disease, or disorder that is responsive to anti-angiogenic therapy and treatment of a condition, disease, or disorder that is responsive

10 to anti-angiogenic therapy in a clinically symptomatic individual.

“Regression” refers to the reduction of tumor mass and size.

“Patient” as used herein refers to a mammalian, preferably human, individual who can benefit from the pharmaceutical formulations and methods of the present invention. There is no limitation on the type of mammal that could benefit from the

15 presently described pharmaceutical formulations and methods.

By the terms “effective amount” or “therapeutically effective amount” of an agent as used herein are meant a sufficient amount of the agent to provide the desired therapeutic effect. Furthermore, an “effective angiogenesis-inhibiting amount” of an agent is a sufficient amount of the agent to at least partially inhibit angiogenesis. Of course, undesirable effects, e.g., side effects, are sometimes manifested along with the desired therapeutic effect; hence a practitioner balances the potential benefits against the potential risks in determining what is an appropriate “effective amount.” As will be pointed out below, the exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, mode of

20 administration, and the like. Thus, it is not possible to specify an exact “effective amount.” However, an appropriate “effective amount” or “effective angiogenesis-inhibiting amount” in any individual case may be determined by one of ordinary skill in the art using only routine experimentation.

25

I. The Pharmaceutical Formulation

30 One aspect of the invention provides a pharmaceutical formulation for treating a patient in need of anti-angiogenic therapy comprising a thrombin inhibitor in an amount effective to inhibit angiogenesis and a pharmaceutically acceptable carrier.

A. Active Agent

In the past few years cDNAs and synthetic genes coding for hirudin variants have been cloned and expressed in microbial hosts, such as but not limited to *E. coli* and yeast. The expression products in yeast (*Saccharomyces cerevisiae*, strain TY 1456) lack the sulphate monoester group at Tyr⁶³ - and were therefore designated "desulphohirudins" - they turned out to exhibit approximately the same biological activity as the natural, sulphated hirudins. Desulphohirudin variant HV1 has been expressed in *Escherichia coli* (European Patent Nos. 158 564 and 168 342) and in *Saccharomyces cerevisiae* (European Patent No. 168 342, 200 655, 225 633, 252 854 and 341 215). Similarly, desulphohirudin HV2 has been expressed in *Escherichia coli* (European Patent No. 158 564) and in *Saccharomyces cerevisiae* (European Patent No. 200 655, PCT-Application No. 86/01224) and des-(Val)₂- desulphohirudin has been expressed in *Escherichia coli* (European Patent No. 158 986).

According to the present invention, the term "hirudin" is intended to embrace desulphohirudin, a hirudin variant or a desulphohirudin variant or a mutant thereof, respectively, described in the literature and in particular a desulphohirudin compound or a mutant thereof obtainable from a transformed microorganism strain containing DNA which codes for a desulphohirudin or a mutant thereof. Such desulphohirudins are, for example, desulphohirudin variant HV1, HV1 modified (a, b), HV2, HV2 modified (a, b, c), HV3, variants of HV3 and des-(Val₂)- desulphohirudin. Also included are synthetic forms such as hirulogs and low molecular weight peptide-based thrombin inhibitors.

Preferred desulphohirudins are those having the formula I (SEQ ID NO: 1):

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (I)

1 5 10 15

Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Xaa Cys Ile Leu Gly Ser

30 20 25 30

Asp Gly Glu Xaa Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Xaa Pro

35 40 45

Gln Ser Xaa Asn Asp Gly Asp Phe Glu Glu Ile Pro Glu Xaa

50 55 60

in which

a) Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62 is the peptide residue Glu-Tyr-Leu-Gln (HV1) (SEQ ID NO:2), or

5 b) Xaa at 27 is Ile or Glu and Xaa at 36, 47, 51 and 62 are as defined in a) (HV1 modified a), or

c) Xaa at 36 is Ile or Glu and Xaa at 27, 47, 51 and 62 are as defined in a) (HV1 modified a), or

d) Xaa at 47 is Ile or Glu and Xaa at 27, 36, 51 and 62 are as defined

10 in a) (HV1 modified a), or

e) Xaa at 51 is Leu or Asp and Xaa at 27, 36, 47 and 62 are as defined in a) (HV1 modified a), or

f) Xaa at 62 is selected from the group consisting of Glu-Tyr, Glu-Tyr-Leu, Glu-Asp-Leu-Gln (SEQ ID NO:3), Glu-Glu-Leu-Gln (SEQ ID NO:4), Glu-Tyr-
15 Lys-Arg (SEQ ID NO:5), Glu-Asp-Lys-Arg (SEQ ID NO:6), Glu-Lys-Leu-Gln (SEQ ID NO:7), Ser-Phe-Arg-Tyr (SEQ ID NO:8), Trp-Glu-Leu-Arg (SEQ ID NO:9), Glu-Tyr-Leu-Gln-Pro (SEQ ID NO:10) and Glu-Tyr-Leu-Gln-Arg (SEQ ID NO:11) and Xaa at 27, 36, 47 and 51 are as defined in a) (HV1 modified b),

20 or having the formula II (SEQ ID NO: 12)

Leu Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (II)
1 5 10 15
Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys Ile Leu Gly Ser
25 20 25 30
Asp Gly Glu Lys Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Lys Pro
35 35 40 45
Gln Ser His Asn Asp Gly Asp Phe Glu Glu Ile Pro Glu Glu Tyr Leu Gln
50 55 60 65

30 or having the formula III (SEQ ID NO: 13)

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (III)
1 5 10 15
35 Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys Ile Leu Gly Ser
20 25 30
Asn Gly Lys Gly Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Xaa Pro
35 35 40 45
40 Glu Ser His Asn Asn Gly Asp Phe Glu Glu Ile Pro Glu Glu Xaa Leu Gln
50 55 60 65

in which

- a) Xaa at 47 is Asn and Xaa at 63 is Tyr (HV2), or
- b) Xaa at 47 is Lys, Arg or His and Xaa at 63 is Tyr (HV2 modified a),
- 5 or
- c) Xaa at 63 is Glu or Asp and Xaa at 47 is Asn (HV2 modified b),

or having the formula IV (SEQ ID NO: 14)

10 Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (IV)
1 5 10 15
Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys Ile Leu Gly Ser
20 25 30
Asn Gly Lys Gly Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Asn Pro
15 35 40 45
Glu Ser His Asn Asn Gly Asp Phe Glu Glu Ile Pro Glu Glu Tyr Leu Gln
50 55 60 65

or having the formula V (SEQ ID NO: 15)

20 Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (V)
1 5 10 15
Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys Ile Leu Gly Ser
20 25 30
25 Gln Gly Lys Asp Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Lys Pro
35 40 45
Gln Ser His Asn Gln Gly Asp Phe Glu Pro Ile Pro Glu Asp Ala Tyr Asp Glu
50 55 60 65

30 HV3 and variants of HV3 which are characterized by a shortening of the primary structure by 1 or 2 amino acids at the N-terminus or by 18, 10, 9, 6, 4 or 2 amino acids at the C-terminus.

