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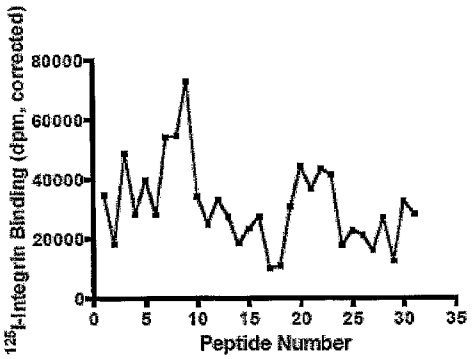
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(54) **Title:** ANGIO-INHIBITORY PEPTIDES DERIVED FROM HUMAN TIMP-2



(57) **Abstract:** The invention provides proteins and polypeptides with anti-angiogenic activity. The proteins may be prepared by recombinant or other synthetic means and may be formulated in compositions as needed to facilitate their use. The invention further provides methods of inhibiting or reducing angiogenesis as well as the treatment of angiogenesis mediated diseases.

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ANGIO-INHIBITORY PEPTIDES DERIVED FROM HUMAN TIMP-2

RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Patent Application 60/728,146, filed October 18, 2005, which is hereby incorporated in its entirety as if fully set forth.

FIELD OF THE INVENTION

This invention relates to proteins and polypeptides with inhibitory activity against angiogenesis. The proteins of the invention may be prepared by recombinant or other synthetic means and may be formulated in compositions as needed to facilitate their use. The invention further relates to methods of inhibiting angiogenesis and the treatment of angiogenesis mediated diseases.

BACKGROUND OF THE INVENTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases the activity of which have been implicated in tumor cell invasion, metastasis formation, and angiogenesis. Endogenous protease inhibitors, referred to as tissue inhibitor of metalloproteinases (TIMPs) have been previously identified, with four recognized members in the mammalian TIMP family: TIMP-1, -2, -3, and -4). See for example Stetler-Stevenson *et al.* J. Biol. Chem. 264:17374, 1989; Stetler-Stevenson *et al.* J. Biol. Chem. 265:13933, 1990; Stetler-Stevenson *et al.* J. Biol. Chem. 264:17374, 1989; and Wingfield *et al.* J. Biol. Chem. 274:21362, 1999. Wingfield *et al.* describe the recombinant expression of TIMP-2.

Recently, Seo *et al.* demonstrated that the natural human protein tissue inhibitor of metalloproteinases-2 (TIMP-2) inhibits angiogenesis in vivo by an activity that is independent of metalloproteinase inhibition (see "TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism" Cell 114:171-180, 2003). The mechanism of TIMP-2 anti-angiogenic activity was shown to be mediated by TIMP-2 binding to the $\alpha 3\beta 1$ integrin receptor to result in activation of protein tyrosine phosphatase

activity. That work was the first demonstration of a direct interaction between a member of the TIMP family and an integrin.

TIMP-2 may also function via inhibition of MMPs (see Murphy *et al.* J. Cell. Physiol. 157:351, 1993) and by a MMP independent mechanism (see Hoegy *et al.* J. Biol. Chem. 276:3203, 2001).

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.

BRIEF SUMMARY OF THE INVENTION

This invention is based in part upon the discovery of proteins (polypeptides) that possess angio-inhibitory, or anti-angiogenic, activity. The proteins of the invention contain or are based on sequences present in human TIMP-2 and may be advantageously used as angio-inhibitory agents.

In a first aspect, the invention provides a protein, or protein mimetic, with anti-angiogenic activity. In some embodiments, a protein, or protein mimetic, of the invention contains a sequence selected from QIKMFKGPEKDI (SEQ ID NO:1); DIEFIYTAPSSAV (SEQ ID NO:2); or GVSLDVGGK (SEQ ID NO:3) in the alternative. The invention also provides proteins, or protein mimetics, containing only one of these three sequences without additional amino acid residues at either the N- or C- terminus of the sequence.

In additional embodiments, the invention provides a protein, or protein mimetic, containing a combination of the above three sequences, such as, but not limited to, a protein, or protein mimetic, containing the sequence KRIQYEIKQIKMFKGPEKDIEFIYTAPSSAVCGVSLDVGGK (SEQ ID NO:7).

