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(54) Title: ANTI–INVASIVE AND ANTI–ANGIOGENIC UROKINASE FRAGMENTS AND THEIR USE

(57) Abstract

A peptide compound having the sequence Lys–Pro–Ser–Ser–Pro–Pro–Glu–Glu [SEQ ID NO:2] or a substitution variant, addition variant or other chemical derivative thereof inhibits cell invasion, endothelial tube formation or angiogenesis *in vitro*. A number of substitution variants and addition variants of this peptide, preferably capped at the N– and C–termini, as well as peptidomimetic derivatives, are useful for treating diseases and conditions mediated by undesired and uncontrolled cell invasion and/or angiogenesis. Pharmaceutical compositions comprising the above peptides and derivatives are administered to subjects in need of such treatment in a dosage sufficient to inhibit invasion and/or angiogenesis. The disclosed compositions and methods are particularly useful for suppressing the growth and metastasis of tumors.

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ANTI-INVASIVE AND ANTI-ANGIOGENIC UROKINASE FRAGMENTS AND THEIR USE

Field of the Invention

The invention in the fields of biochemistry, organic chemistry and medicine relates to peptide compounds and methods of their use to treat diseases and conditions associated with movement, migration and adhesion of cells including diseases that involve angiogenesis such as tumor invasion and metastasis.

Description of the Background Art

Several disease processes have been demonstrated to require the invasion or migration of cells as part of their pathology. These include tumor invasion, tumor metastasis, pathological angiogenesis, inflammation, and endometriosis (Liotta *et al.*, 1991; Fox *et al.*, 1996; Osborn, 1990; Mareel *et al.*, 1990; Aznavoorian *et al.*, 1993; Lennarz & Strittmatter, 1991; Fernandez-Shaw *et al.*, 1995).

In the case of tumor angiogenesis, quiescent endothelial cells can become motile in response to a variety of angiogenic growth factors as well as to changes in the basement membrane induced by tumor cells and various accessory cells found within a tumor (Blood & Zetter, 1990; Liotta *et al.*, 1991; Odedra & Weiss, 1991). Neovascularization of a tumor enables the metastatic spread of aggressive tumor cells by (1) providing a route of escape for the metastatic cells as well as (2) nurturing the tumor by providing a growth-conducive environment (Cornelius *et al.*, 1995; Blood & Zetter, 1990; Weaver *et al.*, 1997; Weinstat-Saslow & Steeg, 1994; Leek *et al.*, 1994).

The process of tumor metastasis may be viewed as bi-directional, comprising the following steps

(1) endothelial cells migrate into a tumor in response to a chemotactic gradient produced by the tumor cells or by accessory cells (stromal cells, leukocytes); and

(2) aggressive tumor cells concomitantly invade toward the developing neovasculature.

The process of invasion may further fuel angiogenesis by the proteolytic release of growth/angiogenic factors bound to extracellular matrix (ECM), including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) as well as other factors including interleukin -8 (IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF). Also generated are proteolytic fragments of the ECM which are themselves chemotactic for both tumor cells and endothelial cells (Fox *et al.*, 1996; Leek *et al.*, 1994; Vlodavsky *et al.*, 1990; Sweeney *et al.*, 1991; Taipale & Keski-Oja, 1997).

It has been suggested that only 1-2% of the total cells in a tumor are capable of metastasis. As this statement is based on a static view of the tumor phenotype, it is probably inaccurate. In reality, metastasis appears to depend on disseminated tumor cells becoming exposed to an environment which supports their spread and survival (Weaver *et al.*, 1997). In the majority of patients presenting with a clinically detectable primary tumor, metastasis has already occurred (Welch, 1997). Metastatic disease occurs when the disseminated foci of tumor cells seed a tissue which supports their growth and propagation, and this secondary spread of tumor cells is responsible for the morbidity and mortality associated with the majority of cancers. Clinical management of metastatic disease is often unsuccessful with conventional cytotoxic therapies. Metastasis differs substantially from the growth of the primary tumor in that it involves the simultaneous outgrowth of many foci which are phenotypically similar from the standpoint of their aggressiveness This outgrowth is dependent on the ability of cells that have metastasized to invade locally and to recruit neovessels.

By preventing interaction of adhesion molecules, the important process of cell migration/invasion and angiogenesis can be diminished or halted, with a number of important consequences for those diseases and conditions which are caused in part by undesirable cell migration, invasion and angiogenesis. In addition to vascular phenomena, such cell migration/invasion is important in tumor metastasis, which can be suppressed by the compositions and methods disclosed herein. Administration of effective amounts of these compositions will also disrupt the molecular interactions required for angiogenesis.

The art recognizes the need for novel treatments of subjects with cancer, in particular patients with metastatic cancer who have the poorest prognosis. Such treatment should be as devoid as possible of undesired side effects such as those associated with conventional chemotherapy and some of the experimental biotherapies. The present invention is directed to this objective. Inhibition of tumor cell invasion and endothelial cell migration (an important component of the angiogenic process) provide a novel approach to treating subjects with metastatic cancer. By inhibiting the local spread of tumor cells and angiogenesis at metastatic sites, metastatic foci should be induced to regress due to deprivation of their blood supply thus encouraging the subsequent expression of the cells' endogenous apoptotic program.

Furthermore, the inhibition of invasion of tissue by leukocytes and the concomitant angiogenesis would be useful for treating inflammation and other disease processes wherein cellular invasiveness is part of the pathogenic process. Inflammation and tumor invasion and metastasis and angiogenesis are known to involve similar mechanisms and extracellular factors (Liotta *et al.*, 1991; Fox *et al.*, 1996; Osborn, 1990; Mareel *et al.*, 1990; Aznavoorian *et al.*, 1993; Lennarz and Strittmatter, 1993).

Blasi *et al.* (U.S. 5,416,006) discloses plasminogen activators and their chemical modification, in particular phosphorylated uPA and tPA as thrombolytic agents. These workers examined phosphorylated uPA by generating tryptic phosphopeptides therefrom and noted the existence of KPSSPPEELK [SEQ ID NO:1] (corresponding to positions 136-145 of uPA). This decapeptide was not tested for any function, nor ascribed any properties of functional relevance. More importantly, as disclosed herein, this peptide (unphosphorylated), capped or uncapped, is inactive in an *in vitro assay* of cell invasion.

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for treating diseases and processes mediated by undesired and uncontrolled cell invasion and/or angiogenesis by administering to an animal a composition comprising an oligopeptide, chemical derivative or peptidomimetic in a dosage sufficient to inhibit the invasion and/or angiogenesis. The present invention is particularly useful for treating or for suppressing the growth of tumors . Administration of the composition to a human or subject with prevascularized metastasized tumors will prevent the growth or expansion of those tumors.

Thus, the present invention is directed to a peptide compound having the sequence Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu (also abbreviated in single letter amino acid code as KPSSPPEE) [SEQ ID NO:2] or a substitution variant, addition variant or other chemical derivative thereof. The preferred peptide, variant or derivative is "capped" at the amino and carboxyl termini, wherein (a) acetyl (abbreviated as "Ac") is bound to the N at the amino-terminus and (b) an amido group (abbreviated as "Am") is bound to the C-terminal carboxyl group. In general, this capped peptide will be written "Ac-KPSSPPEE-Am" throughout this document using the single letter amino acid code and indicating the blocking groups as Ac and Am.

The peptide, variant or derivative of this invention has one or more of the following activities:

(a) at least about 20% of the biological activity of Ac-KPSSPPEE-Am in one or more of the following *in vitro* bioassays: (i) invasion in a Matrigel® assay; (ii) endothelial tube formation on Matrigel®, or (iii) endothelial tube formation on a fibrin matrix in the presence of basic fibroblast growth factor and vascular endothelial growth factor; or

(b) binding activity such that it competes with labeled Ac-KPSSPPEE-Am for binding to a cell or molecule which has a binding site for Ac-KPSSPPEE-Am.

In a preferred embodiment, the peptide or peptide variant is capped at both ends with an N-terminal acetyl group and a C terminal amide group.

A preferred substitution or addition variant of the peptide, or a chemical derivative of the variant, has an amino acid sequence selected from the group consisting of:

(a) SEQ ID NO:2 wherein the Glu at position 7 or 8 or both is replaced by one or any two of the substituent amino acids Gln, Asp or Asn;

(b) SEQ ID NO:2 wherein Ser at position 3 or 4 or both is replaced by one or any two of the substituent amino acids Thr, Ala, Gly, hSer (homoserine) or ValβOH (β-hydroxyvaline);

(c) SEQ ID NO:2 wherein the Lys at position 1 is replaced by His, Arg, Gln, Orn (ornithine), Cit (citrulline) or Hci (homocitrulline);

(d) SEQ ID NO:2 wherein the Pro at position 2, 5 or 6 is replaced by Hyp (hydroxyproline);

(e) an addition variant of SEQ ID NO:2, wherein Leu, Ile, Val, Nva (norvaline), Nle (norleucine), Met, Ala, or Gly is added to the C-terminal Glu or to any C-terminal substituent for Glu at position 8 as disclosed above.

(f) an addition variant of SEQ ID NO:2, wherein any of the following peptides are added to the C-terminal Glu or to the C terminal substituent for Glu at position 8: Leu-$(Gly)_n$; Ile-$(Gly)_n$; Val-$(Gly)_n$; Nva-$(Gly)_n$; or Nle-$(Gly)_n$, wherein n = 1-10.

(g) an addition variant of SEQ ID NO:2 wherein one or more of the following residues or peptides is added to the N-terminal Lys, or to any N-terminal substituent of Lys at position 1 as disclosed: Gly, Lys-$(Gly)_n$; Tyr-$(Gly)_n$; or Gly-$(Gly)_n$, wherein n = 1-10; and

(h) a combination of one or more of (a) - (g).

In a preferred embodiment, the chemical derivative above is a peptidomimetic agent.

Also provided is a multimer of the peptide or variant above, which, when the peptide is not a variant, has the formula: $(KPSSPPEE\text{-}X_m)_n$-KPSSPPEE wherein X is selected from the group consisting of C_1-C_{20} alkyl, C_1-C_{20} alkenyl, C_1-C_{20} alkynyl, C_1-C_{20} polyether containing up to 9 oxygen atoms and Gly_m, and wherein m = 0 or 1, n = 1-100 and z = 1-10.

The invention is further directed to a pharmaceutical composition useful for inhibiting invasion of tumor cells or angiogenesis, comprising (a) any of the above peptides, variants or chemical derivatives including a peptidomimetic or a multimeric peptide and (b) a pharmaceutically acceptable carrier or excipient.

Also included is a method for inhibiting the invasiveness of tumor cells comprising contacting the cells with an effective amount of a peptide, variant or derivative as above.

In another embodiment, a method is provided for inhibiting tumor invasion or metastasis in a subject comprising administering to the subject any of the above pharmaceutical compositions.

Also provided is a method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis comprising administering to the subject an effective amount of a pharmaceutical composition as described above.

In any of the foregoing methods, the disease or condition being treated may be primary tumor growth, tumor invasion or metastasis, atherosclerosis, post-balloon angioplasty vascular restenosis, neointima formation following vascular trauma, vascular graft restenosis, fibrosis associated with a chronic inflammatory condition, lung fibrosis, chemotherapy-induced fibrosis, wound healing with scarring and fibrosis, psoriasis, deep venous thrombosis, or another disease or condition in which angiogenesis is pathogenic. The treatment methods are most preferred for tumor growth, invasion or metastasis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the inhibitory effect of Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am (5μM) on the *in vitro* invasion of both human (PC-3) and rat (Mat BIII) tumor cell lines in a Matrigel® system as described in the Examples. The left bar of each pair is the control group and the right bar represents cells responding in the presence of the peptide.

Figure 2 is a graph showing the effect of various peptides on the *in vitro* invasion of PC-3 cells in a Matrigel® system as described in the Examples. All compounds were tested at a 5 μM. The following peptides were examined: Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am (Ac-KPSSPPEE-Am) (SEQ ID NO:2), also termed Å6, and the following variants: Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Am (Ac-KPSSPPE-Am), Ac-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am (Ac-PSSPPEE-Am) as well as SEQ ID NO:1 (Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Leu-Lys) either capped (Ac-KPSSPPEELK-Am) or uncapped (KPSSPPEELK).

Figure 3 is a graph showing the effect of additional peptides on the *in vitro* invasion of PC3 cells as above, at two different concentrations. In addition to (Ac-KPSSPPEE-Am) which has marked inhibitory activity, additional peptides tested are Å13 (KPSSPPEE, uncapped) and Å14 (Ac-KPPSSPPEEL-Am), both of which have no inhibitory activity, and Å15, (GGKPPSSPPEE-Am, capped only at the C-terminus), which has low inhibitory activity at the higher concentration. The peptides are compared a negative control of basic fibroblast growth factor (bFGF)

Figure 4 is a graph showing the inhibition of human enthdothelial cell proliferation *in vitro* by Ac-KPSSPPEE-Am (Å6) over a three day assay period. Normal cell growth was seen in the presence of 2 ng/mL bFGF as negative control, wherease significant cell proliferation did not occur in the presence of 50μM Å6 peptide.

Figure 5 is a graph showing the dose-related inhibition of human enthdothelial cell migration *in vitro* by Ac-KPSSPPEE-Am (Å6). bFGF is the negative control,

Figure 6 is a graph showing the inhibition of tumor progression (growth) in rats of the MatBIII syngeneic rat breast cancer line by treatment with the Å6 peptide.

Figure 7 is a graph showing that Å6 peptide inhibits metastasis of MatBIII cells breast cancer cells to lymph nodes in syngeneic rats

Figure 8 is a graph showing that Å6 peptide arrests the growth of human breast cancer cells (MCA-MB-231 line) *in vivo* in a nude mouse xenograft model.

Figure 9 is a graph showing that peptide Å6 inhibits the metastatic spread of e human breast cancer cells to lymph nodes in nude mice. Mice treated with this peptide had no detectable macroscopic metastases in the lungs or the lymph nodes.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have discovered a novel peptide and related compounds which act as inhibitors of angiogenesis and invasiveness and have devised various methods for using this peptide for diagnosis, therapy and receptor identification. The peptide is a potent and specific inhibitor of (a) cell invasion, (b) angiogenesis at tumor sites including sites of metastasis, and (c) inflammatory responses.

In addition, the peptide and its derivatives are designed to be highly soluble in aqueous buffer and body fluids but not in lipids. This property limits non-specific partitioning into membranes. Non-specific partitioning of compounds into and across membranes is a frequent cause of toxicity. The compounds of this invention have minimal toxicity because, owing to their possessing Coulombic charge, they are not expected to partition into cells. The target(s) of the compositions are extracellular, and method(s) of this invention are predicated on the compositions acting first in the extracellular space. Therefore, it is desirable to maintain the compounds in the extracellular space.

Additional pharmacological advantage is obtained due to the high solubility limit of the compounds, allowing their delivery in high concentrations in the absence of co-solvents or extraordinary excipients.

Compounds of the invention have been shown by the present inventors (see Example II) to block the invasion of both human and rat tumor cells *in vitro* in the Matrigel® system.

In addition, they block endothelial cell tube formation in response to bFGF and VEGF in either a fibrin matrix or when the endothelial cells are plated on Matrigel®.

The compounds of the invention also inhibit experimental metastasis in a xenograft model in *nu/nu* mice using the human prostatic carcinoma cell line, PC-3, transfected with the green fluorescent protein (GFP) as a reporter. Finally, the compounds also inhibit tumor progression, spontaneous metastasis and angiogenesis in a syngeneic rat model of breast cancer.

The Peptide Compositions

The original inhibitory capped peptide discovered by the present inventors has 8 amino acid residues with a molecular weight of 911 Da. This preferred peptide is characterized by the sequence:

CH_3CO-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-NH_2 [SEQ ID NO:2]

The amino and carboxyl termini are preferably blocked or "capped" with acetyl (CH_3CO-, bound to the amino-terminal N; also abbreviated as "Ac") and amido (-NH_2 bound to the C-terminal carboxyl group; also abbreviated as "Am"), respectively. This peptide will also be referred to below in single letter code indicating the blocking groups as Ac and Am groups: Ac-KPSSPPEE-Am.