Particularly preferred desulphohirudin compounds are those of formula I in which the Xaa groups are as defined under a) or the compound of formula III in which Xaa at 47 is Lys and Xaa at 63 is Tyr.

The most preferred hirudin is desulphohirudin HV1 having the formula I in which Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:16).

5 Another embodiment of the invention relates to the hirudin variant Refludan, or Leu-hirudin, also described as [Leu¹, Thr²]-63-desulphohirudin or lepirudin, which is described in sequence number 4 of European Patent No. EP-B 0 324 712 and in Formula VI (SEQ ID NO: 17).

10 Leu Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu Cys (VI)
1 5 10 15
Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys Ile Leu Gly Ser
20 25 30
Asp Gly Glu Lys Asn Gln Cys Val Thr Gly Glu Gly Thr Pro Lys Pro
15 35 40 45
Gln Ser His Asn Asp Gly Asp Phe Glu Glu Ile Pro Glu Glu Tyr Leu Gln
50 55 60 65

20 A further embodiment relates to hirudin variants having delayed action (e.g. PEG-hirudin, described in European Patent No. EP 0 345 616). Another embodiment of the invention relates to hirudin and hirudin variants, which, compared to the natural isoforms or variants, are truncated at the N-terminus and/or C-terminus. In another embodiment of the invention, a hirudin variant has 80% or more homology (amino acid identity) to a natural isoform of hirudin.

25 The hirudins used in the present invention can be prepared synthetically, e.g., chemically or preferably by recombinant techniques, or by isolation from leeches.

30 According to the present invention the term "mutant" refers to proteins (mutoins) exhibiting antithrombotic activity which differ from native hirudin or desulphohirudin by simple or multiple mutations (cf. European Patent Nos. 352 227 and 352 228). The DNA coding for said mutants which can be prepared by methods known in the art e.g., site-directed mutagenesis, is cloned and expressed in microbial hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*.

Alternatively, hirudins that may be utilized according to the present invention are hirudin fragments (i.e. those with at least the last 8 carboxyterminal amino acids, e.g. the fragment consisting of the last C-terminal amino acids of the known sequence in hirudin), biosynthetic analogues of hirudin (e.g. those with up to 10 to 12 amino acids, some of which are commercially available), the protein NAPc2, and low molecular weight peptide-based thrombin inhibitors.

Preferred thrombin inhibitors include low molecular weight peptide-based thrombin inhibitors. The term "low molecular weight peptide-based thrombin inhibitors" will be well understood by one skilled in the art to include thrombin inhibitors with one to four peptide linkages, and/or with a molecular weight below 1000, and includes those described in the review paper by Claesson in *Blood Coagul. Fibrin.* (1994) 5, 411, as well as those disclosed in U.S. Pat. No. 4,346,078; International Patent Applications WO 93/11152, WO 95/23609, WO 95/35309, WO 96/25426, WO 94/29336, WO 93/18060 and WO 95/01168; and European Patent Nos. 648 780, 468 231, 559 046, 641 779, 185 390, 526 877, 542 525, 195 212, 362 002, 364 344, 530 167, 293 881, 686 642, 669 317 and 601 459.

Preferred low molecular weight peptide-based thrombin inhibitors include those known collectively as the "gatrans". Particular gatrans which may be mentioned include HOOC--CH₂--(R)Cha-Pic-Nag-H (known as inogatran; see International Patent Application WO 93/11152 and the list of abbreviations therein) and HOOC--CH₂--(R)Cgl-Aze-Pab-H (known as melagatran; see International Patent Application WO 94/29336 and the list of abbreviations therein). Particularly preferred thrombin inhibitors include melagatran and ximelagatran. Other argentine derivatives may be useful in the present invention.

A novel class of synthetic peptides has been designed that inhibit the thrombin catalytic site and exhibit specificity for the anion-binding exosite (ABE) of alpha-thrombin. These peptides, called "hirulogs", consist of (i) an active-site specificity sequence with a restricted Arg-Pro scissile bond, (ii) a polymeric linker of glycyl residues from 6 to 18 Å in length, and (iii) an ABE recognition sequence such as that in the hirudin C-terminus, such as but not limited to, Hirulog-1 [(D-Phe)-Pro-Arg-Pro-(Gly)4-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu] (SEQ ID NO:18); the synthetic C-terminal hirudin peptide S-Hir53-64; the pentapeptide (D-Phe)-Pro-Arg-Pro-Gly (SEQ ID NO:19); and bivalirudin.

11a

The hirudin compounds used in the invention can be in the free form but also in the form of their salts. As they contain free amino group in several amino acid residues, the compounds can be in the form of acid addition salts. Suitable acid

addition salts are in particular pharmacologically acceptable salts with conventional therapeutically acceptable acids. Representative inorganic acids are hydrohalic acids (such as hydrochloric acid), and also sulfuric acid, phosphoric acid and pyrophosphoric acid. Representative organic acids are in particular arenesulfonic acids (such as benzenesulfonic or p-toluenesulfonic acid), or lower alkanesulfonic acids (such as methanesulfonic acid), as well as carboxylic acids such as acetic acid, lactic acid, palmitic acid, stearic acid, malic acid, tartaric acid, ascorbic acid and citric acid. As, however, the compound used in the invention also contains free carboxyl groups in several amino acid residues, which carboxyl groups impart acidic character to the entire peptide, they can also be in the form of salts with inorganic or organic bases, e.g., sodium, potassium, calcium or magnesium salts, or also ammonium salts derived from ammonia or a pharmacologically acceptable organic nitrogen-containing base. However, as they contain at the same time free carboxyl groups and free amino groups, they can also be in the form of inner salts. Pharmacologically acceptable salts are preferred. A "pharmaceutically acceptable salt" is a salt that retains the biological effectiveness of the free acids and bases of the specified compound and that is not biologically or otherwise undesirable.

One problem in developing a dosage form containing hirudin is its poor stability in aqueous solutions and in powder form. The poor stability can be seen when hirudin is analyzed by chromatographic methods, such as reverse phase HPLC (RP-HPLC), MONO Q method, or the PROPAX method.