In other embodiments, the invention provides a protein, or protein mimetic, containing a sequence selected from IQYEIKQIKMFKGPEKDI (SEQ ID NO:4), QIKMFKGPEKDIEFIYTA (SEQ ID NO:5), or DIEFIYTAPSSAVCGVSLDVGGK (SEQ ID NO:6) where the first two contain SEQ ID NO:1 and the third contains both SEQ ID NOs: 2 and 3.

Proteins, or protein mimetics, of the invention may also contain portions or fragments of contiguous amino acids found in SEQ ID NOs: 1-7. With respect to SEQ ID NOs: 4-7, the protein, or protein mimetic, would retain the presence of one or more of SEQ ID NOs: 1-3.

The proteins, or protein mimetics, of the invention may be conventional, such that they are linear and composed of L-amino acids, or non-conventional. Non-limiting examples of non-conventional proteins, or protein mimetics, include those that are non-linear and/or contain at least one D-amino acid. Thus in some embodiments, the proteins, or protein mimetics, of the invention are cyclic in structure such that the two ends, such as the N- and C- termini, are directly or indirectly linked to form a circular polypeptide, or mimetic thereof. Circular embodiments of the invention may also contain at least one D-amino acid.

In another aspect, the invention provides methods of preparing a protein, or protein mimetic, of the invention. In some embodiments, a method of preparation is synthetic such that it is not based upon the derivatization of a larger, or equally sized, naturally occurring molecule. Such methods include, but are not limited to, *de novo* synthesis or recombinant production of a protein, or protein mimetic, of the invention.

In embodiments involving recombinant production, a nucleic acid sequence encoding a protein of the invention is provided and may be used to express the protein via a cell based or cell-free system followed by isolation and/or purification of the protein from other components present in the system. A non-limiting example of a cell based expression system is the use of an expression vector containing the protein coding sequence in a cell. Alternatively, a protein coding sequence may be under the control of an appropriate regulatory sequence which directs expression of the coding sequence in a cell-free system.

In an additional aspect of the invention, a composition comprising a protein, or protein mimetic, of the invention is provided. In some embodiments, the composition is a pharmaceutical composition, such as one containing at least one pharmaceutically acceptable excipient or at least one agent to facilitate the therapeutic use of the composition. In other embodiments, the composition may simply be a sterile formulation containing the protein, or protein mimetic.

In a further aspect, the invention provides a method of forming a ligand-receptor complex containing a protein, or protein mimetic, of the invention (as the ligand) and $\alpha 3\beta 1$ integrin (as the receptor). In some embodiments, the integrin is human $\alpha 3\beta 1$

integrin. This aspect of the invention is based upon the observation that proteins of the invention are capable of directly binding to α3β1 integrin as well as competing for α3β1 integrin binding. Without being bound by theory, and offered to improve the understanding of the invention, the binding of a protein, or protein mimetic, of the invention to α3β1 integrin to form a complex is believed to mediate the anti-angiogenic activity of the protein, or protein mimetic.

In some embodiments, the formation of a ligand-receptor complex of the invention occurs on the surface of a cell, such as a human cell, expressing α3β1 integrin to mediate RTK inactivation, Rap1 activation, RECK induction, inhibition of migration, induction of p27 synthesis, nuclear localization of p27, and/or G1 cell cycle arrest or inhibition of proliferation or growth in the cell. The cell may of course be in an *in vivo* context, such that formation of the complex mediates the inhibition or reduction of angiogenesis. In other embodiments, the formation of a complex occurs *in vitro*, such as in cases of detecting the presence of α3β1 integrin by use of a protein, or protein mimetic, of the invention.

In yet another aspect, the invention provides a method of inhibiting or reducing angiogenesis in a subject by administration of a protein, or protein mimetic, of the invention, optionally as present in a composition of the invention, to the subject. The amount of protein, or mimetic, administered would of course be sufficient, or effective, to result in an inhibition or reduction of angiogenesis in the subject. In some embodiments, the subject is a human being who has been determined to be in need of an inhibition or reduction in angiogenesis. Such determinations may be made by skilled practitioners, such as a skilled clinician, provided with the instant invention.