The N-terminal capping function is preferably in a linkage to the terminal amino group and may be selected from the group consisting of:
formyl; alkanoyl, having from 1 to 10 carbon atoms, such as acetyl, propionyl, butyryl; alkenoyl, having from 1 to 10 carbon atoms, such as hex-3-enoyl; alkynoyl, having from 1 to 10 carbon atoms, such as hex-5-ynoyl; aroyl, such as benzoyl or 1-naphthoyl; heteroaroyl, such as 3-pyrroyl or 4-quinoloyl; alkylsulfonyl, such as methanesulfonyl; arylsulfonyl, such as benzenesulfonyl or sulfanilyl; heteroarylsulfonyl, such as pyridine-4-sulfonyl; substituted alkanoyl, having from 1 to 10 carbon atoms, such as 4-aminobutyryl; substituted alkenoyl, having from 1 to 10 carbon atoms, such as 6-hydroxy-hex-3-enoyl; substituted alkynoyl, having from 1 to 10 carbon atoms, such as 3-hydroxy-hex-5-ynoyl; substituted aroyl, such as 4-chlorobenzoyl or 8-hydroxy-naphth-2-oyl; substituted heteroaroyl, such as 2,4-dioxo-1,2,3,4-tetrahydro-3-methyl-quinazolin-6-oyl; substituted alkylsulfonyl, such as 2-aminoethanesulfonyl; substituted arylsulfonyl, such as 5-dimethylamino-1-naphthalenesulfonyl; substituted heteroarylsulfonyl, such as 1-methoxy-6-isoquinolinesulfonyl; carbamoyl or thiocarbamoyl; substituted carbamoyl (R'-NH-CO) or substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkyl, alkenyl, alkynyl, aryl, heteroaryl, substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, or substituted heteroaryl; substituted carbamoyl (R'-NH-CO) and substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkanoyl, alkenoyl, alkynoyl,

aroyl, heteroaroyl, substituted alkanoyl, substituted alkenoyl, substituted alkynoyl, substituted aroyl, or substituted heteroaroyl, all as above defined;

Lys-$(Gly)_n$ where n = 1 4; or Tyr-$(Gly)_n$ where n = 1 4.

The C-terminal capping function can either be in an amide bond with the terminal carboxyl or in an ester bond with the terminal carboxyl. Capping functions that provide for an amide bond are designated as NR^1R^2 wherein R^1 and R^2 may be independently drawn from the following group: hydrogen; alkyl, preferably having from 1 to 10 carbon atoms, such as methyl, ethyl, isopropyl; alkenyl, preferably having from 1 to 10 carbon atoms, such as prop-2-enyl; alkynyl, preferably having from 1 to 10 carbon atoms, such as prop-2-ynyl; substituted alkyl having from 1 to 10 carbon atoms, such as hydroxyalkyl, alkoxyalkyl, mercaptoalkyl, alkylthioalkyl, halogenoalkyl, cyanoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkanoylalkyl, carboxyalkyl, carbamoylalkyl; substituted alkenyl having from 1 to 10 carbon atoms, such as hydroxyalkenyl, alkoxyalkenyl, mercaptoalkenyl, alkylthioalkenyl, halogenoalkenyl, cyanoalkenyl, aminoalkenyl, alkylaminoalkenyl, dialkylaminoalkenyl, alkanoylalkenyl, carboxyalkenyl, carbamoylalkenyl; substituted alkynyl having from 1 to 10 carbon atoms, such as hydroxyalkynyl, alkoxyalkynyl, mercaptoalkynyl, alkylthioalkynyl, halogenoalkynyl, cyanoalkynyl, aminoalkynyl, alkylaminoalkynyl, dialkylaminoalkynyl, alkanoylalkynyl, carboxyalkynyl, carbamoylalkynyl; aroylalkyl having up to 10 carbon atoms, such as phenacyl or 2-benzoylethyl; aryl, such as phenyl or 1-naphthyl; heteroaryl, such as 4-quinolyl; alkanoyl having from 1 to 10 carbon atoms, such as acetyl or butyryl; aroyl, such as benzoyl; heteroaroyl, such as 3-quinoloyl;

OR' or NR'R'' where R' and R'' are independently hydrogen, alkyl, aryl, heteroaryl, acyl, aroyl, sulfonyl, sulfinyl, or SO_2-R''' or SO-R''' where R''' is substituted or unsubstituted alkyl, aryl, heteroaryl, alkenyl, or alkynyl.

Capping functions that provide for an ester bond are designated as OR, wherein R may be: alkoxy; aryloxy; heteroaryloxy; aralkyloxy; heteroaralkyloxy; substituted alkoxy; substituted aryloxy; substituted heteroaryloxy; substituted aralkyloxy; or substituted heteroaralkyloxy.

Either the N-terminal or the C-terminal capping function, or both, may be of such structure that the capped molecule functions as a prodrug (a pharmacologically inactive derivative of the parent drug molecule) that undergoes spontaneous or enzymatic transformation within the body in order to release the active drug and that has improved delivery properties over the parent drug molecule (Bundgaard, 1985).

Judicious choice of capping groups allows the addition of other activities on the peptide. For example, the presence of a sulfhydryl group linked to the N- or C-terminal cap will permit conjugation of the derivatized peptide to other molecules.

Production of Peptides and Derivatives

General Chemical Synthetic Procedures

The peptides of the invention may be prepared using recombinant DNA technology. However, given their length, they are preferably prepared using solid-phase synthesis, such as that generally described by Merrifield, *J. Amer. Chem. Soc.*, *85*:2149-54 (1963), although other equivalent chemical syntheses known in the art are also useful. Solid-phase peptide synthesis may be initiated from the C-terminus of the peptide by coupling a protected α-amino acid to a suitable resin. Such a starting material can be prepared by attaching an α-amino-protected amino acid by an ester linkage to a chloromethylated resin or to a hydroxymethyl resin, or by an amide bond to a BHA resin or MBHA resin.

The preparation of the hydroxymethyl resin is described by Bodansky *et al.*, 1966. Chloromethylated resins are commercially available from BioRad Laboratories, Richmond, Calif. and from Lab. Systems, Inc. The preparation of such a resin is described by Stewart *et al.*, 1969. BHA and MBHA resin supports are commercially available and are generally used only when the desired polypeptide being synthesized has an unsubstituted amide at the C-terminus.

The amino acids can be coupled to the growing peptide chain using techniques well known in the art for the formation of peptide bonds. For example, one method involves converting the amino acid to a derivative that will render the carboxyl group of the amino acid more susceptible to reaction with the free N-terminal amino group of the growing peptide chain. Specifically, the C-terminal of the protected amino acid can

be converted to a mixed anhydride by the reaction of the C-terminal with ethyl chloroformate, phenyl chloroformate, sec-butyl chloroformate, isobutyl chloroformate, or pivaloyl chloride or the like acid chlorides. Alternatively, the C-terminal of the amino acid can be converted to an active ester, such as a 2,4,5-trichlorophenyl ester, a pentachlorophenyl ester, a pentafluorophenyl ester, a p-nitrophenyl ester, a N-hydroxysuccinimide ester, or an ester formed from 1-hydroxybenzotriazole. Another coupling method involves the use of a suitable coupling agent, such as N,N'-dicyclohexylcarbodiimide or N,N'-diisopropylcarbodiimide. Other appropriate coupling agents, apparent to those skilled in the art, are disclosed in Gross *et al.* 1979, which is hereby incorporated by reference.

The α-amino group of each amino acid employed in the peptide synthesis must be protected during the coupling reaction to prevent side reactions involving their active α-amino function. Certain amino acids contain reactive side-chain functional groups (*e.g.*, sulfhydryl, amino, carboxyl, and hydroxyl) and such functional groups must also be protected with suitable protecting groups to prevent a chemical reaction from occurring at either (1) the α-amino group site or (2) a reactive side chain site during both the initial and subsequent coupling steps.

In the selection of a particular protecting group to be used in synthesizing the peptides, the following general rules are typically followed. Specifically, an α-amino protecting group (1) should render the α-amino function inert under the conditions employed in the coupling reaction, (2) should be readily removable after the coupling reaction under conditions that will not remove side-chain protecting groups and will not alter the structure of the peptide fragment, and (3) should substantially reduce the possibility of racemization upon activation, immediately prior to coupling.

On the other hand, a side-chain protecting group (1) should render the side chain functional group inert under the conditions employed in the coupling reaction, (2) should be stable under the conditions employed in removing the α-amino protecting group, and (3) should be readily removable from the desired fully-assembled peptide under reaction conditions that will not alter the structure of the peptide chain.

It will be apparent to those skilled in the art that the protecting groups known to be useful for peptide synthesis vary in reactivity with the agents employed for their removal. For example, certain protecting groups, such as triphenylmethyl and 2-(p-biphenyl)isopropyloxycarbonyl, are very labile and can be cleaved under mild acid conditions. Other protecting groups, such as t-butyloxycarbonyl (BOC), t-amyloxycarbonyl, adamantyl-oxycarbonyl, and p-methoxybenzyloxycarbonyl, are less labile and require moderately strong acids for their removal, such as trifluoroacetic, hydrochloric, or boron trifluoride in acetic acid. Still other protecting groups, such as benzyloxycarbonyl (CBZ or Z), halobenzyloxycarbonyl, p-nitrobenzyloxycarbonyl cycloalkyloxycarbonyl, and isopropyloxycarbonyl, are even less labile and require even stronger acids, such as hydrogen fluoride, hydrogen bromide, or boron trifluoroacetate in trifluoroacetic acid, for their removal. Suitable protecting groups, known in the art are described in Gross *et al.* 1981.

Among the classes of amino acid protecting groups useful for protecting the α-amino group or for protecting a side chain group are included the following.

(1) For an α-amino group, three typical classes of protecting groups are:

(a) aromatic urethane-type protecting groups, such as fluorenylmethyloxycarbonyl (FMOC), CBZ, and substituted CBZ, such as, p-chlorobenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, and p-methoxybenzyloxycarbonyl, o-chlorobenzyloxycarbonyl, 2,4-dichlorobenzyloxycarbonyl, 2,6-dichlorobenzyloxycarbonyl, and the like;

(b) aliphatic urethane-type protecting groups, such as BOC, t-amyloxycarbonyl, isopropyloxycarbonyl, 2-(p-biphenyl)isopropyloxycarbonyl, allyloxycarbonyl and the like; and

(c) cycloalkyl urethane-type protecting groups, such as cyclopentyloxycarbonyl, adamantyloxycarbonyl, and cyclohexyloxycarbonyl.

The preferred α-amino protecting groups are BOC and FMOC.

(2) For the side chain amino group present in Lys, protection may be by any of the groups mentioned above in (1) such as BOC, 2-chlorobenzyloxycarbonyl and the like.

(3) For the guanidino group of Arg, protection may be provided by nitro, tosyl, CBZ, adamantyloxycarbonyl, 2,2,5,7,8-pentamethylchroman-6-sulfonyl, 2,3,6-trimethyl-4-methoxyphenylsulfonyl, or BOC groups.

(4) For the hydroxyl group of Ser or Thr, protection may be, for example, by t-butyl; benzyl (BZL); or substituted BZL, such as p-methoxybenzyl, p-nitrobenzyl, p-chlorobenzyl, o-chlorobenzyl, and 2,6-dichlorobenzyl.

(5) For the carboxyl group of Asp or Glu, protection may be, for example, by esterification using such groups as BZL, t-butyl, cyclohexyl, cyclopentyl, and the like.

(6) For the imidazole nitrogen of His, the benzyloxymethyl (BOM) or tosyl moiety is suitably employed as a protecting group.

(7) For the phenolic hydroxyl group of Tyr, a protecting group such as tetrahydropyranyl, tert-butyl, trityl, BZL, chlorobenzyl, 4-bromobenzyl, and 2,6-dichlorobenzyl are suitably employed. The preferred protecting group is bromobenzyloxycarbonyl.

(8) For the side chain amino group of Asn or Gln, xanthyl (Xan) is preferably employed.

(9) For Met, the amino acid is preferably left unprotected.

(10) For the thio group of Cys, p-methoxybenzyl is typically employed.

The first C-terminal amino acid of the growing peptide chain, *e.g.*, Glu, is typically protected at the α-amino position by an appropriately selected protecting group such as BOC. The BOC-Glu-(γ-cyclohexyl)-OH can be first coupled to a benzylhydrylamine resin using isopropylcarbodiimide at about 25°C for two hours with stirring or to a chloromethylated resin according to the procedure set forth in Horiki *et al.*, 1978. Following the coupling of the BOC-protected amino acid to the resin support, the α-amino protecting group is usually removed, typically by using trifluoroacetic acid (TFA) in methylene chloride or TFA alone. The α-amino group

de-protection reaction can occur over a wide range of temperatures, but is usually carried out at a temperature between about 0°C and room temperature.

Other standard α-amino group de-protecting reagents, such as HCl in dioxane, and conditions for the removal of specific α-amino protecting groups are within the skill of those working in the art, such as those described in Lübke *et al.*, 1975, which is hereby incorporated by reference. Following the removal of the α-amino protecting group, the unprotected α-amino group, generally still side-chain protected, can be coupled in a stepwise manner in the intended sequence.

An alternative to the stepwise approach is the fragment condensation method in which pre-formed peptides of short length, each representing part of the desired sequence, are coupled to a growing chain of amino acids bound to a solid phase support. For this stepwise approach, a particularly suitable coupling reagent is N,N'-dicyclohexylcarbodiimide or diisopropylcarbodiimide. Also, for the fragment approach, the selection of the coupling reagent, as well as the choice of the fragmentation pattern needed to couple fragments of the desired nature and size are important for success and are known to those skilled in the art.

Each protected amino acid or amino acid sequence is usually introduced into the solid-phase reactor in amounts in excess of stoichiometric quantities, and the coupling is suitably carried out in an organic solvent, such as dimethylformamide (DMF), CH_2Cl_2 or mixtures thereof. If incomplete coupling occurs, the coupling procedure is customarily repeated before removal of the N-amino protecting group in preparation for coupling to the next amino acid. Following the removal of the α-amino protecting group, the remaining α-amino and side-chain-protected amino acids can be coupled in a stepwise manner in the intended sequence. The success of the coupling reaction at each stage of the synthesis may be monitored. A preferred method of monitoring the synthesis is by the ninhydrin reaction, as described by Kaiser *et al.*, 1970. The coupling reactions can also be performed automatically using well-known commercial methods and devices, for example, a Beckman 990 Peptide Synthesizer.

Upon completion of the desired peptide sequence, the protected peptide must be cleaved from the resin support, and all protecting groups must be removed. The

cleavage reaction and removal of the protecting groups is suitably accomplished concomitantly or consecutively with de-protection reactions. When the bond anchoring the peptide to the resin is an ester bond, it can be cleaved by any reagent that is capable of breaking an ester linkage and of penetrating the resin matrix. One especially useful method is by treatment with liquid anhydrous hydrogen fluoride. This reagent will usually not only cleave the peptide from the resin, but will also remove all acid-labile protecting groups and, thus, will directly provide the fully de-protected peptide. When additional protecting groups that are not acid-labile are present, additional de-protection steps must be carried out. These steps can be performed either before or after the hydrogen fluoride treatment described above, according to specific needs and circumstances.

When a chloromethylated resin is used, the hydrogen fluoride cleavage/de-protection treatment generally results in the formation of the free peptide acids. When a benzhydrylamine resin is used, the hydrogen fluoride treatment generally results in the free peptide amides. Reaction with hydrogen fluoride in the presence of anisole and dimethylsulfide at 0°C for one hour will typically remove the side-chain protecting groups and, concomitantly, release the peptide from the resin.