RP-HPLC method: A LiChroCART 125-4 column is used (Merck LiChrospher 100 RP-18 5 .mu.m). Solvent A is 0.5% ammonium acetate in acetonitrile/water (10:90), (v:v); solvent B is 0.5% ammonium acetate in acetonitrile/water (25:75). The elution is performed at 45°C using a flow rate of 0.5 ml/min. The binary elution is a linear gradient starting at time zero with 23% solvent B and reaching 46% solvent B after 24 minutes. After 2 min at 70% solvent B the column is equilibrated for 7 min at 23% solvent B.

MONO Q METHOD: The stability of hirudin can be analysed by FPLC (fine protein liquid chromatography) using a Mono-Q column (10 μm particle size, 5.0 x 50 mm, purchased from Pharmacia). The method was developed by Ciba-Geigy, Basel. Solvent A is 50 mM HCOONH₄ in H₂O pH 4.5, and solvent B is 50 mM HCOONH₄

in H₂O, pH 3.5. The elution is performed at room temperature (22°C) using a flow rate of 1.4 ml/min. The binary elution for the first 5 min is at constant flow of 20% B, followed by a linear gradient from 20% B to 75% B over 10 minutes, and 2 minutes at 100% B after which the column is equilibrated for 2 minutes at the starting conditions 5 of 20% B.

PROPAC METHOD: A recently published method for hirudin analysis can also be used (Tuong, A., *et al.*, "Characterisation of the Deamidated Forms of Recombinant Hirudin" *Biochemistry*, 31:8291-8299 (1992)). In this method a ProPac PA1 anion-exchange column (250 x 4 mm i.d.) from Dionex is used. Solvent A is 20 mM Tris-HCl, pH 7.0 and solvent B is 0.5M NaCL in A. Isocratic elution for 5 min at 28% is followed by a linear gradient from 28% B to 54% B over 60 minutes at a flow rate of 1.3 ml/min.

Potassium phosphate can be used to increase the stability of hirudin (U.S. Patent No. 6,436,901 B1). Accordingly, one embodiment of the present invention 15 provides a freeze-dried pharmaceutical composition comprising hirudin, potassium phosphate and a sugar. A freeze-dried composition of the invention may be produced by forming an aqueous solution of the ingredients and then freeze-drying it in a conventional manner.

The potassium phosphate is preferably dipotassium hydrogen phosphate. It 20 may be used, in the solution before freeze-drying, at a molarity of from 0.1 to 0.5, preferably from 0.1 to 0.3.

Suitable sugars include, but are not limited to, mannitol, trehalose, sucrose, sorbitol, fructose, glucose, maltose, lactose and dextran. The preferred sugars are mannitol and trehalose. The amount of sugar in the solution before freeze-drying may 25 be such as to produce a concentration of from 5 to 50% (w/v) and preferably from 5 to 20% (w/v).

The solution before freeze-drying is preferably isotonic. The pH of the solution before freeze drying may be from 4 to 10, preferably from 6 to 9 and most preferably from 6.5 to 8. If desired a citrate buffer may be added to the solution 30 before freeze-drying, e.g., by adding citric acid. The molarity of the citrate may be from 0.1 to 0.5, preferably from 0.1 to 0.3.

The concentration of hirudin in the solution before freeze-drying may be from

0.1 to 500 mg/ml, preferably from 20 to 250 mg/ml.

The freeze-dried product is stable for long periods of time without the need for refrigerated storage. In addition, after the product has been redissolved in water, the resulting solution is also stable for long periods although the stability in solution is not 5 as good as the stability of the freeze-dried powder.

The solutions made by redissolving the freeze-dried product may be used in the production of standard ampoules, pre-filled double camber syringes, or multi-administration systems. The solutions may of course also be used immediately for administration.

10 The compounds of this invention may be in the form of a "pharmaceutically acceptable prodrug," i.e., a compound that may be converted under physiological conditions or by solvolysis to the specified compound or to a pharmaceutically acceptable salt of such compound. Prodrugs may be identified using routine techniques known in the art. Various forms of prodrugs are known in the art. For 15 examples of such prodrug derivatives, see, for example, a) *Design of Prodrugs*, edited by H. Bundgaard, (Elsevier, 1985) and *Methods in Enzymology*, Vol. 42, p. 309-396, edited by K. Widder, et al. (Academic Press, 1985); b) *A Textbook of Drug Design and Development*, edited by Krogsgaard-Larsen and H. Bundgaard, Chapter 5 "Design and Application of Prodrugs", by H. Bundgaard p. 113-191 (1991); c) H. 20 Bundgaard, *Advanced Drug Delivery Reviews*, 8, 1-38 (1992); d) H. Bundgaard, et al., *Journal of Pharmaceutical Sciences*, 77:285 (1988); and e) N. Kakeya, et al., *Chem. Pharm. Bull.*, 32: 692 (1984), each of which is specifically incorporated herein by reference.

25 The amount of desulphohirudin in the formulation is preferably a relatively low, effective single unit dose that may be repeatedly administered over time, e.g., over a period of days, weeks, or months. Of course, the actual amount in any unit dose and the administration schedule will depend upon a number of factors, such as the patient and the type of condition, disease, or disorder that is responsive to inhibition of angiogenesis. The amount of the desulphohirudin, however, is a 30 quantity that is effective to inhibit angiogenesis, but insufficient to cause substantial cellular death by a direct cytotoxic mechanism, in contrast to cellular death as a result of inhibition of angiogenesis. In the context of desulphohirudin, the required

amount at least partially inhibits the formation of new blood vessels.

Preferably, the effective angiogenesis-inhibiting amount of desulphohirudin represents from about 0.0001 mg to about 200 mg desulphohirudin per kilogram body weight of the patient, more preferably from about 0.0001 to about 120 mg desulphohirudin per kilogram body weight of the patient, still more preferably from about 0.0001 mg to about 15 mg desulphohirudin per kilogram body weight of the patient, yet still more preferably from about 0.5 mg to about 15 mg desulphohirudin per kilogram body weight of the patient, and most preferably from about 1 mg to about 13 mg desulphohirudin per kilogram body weight of the patient. In terms of weight percent, the formulations will preferably comprise the desulphohirudin in an amount of from about 0.0001 wt. % to about 10 wt. %, more preferably from about 0.001 wt. % to about 1 wt. %, and most preferably from about 0.01 wt. % to about 0.5 wt. %.