The invention further provides for a method of treating an angiogenesis-mediated disease in a subject by administration of a protein, or protein mimetic, of the invention to the subject. Non-limiting examples of an angiogenesis mediated disease include chronic diseases and angioproliferative diseases. The amount of protein, or mimetic, administered would of course be sufficient, or effective, to result in efficacious treatment of the subject, such as to inhibit or reduce the amount and/or severity of angiogenesis or a disease symptom in the subject. In some embodiments, the subject is a human being who has been diagnosed as having an angiogenesis mediated disease. Such determinations may be made by skilled practitioners, such as a skilled clinician, provided with the instant invention.

In a further aspect, the invention provides for a method in which formation of a complex comprising a peptide or mimetic of the invention and an α3β1 integrin is used to screen for compounds that compete for peptide or mimetic binding to the integrin. In some embodiments, the integrin is human α3β1 integrin. The compounds may be all or part of a library of synthetic and/or naturally occurring compounds. In some embodiments, the compound may be considered a "test" compound, such as one suspected of being able to bind α3β1 integrin or optionally one designed to bind α3β1 integrin.

The compounds identified by such methods are may be modulators of α3β1 integrin activity or function. In some embodiments, an identified compound may be either an agonist or antagonist of α3β1 integrin activation and/or function to inhibit or promote angiogenesis. Generally, an agonist is a compound that binds a receptor (forming a ligand-receptor complex) which elicits the complete or full physiological activity of the receptor. An antagonist is generally considered a compound that binds a receptor (forming a ligand-receptor complex) which does not elicit any receptor response, such that the receptor behaves as if it were unoccupied.

Alternatively, the identified compounds may be an inverse agonist or partial agonist. Generally an inverse agonist is possible in cases of a receptor which produces some basic physiological (e.g. signaling) activity even the absence of an agonist. Thus the receptor may be considered as having "constitutive activity" such that an agent which binds the receptor and suppresses this activity to some degree is an inverse agonist. A partial agonist is generally a compound which binds or otherwise interacts with a receptor, but only elicits a small degree of the physiological response of the receptor in comparison to a "full" agonist. The reduced response is seen even if a large number (or high proportion) of the receptors are occupied by a partial agonist.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the drawings and detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the results of a peptide array analysis for TIMP-2 peptides that bind radiolabeled α3β1 integrin.

Figure 2 illustrates the inhibition of α3β1 integrin binding to TIMP-2 in the presence of various peptides. N-term TIMP-2 denotes a recombinant human TIMP-2 containing only the amino-terminal domain (lacking the C-terminal domain). Peptide 8, Peptide 8-9, Peptide 9, and P647 (Peptide 647) are described in the examples below. Peptide 647 SCR denotes a control peptide with the same amino acids as P647 except that the position of the amino acid residues in the peptide are randomly assigned such that although 647 SCR has the same overall amino acid composition, it does not have the same sequence. Loop 6 refers to a peptide comprising the sequence between about position 145 and 166 of TIMP-2.

Figure 3 illustrates the ability of peptides of the invention to inhibit VEGF-A mediated HMVEC cell proliferation. AT2 denotes the Ala+TIMP-2 mutant while NT2 denotes a recombinant human TIMP-2 containing only the amino-terminal domain (lacking C-terminal domain). The tested peptides are individually referred to as "T2 peptide" to indicate their origin from TIMP-2. Peptides 8, 8-9, 9, P647, P663, and p665 are described in the examples below. Loop 6 is as described for Figure 2, and loop-6-4 peptide is the disulfide bonded form of loop 6 peptide wherein the sequence is the same except the peptide has been oxidized to form the disulfide between the two cysteine residues.

Figure 4 illustrates the ability of peptides of the invention to inhibit FGF-A (or basic FGF) mediated HMVEC cell proliferation. The abbreviations are as described for Figure 3.