When it is desired to cleave the peptide without removing protecting groups, the protected peptide-resin can be subjected to methanolysis, thus yielding a protected peptide in which the C-terminal carboxyl group is methylated. This methyl ester can be subsequently hydrolyzed under mild alkaline conditions to give the free C-terminal carboxyl group. The protecting groups on the peptide chain can then be removed by treatment with a strong acid, such as liquid hydrogen fluoride. A particularly useful technique for methanolysis is that of Moore *et al.*, 1977, in which the protected peptide-resin is treated with methanol and potassium cyanide in the presence of a crown ether.

Other methods for cleaving a protected peptide from the resin when a chloromethylated resin is employed include (1) ammoniolysis and (2) hydrazinolysis. If desired, the resulting C-terminal amide or hydrazide can be hydrolyzed to the free C-terminal carboxyl moiety, and the protecting groups can be removed conventionally. The protecting group present on the N-terminal α-amino group may

be removed either before, or after, the protected peptide is cleaved from the support. Purification of the peptides of the invention is typically achieved using chromatographic techniques, such as preparative HPLC (including reverse phase HPLC), gel permeation, ion exchange, partition chromatography, affinity chromatography (including monoclonal antibody columns), and the like, or other conventional techniques such as countercurrent distribution or the like.

Amino Acid Substitution and Addition Variants

Also included in this invention are peptides in which at least one amino acid residue and preferably, only one, has been removed and a different residue inserted in its place. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1979, and Creighton, T.E., *Proteins: Structure and Molecular Principles*, W.H. Freeman & Co., San Francisco, 1984, which are hereby incorporated by reference. The types of substitutions which may be made in the peptide molecule of the present invention are conservative substitutions and are defined herein as exchanges within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: *e.g.*, Ala, Ser, Thr, Gly;
2. Polar, negatively charged residues and their amides: *e.g.*, Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: *e.g.*, His, Arg, Lys;

Pro, because of its unusual geometry, tightly constrains the chain. Substantial changes in functional properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above groups (or two other amino acid groups not shown above), which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Most substitutions according to the present invention are those which do not produce radical changes in the characteristics of the peptide molecule. Even when it is difficult to predict the exact effect of a substitution in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays, preferably the biological assays described below. Modifications of peptide properties including redox or thermal stability,

hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

One group of preferred substitution variants of KPSSPPEE have the Glu at position 7 or 8 (or both) of SEQ ID NO:2 replaced by one or any two of Gln, Asp or Asn.

Other derivatives may further include substitution of the Ser at position 3 or 4 (or both) of SEQ ID NO:2 with one or any two of the following: Thr, Ala, Gly, hSer or ValβOH.

Furthermore, the Lys at position 1 of SEQ ID NO:2 may be replaced by His, Arg, Gln, Orn , Cit or Hci .

Other derivatives have Pro at position 2, 5 or 6 replaced by Hyp (hydroxyproline).

It is noteworthy that any and all combinations of the foregoing substitutions are within the scope of this invention.

Also included in this invention are addition variants wherein two or more residues are added to the C-terminus after Glu (or after any of its above substituents) in SEQ ID NO:2. These residues may be Leu-$(Gly)_n$, Ile-$(Gly)_n$, Val-$(Gly)_n$, Nva-$(Gly)_n$, or Nle-$(Gly)_n$, wherein Nva is norvaline, Nle is norleucine, and n = 1-10.

Also included in this invention are addition variants wherein one or more residues is/are added to the N-terminus before Lys (or any of its above substituents) in SEQ ID NO:2. These residues may be Gly, Lys-$(Gly)_n$, Tyr-$(Gly)_n$, or Gly-$(Gly)_n$ wherein n = 1-10.

Another preferred derivative of this invention is a 9-mer addition variant wherein any one of the following amino acids is added to the C-terminus after Glu (or any of its above substituents) in SEQ ID NO:2: Leu, Ile, Val, Nva, Nle, Met, Ala, or Gly.

Uncapped peptides of any of the foregoing sequences having free N- and C-termini, for example, uncapped NH_2-KPSSPPEE-OH [SEQ ID NO:2].

Chemical Derivatives

"Chemical derivatives" of KPSSPPEE [SEQ ID NO:2] contain additional chemical moieties not normally a part of the peptide. Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

The capped peptides discussed above are examples of preferred chemical derivatives of the "natural" uncapped peptide. Any of the above combination of substitution or addition variants may be capped with any of the capping groups disclosed herein.

Other examples of chemical derivatives of the peptide follow.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino group of lysine (Creighton, *supra,* pp. 79-86), acetylation of the N-terminal amine, and amidation of the C-terminal carboxyl groups.

For every single peptide sequence disclosed herein, this invention includes the corresponding retro-inverso sequence wherein the direction of the peptide chain has been inverted and wherein all the amino acids belong to the D-series. For example the

retro-inverso analogue of the natural L-series peptide KPSSPPEE is EEPPSSPK which is composed of D-series amino acids and in which E is the N-terminus and K is the C-terminus. For example the retro-inverso analogue of the natural L-series capped peptide Ac-KPSSPPEE-Am is Ac-EEPPSSPK-Am which is composed of D-series amino acids and in which the N-terminal E is acetylated and the C-terminal K is amidated. The complete range of N-terminal capping groups and the complete range C-terminal capping groups specified for the L-series peptides are also intended for the D-series peptides.

Also included are peptides wherein one or more D-amino acids has/have been substituted for one or more L-amino acids. Additionally, modified amino acids or chemical derivatives of amino acids may be provided such that the peptide contains additional chemical moieties or modified amino acids not normally a part of a natural protein. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

Multimeric Peptides

The present invention also includes longer peptides in which the basic peptidic sequence of about 7-9 amino acids is repeated from about two to about 100 times, with or without intervening spacers or linkers. A multimer of the peptide KPSSPPEE is shown by the following formula $(KPSSPPEE\text{-}X_m)_n$-KPSSPPEE wherein m= 0 or 1, n = 1-100. X is a spacer group, preferably C_1-C_{20} alkyl, C_1-C_{20} alkenyl, C_1-C_{20} alkynyl, C_1.C_{20} polyether containing up to 9 oxygen atoms or Gly_z. (z=1-10).

It is understood that such multimers may be built from any of the peptide variants described herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers, both KPSSPPEE and the disclosed variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced chemically, the oligomers preferably have from 2-8 repeats of the basic peptide sequence. When produced recombinantly, the multimers may have as many repeats as the expression system permits, for example from two to about 100 repeats.

Peptidomimetics

A preferred type of chemical derivative of the peptides described herein is a peptidomimetic compound which mimics the biological effect of KPSSPPEE, capped or uncapped. A peptidomimetic agent may be an unnatural peptide or a non-peptide agent which has the stereochemical properties of KPSSPPEE, capped or uncapped, such that it has the binding activity or biological activity of KPSSPPEE, capped or uncapped. Hence, this invention includes compounds wherein a peptidomimetic compound is coupled to a peptide, for example,

—PPEE

wherein X is a peptidomimetic which mimics KPSS; the peptide portion may include a normal or a retro-inverso sequence.

Peptidomimetic compounds, either agonists, substrates or inhibitors, have been described for a number of bioactive peptides such as opioid peptides, VIP, thrombin, HIV protease, *etc.* Methods for designing and preparing peptidomimetic compounds are known in the art (Kempf DJ, *Methods Enzymol 241*:334-354 (1994); Hruby, V.J., *Biopolymers 33*:1073-82 (1993); Wiley, R.A. *et al.*, *Med. Res. Rev. 13*:327-384 (1993); Claeson, G., *Blood Coagul. Fibrinolysis 5*:411-436 (1994), which references are incorporated by reference in their entirety). These methods are used to prepare capped or uncapped KPSSPPEE peptidomimetics which possess at least the binding capacity and specificity of the peptide and preferably also possess the biological activity. Knowledge of peptide chemistry and general organic chemistry available to those skilled in the art are sufficient for the design and testing of such compounds.

For example, such peptidomimetics may be identified by inspection of the cystallographically-derived three-dimensional structure of a peptide of the invention, for example KPSSPPEE, capped or uncapped, either free or bound in complex with its receptor(s). Alternatively, the structure of a peptide of the invention bound to its receptor(s) can be gained by the techniques of nuclear magnetic resonance spectroscopy. The better knowledge of the stereochemistry of the interaction of, say, KPSSPPEE, capped or uncapped, with its receptor will permit the rational design of such peptidomimetic agents.

All the foregoing peptides, variants and chemical derivatives including peptidomimetics and multimeric peptides must have the biological activity and/or the binding activity of KPSSPPEE as follows: at least about 20% of the activity of Ac-KPSSPPE-Am in an *in vitro* assay of cell invasiveness or an *in vitro* assay of endothelial tube formation and/or angiogenesis . These activities are characterized in greater detail below. Alternatively, or in addition, the peptide, variant or chemical derivatives should compete with labeled Ac-KPSSPPEE-Am for binding to a ligand or binding partner for Ac-KPSSPPEE-Am, whether this be a cellular receptor (tested in a binding assay with whole cells or fractions thereof), an isolated receptor or any other Ac-KPSSPPEE-Am-binding molecule.

Moreover, the peptides, variants or derivatives of the present invention do not have biological activities previously associated with urokinase plasminogen activator (uPA). That is they do no block the binding of uPA to the uPA receptor. These peptides lack thrombolytic activity, a hallmark of uPA.

DIAGNOSTIC AND PROGNOSTIC COMPOSITIONS

Further, the peptides can be labeled for detection and used, for example, to detect a binding site for the peptide on the surface or in the interior of a cell. Thus, the fate of the peptide can be followed *in vitro* or *in vivo* by using the appropriate method to detect the label. The labeled peptide may also be utilized *in vivo* for diagnosis and prognosis, for example to image occult metastatic foci or for other types of *in situ* evaluations.

Example of suitable detectable labels are radioactive, fluorogenic, chromogenic, or other chemical labels. Useful radiolabels. which are detected by a gamma counter or a scintillation counter or by autoradiography include ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C. In addition, ^{131}I is also useful as a therapeutic isotope (see below).

Common fluorescent labels include fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The fluorophore, such as the dansyl group, must be excited by light of a particular wavelength to fluoresce. See, for example, Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Sixth Edition, Molecular Probes, Eugene, OR., 1996). In general, a fluorescent reagent is selected based on its ability

to react readily with an amino function. Examples of such fluorescent probes include the Bodipy (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) fluorophores which span the visible spectrum (US 4,774,339; US 5,187,288; US 5,248,782; US 5,274,113; US 5,433,896; US 5,451,663). A preferred member of this group is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid.

Fluorescein, fluorescein derivatives and fluorescein-like molecules such as Oregon Green™ and its derivatives, Rhodamine Green™ and Rhodol Green™, are coupled to amine groups using the isocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups. The long wavelength rhodamines, which are basically Rhodamine Green™ derivatives with substituents on the nitrogens, are among the most photostable fluorescent labeling reagents known. Their spectra are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications. This group includes the tetramethylrhodamines, X-rhodamines and Texas Red derivatives. Other preferred fluorophores for derivatizing the peptide according to this invention are those which are excited by ultraviolet light. Examples include cascade blue, coumarin derivatives, naphthalenes (of which dansyl chloride is a member), pyrenes and pyridyloxazole derivatives.

In yet another approach, one or more amino groups is allowed to react with reagents that yield fluorescent products, for example, fluorescamine, dialdehydes such as *o*-phthaldialdehyde, naphthalene-2,3-dicarboxylate and anthracene-2,3-dicarboxylate. 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivatives, both chloride and fluoride, are useful to modify amines to yield fluorescent products.

Those skilled in the art will recognize that known fluorescent reagents modify groups other than amines, such as thiols , alcohols, aldehydes, ketones, carboxylic acids and amides. Hence, fluorescent substrates can readily be designed and synthesized using these other reactive groups.

The peptide can also be labeled for detection using fluorescence-emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the peptide using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). The peptide can be made detectable by coupling it to a chemiluminescent compound. The presence of the

chemiluminescent-tagged peptide is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescers are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the peptide. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

In yet another embodiment, colorimetric detection is used, based on chromogenic compounds (chromophores) with high extinction coefficients.

In situ detection of the labeled peptide may be accomplished by removing a histological specimen from a subject and examining it by microscopy under appropriate conditions to detect the label. Those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

The term "diagnostically labeled" means that the peptide has attached to it a diagnostically detectable label. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes, paramagnetic isotopes, and compounds which can be imaged by positron emission tomography (PET). Those of ordinary skill in the art will know of other suitable labels for binding to the peptides used in the invention, or will be able to ascertain such, by routine experimentation. Furthermore, the binding of these labels to the peptide or derivative can be done using standard techniques known to those of ordinary skill in the art.

For diagnostic *in vivo* radioimaging, the type of detection instrument available is a major factor in selecting a given radionuclide. The radionuclide chosen must have a type of decay which is detectable by a given type of instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention. Another factor in selecting a radionuclide for *in vivo*

diagnosis is that the half-life of a radionuclide be long enough so that it is still detectable at the time of maximum uptake by the target issue, but short enough so that deleterious radiation of the host is minimized. In one preferred embodiment, a radionuclide used for *in vivo* imaging does not emit particles, but produces a large number of photons in a 140-200 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radionuclides may be bound to peptide either directly or indirectly by using an intermediary functional group. Intermediary functional groups that are often used to bind radioisotopes, which exist as metallic ions, to peptides are the chelating agents, DTPA and EDTA. Examples of metallic ions which can be bound to peptides are ^{99}Tc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, ^{125}I, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl. Generally, the dosage of peptide labeled for detection for diagnostic use will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, counterindications, if any, and other variables, to be adjusted by the individual physician. Dosage can vary from 0.01 mg/kg to 100 mg/kg.

In another embodiment, the peptides or derivatives of the present invention are used as affinity ligands for binding the peptide's receptor in assays, preparative affinity chromatography or solid phase separation. Such compositions may also be used to enrich, purify or isolate cells to which the peptide or derivative binds, preferably through a specific receptor-ligand interaction. The peptide or derivative is immobilized using common methods known in the art, *e.g.* binding to CNBr-activated Sepharose® or Agarose®, NHS-Agarose® or Sepharose®, epoxy-activated Sepharose® or Agarose®, EAH-Sepharose® or Agarose®, streptavidin-Sepharose® or Agarose® in conjunction with biotinylated peptide or derivatives. In general the peptides or derivatives of the invention may be immobilized by any other method which is capable of immobilizing these compounds to a solid phase for the indicated purposes. See, for example *Affinity Chromatography: Principles and Methods (Pharmacia LKB Biotechnology)*. Thus, one embodiment is a composition comprising any of the peptides, derivatives or peptidomimetics described herein, bound to a solid support or a resin. The compound may be bound directly or via a spacer, preferably an aliphatic chain having about 2-12 carbon atoms.

By "solid phase" or "solid support" or "carrier" is intended any support or carrier capable of binding the peptide or derivative. Well-known supports, or carriers, in addition to Sepharose® or Agarose® described above are glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses such as nitrocellulose, polyacrylamides, polyvinylidene difluoride, other agaroses, and magnetite, including magnetic beads.. The carrier can be totally insoluble or partially soluble. The support material may have any possible structural configuration so long as the coupled molecule is capable of binding to receptor material. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube or microplate well, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, bottom surface of a microplate well, *etc.*

Antibodies and Their Uses

The present invention also provides antibodies specific for an epitope defined by the peptide sequence KPSSPPEE or specific for a chemical derivative thereof or a peptidomimetic thereof. Such antibodies may be polyclonal, monoclonal, bispecific, chimeric or antiidiotypic, and include antigen-binding fragments thereof. Any immunoassay known in the art may be used to detect the binding of such an antibody to a peptide, chemical derivative thereof or peptide oligomer according to this invention. Preferred assays are enzyme immunoassays or radioimmunoassay. The following references (incorporated by reference in their entirety) describe the production, purification, testing and use of antibodies: Hartlow, E. *et al.*, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988; Campbell, A., In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13 (Burdon, R., *et al.*, eds.), Elsevier, Amsterdam (1984)); Work, T.S. *et al.*, *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, NY, 1978; Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986; Butler, J.E. (ed.), *Immunochemistry of Solid-Phase Immunoassay*, CRC Press, Boca Raton, 1991; Butler, J.E., In: *STRUCTURE OF ANTIGENS*, Vol. 1, Van Regenmortel, M., ed., CRC Press, Boca Raton 1992, pp.