B. Solvent

In order to use a compound, or a pharmaceutically acceptable salt or *in vivo* cleavable prodrug thereof, for the therapeutic treatment (including prophylactic treatment) of mammals including humans, it is normally formulated in accordance with standard pharmaceutical practice as a pharmaceutical composition. According to one embodiment of the invention there is provided a pharmaceutical composition that comprises desulphohirudin or a desulphohirudin variant, or a pharmaceutically acceptable salt or *in vivo* cleavable prodrug thereof, as defined hereinbefore in association with a pharmaceutically acceptable solvent or carrier. By "pharmaceutically acceptable" solvent or carrier is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the desulphohirudin without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical formulation in which it is contained.

Suitable solvents include, without limitation: alcohols, e.g., monohydric alcohols such as 1-butanol, 2-butanol, 3-methyl-1-butanol, 2-methyl-1-propanol, and ethanol, as well as polyhydric alcohols such as propylene glycol; ethyl ether; ethyl formate; ethyl acetate; methyl acetate; isobutyl acetate; isopropyl acetate; methylethyl

ketone; dimethylacetamide (DMA); 1,1-dimethyloxymethane; 2,2-dimethyloxyp propane; dimethyl sulfoxide (DMSO); and the like. The solvent may be an organic solvent or an inorganic solvent. Furthermore, the solvent may be aqueous or nonaqueous. As will be readily appreciated by one of ordinary skill in the art, the 5 choice of solvent will depend on the desired solubility, the nature of the desulphatohirudin, and the desired release characteristics.

The amount of solvent present in the formulation will vary depending on the type of tumor (solid or soft), the solubility of desulphatohirudin, the solvent chosen, and the desired form of the formulation (e.g., solution, suspension, etc.). Those of 10 ordinary skill in the art routinely consider such factors in determining the appropriate amount of a solvent in a pharmaceutical formulation. Thus, the amount of solvent included in the pharmaceutical formulation may be established by those skilled in the art without undue experimentation. In one embodiment, the amount of solvent contained in the formulation is from about 0.01% by volume to about 50% by volume 15 (v/v) of formulation.

In one embodiment, the desulphatohirudin (optionally together with a solvent) is added to a carrier, i.e., a solution such as but not limited to a sodium chloride solution (e.g., normal saline), a dextrose solution, lactated Ringer's solution, water, or a mixture thereof. Carriers may include excipients and other additives such as 20 diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like. The desulphatohirudin, solvent, and any additional components are added to the carrier and mixed.

C. Formulations

The pharmaceutical formulations of the present invention are not limited with 25 regard to the particular form used. A nonlimiting list of preferred delivery forms of the present invention includes solutions, suspensions, dispersions, emulsions, and controlled release formulations such as microspheres. For systemic administration, solutions and suspensions are preferred. For local administration, any delivery form, e.g., solution, suspension, dispersion, emulsion, or microspheres, may be used.

30 In general, the pharmaceutical formulation may include one or more additional components. Such additional components include, for example, antimicrobials, buffers, antioxidants, tonicity-adjusting agents, and detergents. Antimicrobial agents

are used to deter the growth of microorganisms, particularly in multiple dose formulations (i.e., formulation for a multiple dose vial). Suitable antimicrobial agents include phenylmercuric nitrate, thimerosal, benzethonium chloride, benzalkonium chloride, phenol, cresol, chlorobutanol, and combinations thereof. Buffers are used

5 primarily to stabilize a solution against potential chemical degradation resulting from a change in the formulation's pH. Suitable buffers include acid salts of citrates, acetates, and phosphates. Antioxidants are used for preservation, as many drugs are susceptible to degradation through oxidation. Examples of antioxidants include sodium bisulfite, acetone sodium bisulfite, sodium formaldehyde, sulfoxylate,

10 thiourea, ascorbic acid, sodium salt of ethylenediamine tetraacetic acid, and storage in an inert gas (e.g., nitrogen). Tonicity-adjusting agents are used to control tonicity to ensure that the resulting formulation is not excessively hypotonic or hypertonic relative to the physiological state. Examples of tonicity agents include electrolytes (e.g. sodium chloride) and mono- or disaccharides (e.g., dextrose for

15 monosaccharides). Examples of detergents include, for example, anionic detergents; nonionic detergents, e.g., polyoxyethylated sorbitans (available under the TWEEN™ name from ICI Americas, Inc.); sorbitan esters; polysorbates; polyoxyethylene ethers (e.g., available under the TRITON™ name from Union Carbide Corp., Midland Mich.); cationic and zwitterionic detergents; and combinations thereof.

20 The amounts of additional components that are not active agents will vary depending on the solvents chosen, desired form of the formulation, and other factors. Those of ordinary skill in the art routinely consider such factors. Thus, the amount of any particular additional component can be established by those skilled in the art without undue experimentation. Typically, the amount of any such component

25 preferably does not exceed 10% by volume of the total formulation.

The compositions of the invention may be in a form suitable for oral use (for example as tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs), for topical use (for example as creams, ointments, gels, or aqueous or oily solutions or suspensions), for

30 administration by inhalation (for example as a finely divided powder or a liquid aerosol), for administration by insufflation (for example as a finely divided powder) or for parenteral administration (for example as a sterile aqueous or oily solution for intravenous, subcutaneous, or intramuscular dosing or as a suppository for rectal

dosing). For example, compositions intended for oral use may contain, for example, one or more coloring, sweetening, flavoring and/or preservative agents.

Suitable pharmaceutically-acceptable excipients for a tablet formulation include, for example, inert diluents such as lactose, sodium carbonate, calcium phosphate or calcium carbonate, granulating and disintegrating agents such as corn starch or algenic acid; binding agents such as starch; lubricating agents such as magnesium stearate, stearic acid or talc; preservative agents such as ethyl or propyl p-hydroxybenzoate, and anti-oxidants, such as ascorbic acid. Tablet formulations may be uncoated or coated either to modify their disintegration and the subsequent absorption of the active ingredient within the gastrointestinal tract, or to improve their stability and/or appearance, in either case, using conventional coating agents and procedures well known in the art.

Compositions for oral use may be in the form of hard gelatin capsules in which the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules in which the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin, or olive oil.

Aqueous suspensions generally contain the active ingredient in finely powdered form together with one or more suspending agents, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as lecithin or condensation products of an alkylene oxide with fatty acids (for example polyoxethylene stearate), or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives (such as ethyl or propyl p-hydroxybenzoate, anti-oxidants (such as ascorbic acid), coloring agents, flavoring agents, and/or sweetening agents (such as sucrose, saccharine or aspartame).