Figure 5 illustrates the ability of peptides of the invention to inhibit VEGF-A stimulated angiogenesis *in vivo*. The assay utilized is the directed *in vivo* angiogenesis assay (DIVAa).

DETAILED DESCRIPTION OF MODES OF PRACTICING THE INVENTION

This invention provides proteins, or protein mimetics, which may be used for their angio-inhibitory/anti-angiogenic activity and/or their binding to α3β1 integrin. As used herein, the term "protein" refers to polypeptides and peptides composed of amino acids linked by peptide bonds (linkages). The terms protein, polypeptide, and peptide are used interchangeably herein unless otherwise indicated. In some embodiments, a polypeptide of the invention may be composed of only L-amino acids or may contain at least one D-amino acid, up to the entire content being composed of D-amino acids. Of course such a

polypeptide of the invention would retain a functional activity as described herein. In other embodiments, a polypeptide of the invention may also include the presence of one or more related organic acids, such as, but not limited to, para-aminobenzoic acid (PABA).

Peptides of the invention are optionally acetylated at the amino terminal group. Similarly, peptides may include a carboxamide amino acid at the C-terminal. Thus, peptides which have the sequences described herein, but which have been modified to include an amino-terminal N-acyl or aryl group and/or a carboxyl-terminal amide or alkyl amide group are also included in the present invention.

The term "protein mimetic" refers to molecular analogs used as non-peptide agents with properties analogous to that of a template protein (or polypeptide or peptide). See for example, Fauchere, J. (1986) Adv. Drug Res. 15:29; and Evans et al. (1987) J. Med. Chem. 30:1229). Given the equivalence of protein and peptide, other equivalent and interchangeable terms for protein mimetic include, but are not limited to, "peptide mimetic", "peptide equivalent", "peptide analog", and "peptidomimetic". A peptide mimetic of the invention may be developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent functional effect, including a therapeutic or prophylactic effect.

A peptide mimetic is structurally similar to an active (biologically, chemically, or pharmacologically) polypeptide of the invention as provided herein but has one or more peptide bonds replaced by a linkage selected from -CH_2NH-, -CH_2S-, -CH_2CH_2-, -CH=CH- (*cis* and *trans*), -$COCH_2$-, -$CH(OH)CH_2$-, and-CH_2SO-, by means known in the art. See for example, Spatola, A. F. in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3,"Peptide Backbone Modifications" (general review); Morley, J.S., Trends Pharm. Sci. (1980) pp. 463-468 (general review); Hudson, D. et al., Int J Pent Prot Res (1979) 14:177- 185 (-CH_2NH-, -CH_2CH_2-); Spatola, A. F. et al., Life Sci. (1986) 38: 1243-1249 (-CH_2S-); Hann, M. M., J. Chem. Soc. Perkin Trans. (1982) 307-314 (-CH=CH-, *cis* and *trans*); Almquist, R. G. et al., J. Med. Chem. (1980) 23: 1392-1398 (-$COCH_2$-); Jennings-White, C. et al., Tetrahedron Lett. (1982) 23: 2533 (-$COCH_2$-); Szelke, M. et al., European Patent Application EP 45665 (1982) Chem. Abstr: 97: 39405 (1982) (-$CH(OH)CH_2$-); Holladay, M. W. et al., Tetrahedron Lett. (1983) 24: 4401-4404 (-$C(OH)CH_2$-); and Hruby, V. J., Life Sci. (1982) 31: 189-199 (-CH_2S-). All or part of a peptide's backbone may also be replaced by conformationally constrained cyclic alkyl or aryl substituents to restrict mobility of amino

acid side chains as specified in the sequences herein. See for example, Bondinell et al.. "Design of a potent and orally active nonpeptide platelet fibrinogen receptor (GPIIb/IIIa) antagonist." Bioorg. Med. Chem. 2:897 (1994); Keenan et al. "Discovery of potent nonpeptide vitronectin receptor ($\alpha v\beta 3$) antagonists." J. Med. Chem. 40:2289 (1997); and Samanen et al. "Potent, selective, orally active3-oxo-1,4-benzodiazepine GPIIb/IIIa integrin antagonists." J. Med. Chem. 39:4867 (1996).