209-259; Butler, J.E., In: van Oss, C.J. *et al.*, (eds), *IMMUNOCHEMISTRY*, Marcel Dekker, Inc., New York, 1994, pp. 759-803; Voller, A. *et al.* (eds)., *Immunoassays for the 1980's*, University Park Press, Baltimore, 1981.

Antibodies of this invention are used to detect the presence of or measure the amount of the peptide epitope in a biological material or other sample by direct or competitive immunoassay. The antibodies can be coupled to a solid support and used in affinity chromatography to isolate and purify material containing the peptide epitope. Conversely, as described above, the peptide, variant or chemical derivative of this invention, bound to a solid support, is used to enrich or purify specific antibodies. Antiidiotypic antibodies can be used to gain a knowledge of the structure of a peptide, variant or chemical derivative of this invention when bound to a receptor for it.

Biological Assay of Anti-Invasive Activity

The compositions of the invention are tested for their anti-invasive capacity in a Matrigel® invasion assay system as described in detail by Kleinman *et al.*, 1986 and Parish *et al.*, 1992, which references are hereby incorporated by reference in their entirety. The assay is performed with a cell line, more preferably a tumor cell line, most preferably the rat breast cancer (Mat BIII) line or the human prostate cancer (PC-3) line (Xing and Rabbani, 1996; Hoosein *et al.*, 1991).

Matrigel® is a reconstituted basement membrane containing type IV collagen, laminin, heparan sulfate proteoglycans such as perlecan, which bind to and localize bFGF, vitronectin as well as transforming growth factor-β (TGFβ), urokinase-type plasminogen activator (uPA), tissue plasminogen activator (tPA), and the serpin known as plasminogen activator inhibitor type 1 (PAI-1) (Chambers *et al.*, 1995).

It is accepted in the art that results obtained in this assay for compounds which target extracellular receptors or enzymes are predictive of the efficacy of these compounds *in vivo* (Rabbani *et al.*, 1995).

Biological Assay of Anti-Angiogenic Activity

The compounds of this invention are tested for their anti-angiogenic activity in one of two different assay systems *in vitro*.

Endothelial cells, for example, human umbilical vein endothelial cells (HUVEC) or human microvascular endothelial cells (HMVEC) which can be prepared or obtained commercially, are mixed at a concentration of 2 x 10^5 cells/mL with fibrinogen (5mg/mL in phosphate buffered saline (PBS) in a 1:1 (v/v) ratio. Thrombin is added (5 units/ mL final concentration) and the mixture is immediately transferred to a 24-well plate (0.5 mL per well). The fibrin gel is allowed to form and then VEGF and bFGF are added to the wells (each at 5 ng/mL final concentration) along with the test compound. The cells are incubated at 37°C in 5% CO_2 for 4 days at which time the cells in each well are counted and classified as either rounded, elongated with no branches, elongated with one branch, or elongated with 2 or more branches. Results are expressed as the average of 5 different wells for each concentration of compound. Typically, in the presence of angiogenic inhibitors, cells remain either rounded or form undifferentiated tubes (*e.g.* 0 or 1 branch).

This assay is recognized in the art to be predictive of angiogenic (or anti-angiogenic) efficacy *in vivo* (Min *et al.*, 1996).

In an alternate assay, endothelial cell tube formation is observed when endothelial cells are cultured on Matrigel® (Schnaper *et al.*, 1995). Endothelial cells (1 x 10^4 cells/well) are transferred onto Matrigel®-coated 24-well plates, and tube formation is quantitated after 48 hrs. Inhibitors are tested by adding them either at the same time as the endothelial cells or at various time points thereafter.

This assay models angiogenesis by presenting to the endothelial cells a particular type of basement membrane, namely the layer of matrix which migrating and differentiating endothelial cells might be expected to first encounter. In addition to bound growth factors, the matrix components found in Matrigel® (and in basement membranes *in situ*) or proteolytic products thereof may also be stimulatory for endothelial cell tube formation which makes this model complementary to the fibrin gel angiogenesis model previously described (Blood and Zetter, 1990; Odedra and Weiss, 1991). The compounds of this invention inhibit endothelial cell tube formation in both assays, which suggests that the compounds will also have anti-angiogenic activity.

In Vivo Testing of Compositions in Animal Models of Human Tumors

The peptides, peptidomimetics and conjugates are tested for therapeutic efficacy in several well established rodent models which are considered to be highly representative of a broad spectrum of human tumors. The approaches are described in detail in Geran, R.I. *et al.*, "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems (Third Edition)", *Canc. Chemother. Reports*, Part 3, 3:1-112, which is hereby incorporated by reference in its entirety. All general test evaluation procedures, measurements and calculations are performed in accordance with this reference, including mean survival time, median survival time, calculation of approximate tumor weight from measurement of tumor diameters with vernier calipers; calculation of tumor diameters: calculation of mean tumor weight from individual excised tumors; and ratios between treated and control groups ratio for any measure (T/C ratios).

A. Rat Model of Tumor Progression

The effects of the compounds are tested on tumor progression in a rat syngeneic model of breast cancer (Xing and Rabbani, 1996). The MatBIII cell line is a highly aggressive tumor cell line which expresses high levels of uPA and uPAR and leads to macroscopic metastasis to the lymph nodes when inoculated *in vivo*.

Mat BIII rat breast tumor cells, *e.g.*, 10^5-10^6 cells in PBS, 0.1-0.2 mL per rat, are inoculated into the mammary fat pads of female Fisher rats. The test compound is dissolved in PBS (200 mM stock), sterile filtered and dispensed *in vivo* at a dose of up to about 100 mg/kg/day), preferably through a 14-day Alza osmotic mini-pump implanted intraperitoneally at the time of inoculation. Control animals receive vehicle (PBS) alone. Alternatively, the test compound is administered by systemic, preferably intraperitoneal (IP) injection. Animals are euthanized at day 14 and examined for metastasis in the spleen, lungs, liver, kidney and lymph nodes, preferably by counting of macroscopic lesions. Primary tumors and metastases may be embedded in paraffin and processed for histological or immunohistochemical analysis by conventional means. Primary tumor size can be measured with calipers.

B. 3LL Lewis Lung Carcinoma: Primary Tumor Growth

This tumor line arose spontaneously in 1951 as carcinoma of the lung in a C57BL/6 mouse (*Cancer Res 15*:39, 1955. See, also Malave, I. *et al.*, *J. Nat'l. Canc. Inst. 62*:83-88 (1979)). It is propogated by passage in C57BL/6 mice by subcutaneous (sc) inoculation and is tested in semiallogeneic C57BL/6 x DBA/2 F_1 mice or in allogeneic C3H mice. Typically six animals per group for subcutaneously (sc) implant, or ten for intramuscular (im) implant are used. Tumor may be implanted sc as a 2-4 mm fragment, or im or sc as an inoculum of suspended cells of about 0.5-2 x 10^6-cells. Treatment begins 24 hours after implant or is delayed until a tumor of specified size (usually approximately 400 mg) can be palpated. The test compound is administered ip daily for 11 days

Animals are followed by weighing, palpation, and measurement of tumor size. Typical tumor weight in untreated control recipients on day 12 after im inoculation is 500-2500 mg. Typical median survival time is 18-28 days. A positive control compound, for example cyclophosphamide at 20 mg/kg/injection per day on days 1-11 is used. Results computed include mean animal weight, tumor size, tumor weight, survival time For confirmed therapeutic activity, the test composition should be tested in two multi-dose assays.

C. 3LL Lewis Lung Carcinoma: Primary Growth and Metastasis Model

This model has been utilized by a number of investigators. See, for example, Gorelik, E. *et al.*, *J. Nat'l. Canc. Inst. 65*:1257-1264 (1980); Gorelik, E. *et al.*, *Rec. Results Canc. Res. 75*:20-28 (1980); Isakov, N. *et al.*, *Invasion Metas. 2*:12-32 (1982); Talmadge J.E. *et al.*, *J. Nat'l. Canc. Inst. 69*:975-980 (1982); Hilgard, P. *et al.*, *Br. J. Cancer 35*:78-86(1977)). Test mice are male C57BL/6 mice, 2-3 months old. Following sc, im, or intra-footpad implantation, this tumor produces metastases, preferentially in the lungs. With some lines of the tumor, the primary tumor exerts anti-metastatic effects and must first be excised before study of the metastatic phase (see also U.S. 5,639,725).

Single-cell suspensions are prepared from solid tumors by treating minced tumor tissue with a solution of 0.3% trypsin. Cells are washed 3 times with PBS (pH 7.4) and suspended in PBS. Viability of the 3LL cells prepared in this way is

generally about 95-99% (by trypan blue dye exclusion). Viable tumor cells (3×10^4 - 5×10^6) suspended in 0.05 ml PBS are injected subcutaneously, either in the dorsal region or into one hind foot pad of C57BL/6 mice. Visible tumors appear after 3-4 days after dorsal sc injection of 10^6 cells. The day of tumor appearance and the diameters of established tumors are measured by caliper every two days.

The treatment is given as one or two doses of peptide or derivative, per week. In another embodiment, the peptide is delivered by osmotic minipump.

In experiments involving tumor excision of dorsal tumors, when tumors reach about 1500 mm^3 in size, mice are randomized into two groups: (1) primary tumor is completely excised; or (2) sham surgery is performed and the tumor is left intact. Although tumors from 500-3000 mm^3 inhibit growth of metastases, 1500 mm^3 is the largest size primary tumor that can be safely resected with high survival and without local regrowth. After 21 days, all mice are sacrificed and autopsied.

Lungs are removed and weighed. Lungs are fixed in Bouin's solution and the number of visible metastases is recorded. The diameters of the metastases are also measured using a binocular stereoscope equipped with a micrometer-containing ocular under 8X magnification. On the basis of the recorded diameters, it is possible to calculate the volume of each metastasis. To determine the total volume of metastases per lung, the mean number of visible metastases is multiplied by the mean volume of metastases. To further determine metastatic growth, it is possible to measure incorporation of ^{125}IdUrd into lung cells (Thakur, M.L. *et al., J. Lab. Clin. Med. 89*:217-228 (1977). Ten days following tumor amputation, 25 μg of fluorodeoxyuridine is inoculated into the peritoneums of tumor-bearing (and, if used, tumor-resected mice). After 30 min, mice are given 1 μCi of ^{125}IdUrd (iododeoxyuridine). One day later, lungs and spleens are removed and weighed, and a degree of ^{125}IdUrd incorporation is measured using a gamma counter.

In mice with footpad tumors, when tumors reach about 8-10 mm in diameter, mice are randomized into two groups: (1) legs with tumors are amputated after ligation above the knee joints; or (2) mice are left intact as nonamputated tumor-bearing controls. (Amputation of a tumor-free leg in a tumor-bearing mouse has no known effect on subsequent metastasis, ruling out possible effects of anesthesia, stress

or surgery). Mice are killed 10-14 days after amputation. Metastases are evaluated as described above.

Statistics: Values representing the incidence of metastases and their growth in the lungs of tumor-bearing mice are not normally distributed. Therefore, non-parametric statistics such as the Mann-Whitney U-Test may be used for analysis.

Study of this model by Gorelik *et al.* (1980, *supra*) showed that the size of the tumor cell inoculum determined the extent of metastatic growth. The rate of metastasis in the lungs of operated mice was different from primary tumor-bearing mice. Thus in the lungs of mice in which the primary tumor had been induced by inoculation of larger doses of 3LL cells (1-5 x 10^6) followed by surgical removal, the number of metastases was lower than that in nonoperated tumor-bearing mice, though the volume of metastases was higher than in the nonoperated controls. Using ^{125}IdUrd incorporation as a measure of lung metastasis, no significant differences were found between the lungs of tumor-excised mice and tumor-bearing mice originally inoculated with 1 x 10^6 3LL cells. Amputation of tumors produced following inoculation of 1 x 10^5 tumor cells dramatically accelerated metastatic growth. These results were in accord with the survival of mice after excision of local tumors. The phenomenon of acceleration of metastatic growth following excision of local tumors had been repeatedly observed (for example, see U.S. 5,639,725). These observations have implications for the prognosis of patients who undergo cancer surgery.

D. Experimental Metastasis Models

The compounds of this invention are also tested for inhibition of late metastasis using an experimental metastasis model (Crowley *et al.*, 1993). Late metastasis involves the steps of attachment and extravasation of tumor cells, local invasion, seeding, proliferation and angiogenesis.

Human prostatic carcinoma cells (PC-3) transfected with a reporter gene, preferably the green fluorescent protein (GFP) gene, but as an alternative with a gene encoding the enzymes chloramphenicol acetyl-transferase (CAT), luciferase or LacZ. This permits utilization of either of these markers (fluorescence detection of GFP or histochemical colorimetric detection of enzymatic activity) for following the fate of these cells. Cells are injected, preferably iv, and metastases identified after about 14

days, particularly in the lungs but also in regional lymph nodes, femurs and brain. This mimics the organ tropism of naturally occurring metastases of prostate cancer. For example, GFP-expressing PC-3 cells (1 x 10^6 cells per mouse) are injected iv into the tail veins of nude *(nu/nu)* mice. Animals are also implanted with mini-pumps (sub-dermally on the back) dispensing either the test compound (at least about 100 mg/kg/day) or vehicle. The animals are euthanized after 14 days and their organs prepared for histological examination. Single metastatic cells and foci are visualized and quantitated by fluorescence microscopy or light microscopic histochemistry or by grinding the tissue and quantitative colorimetric assay of the detectable label.

E. Human Tumor Xenograft in Nude Mice

A preferred model employs an aggressive human breast cancer cell line, MDA-MB-231. This line is highly invasive *in vitro* and expresses very high levels of uPA and uPAR. Despite its aggressive phenotype *in vitro,* this cell line, like most other human breast cancer cell lines, grows poorly and does not metastasize reproducibly when inoculated subcutaneously into nude mice. When MDA-MB-231 are co-inoculated with Matrigel or fibroblasts into the mammary fat pad of a nude mouse, tumor growth and metastasis are enhanced significantly, resulting in a useful model (Price (1996) *Breast Cancer Research and Treatment* 39: 93-102)

MDA-MB-231 cells, *e.g.*, 1 x 10^5 cells in 0. 2 mL of cold Matrigel are inoculated into mammary fat pads of female Balb/C *(nu/nu)* mice and are allowed to implant and grow for a period, usually about 25-30 days depending on cell dose. Treatment with a test compound is initiated by IP injections, b.i.d. (In the Example below, 77 mg/kg/day of the Å6 peptide was given). The animals are 2-4 weeks later and examined for macroscopic metastases. In addition, primary tumors are removed and weighed, and may be paraffin embedded for histological evaluation. Metastases typically occur in lymph nodes and are quantitated by counting macroscopic lesions .

For a compound to be useful in accordance with this invention, it should demonstrate anti-tumor activity in the above models, for example, blocking tumor progression, angiogenesis and/or metastasis.

Angiogenesis

Angiogenesis is measured by determining microvessel density using immunostaining for CD31 (also known as platelet-endothelial cell adhesion molecule or PECAM). Results are reported as the average microvessel density of 5 fields each from 5 different sections (Penfold *et al.*, 1996). Typically, the whole tumor is excised, sectioned and the sections examined histologically for microvessel density using appropriate stains or labels for other makers. Endothelial tube formation is another assay that is related to angiogenesis, and is described in the Examples.