Oily suspensions may be formulated by suspending the active ingredient in a

vegetable oil (such as arachis oil, olive oil, sesame oil or coconut oil) or in a mineral oil (such as liquid paraffin). The oily suspensions may also contain a thickening agent such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set out above, and flavoring agents may be added to provide a palatable oral 5 preparation. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water generally contain the active ingredient together with a dispersing or wetting agent, suspending agent and one or more preservatives. 10 Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients such as sweetening, flavoring and coloring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or 15 arachis oil, or a mineral oil, such as for example liquid paraffin or a mixture of any of these. Suitable emulsifying agents may be, for example, naturally-occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soya bean, lecithin, an esters or partial esters derived from fatty acids and hexitol anhydrides (for example sorbitan monooleate) and condensation products of the said 20 partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening, flavoring and preservative agents.

Syrups and elixirs may be formulated with sweetening agents such as glycerol, propylene glycol, sorbitol, aspartame or sucrose, and may also contain a demulcent, preservative, flavoring and/or coloring agent. 25 The pharmaceutical compositions may also be in the form of a sterile injectable aqueous or oily suspension, which may be formulated according to known procedures using one or more of the appropriate dispersing or wetting agents and suspending agents, which have been mentioned above. A sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic 30 parenterally-acceptable diluent or solvent, for example a solution in 1,3-butanediol.

Suppository formulations may be prepared by mixing the active ingredient with a suitable non-irritating excipient which is solid at ordinary temperatures but

liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Suitable excipients include, for example, cocoa butter and polyethylene glycols.

Topical formulations, such as creams, ointments, gels and aqueous or oily solutions or suspensions, may generally be obtained by formulating an active 5 ingredient with a conventional, topically acceptable, vehicle or diluent using conventional procedures well known in the art.

Compositions for administration by insufflation may be in the form of a finely divided powder containing particles of average diameter of, for example, 30 μm or much less, the powder itself comprising either active ingredient alone or diluted with 10 one or more physiologically acceptable carriers such as lactose. The powder for insufflation is then conveniently retained in a capsule containing, for example, 1 to 50 mg of active ingredient for use with a turbo-inhaler device, such as is used for insufflation of the known agent sodium cromoglycate.

Compositions for administration by inhalation may be in the form of a 15 conventional pressurized aerosol arranged to dispense the active ingredient either as an aerosol containing finely divided solid or liquid droplets. Conventional aerosol propellants such as volatile fluorinated hydrocarbons or hydrocarbons may be used and the aerosol device is conveniently arranged to dispense a metered quantity of active ingredient.

20 For further information on formulations, see Chapter 25.2 in Volume 5 of *Comprehensive Medicinal Chemistry* (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990, which is specifically incorporated herein by reference.

The amount of a compound of this invention that is combined with one or more excipients to produce a single dosage form will necessarily vary depending upon 25 the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans may contain, for example, from 0.5 mg to 2 g of desulphohirudin compounded with an appropriate and convenient amount of excipients which may vary from about 5 to about 98 percent by weight of the total composition. Dosage unit forms will generally contain about 1 mg to about 500 mg 30 of an active ingredient. For further information on routes of administration and dosage regimes, see Chapter 25.3 in Volume 5 of *Comprehensive Medicinal Chemistry* (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990,

which is specifically incorporated herein by reference.

The pharmaceutical formulations of the present invention may be sustained-release formulations. The term "sustained release" as used herein refers to a drug formulation that provides for gradual release of a drug over an extended period of time, and that preferably, although not necessarily, results in substantially constant drug levels at the desired site over an extended period of time. That is, the formulation would release the desulphohirudin over an extended period of time relative to an immediate-release or nonsustained-release formulation. For example, in one embodiment the pharmaceutical formulations of the present invention release desulphohirudin over a time of from about 4 hours to about 24 hours.

Sustained-release formulations generally include one or more components designed to slow or control the release of the desulphohirudin over time. The invention is not limited with regard to the particular component(s) used to effect sustained release, so long as the component or components are pharmaceutically acceptable. Preferred sustained-release components include polysaccharides, salts of polysaccharides, microspheres of polysaccharides, dextrans, gums, celluloses, gels, silicones, liposomes, and combinations thereof. It is particularly preferred that the sustained-release component be a polysaccharide. Examples of suitable polysaccharides include, but are not limited to, chitin (poly-N-acetyl-D-glucosamine), chitosan (deacetylated chitin), and combinations thereof. Derivatives of these carriers are contemplated as well, including, for example, derivatives formed by adding or removing sugar molecules (i.e. by increasing or decreasing the weight average molecular weight of the carrier). In one embodiment, the carriers have a weight average molecular weight of about 500 daltons to about 100,000 daltons, more preferably of about 10,000 daltons to about 60,000 daltons.

The component providing sustained release can be covalently bonded to the desulphohirudin, thereby providing a sustained-release conjugate. For example, chitin may be covalently bound to desulphohirudin. Methods for producing these conjugates are well known to those skilled in the art, and are described in detail in the pertinent texts and literature.

The amount of the component or components providing sustained release will depend on several factors, such as whether the formulation is intended to contain a

single dose or multiple doses, and the injectability (e.g., viscosity) of the resulting formulation. In addition, the amount should be considered in light of the environment of the target area in which the pharmaceutical formulation will be administered. For example, it is desirable that low density organs, e.g., the prostate, brain, and bladder, 5 receive a low viscosity formulation, whereas dense tissue, e.g., solid tumors and breast tumors, receive a high viscosity pharmaceutical formulation. Those of ordinary skill in the art routine consider these and other factors in providing sustained-release formulations.

Generally, the total amount of a sustained-release component in the 10 formulation is preferably between from about 0.01% w/v to about 50% w/v of the total formulation. It is particularly preferred that the biocompatible carrier is present in an amount of from about 0.5% w/v to about 30% w/v.

II. Methods of Treatment

The present invention also provides a method for inhibiting angiogenesis in a 15 patient in need thereof comprising administering to the patient an effective angiogenesis-inhibiting amount of desulphohirudin. The method is used to treat a patient suffering from a condition, disease, or disorder that is treatable by at least partial inhibition of angiogenesis. In general, patients suffering from a neoplastic disease, i.e., a cellular proliferative disease, benefit from administration of the present 20 compositions. Generally, cellular proliferative diseases include not only sarcomas, carcinomas, lymphomas, and malignant melanomas, but also noncancerous melanomas and other benign growths caused by rapidly dividing cells.