In one aspect, the invention provides a protein comprising a sequence selected from QIKMFKGPEKDI (SEQ ID NO:1); DIEFIYTAPSSAV (SEQ ID NO:2); or GVSLDVGGK (SEQ ID NO:3). In another aspect, the invention provides a mimetic of such proteins. In some embodiments, the protein or mimetic thereof has a length of less than 48 amino acid residues. Thus a protein of 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, or 13 residues comprising any one of SEQ ID NOs: 1-3; a protein of 12 residues comprising SEQ ID NO: 1 or 3; and a protein of 11, 10, or 9 residues comprising SEQ ID NO:3 are provided by the invention. Thus proteins of less than about 45, less than about 40, less than about 35, less than about 30, less than about 25, less than about 20, and less than about 15 amino acids are provided by the invention.

In some embodiments, such proteins of the invention are, or comprise, SEQ ID NO: 4, 5, or 6 in the alternative. In other embodiments, such proteins are, or comprise, all or part of SEQ ID NO:7. Such embodiments include fragments or portions of SEQ ID NO:7 comprising a contiguous sequence including any one or more of SEQ ID NOs: 1-6. Thus a protein of the invention may comprise positions 9-20 of SEQ ID NO:7 (which is the sequence of SEQ ID NO:1) attached to i) 1, 2, 3, 4, 5, 6, 7, or all 8 N-terminal residues, as found in positions 1-8 of SEQ ID NO:7 to result in the sequences of positions 8-20, 7-20, 6-20, 5-20, 4-20, 3-20, 2-20, or 1-20 of SEQ ID NO:7 (which extend the sequence of positions 9-20 of SEQ ID NO:7 at the N-terminus); or ii) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or all 21 C-terminal residues, as found in positions 21-41 of SEQ ID NO:7 to result in the sequences of positions 9-21, 9-22, 9-23, 9-24, 9-25, 9-26, 9-27, 9-28, 9-29, 9-30, 9-31, 9-32, 9-33, 9-34, 9-35, 9-36, 9-37, 9-38, 9-39, 9-40, or 9-41 of SEQ ID NO:7 (which extend the sequence of positions 9-20 of SEQ ID NO:7 at the C-terminus).

A non-limiting example of i) as described herein is a peptide represented by SEQ ID NO:4, while a non-limiting example of ii) as described herein is a peptide

represented by SEQ ID NO:5. Of course the invention also provides any combination of both i) and ii) such that positions 9-20 of SEQ ID NO:7 are extended from 1-8 residues at the N-terminus as described above in combination with being extended from 1-21 residues at the C-terminus as described above. This provides for each of a plurality of proteins containing the sequence of positions 9-20 of SEQ ID NO:7 and ranging from 12 to 41 amino acids long.

Other fragments or portions of SEQ ID NO:7 include, but are not limited to, proteins comprising positions 19-31 of SEQ ID NO:7 (which is the sequence of SEQ ID NO:2) attached to i) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or all 18 N-terminal residues, as found in positions 1-18 of SEQ ID NO:7 to result in the sequences of positions 18-31, 17-31, 16-31, 15-31, 14-31, 13-31, 12-31, 11-31, 10-31, 9-31, 8-31, 7-31, 6-31, 5-31, 4-31, 3-31, 2-31, or 1-31 of SEQ ID NO:7 (which extend the sequence of positions 19-31 of SEQ ID NO:7 at the N-terminus); or ii) 1, 2, 3, 4, 5, 6, 7, 8, 9, or all 10 C-terminal residues, as found in positions 32-41 of SEQ ID NO:7 to result in the sequences of positions 19-32, 19-33, 19-34, 19-35, 19-36, 19-37, 19-38, 19-39, 19-40, or 19-41 of SEQ ID NO:7 (which extend the sequence of positions 19-31 of SEQ ID NO:7 at the C-terminus). Again, the invention provides any combination of both i) and ii) such that positions 19-31 of SEQ ID NO:7 are extended from 1-18 residues at the N-terminus as described above in combination with being extended from 1-10 residues at the C-terminus as described above. This provides for each of a plurality of proteins containing the sequence of positions 19-31 of SEQ ID NO:7 and ranging from 13 to 41 amino acids long.