Pharmaceutical and Therapeutic Compositions and Their Administration

The compounds that may be employed in the pharmaceutical compositions of the invention include all of those compounds described above, as well as the pharmaceutically acceptable salts of these compounds. Pharmaceutically acceptable acid addition salts of the compounds of the invention containing a basic group are formed where appropriate with strong or moderately strong, non-toxic, organic or inorganic acids in the presence of a basic amine by methods known to the art. Exemplary of the acid addition salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate and nitrate salts.

Pharmaceutically acceptable base addition salts of compounds of the invention containing an acidic group are prepared by known methods from organic and inorganic bases and include, for example, nontoxic alkali metal and alkaline earth bases, such as calcium, sodium, potassium and ammonium hydroxide; and nontoxic organic bases such as triethylamine, butylamine, piperazine, and tri(hydroxymethyl)methylamine.

As stated above, the compounds of the invention possess the ability to inhibit invasiveness or angiogenesis, properties that are exploited in the treatment of cancer, in particular metastatic cancer. A composition of this invention may be active *per se*, or may act as a "pro-drug" that is converted *in vivo* to the active form.

The compounds of the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms, such as capsules,

impregnated wafers, tablets or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may be employed.

Preferably, the compounds of the invention are administered systemically, *e.g.*, by injection. When used, injection may be by any known route, preferably intravenous, subcutaneous, intramuscular, intracranial or intraperitoneal. Injectables can be prepared in conventional forms, either as solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline, water, dextrose, glycerol and the like. Similarly, the carrier or diluent may include any prolonged release material, such as glyceryl monostearate or glyceryl distearate, alone or with a wax. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, soft gelatin capsule, sterile injectable liquid (e.g., a solution), such as an ampoule, or an aqueous or nonaqueous liquid suspension. A summary of such pharmaceutical compositions may be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton Pennsylvania (Gennaro 18th ed. 1990).

The pharmaceutical preparations are made following conventional techniques of pharmaceutical chemistry involving such steps as mixing, granulating and compressing, when necessary for tablet forms, or mixing, filling and dissolving the ingredients, as appropriate, to give the desired products for oral, parenteral, topical, transdermal, intravaginal, intranasal, intrabronchial, intracranial, intraocular, intraaural and rectal administration. The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and so forth.

Though the preferred routes of administration are systemic the pharmaceutical composition may be administered topically or transdermally, e.g., as an ointment, cream or gel; orally; rectally; *e.g.*, as a suppository, parenterally, by injection or continuously by infusion; intravaginally; intranasally; intrabronchially; intracranially intra-aurally; or intraocularly.

For topical application, the compound may be incorporated into topically applied vehicles such as a salve or ointment. The carrier for the active ingredient may be either in sprayable or nonsprayable form. Non-sprayable forms can be semi-solid or solid forms comprising a carrier indigenous to topical application and having a dynamic viscosity preferably greater than that of water. Suitable formulations include, but are not limited to, solution, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like. If desired, these may be sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers, or salts for influencing osmotic pressure and the like. Preferred vehicles for non-sprayable topical preparations include ointment bases, e.g., polyethylene glycol-1000 (PEG-1000); conventional creams such as HEB cream; gels; as well as petroleum jelly and the like.

Also suitable for topic application are sprayable aerosol preparations wherein the compound, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, perfumes, and/or antioxidants in addition to the compounds of the invention.

For the preferred topical applications, especially for humans, it is preferred to administer an effective amount of the compound to an infected area, *e.g.*, skin surface, mucous membrane, eyes, *etc.* This amount will generally range from about 0.001 mg to about 1 g per application, depending upon the area to be treated, the severity of the symptoms, and the nature of the topical vehicle employed.

The compositions of the invention may further comprise one or more additional compounds that are anti-tumor agents, such as mitotic inhibitors, *e.g.*, vinblastine; alkylating agents, *e.g.*, cyclophosphamide; folate inhibitors, *e.g.*, methotrexate, piritrexim or trimetrexate; antimetabolites, *e.g.*, 5-fluorouracil and cytosine arabinoside; intercalating antibiotics, *e.g.*, adriamycin and bleomycin; enzymes or enzyme inhibitors, *e.g.*, asparaginase; topoisomerase inhibitors, *e.g.*, etoposide; or biological response modifiers, *e.g.*, interferon. In fact, pharmaceutical compositions comprising any known cancer therapeutic in combination with the peptides disclosed herein are within the scope of this invention.

The composition of the invention may also comprise one or more other medicaments, preferably anti-infectives such as antibacterial, anti-fungal, anti-parasitic, anti-viral, and anti-coccidial agents. Exemplary antibacterial agents include, for example, sulfonamides such as sulfamethoxazole, sulfadiazine or sulfadoxine; DHFR inhibitors such as trimethoprim, bromodiaprim or trimetrexate; penicillins; cephalosporins; aminoglycosides; bacteriostatic inhibitors of protein synthesis; the quinolonecarboxylic acids and their fused isothiazole analogs; and the like.

Other Therapeutic Compositions

In another embodiment, the compounds of this invention are "therapeutically conjugated" and used to deliver a therapeutic agent to the site of where the compounds home and bind, such as sites of tumor metastasis or foci of infection/inflammation. The term "therapeutically conjugated" means that the compound, preferably a peptide, peptide derivative, or peptidomimetic, is conjugated to a therapeutic agent. The therapeutic agents used in this manner act are directed either to the underlying cause or the components of the processes of tumor invasion, angiogenesis or inflammation. Examples of agents used to treat inflammation are the steroidal and non-steroidal anti-inflammatory drugs, many of which inhibit prostaglandin synthesis.

Other therapeutic agents which can be coupled to the compounds according to the method of the invention are drugs, radioisotopes, lectins and other toxins. The therapeutic dosage administered is an amount which is therapeutically effective, and will be known to one of skill in the art. The dose is also dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired, such as, for example, anti-inflammatory effects or anti-bacterial effect.

Lectins are proteins, commonly derived from plants, that bind to carbohydrates. Among other activities, some lectins are toxic. Some of the most cytotoxic substances known are protein toxins of bacterial and plant origin (Frankel, A.E. *et al., Ann. Rev. Med. 37*:125-142 (1986)). These molecules binding the cell surface and inhibition cellular protein synthesis. The most commonly used plant toxins are ricin and abrin; the most commonly used bacterial toxins are diphtheria toxin and Pseudomonas exotoxin A. In ricin and abrin, the binding and toxic functions are

contained in two separate protein subunits, the A and B chains. The ricin B chain binds to the cell surface carbohydrates and promotes the uptake of the A chain into the cell. Once inside the cell, the ricin A chain inhibits protein synthesis by inactivating the 60S subunit of the eukaryotic ribosome Endo, Y. *et al., J. Biol. Chem. 262*: 5908-5912 (1987)). Other plant derived toxins, which are single chain ribosomal inhibitory proteins, include pokeweed antiviral protein, wheat germ protein, gelonin, dianthins, momorcharins, trichosanthin, and many others (Strip, F. *et al., FEBS Lett. 195*:1-8 (1986)). Diphtheria toxin and Pseudomonas exotoxin A are also single chain proteins, and their binding and toxicity functions reside in separate domains of the same protein chain with full toxin activity requiring proteolytic cleavage between the two domains. Pseudomonas exotoxin A has the same catalytic activity as diphtheria toxin. Ricin has been used therapeutically by binding its toxic α-chain, to targeting molecules such as antibodies to enable site-specific delivery of the toxic effect. Bacterial toxins have also been used as anti-tumor conjugates. As intended herein, a toxic peptide chain or domain is bound to a compound of this invention and delivered in a site-specific manner to a target site where the toxic activity is desired, such as a metastatic focus. Conjugation of toxins to protein such as antibodies or other ligands are known in the art (Olsnes, S. *et al., Immunol. Today 10*:291-295 (1989); Vitetta, E.S. *et al., Ann. Rev. Immunol. 3*:197-212 (1985)).

Examples of therapeutic radioisotopes which can be bound to the compound for use in accordance with the methods of the invention, are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{217}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, and ^{109}Pd.

Cytotoxic drugs that interfere with critical cellular processes including DNA, RNA, and protein synthesis, have been conjugated to antibodies and subsequently used for in vivo therapy. Such drugs, including but are not limited to daunorubicin, doxorubicin, methotrexate, and Mitomycin C are also coupled to the compounds of this invention and use therapeutically in this form.

Therapeutic Methods

This invention includes methods for inhibiting cellular invasion, chiefly by tumor cells, or angiogenesis, primarily induced by tumor cells in a subject. By inhibiting invasion by cells or angiogenesis, the method results in inhibition of tumor

metastasis. In this method, a vertebrate subject, preferably a mammal, more preferably a human, is administered an amount of the compound effective to inhibit invasion or angiogenesis. The compound or pharmaceutically acceptable salt thereof is preferably administered in the form of a pharmaceutical composition as described above.

Doses of the compounds preferably include pharmaceutical dosage units comprising an effective amount of the peptide. By an effective amount is meant an amount sufficient to achieve a steady state concentration *in vivo* which results in a measurable reduction in any relevant parameter of disease and may include growth of primary or metastatic tumor, any accepted index of inflammatory reactivity, or a measurable prolongation of disease-free interval or of survival. For example, a reduction in tumor growth in 20 % of patients is considered efficacious (Frei III, E., *The Cancer Journal* 3:127-136 (1997)). However, an effect of this magnitude is not considered to be a minimal requirement for the dose to be effective in accordance with this invention.

In one embodiment, an effective dose is at least equal to, preferably 10-fold and more preferably 100-fold higher than the 50% inhibitory concentration (IC_{50})of the compound in an *in vivo* assay as described herein.

The amount of active compound to be administered depends on the precise peptide or derivative selected, the disease or condition, the route of administration, the health and weight of the recipient, the existence of other concurrent treatment, if any, the frequency of treatment, the nature of the effect desired, for example, inhibition of tumor metastasis, and the judgment of the skilled practitioner.

A preferred dose for treating a subject, preferably mammalian, more preferably human, with a tumor is an amount of up to about 100 milligrams of active compound per kilogram of body weight.

Typical single dosages of the peptide are between about 1 μg and about 100mg/kg body weight. For topical administration, dosages in the range of about 0.01-20% concentration of the compound, preferably 1-5%, are suggested. A total daily dosage in the range of about 10 milligrams to about 7 grams is preferred for oral administration. The foregoing ranges are, however, suggestive, as the number of

variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are expected.

An effective amount or dose of the peptide for inhibiting invasion *in vitro* is in the range of about 1 picogram to about 0.5 nanograms per cell. Effective doses and optimal dose ranges may be determined *in vitro* using the methods described herein.

The compounds of the invention may be further characterized as producing an inhibitory effect on cell migration and invasion, on angiogenesis, on tumor metastasis or on inflammatory reactions. The compounds are especially useful in producing an anti-tumor effect in a mammalian host, preferably human, harboring a tumor.

The foregoing compositions and treatment methods are useful for inhibiting cell migration and invasion or migration-induced cell proliferation in a subject having a disease or condition associated with undesired cell invasion, migration-induced proliferation, angiogenesis or metastasis. Such diseases or conditions may include primary growth or solid tumors or leukemias and lymphomas, metastasis, invasion and/or growth of tumor metastases, atherosclerosis, myocardial angiogenesis, post-balloon angioplasty vascular restenosis, neointima formation following vascular trauma, vascular graft restenosis, coronary collateral formation, deep venous thrombosis, ischemic limb angiogenesis, telangiectasia, pyogenic granuloma, corneal diseases, rubeosis, neovascular glaucoma, diabetic and other retinopathy, retrolental fibroplasia, diabetic neovascularization, macular degeneration, endometriosis, arthritis, fibrosis associated with chronic inflammatory conditions including psoriasis scleroderma, lung fibrosis, chemotherapy-induced fibrosis, wound healing with scarring and fibrosis; peptic ulcers, fractures, keloids, and disorders of vasculogenesis, hematopoiesis, ovulation, menstruation, pregnancy and placentation, or any other disease or condition in which invasion or angiogenesis is pathogenic.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Synthesis of Acetyl-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-NH_2

The starting material was p-methyl-benzhydrylamine resin substituted at a level of 0.70 mEq per gram of resin. Each of the L-amino acids, starting with glutamic acid, was added in sequence in a synthesis cycle consisting of the three steps of TFA deprotection, coupling and capping. The completed peptide was subjected to HF cleavage and then purified.

1. TFA De-protection

The starting resin was conditioned before adding the first glutamic acid, or, in the case of subsequent cycles, the BOC protecting group was removed from the α-amino nitrogen of the starting material by treating the resin with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) (two to three volumes per resin volume). The mixture was stirred at room temperature for 30 minutes and then drained. The resin was then washed once with an equal volume of isopropanol for one minute and washed twice with an equal volume of methanol, each wash taking one minute.

2. Coupling

The de-protected resin was washed twice with an equal volume of 10% triethylamine in DCM, each wash taking one minute, and washed twice with an equal volume of methanol, each wash taking one minute, and washed twice with an equal volume of DCM, each wash taking one minute. A BOC-protected amino acid (three equivalents, dissolved in DCM or in a mixture of DCM and N,N'-dimethylformamide (DMF)) and 1-hydroxybenzotriazole (1M solution in DMF, three equivalents) was added to the resin, and the mixture was stirred for a few seconds. Dicyclohexylcarbodiimide (DCC) (1M solution in DCM, three equivalents) was then added, and the whole mixture was stirred for 60-120 minutes. The resin was washed twice with an equal volume of methanol and then washed twice with an equal volume of DCM. A small sample was taken for a ninhydrin test to assess the completeness of coupling. Generally, if incomplete, the coupling step 2 is repeated. If complete, the synthesis is continued with the capping step 3.

All amino acids were used as α-BOC derivatives. Side chain protecting groups were as follows:

Amino acid	Protecting group
Histidine	Benzyloxymethyl
Asparagine	Xanthyl
Glutamine	Xanthyl
Serine	O-benzyl
Threonine	O-benzyl
Tyrosine	2-Bromo-Z
Lysine	2-Chloro-Z
Glutamic acid	Cyclohexyl
Aspartic acid	Cyclohexyl

3. Capping

The resin was stirred with an equal volume of acetic anhydride (20% solution in DCM) for 5 minutes at room temperature. The resin was washed twice with an equal volume of methanol and then washed twice with an equal volume of DCM.

4. HF Cleavage

The resin bearing the desired amino acid sequence (1.0 gram) was placed in a Teflon reaction vessel, and anhydrous anisole (1 mL) was added. The vessel was cooled with liquid N_2 , and anhydrous HF (10 mL) was distilled into it. The temperature was raised with ice water to 0°C. The mixture was stirred at this temperature for one hour, and then the HF was distilled off at 0°C. The residue was washed with anhydrous ether, and the peptide was extracted with a 1:1 mixture of CH_3CN:H_2O.

5. Purification

The lyophilized powder was dissolved in 0.1% TFA buffer and loaded onto a Waters C18 preparative column (2 inches diameter, 15-20 μm particle size, 300Å pore size). The loaded column was eluted with a two-component eluent applied as a linear gradient, starting with 0% of solution A in solution B and finishing with 40% of solution A in solution B. Solution A was 0.1% TFA in H_2O, and solution B was 0.1% TFA in CH_3CN. Fractions exhibiting purity equal to or better than that desired were pooled and lyophilized to render the purified final product as the trifluoroacetate salt.

EXAMPLE II

Anti-Invasive Activity of Capped KPSSPPEE and Related Peptides

Several peptides were tested for anti-invasive capacity in a Matrigel® invasion assay system as indicated above (Kleinman *et al.*, *supra*; Parish *et al.*, *supra*)

Cells of the rat breast cancer line Mat BIII and the human prostate cancer line PC-3 were used. Tumor cells (5 x 10^5/mL, in a volume of 200 µL) in serum-free RPMI 1640 medium were added to a disposable transwell invasion chamber coated with Matrigel® (Becton Dickinson, Lincoln Park, NJ). The invasion chambers were placed in 24-well tissue culture plates filled with serum-free RPMI-1640 and the plates were placed in an atmosphere of 5% CO_2 in humidified air at 37°C for 48-72 hours. The chambers were then removed, inverted, and the cells which had invaded (and now appeared on the bottom face of the invasion chamber) were fixed and stained using Diff-Quik® (Scientific Products). Cells were counted in 10 different fields on each filter and an average obtained. Typically, 3-5 replicates were performed at each concentration of compound tested.