Nonlimiting examples of cellular proliferative diseases for which the present invention is suited include adrenocortical cancer, bladder cancer, bone cancer, brain 25 cancer, breast cancer, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, esophageal cancer, eye cancer, gallbladder cancer, gastric cancer, head and neck cancer, laryngeal cancer, liver cancer, lung cancer, melanoma, myeloproliferative disorders such as myeloma, neck cancer, nonmelanoma skin cancer, ovarian cancer, prostate cancer, pancreatic cancer, rectal cancer, and testicular 30 cancer. Diseases for which the present invention is particularly well suited to treat include those diseases selected from the group consisting of brain cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, rectal cancer, head and neck

cancer, liver cancer, lung cancer myeloma, prostate cancer, and ovarian cancer.

Further, included within the scope of the present invention is the co-administration of desulphatohirudin with an anticancer agent or a cytotoxic agent. Such agents suitably include, but are not limited to, antimetabolites (e.g., 5-fluorouracil, methotrexate, fludarabine), antimicrotubule agents (e.g., vincristine, vinblastine, taxanes such as paclitaxel, docetaxel), an alkylating agent (e.g., cyclophosphamide, melphalan, biochloroethylnitrosurea, hydroxyurea), platinum agents (e.g. cisplatin, carboplatin, oxaliplatin, JM-216, CI-973), anthracyclines (e.g., doxorubicin, daunorubicin), antibiotics (e.g., mitomycin, idarubicin, adriamycin, 10 daunomycin), topoisomerase inhibitors (e.g., etoposide, camptothecins) or any other cytotoxic agents. (estramustine phosphate, prednimustine). It is anticipated that desulphatohirudin used in combination with various anticancer drugs can give rise to a significantly enhanced cytotoxic effect on cancerous cells, thus providing an increased therapeutic effect. Specifically, a significantly increased growth-inhibitory 15 effect can be obtained utilizing lower concentrations of the anticancer drugs compared to the treatment regimes in which the drugs are used alone. Thus, there is the potential to provide therapy wherein adverse side effects associated with the anticancer drugs are considerably reduced than normally observed with the anticancer drugs used alone in larger doses. Possible dose ranges of these co-administered 20 anticancer agents are about 0.1 to 20 mg/kg/day.

The term "co-administration" is meant to refer to any administration route in which two or more agents (i.e., desulphatohirudin and one or more additional anticancer or cytotoxic agents) are administered to a patient or subject. For example, the agents may be administered together, or before or after each other. The agents 25 may be administered by different routes, e.g., one agent may be administered intravenously while the second agent is administered intramuscularly, intravenously or orally. The agents may be administered simultaneously or sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations in the body. The agents also may be in an admixture, as, for example, 30 in a single tablet. In sequential administration, one agent may directly follow administration of the other or the agents may be given episodically, i.e., one can be given at one time followed by the other at a later time, typically within a week. An example of a suitable co-administration regimen is where desulphatohirudin is

administered from 0.5 to 7 days prior to administration of a cytotoxic agent.

According to the present invention, suitable methods of administering the therapeutic composition of the present invention to a patient include any route of *in vivo* administration that is suitable for delivering the composition into a patient. The 5 preferred routes of administration will be apparent to those of skill in the art, depending on the type of condition to be prevented or treated, and/or the target cell population. Preferred methods of *in vivo* administration include, but are not limited to, intravenous administration, intraperitoneal administration, intramuscular administration, intranodal administration, intracoronary administration, intraarterial 10 administration (e.g., into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, intraarticular administration, intraventricular administration, inhalation (e.g., aerosol), intracranial, intraspinal, intraocular, intranasal, oral, bronchial, rectal, topical, vaginal, urethral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue.

15 Parenteral administration may be carried out in any number of ways, but it is preferred that the use of a syringe, catheter, or similar device, be used to effect parenteral administration of the formulations described herein. The formulation may be injected systemically such that the desulphatohirudin travels substantially throughout the entire bloodstream.

20 In addition, the formulation may also be injected locally to a target site, i.e., injected to a specific portion of the body for which inhibition of angiogenesis is desired. An advantage of local administration via injection is that it limits or avoids exposure of the entire body to the desulphatohirudin. It must be noted that in the present context, the term local administration includes regional administration, e.g., 25 administration of a formulation directed to a portion of the body through delivery to a blood vessel serving that portion. Local delivery may be direct, i.e., intratumoral. Local delivery may also be nearly direct, i.e., intralesional or intraperitoneal, that is, to an area that is sufficiently close to a tumor so that the desulphatohirudin exhibits the desired pharmacological activity. Thus, when local delivery is desired, the 30 pharmaceutical formulations are preferably delivered intralesionally, intratumorally, or intraperitoneally.

It is intended that, by local delivery of the presently described pharmaceutical formulations, a higher concentration of the desulphatohirudin may be retained at the target site. There are several advantages to having high concentrations delivered directly at the target site. First, since the desulphatohirudin is localized, there is less 5 potential for toxicity to the patient since minimal systemic exposure occurs. Second, drug efficacy is improved since the target site is exposed to higher concentrations of drug. Third, relatively fast delivery ensures both solubility of the drug and little or no degradation of the desulphatohirudin before reaching the target site. Fourth, the method is relatively noninvasive, which is ideal for unresectable tumors such as brain 10 tumors, liver tumors, and pancreatic tumors.

With local administration, it is preferred that the pharmaceutical formulations of the present invention be directed to the target area with the assistance of computerized tomography (CT), ultrasound, or similar method in order to ensure correct placement. Once the initial dose is administered, the patient may be given 15 other doses either immediately or after a period of time. Such a dosing schedule is easily determined by one of ordinary skill in the art once the nature of the condition, disorder, or disease, strength of the patient, expected effects of the formulation, and so forth, are taken into consideration.

The amount of desulphatohirudin administered will, of course, be dependent 20 on the subject being treated, the subject's weight, the manner of administration, and the judgment of the prescribing physician. The amount of desulphatohirudin administered, however, will be an effective angiogenesis-inhibiting amount. Preferably, the desulphatohirudin is administered in an amount of from about 0.0001 25 mg/kg to about 200 mg/kg (milligrams of drug per kilogram body weight of the patient), more preferably from about 0.0001 mg/kg to 120 mg/kg, still more preferably from about 0.0001 mg/kg to about 15 mg/kg, yet still more preferably from about 0.5 mg/kg to about 15 mg/kg, and most preferably from about 1 mg/kg to about 13 mg/kg. Depending on the patient's response, additional dosages within this range may be administered.

30 The size of the dose for therapeutic or prophylactic purposes of desulphatohirudin will naturally vary according to the nature and severity of the conditions, the age and sex of the animal or patient and the route of administration,

according to well known principles of medicine. For example, the method may comprise at least one of an hourly administration, a daily administration, a weekly administration, or a monthly administration of one or more compositions described herein.