Moreover, the invention provides for each of a plurality of proteins containing the sequence of positions 33-41 of SEQ ID NO:7 (which is the sequence of SEQ ID NO:3) attached to from 1 to all 32 of the N-terminal residues, as found in positions 1-32 of SEQ ID NO:7 to result in the sequences of positions 32-41, 31-41, 30-41, 29-41, 28-41, 27-41, 26-41, 25-41, 24-41, 23-41, 22-41, 21-41, 20-41, 19-41, 18-41, 17-41, 16-41, 15-41, 14-41, 13-41, 12-41, 11-41, 10-41, 9-41, 8-41, 7-41, 6-41, 5-41, 4-41, 3-41, 2-41, or 1-41 of SEQ ID NO:7 (which extend the sequence of positions 33-41 of SEQ ID NO:7 at the N-terminus). A non-limiting example is a protein comprising the sequence of SEQ ID NO:6.

The peptides of this invention may be produced by recognized methods, such as recombinant and synthetic methods that are well known in the art. Recombinant techniques are generally described in Sambrook, et al., Molecular Cloning: A Laboratory Manual, (2nd ed.) Vols. 1-3, Cold Spring Harbor Laboratory, (1989). Techniques for the synthesis of peptides are well known and include those described in Merrifield, J. Amer.

Chem. Soc. 85: 2149-2456 (1963), Atherton, et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press (1989), and Merrifield, Science 232: 341-347 (1986).

In other embodiments, the invention provides a protein comprising a sequence selected from QIKMFKGPEKDI (SEQ ID NO:1), with a deletion of from 1 to 4 amino acid residues from the N or C terminus in the alternative; DIEFIYTAPSSAV (SEQ ID NO:2), with a deletion of from 1 to 5 amino acid residues from the N or C terminus in the alternative; or GVSLDVGGK (SEQ ID NO:3), with a deletion of from 1 to 3 amino acid residues from the N or C terminus in the alternative. Such a protein, however, has a length of less than 48 amino acid residues and retains the angio-inhibitory/anti-angiogenic activity and/or $\alpha 3\beta 1$ integrin binding activity as described herein.

In yet additional embodiments, the invention provides a protein comprising a sequence selected from a derivative of QIKMFKGPEKDI (SEQ ID NO:1) wherein at least one residue of SEQ ID NO:1 is conservatively substituted; a derivative of DIEFIYTAPSSAV (SEQ ID NO:2) wherein at least one residue of SEQ ID NO:2 is conservatively substituted; or a derivative of GVSLDVGGK (SEQ ID NO:3) wherein at least one residue of SEQ ID NO:3 is conservatively substituted. Such a protein, however, retains the angio-inhibitory/anti-angiogenic activity and/or $\alpha 3\beta 1$ integrin binding activity as described herein.

As referred to herein, amino acid residues are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or 1; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H;Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G. As used herein, conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. Thus amino acid side chains may be grouped such that one may substitute for another as a "conservative" substitution. Non-limiting examples include, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side

chains is cysteine and methionine. In some embodiments of the invention, conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine.

With respect to mimetics, a protein of the invention may contain one or more D-amino acids and/or be circular in structure. So in some embodiments, a protein of the invention may contain one or more D-amino acid residue up to a protein comprised of all D-amino acid residues. Thus one or more of the residues in any protein of the invention, including those discussed above, may contain one or more D-amino acid residues to form a mimetic of the invention. In other embodiments, the mimetic is a circularized form of a protein of the invention. A circular protein may, of course, optionally contain one or more D-amino acid residues.

The choice of including an (L)-or a (D)-amino acid into a peptide of the present invention depends, in part, on the desired characteristics of the peptide. For example, the incorporation of one or more (D)-amino acids can confer increasing stability on the peptide *in vitro* or *in vivo*. In some cases it is desirable to design a peptide which retains activity for a short period of time, for example, when designing a peptide to administer to a subject. In these cases, the incorporation of one or more (L)-amino acids in the peptide can allow endogenous peptidases in the subject to digest the peptide *in vivo*, thereby limiting the subject's exposure to an active peptide.