As shown in Figure 1, the capped octapeptide, Ac-KPSSPPEE-Am (also designated Å6), inhibited the invasion of both rat and human tumor cell lines. This peptide was not cytotoxic to the cells nor did it inhibit cell proliferation. Thus the observed effect was not a side effect of cytotoxicity and could be ascribed to a mechanism of action distinct from that of cytotoxic or cytostatic agents.

Tests were also conducted on shorter related, capped peptides having the sequence Ac-PSSPPEE-Am (a deletion variant of SEQ ID NO:2 which lacks the N-terminal Lys) and Ac-KPSSPPE-Am (a deletion variant of SEQ ID NO:2 lacking one of the C- terminal Glu residues). Also tested was a similar longer peptide, KPSSPPEELK (SEQ ID NO: 1) (Blasi *et al.*, US 5,416,006) and its capped counterpart, Ac-KPSSPPEELK-Am).

It is noteworthy that all the peptides other than Ac-KPSSPPEE-Am showed little or no activity in this assay, indicating that SEQ ID NO: 2 was the minimal required size for activity (Figure 2). The results also indicated that addition of Leu and Lys at the C terminus of KPSSPPEE abrogated its biological activity, regardless of whether the termini were capped or uncapped.

Additional results are shown in Figure 3 for peptides desiganted Å13 (KPSSPPEE, uncapped) , Å14 (Ac-KPPSSPPEEL-Am), and Å15, (GGKPPSSPPEE-Am, capped only at the C-terminus). Neither Å13 nor Å14 were inhibitory, whereas, at the higher concentration tested, Å15 showed some activity.

EXAMPLE III

Inhibition of Endothelial Cell Proliferation and Migration

Studies were performed to evaluate the activity of peptide Å6 as an inhibitor of cell proliferation and migration *in vitro*. Such assay formats may be used for either tumor cells or endothelial cells. In this case, cells (20, 000 per well) were seeded into a 24-well plate. Cell growth may be stimulated using serum, cytokines, or matrix components coated onto the plates prior to the addition of cells. All proliferation assays are performed during log-phase growth (typically <70% confluent cells) which is established by determining growth curves for each cell line. The typical volume in each well is 0.5 mL and this media is changed every 48 hours (including replacement of any inhibitors being tested). At the completion of the assay, each well is washed 3x with PBS (0.5 mL per well for 5 minutes) and the cell-number is determined using a colorimetric assay, such as the MTS assay (Promega) or CyQuant (Pierce), as per manufacturers instructions. Each condition (inhibitor concentration, day of assay, *etc.*) is tested in triplicate and the means of triplicate wells are shown.

In this study, cells were stimulated with 2 ng/ml basic fibroblast growth factor (bFGF). The results, shown in Figure 4, indicate that Å6 was a potent inhibitor of cell proliferation.

Migration assays were similar to those described in Example II , above. Transwells (Costar, 8.0 μm pore size were are coated with type I collagen (50 μg/mL) by adding 200 μL of the collagen solution per transwell, then incubating overnight at 37°C. The transwells were assembled in a 24-well plate and a chemoattractant, in this case bFGF, was added to the bottom chamber in a total volume of 0.8 mL in the F12K medium supplemented with 10% FBS and antibiotics. This is the appropriate medium for human vascular endothelial cells, but may be varied for other cell types. Endothelial cells, which were detached from monolayer culture using trypsin, were

diluted with serum-free medium to a final concentration of 1 x 10^6 cells/mL and 0.2 mL of this cell suspension was added to the upper chamber of each transwell. The test inhibitors, the Å6 peptide, was added to both the upper and lower chambers, and the migration was allowed to proceed for 5 hrs in a humidified atmosphere at 37°C. The transwells were removed from the plate and the upper chamber wiped clean with a cotton swab. Fixative was added to the upper chamber for 5 minutes followed by washing (3 x 5 minutes per wash) with PBS. Cells were stained with Toluidine Blue and counted under a high-power field (400x) to determine the number of cells migrated.

The results (Figure 5) show that, compared to bFGF controls, addition of Å6 at concentrations between 1 and 1000 μM caused from 20-80% inhibition of migration.

EXAMPLE IV

Inhibition of Angiogenesis *In Vivo* by Capped KPSSPPEE (the Å6 Peptide) and Related Compounds

Angiogenesis induced by tumor growth and metastasis *in vivo* is examined in the models systems described above. Mice injected with 3LL cells are treated either with peptide or with vehicle and are sacrificed at various time points. Angiogenesis is assessed by determining microvessel density (MVD) using an antibody specific for microvascular endothelium or other markers of growing blood vessels, such as PECAM (CD31). Such an antibody is employed in conventional immunohistological methods to immunostain tissue sections as described by Penfold *et al.*, *supra*. A large number of such antibodies is commercially available, for example the JC70 mAb. The MVD are correlated with other measures of tumor behavior including lymph node status and primary tumor size and rate of growth. In humans as reported by Penfold *et al., supra*, tumor MVD correlates with lymph node metastasis and is independent of tumor size, growth rate or type of histological differentiation. Only MVD showed a significant association with lymph node metastasis.

The compounds are given i.v., i.p., or by osmotic minipump. Typical dosages are 100-250 mg/kg/day. At various time points, two animals are sacrificed, and the tumor tissue and surrounding tissue is prepared for histological examination. Results are reported as the average microvessel density of 5 fields each from 5 different

sections. The following seven compounds are tested: Ac-KPSSPPEE-Am (SEQ ID NO:2), Ac-KPTTPPEE-Am (disubstitution variant at positions 3 and 4), Ac-KPSSPPDD-Am (disubstitution variant at positions 7 and 8), Ac-RPSSPPEE-Am (substitution variant at position 1), Ac-PSSPPEE-Am (deletion variant, position 1 of SEQ ID NO:2 deleted) , Ac-KPSSPPE-Am (deletion variant, position 8 of SEQ ID NO:2 deleted), and Ac-KPPSSPPEELK-Am (SEQ ID NO:1).

The following results are obtained. In the rats treated with Ac-KPSSPPEE-Am, Ac-KPTTPPEE-Am, Ac-KPSSPPDD-Am and Ac-RPSSPPEE-Am, there is a significant reduction in the number of microvessels in the region of the primary tumor at the subcutaneous inoculation site as compared to controls. Peptides Ac-PSSPPEE-Am, Ac-KPSSPPE-Am and Ac-KPPSSPPEELK-Am had no significant effect on angiogenesis. Therefore, the four indicated compounds have anti-angiogenic activity which is responsible at least in part for their effectiveness as antitumor agents.

EXAMPLE V

Inhibition of Tumor Growth and Metastasis *In Vivo* by Capped KPSSPPEE (the Å6 Peptide and Related Compounds

The rat syngeneic breast cancer system employs Mat BIII rat breast cancer cells. 1 x 10^6 tumor cells suspended in 0.1 mL PBS are inoculated into the mammary fat pads of 10 female Fisher rats. At the time of inoculation, a 14-day Alza osmotic mini-pump is implanted intraperitoneally to dispense the peptide. The peptide is dissolved in PBS (200 mM stock), sterile filtered and placed in the minipump to achieve a dispensing rate of about 100 mg/kg/day. Control animals receive vehicle (PBS) alone in the minipump. Animals are euthanized at day 14.

In the present study, MatBIII cells (Xing and Rabbani, 1996) (1 x 10^5 cells in 0.2 mL sterile PBS) were inoculated into the mammary fat pads of female Fisher rats. Å6 was delivered via continuous infusion using surgically implanted mini-pumps or, in a second experiment, via IP administration. The total dose (77 mg/ kg/ day) was identical in both studies although in the IP study, the animals received one-half of the total dose in each IP injection b.i.d. The rats were euthanized on day 15 and

examined for macroscopic metastases. These metastases, as well as the tumors resulting from the primary challenge, were paraffin embedded and sections were prepared for histological and immunohistochemical analysis. Tumors resulting from the primary challenge were evaluated using calipers and the metastases were evaluated by simple counting.

The results of primary tumor growth are shown in Figure 6. The Å6 peptide yielded highly significant inhibition of the local growth of the breast tumor as seen over three time points. Figure 7 shows that spread of the tumor to lymph nodes was completely inhibited by the peptide.

Histological analysis showed that Å6 treatment induced massive tumor cell apoptosis, probably through an anti-angiogenic mechanism of action, which deprives tumor cells of survival factors by inhibiting neovessel formation. This is supported by the fact that the Å6 peptide was neither cytotoxic nor did it induce apoptosis in tumor cells *in vitro*.

In other studies following results are expected. In the rats treated with Ac-KPSSPPEE-Am, Ac-KPTTPPEE-Am, Ac-KPSSPPDD-Am and Ac-RPSSPPEE-Am, there is a significant reduction in the size of the primary tumor and in the number of metastases in the spleen, lungs, liver, kidney and lymph nodes (enumerated as discrete foci). Upon histological and immunohistochemical analysis, it is seen that in treated animals, there is increased necrosis and signs of apoptosis. Large necrotic areas are seen in tumor regions lacking in neovascularization.

In contrast, treatment with peptides Ac-PSSPPEE-Am, Ac-KPSSPPE-Am and Ac-KPPSSPPEELK-Am fail to cause a significant change in tumor size or metastasis.

EXAMPLE VI

Inhibition of Growth and Metastasis of Xenografted Human Tumor Cells

Using the model described above (see also Price, *supra*), MDA-MB-231 cells (1×10^5 cells in 0. 2 mL of cold Matrigel) were inoculated into mammary fat pads of female Balb/C *(nu/nu)* mice. The tumor cells were allowed to implant and grow for 27 days. On day 28, Å6 peptide treatment commenced. The peptide was given b.i.d. via IP injection at a dosage of 75 mg/kg/day. Primary tumor growth was measured

every 2 days. The mice were euthanized on day 50 and examined for macroscopic metastases. The primary tumors were removed and weighed, then paraffin embedded for histological evaluation. Metastases were observed in the lymph nodes and were quantitated by counting macroscopic lesions.

The results are shown in Figures 8 and 9. The Å6 peptide was highly effective in arresting the growth of these aggressive tumor cells (Figure 8). The mice treated with the Å6 peptide had no macroscopic metastases in any site examined. The quantitation of lymph node metastases is shown in Figure 9.

Just as in the rat model, histological analysis showed that Å6 treatment induced massive tumor cell apoptosis that was mediated by an anti-angiogenic mechanism.

Thus, it was concluded that the Ac-KPSSPPEE-Am peptide is a potent anticancer agent at the level of the primary tumor and, more importantly for human subjects, suppresses the development of metastases.

EXAMPLE VIII

Inhibition of Experimental Metastasis of Tumor Cells *In Vivo*

The peptide compounds described in the Examples above are tested for efficacy *in vivo* in the 3LL model (also described above).

In another model, PC-3 cells transfected with the gene encoding the enzyme chloramphenicol acetyl-transferase (CAT) are inoculated into mice i.v. at doses of 1 x 10^6 cells per mouse. These mice are implanted with a minipump, as above, which dispenses 100 mg/kg/day of the peptide or vehicle over a period of 14 or 21 days. At termination of treatment, the animals are euthanized and the tumor marker probe is assayed in regional lymph nodes, femurs, lungs, and brain.

The following results are obtained. In the mice treated with Ac-KPSSPPEE-Am, Ac-KPTTPPEE-Am, Ac-KPSSPPDD-Am and Ac-RPSSPPEE-Am, metastasis is markedly inhibited. These results indicate that these compounds interfere with the metastatic process. In contrast, mice treated with peptides Ac-PSSPPEE-Am, Ac-KPSSPPE-Am and Ac-KPPSSPPEELK-Am have no reduction in metastases.

EXAMPLE IX

The Å6 Peptide Inhibits Endothelial Tube Formation *In Vitro*

For this assay, Matrigel (0.375 mg/mL; 250μL/well) was added to a 24 well tissue culture plate and was allowed to gel for 30 min. at 37°C. Endothelial cells at 20,000/well in F12K/10%FBS/antibiotic medium (see above) were added to these coated wells. An angiogenic stimulator, in this case bFGF was added to certain test wells. A compound being tested for inhibitory activity is added at time 0, or may be added at any time thereafter. Medium and inhibitors were replaced every 24 hrs if the assay is allowed to go that long. Tube formation was quantitated by measuring the total tube length in each well, using microscopic detection.

The results indicated that Å6 peptide significantly blocked endothelial tube formation and also promoted dissociation of preformed tubes. This is further evidence of the potency of this peptide and other peptides of this invention as anti-angiogenic agents.

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Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A peptide compound having the sequence

Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu [SEQ ID NO:2]

or a substitution variant, addition variant or other chemical derivative thereof, which peptide, variant or derivative is capped or uncapped,

wherein said peptide, variant or derivative has one or more of the following activities:

(a) has at least about 20% of the biological activity of Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am in one or more of the following *in vitro* bioassays: (i) invasion in a Matrigel® assay; (ii) endothelial tube formation on Matrigel®, or (iii) endothelial tube formation on a fibrin matrix in the presence of basic fibroblast growth factor and vascular endothelial growth factor; or

(b) competes with labeled Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am for binding to a cell or molecule which has a binding site for Ac-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Am.

2. A peptide according to claim 1 capped with an N-terminal acetyl group and a C terminal amide group.

3. A substitution or addition variant of a peptide according to claim 1, or a chemical derivative of the variant, which variant has a sequence selected from the group consisting of:

(a) SEQ ID NO:2 wherein the Glu at position 7 or 8 or both is replaced by one or any two of the substituent amino acids Gln, Asp or Asn;

(b) SEQ ID NO:2 wherein Ser at position 3 or 4 or both is replaced by one or any two of the substituent amino acids Thr, Ala, Gly, hSer or ValβOH;

(c) SEQ ID NO:2 wherein the Lys at position 1 is replaced by His, Arg, Gln, Orn, Cit or Hci;

(d) SEQ ID NO:2 wherein the Pro at position 2, 5 or 6 is replaced by Hyp;

(e) an addition variant of SEQ ID NO:2, wherein Leu, Ile, Val, Nva, Nle, Met, Ala, or Gly is added to the C-terminal Glu or to any of said substituents for Glu at position 8;

(f) an addition variant of SEQ ID NO:2, wherein any of the following peptides are added to the C-terminal Glu or to any of said substituents for Glu at position 8:

Leu-$(Gly)_n$; Ile-$(Gly)_n$; Val-$(Gly)_n$; Nva-$(Gly)_n$; or Nle-$(Gly)_n$, wherein n = 1-10;

(g) an addition variant of SEQ ID NO:2 wherein one or more of the following residues or peptides is added to the N-terminal Lys or to any of said N-terminal substituents of Lys at position 1:

Gly, Lys-$(Gly)_n$; Tyr-$(Gly)_n$; or Gly-$(Gly)_n$, wherein n = 1-10; and

(h) a combination of one or more of (a) - (g).

4. A compound according to claim 1 which is a peptidomimetic agent.

5. A multimer of a peptide or variant according to claim 1 which, when the peptide is not a variant, has the formula:

(Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-X_m)$_n$-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu

wherein X is selected from the group consisting of C_1-C_{20} alkyl, C_1-C_{20} alkenyl, C_1-C_{20} alkynyl, C_1.C_{20} polyether containing up to 9 oxygen atoms and Gly_z, and wherein m = 0 or 1, n = 1-100 and z =1-10.

6. A pharmaceutical composition useful for inhibiting (i) invasion of tumor cells or (ii) angiogenesis, comprising

(a) a peptide, variant or derivative according to claim 1; and

(b) a pharmaceutically acceptable carrier or excipient.