5 Throughout this application, various patents are referenced by number and publications are referenced by author and date. The disclosures of these patents and publications are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of this invention described and claimed herein.

10 The foregoing description is considered as illustrative only of the principles of the invention. Further, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and process shown as described above. Accordingly, all suitable modifications and equivalents may be resorted to falling within the scope of the
15 invention as defined by the claims that follow. The words "comprise," "comprising," "include," "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof.

Experimental Data

A study was conducted consisting of 4 experimental groups, each containing 10 male NMRI mice. At day 0, ASPC-1 tumor cells were orthotopically implanted in the pancreas (tail) of all the mice. During the course of the study, the growth of the orthotopically implanted ASPC-1 tumors and metastasis was monitored on days 2, 7, 16, 23, 29 and 35 using in vivo luminescence imaging. At day 16, the animals were randomized into 4 groups. Treatment was initiated at day 19. Animals in group 1, a control group, received 0.9% NaCl once weekly intravenously up to study end. Animals in group 2 received 8 mg/kg doxorubicin one weekly intravenously for two weeks. Animals in group 3 received 10 mg/kg desulphatohirudin ('desirudin') twice daily s.c through the end of the study. Animals of group 4 received 8 mg/kg doxorubicin once weekly intravenously for two weeks and 10 mg/kg desirudin twice daily s.c. through the end of the study. The animals were weighed throughout the study. After 35 days, the study was terminated as planned and necropsy performed. Primary tumor volumes were determined.

The desirudin which was used is a variant marketed under Iprivask™ (desirudin for injection) which has the following structural formula:

20	Val	Val	Tyr	Thr	Asp	Cys	Thr	Glu	Ser	Gly	Gln	Asn	Leu	Cys	Leu	Cys
	1			5				10					15			
	Glu	Gly	Ser	Asn	Val	Cys	Gly	Gln	Gly	Asn	Lys	Cys	Ile	Leu	Gly	Ser
					20				25				30			
25	Asp	Gly	Glu	Lys	Asn	Gln	Cys	Val	Thr	Gly	Glu	Gly	Thr	Pro	Lys	Pro
					35				40				45			
	Gln	Ser	His	Asn	Asp	Gly	Asp	Phe	Glu	Glu	Ile	Pro	Glu	Glu-Tyr-Leu-Gln		
							50		55			60				

30 This is the same sequence as variant (a) of SEQ ID NO:1 (HV1). The 4 peptide sequence identified in variant (a) is the c-terminal part of the 65 peptide sequence which collectively results in the HV1 variant. While only the 4 peptide sequence is listed in the sequence listing, and is identified as SEQ ID NO:2, it is submitted that by SEQ ID NO:2, one means SEQ ID NO:2 as incorporated with the other substitutes of variant (a) into SEQ ID NO:1, thereby defining the full 65 peptide sequence of variant HV1.

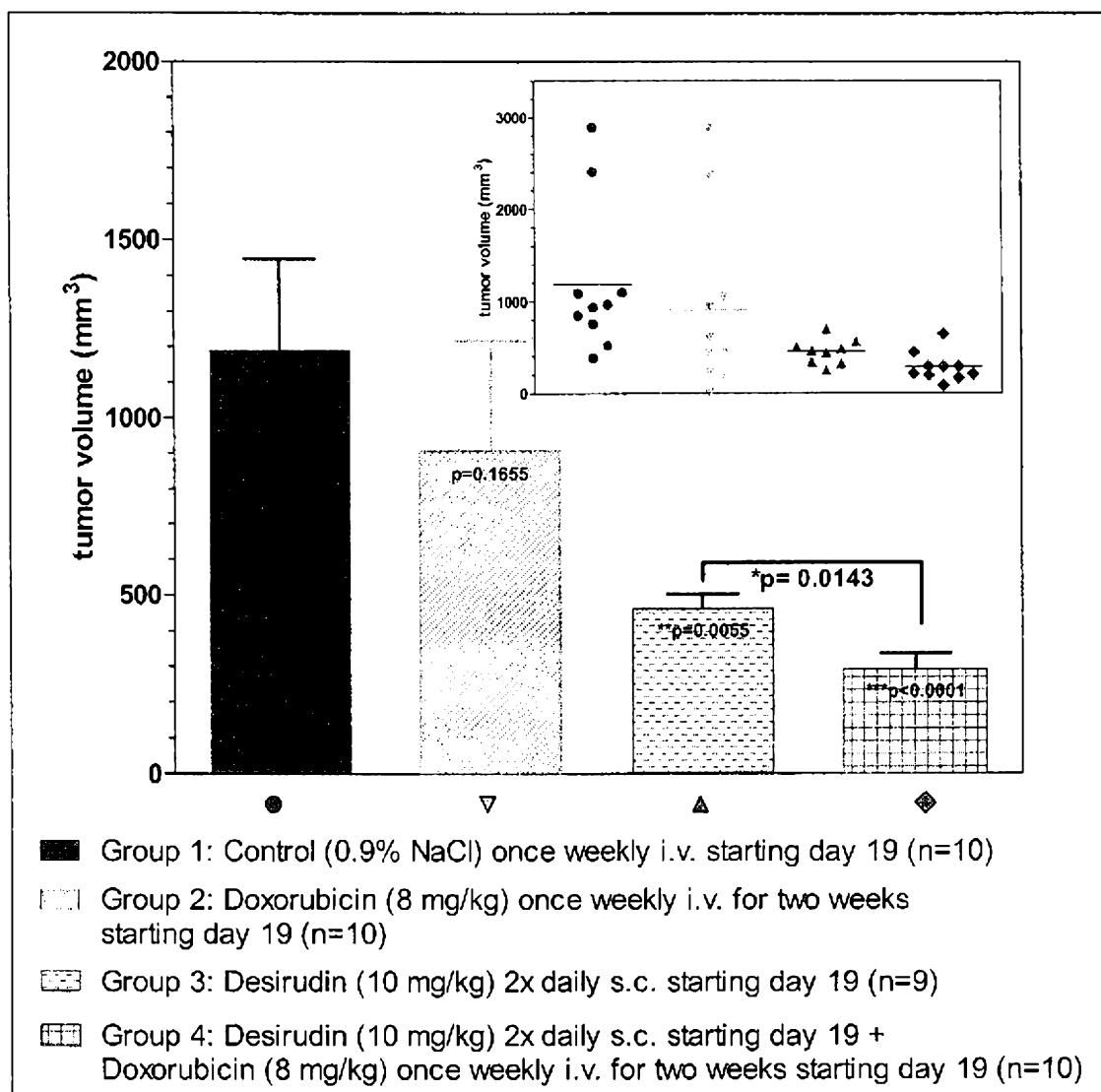
At the start of treatment, all animal weights remained stable. However, the group 2 animals treated with doxorubicin and the group 4 animals treated with doxorubicin and desirudin experienced a mean weight loss by the end of treatment of 12.2% and 17.4%, respectively. No other drug-related side effects were observed.