Peptides and mimetics of the invention may be in a "substantially pure," or "isolated" form, which refers separation from proteins or other contaminants with which they are naturally associated or with which they are associated during synthesis. In some embodiments, a peptide or mimetic makes up at least about 50% of the total polypeptide content (by weight or number) of the composition containing the peptide. In other embodiments, the peptide or mimetic makes up at least about 60%, at least about 75%, at least about 90%, or at least about 95% of the total polypeptide content (by weight or number).

A peptide or mimetic may be prepared under sterile, aseptic, or antiseptic conditions. Alternatively, a peptide or mimetic may be sterilized by, e. g., using heat, filtration, irradiation, or other means. A peptide or mimetic optionally may be in the form of a salt or stored or alternatively used in solid form (e.g., as a powder, such as a lyophilized powder, as non-limiting examples). Additional alternatives include preparation as a sterile solution (e.g., a sterile aqueous solution, such as a buffered aqueous solution, as non-limiting examples.

The present invention also provides a pharmaceutical composition comprising a peptide or mimetic as described herein. The composition may be formulated to contain a pharmaceutically acceptable excipient or carrier in some embodiments. Of course it remains possible to administer a peptide or mimetic of the invention alone, rather than in the form of a pharmaceutical formulation in some cases.

Pharmaceutically acceptable carriers typically include carriers known to those of skill in the art, including pharmaceutical adjuvants. Generally these pharmaceutically acceptable carriers will include water, saline, Ringers solution, Ringer's lactate, 5% dextrose, buffers, and other compounds described, e. g., in the MERCK INDEX, Merck & Co., Rahway, NJ. See, also, Bioreversible Carriers in Drug Design, Theory and Application, Roche (ed.), Pergamon Press, (1987). The peptides may be mixed with a variety of carrier compounds depending on the form of preparation desired for administration.

These formulations typically comprise the pharmacological agent (i. e., the peptide) in a therapeutically or pharmaceutically effective dose together with one or more pharmaceutically or therapeutically acceptable carriers and optionally other therapeutic ingredients. Various considerations are described, e. g., in Gilman et al. (eds) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; Novel Drug Delivery Systems, 2nd Ed., Norris (ed.) Marcel Dekker Inc. (1989), and Remington's Pharmaceutical Sciences. Methods for administration are also discussed therein. In particular, the pharmaceutical compositions of the invention may be administered intravenously, subcutaneously, orally, transdermally, intramuscularly, topically (e. g., by intravascular injection into vessels infiltrating a tumor or tumor metastasis), or by intracavity or peristaltic administration.

The compositions may also be formulated for delayed release or alternatively for rapid release. Other compositions of the invention include those for treatment of ocular conditions, wherein the composition is suitably formulated for administration to the eye. Non-limiting examples include formulations with viscosities suitable for ocular use or for intravitreous injection.

The term "effective amount" or "effective dose" as used herein typically refers to the amount of the active ingredient, e.g. a peptide or mimetic of the invention, which is required to achieve the desired activity or result. As a non-limiting example in therapeutic applications, an effective amount will be the amount required to be administered to a patient to result in treatment of the particular disorder for which treatment is sought.

The term "treatment of a disorder" denotes the reduction or elimination of symptoms of a particular disorder. Effective amounts may be readily determined by the skilled person depending upon the nature of the disorder, the peptides used, the mode of administration, and the size and health of the patient.

In one embodiment, the effective amount of a peptide or mimetic of the invention ranges from about 1 μg to about 1 g of peptide for a 70 kg patient, and another embodiment, from about 1 pg to about 10 mg. In additional embodiments, the concentration of peptide (or mimetic) administered ranges from about 0.1 μM to about 10 mM, or from about 0.5 μM to 1 mM, or from about 5 μM to 100 μM.