7. A pharmaceutical composition useful for inhibiting (i) invasion of tumor cells or (ii) angiogenesis, comprising

(a) a peptide, variant or derivative according to claim 2; and

(b) a pharmaceutically acceptable carrier or excipient.

8. A pharmaceutical composition useful for inhibiting (i) invasion of tumor cells or (ii) angiogenesis, comprising

(a) a peptide, variant or derivative according to claim 3; and

(b) a pharmaceutically acceptable carrier or excipient.

9. A pharmaceutical composition useful for inhibiting (i) invasion of tumor cells or (ii) angiogenesis, comprising

(a) a peptidomimetic according to claim 4; and

(b) a pharmaceutically acceptable carrier or excipient.

10. A pharmaceutical composition useful for inhibiting (i) invasion of tumor cells or (ii) angiogenesis, comprising

(a) a peptide multimer according to claim 5; and

(b) a pharmaceutically acceptable carrier or excipient.

11. A method for inhibiting the invasiveness of tumor cells comprising contacting said cells with an effective amount of a peptide, variant or derivative according to claim 1.

12. A method for inhibiting the invasiveness of tumor cells comprising contacting said cells with an effective amount of a peptide, variant or derivative according to claim 2.

13. A method for inhibiting the invasiveness of tumor cells comprising contacting said cells with an effective amount of a peptide, variant or derivative according to claim 3.

14. A method for inhibiting the invasiveness of tumor cells comprising contacting said cells with an effective amount of a peptidomimetic according to claim 4.

15. A method for inhibiting the invasiveness of tumor cells comprising contacting said cells with an effective amount of a peptide multimer according to claim 5.

16. A method for inhibiting tumor invasion or metastasis in a subject comprising administering to said subject a pharmaceutical composition according to claim 6.

17 A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis, comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 6.

18. A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis, comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 7.

19. A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis, comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 8.

20. A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis, comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 9.

21. A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced proliferation, or angiogenesis, comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 10.

22. A method according to any of claims 17-21 wherein said disease or condition is primary tumor growth, tumor invasion or metastasis, atherosclerosis, post-balloon angioplasty vascular restenosis, neointima formation following vascular trauma, vascular graft restenosis, fibrosis associated with a chronic inflammatory condition, lung fibrosis, chemotherapy-induced fibrosis, wound healing with scarring and fibrosis, psoriasis, deep venous thrombosis, or another disease or condition in which angiogenesis is pathogenic.

23. A method according to claim 22 wherein said disease is tumor growth, invasion or metastasis.

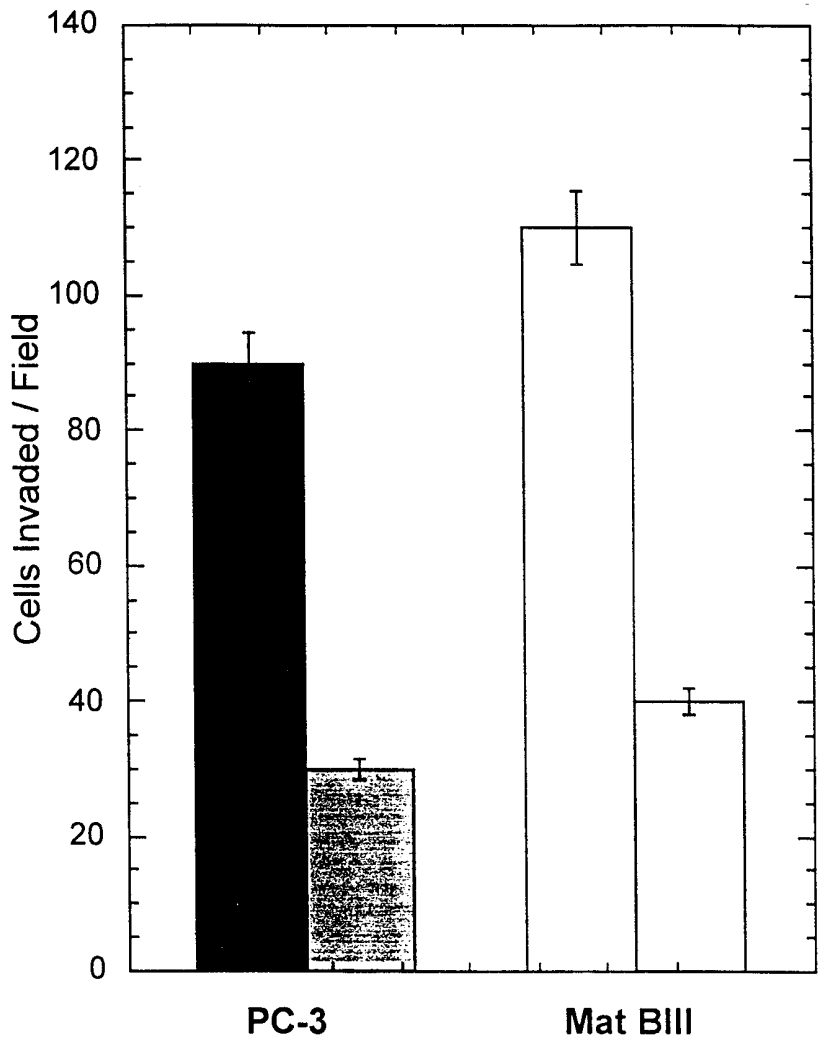

FIG. 1

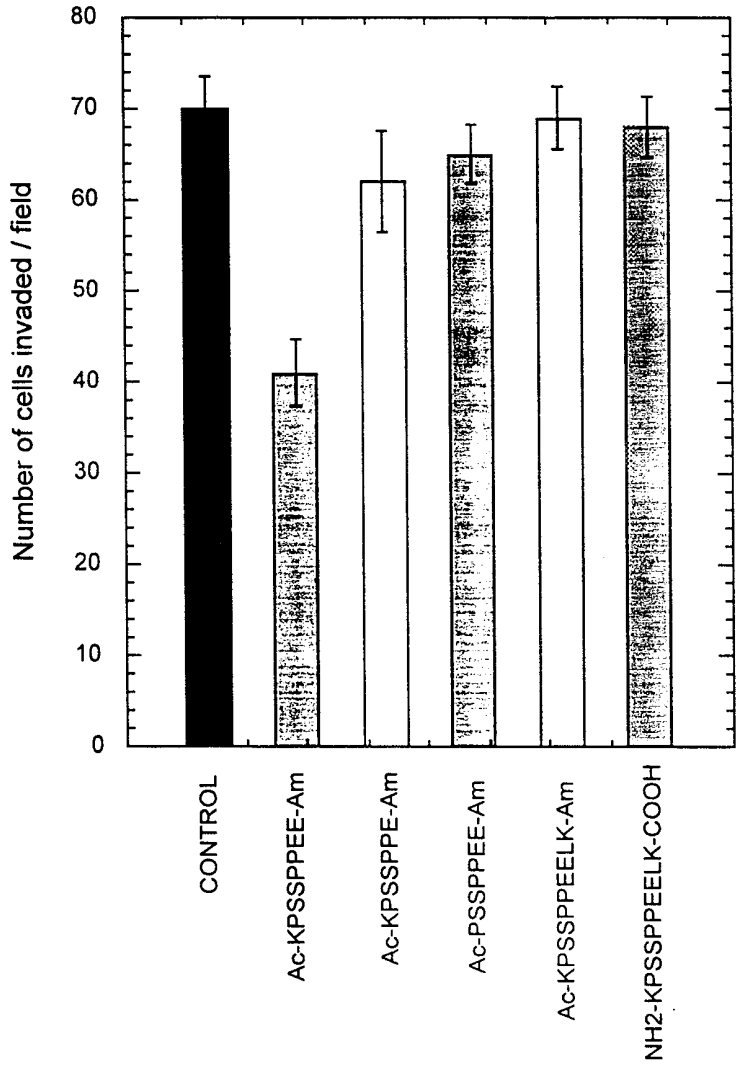
Number of cells invaded / field
80
70
60
50
40
30
20
10
0
CONTROL
Ac-KPSSPPEE-Am
Ac-KPSSPPE-Am
Ac-PSSPPEE-Am
Ac-KPSSPPEELK-Am
NH2-KPSSPPEELK-COOH

FIG. 2

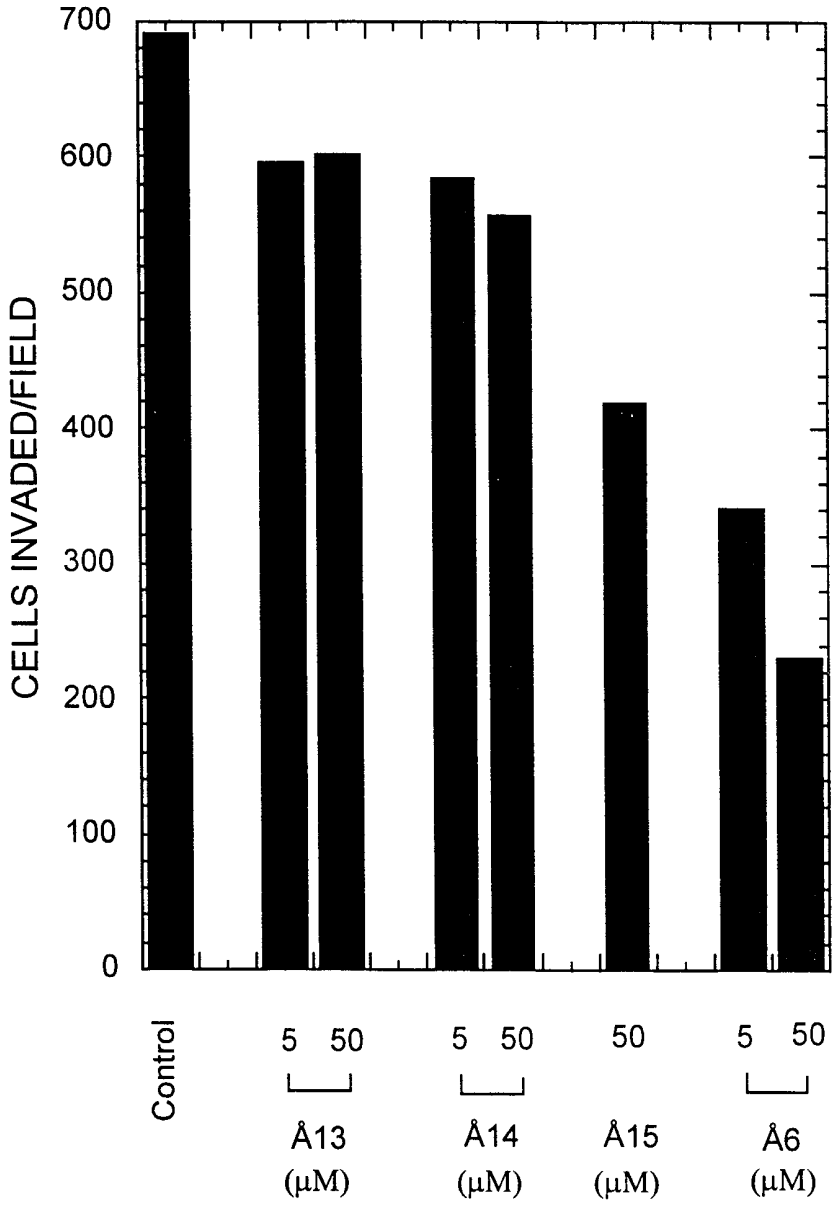
700
600
500
400
300
200
100
0
CELLS INVADED/FIELD
Control
5 50
Å13
(μM)
5 50
Å14
(μM)
50
Å15
(μM)
5 50
Å6
(μM)

FIG. 3

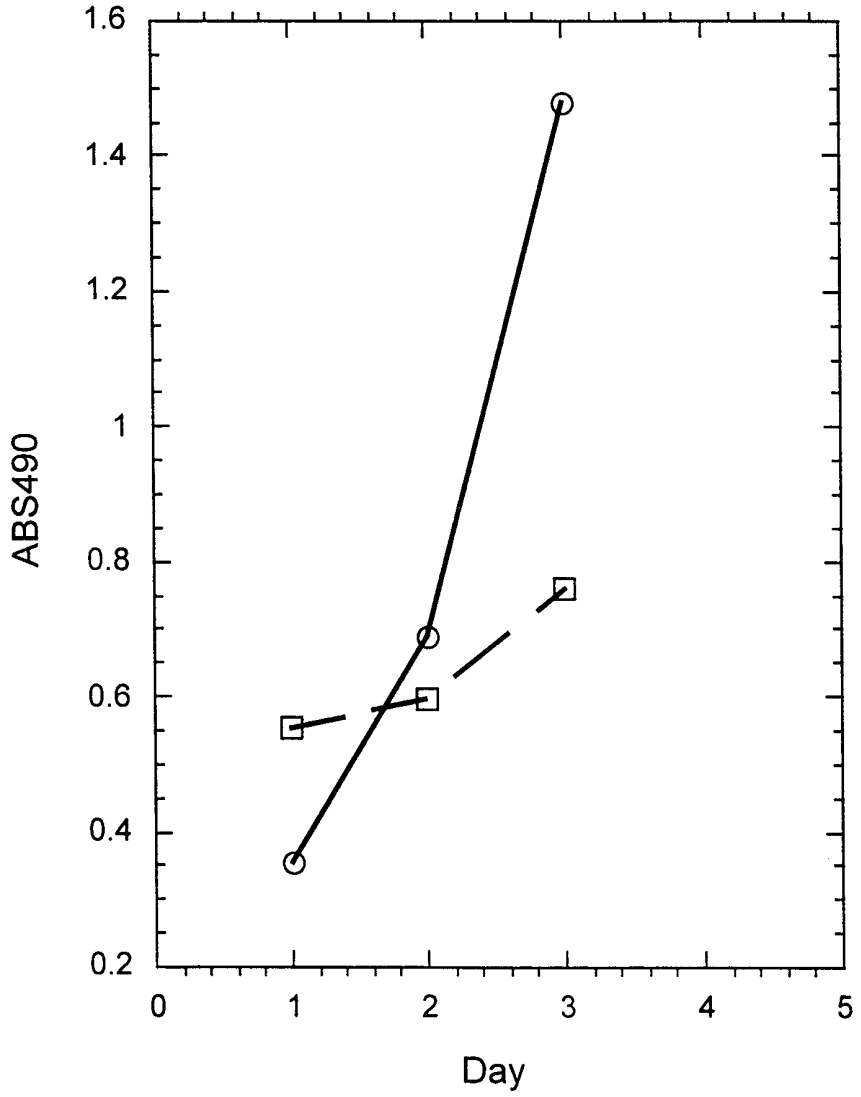

FIG. 4

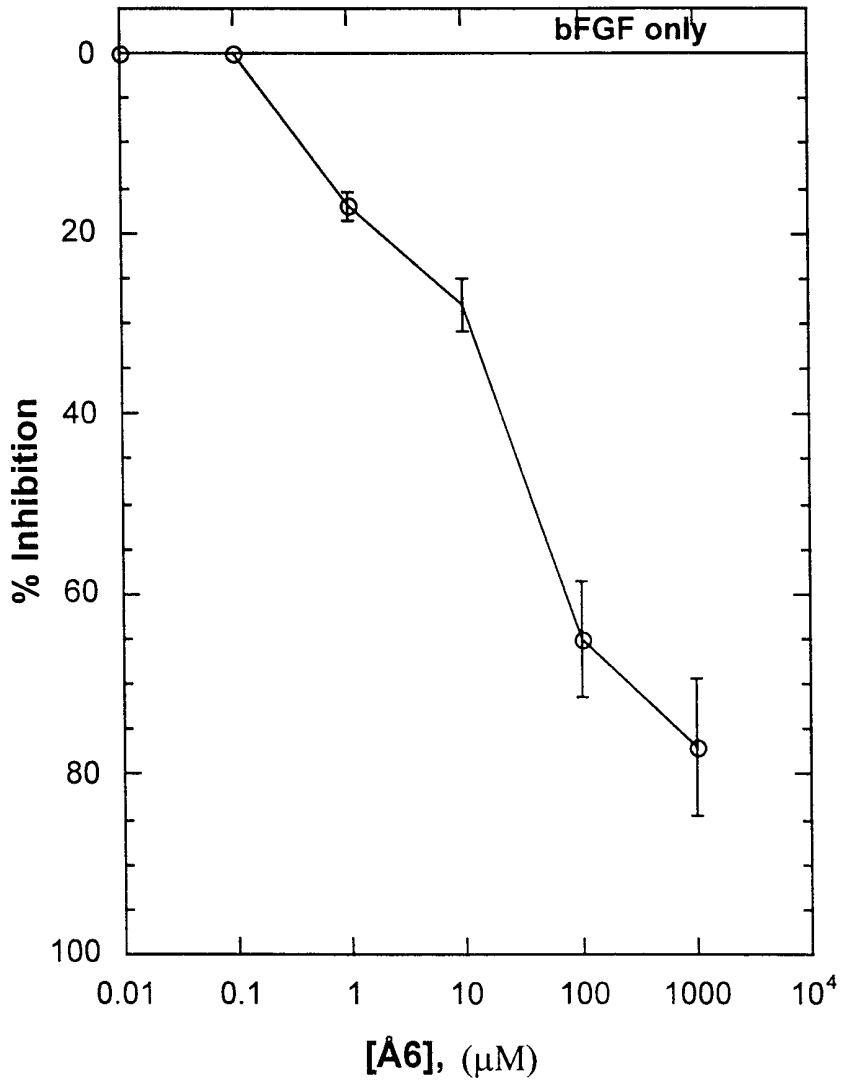
bFGF only
0
20
40
60
80
100
% Inhibition
0.01
0.1
1
10
100
1000
10^4
[Å6], (μM)