5

The luciferase activity of the primary tumors, and of several organs, was measured post necropsy. There was an apparent lack of correlation between luciferase assays and tumor sizes measured. Due to high variances, the effects of the different treatments as measured by luciferase signal were not statistically significant.

10

Primary tumor volumes measured after the end of the study are reported graphically below:



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Compared to a mean pancreatic tumor volume of 1189 mm³ for group 1, the control group, group 2 tumor volume decreased to a mean of 909.1mm³, a decrease of 23.5%. Group 3 animals, treated with desirudin, experienced a mean tumor volume decrease to 455.0 mm³, representing a decrease in tumor volume as compared to the control group 1 of 61.7%. Group 4 animals, who were treated with both doxorubicin and desirudin, experienced a mean tumor volume shrinkage to 282.8 mm³, representing a decrease in tumor volume as compared to control group 1 of 76.2%.

As can be seen from the above graph, both desirudin alone and desirudin with a co-administration of doxorubicin are highly effective in inhibiting the growth of the primary tumor. Thus, desirudin, with or without co-administration of doxorubicin, appears very effective in inhibiting the growth of a primary tumor even when the drug treatment is initiated 13 days after tumor cell implantation.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of effecting regression of tumor mass and size in a metastasized tumor in a subject, the method comprising administering to the subject a pharmaceutical formulation comprising an effective amount of a desulphohirudin variant selected from the group consisting of

5 a) SEQ ID NO:1 wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) Xaa at 66 not present,

10 b) SEQ ID NO:1 wherein Xaa at 27 is Ile or Glu and Xaa at 36, 47, 51 and 62-66 are as defined in a),

15 c) SEQ ID NO:1 wherein Xaa at 36 is Ile or Glu and Xaa at 27, 47, 51 and 62-66 are as defined in a),

 d) SEQ ID NO:1 wherein Xaa at 47 is Ile or Glu and Xaa at 27, 36, 51 and 62-66 are as defined in a), and

20 e) SEQ ID NO:1 wherein Xaa at 51 is Leu or Asp and Xaa at 27, 36, 47 and 62-66 are as defined in a),

 to effect regression in the subject of a metastasized tumor selected from the group consisting of adrenocortical cancer, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, eye cancer, gallbladder cancer, gastric cancer, head and neck cancer, laryngeal cancer, liver cancer, myeloproliferative disorders, neck cancer, nonmelanoma skin cancer, ovarian cancer and testicular cancer.

2. The method of claim 1, wherein said desulphohirudin variant is SEQ ID NO. 1, wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) and Xaa at 66 is not present.

3. The method of claim 1 or 2 wherein the pharmaceutical formulation is administered via injection.

4. The method of claim 3, wherein the pharmaceutical formulation is systemically injected.
5. The method of claim 3, wherein the pharmaceutical formulation is locally injected.
- 5 6. The method of any one of the previous claims, wherein the desulphatohirudin variant is co-administered with a cytotoxic agent comprising an anthracycline selected from the group consisting of doxorubicin and daunorubicin and the co-administration results in increased therapeutic effect as compared to administration of the cytotoxic agent alone.
- 10 7. The method of any one of the previous claims, wherein the pharmaceutical formulation further includes potassium phosphate.
8. The method of any one of the previous claims, wherein the pharmaceutical formulation further includes a divalent or trivalent metal ion.
9. The method of any one of the previous claims, wherein the pharmaceutical 15 formulation further includes a sugar.
10. The method of any one of the previous claims, wherein the pharmaceutical formulation provides a sustained-release profile in vivo.
11. A method of effecting regression of tumor mass and size in a metastasized pancreatic tumor in a subject, the method comprising administering to the subject 20 a pharmaceutical formulation comprising an effective amount of a desulphatohirudin variant selected from the group consisting of
 - a) SEQ ID NO:1 wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) Xaa at 66 not present,
 - 25 b) SEQ ID NO:1 wherein Xaa at 27 is Ile or Glu and Xaa at 36, 47, 51 and 62-66 are as defined in a),

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c) SEQ ID NO:1 wherein Xaa at 36 is Ile or Glu and Xaa at 27, 47, 51 and 62-66 are as defined in a),

d) SEQ ID NO:1 wherein Xaa at 47 is Ile or Glu and Xaa at 27, 36, 51 and 62-66 are as defined in a), and

5 e) SEQ ID NO:1 wherein Xaa at 51 is Leu or Asp and Xaa at 27, 36, 47 and 62-66 are as defined in a),

to effect regression in the subject of a metastasized pancreatic tumor.

12. The method of claim 11, wherein said desulphohirudin variant is SEQ ID NO. 1, wherein Xaa at 27, 36 and 47 are each Lys, Xaa at 51 is His and Xaa at 10 62-65 is the peptide residue Glu-Tyr-Leu-Gln (SEQ ID NO:2) and Xaa at 66 is not present.

13. The method of claim 11 or 12 wherein the pharmaceutical formulation is administered via injection.

14. The method of any one of claims 11 to 13, wherein the pharmaceutical 15 formulation is systemically injected.

15. The method of any one of claims 11 to 13, wherein the pharmaceutical formulation is locally injected.

16. The method of any one of claims 11 to 15 wherein the desulphohirudin variant is co-administered with a cytotoxic agent comprising an anthracycline 20 selected from the group consisting of doxorubicin and daunorubicin and the co-administration results in increased therapeutic effect as compared to administration of the cytotoxic agent alone.

17. The method of any one of claims 11 to 16, wherein the pharmaceutical formulation further includes potassium phosphate.

18. The method of any one of claims 11 to 17, wherein the pharmaceutical formulation further includes a divalent or trivalent metal ion.
19. The method of any one of claims 11 to 18, wherein the pharmaceutical formulation further includes a sugar.
- 5 20. The method of any one of claims 11 to 19, wherein the pharmaceutical formulation provides a sustained-release profile in vivo.
21. The method of any one of the previous claims substantially as hereinbefore described.

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CANYON PHARMACEUTICALS, INC.

WATERMARK PATENT & TRADE MARK ATTORNEYS

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SEQUENCE LISTING

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<140> US 11/099,370
<141> 2005-04-04
<150> US 60/561,741
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20 25 30

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Gln
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