FIG. 5

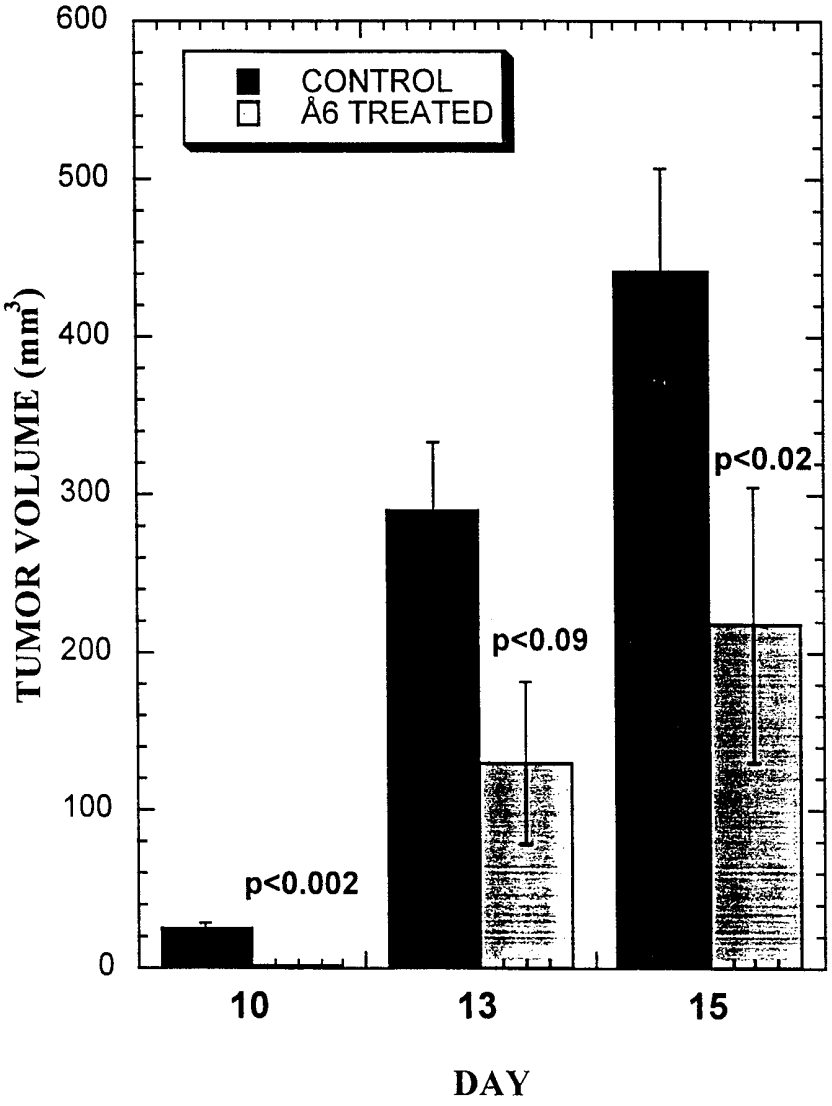
600
500
400
300
200
100
0
TUMOR VOLUME (mm3)
CONTROL
Å6 TREATED
p<0.002
p<0.09
p<0.02
10
13
15
DAY

FIG. 6

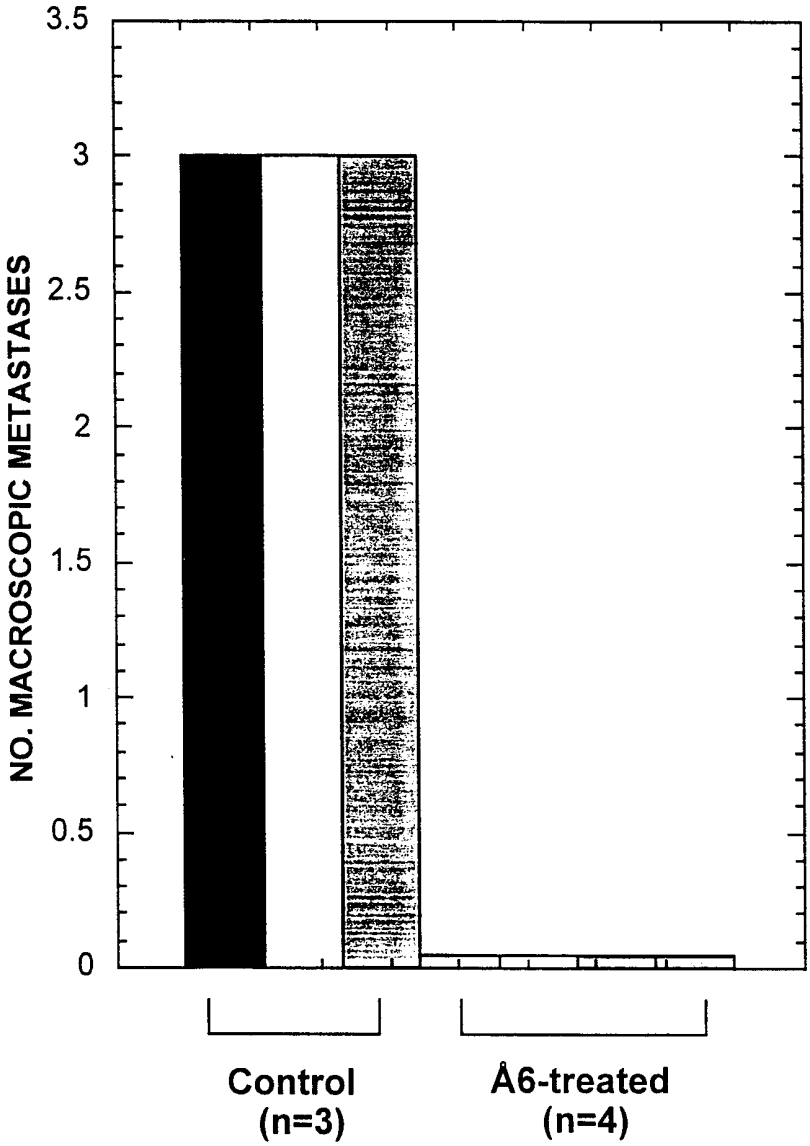
3.5
3
2.5
2
1.5
1
0.5
0
NO. MACROSCOPIC METASTASES
Control
(n=3)
Å6-treated
(n=4)

FIG. 7

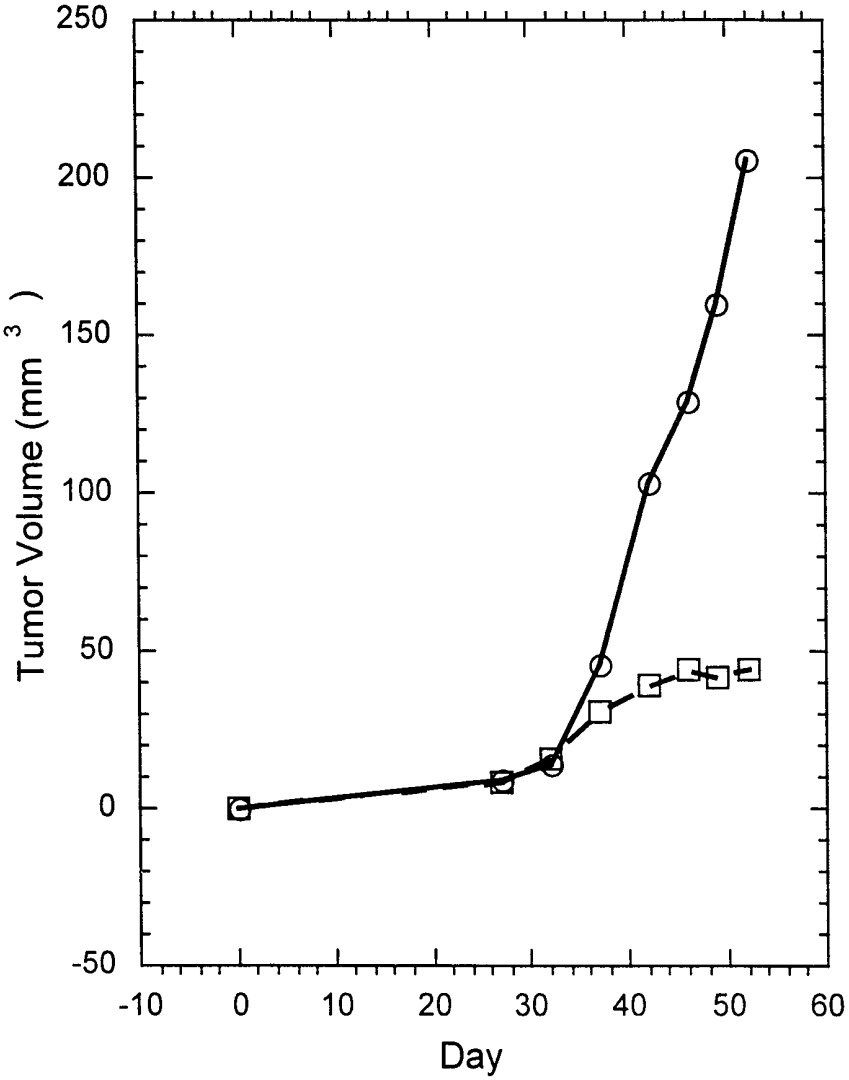

FIG. 8

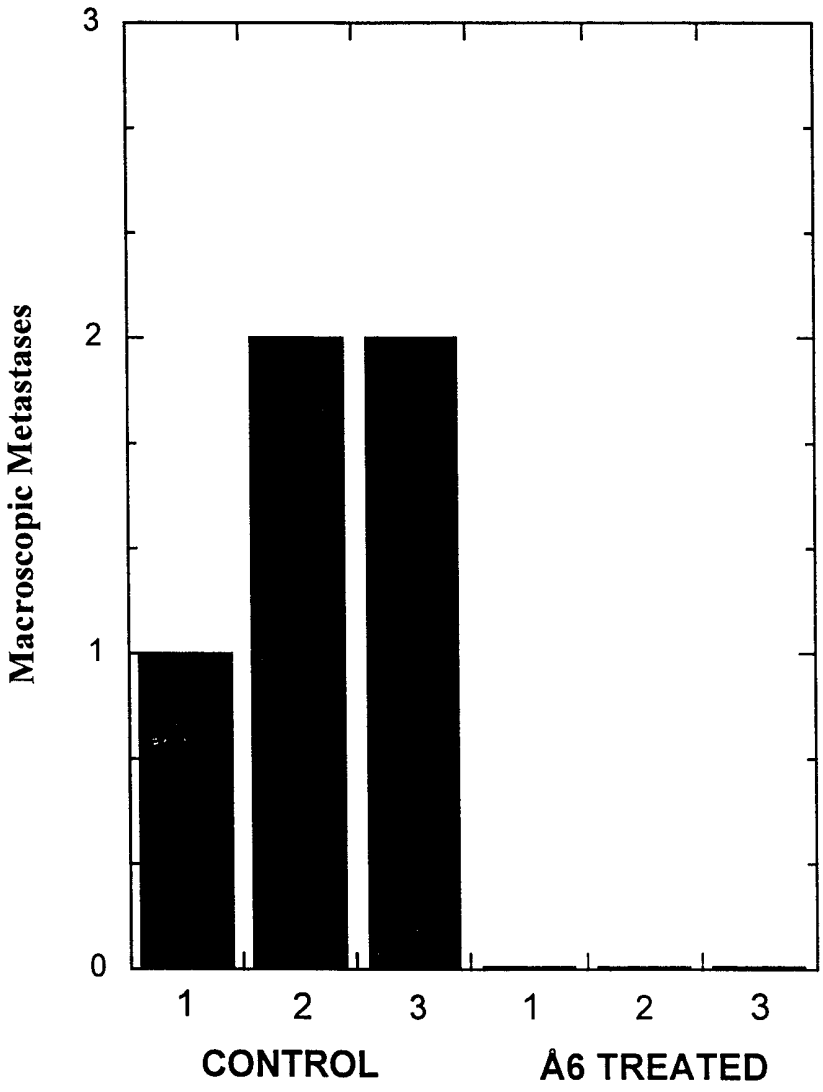

FIG. 9

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/15437

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/72 A61K38/49

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25422 A (NISSIN FOOD PRODUCTS LTD ;KOBAYASHI HIROSHI (JP); TERAO TOSHIHIKO) 17 July 1997 See abstract and claims 4 and 6 ---	1,6,11, 16,17, 22,23
X	WO 90 12872 A (BLASI FRANCESCO ;STOPPELLI MARIA PATRIZIA (IT); MASTRONICOLA MARIA) 1 November 1990 see table 5 ---	1
	-/--	

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1998	17/11/1998

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

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

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/15437

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	SMITH E.A.: "Peptides, chemistry and biology, Proceedings 12th APS, 1991 Cambridge" 1992 , ESCOM , LEIDEN XP002083073 Liu e.a.: The inhibition of fibrin stimulated t_PA-induced plasminogen activation by the A chain fragment 149-157 of urokinase; see pages 810-811, esp. table 1 ---	1
A	WO 97 05257 A (CHIRON CORPORATION) 13 February 1997 see the whole document ---	1-23
A	WO 94 28014 A (CHIRON CORPORATION) 8 December 1994 see the whole document -----	1-23

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 15437

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

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

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 11-23 encompass a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

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

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

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

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

Remark on Protest

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

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.:
3,5,8,10,13,15,19,21 (complete), 1,2,4,6,7,9,11,12,14,16-18,20,22,23 (all partially)

The scope of the claims 1,2 and 4 is unclear and speculative. Due to the use of expressions like "substitution variant", "addition variant" and "chemical derivative" (claim 1) and "peptidomimetic agent" (claim 4) said claims lack any structually constant entity or even any indication concerning the (minimal) size of the peptide. Hence the claims cannot be considered to represent a clear and concise definition of patentable subject-matter (Art.6 PCT).
Therefore a meaningful and economically feasible search could not encompass the complete subject-matter of said claims. Consequently the search has been directed to the compounds encompassed by the claims 3 and 5 and their use (Art.17(2)(a)(ii) and (b) PCT, PCT Guidelines CIII,2.1 and CIII,3,7).

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 98/15437

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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