The invention further provides a method of forming a ligand-receptor complex comprising a peptide or mimetic of the invention. Such a method may comprise contacting a protein or mimetic of the invention (as a ligand) with α3β1 integrin (as the receptor) under conditions that allow the formation of a ligand-receptor complex comprising at least these two components. The formation of the complex may optionally be in the presence of another ligand for the α3β1 integrin such that the peptide or mimetic competes for binding to the integrin. In some embodiments, the peptide or mimetic out competes binding by another ligand or ligands by at least 5 fold, optionally at least 10 fold, at least 50 fold, or at least 100 fold.

In other embodiments of the invention, combinations of peptides or mimetics or combinations of peptide(s) and mimetic(s) may be used in the practice of the invention.

The formation of a ligand-receptor complex comprising a peptide or mimetic of the invention may occur where the α3β1 integrin is present on a cell, optionally a human cell. Complex formation may also occur where the peptide or mimetic is conjugated to a heterologous polypeptide or a non-peptide substrate. The formation of a complex containing a peptide or mimetic of the invention may be used to inhibit or reduce α3β1 integrin binding to another ligand, such as by at least 5 fold, optionally at least 10 fold, at least 50 fold, or at least 100 fold as non-limiting examples.

Formation of the complex in a cellular context may be used to mediate RTK inactivation, Rap1 activation, RECK induction, inhibition of cell migration, induction of p27 synthesis, nuclear localization of p27, and/or G1 cell cycle arrest or inhibition of proliferation or growth in a cell. Non-limiting examples of such cells include an epithelial cell, an endothelial cell, a cancer or tumor cell, or a malignant cell as well as a cell involved in a disease condition as described herein. Inhibition of cell motility may be that of an

endothelial cell, and such inhibition may occur even in the absence of proliferation. As used herein, cell motility refers to the movement or cells across a substrate and can be measured using methods known to the skilled artisan. Inhibitors of cell motility have a variety of uses, such as inhibiting the motility of tumor cells invading surrounding tissue from a primary tumor to prevent tumor metastasis.

In some embodiments, contacting of a peptide or mimetic and an $\alpha 3\beta 1$ integrin to form a complex occurs *in vivo*, such as in a human or in a non-human animal subject as non-limiting examples.

The invention further provides a method of inhibiting or reducing angiogenesis in a subject by use of a protein or mimetic of the invention, optionally as present in a composition of the invention. Such a method may comprise administering a protein or mimetic of the invention to the subject, such as a human or non-human animal subject in need of the protein or mimetic to bind $\alpha 3\beta 1$ integrin or inhibit, or reduce, angiogenesis.

Angiogenesis refers to the generation of new blood vessels into a tissue or organ, a process that involves endothelial cell proliferation. Under normal physiological conditions, humans or non-human animals undergo angiogenesis only in restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development, and formation of the corpus luteum, endometrium and placenta. However, persistent, unregulated angiogenesis occurs in a multiplicity of disease states, including tumor growth, tumor metastasis, and abnormal growth by endothelial cells. Angiogenesis supports the pathological damage seen in these conditions.

The inhibition or reduction of angiogenesis may be used as part of a method to treat an angiogenesis-mediated disease in a subject. Such a method may comprise administering a peptide or mimetic of the invention, optionally as present in a composition of the invention, to the subject. The subject may again be a human patient or a non-human animal subject in need of treatment. Such patients or subjects include those afflicted with an angiogenesis-mediated disease.

Non-limiting examples of an angiogenesis mediated disease include chronic diseases and angioproliferative diseases as well as macular degeneration (dry or wet form), atherosclerosis plaque formation, and restenosis. Non-limiting examples of a chronic disease include psoriasis, rheumatoid arthritis, and cancer (including solid tumor formation, in which case a peptide or mimetic of the invention may be used to induce tumor regression

or inhibit further tumor growth). Non-limiting examples of an angioproliferative disease include retinopathy, vasculopathy, hemangiomas, diabetic retinopathy, and retinopathy of prematurity.

The inhibition of angiogenesis according to the invention is particularly important with respect to cancer because of the important role neovascularization plays in tumor growth. In the absence of neovascularization of tumor tissue, the tumor tissue does not obtain the required nutrients, slows in growth, ceases additional growth, regresses and/or ultimately becomes necrotic, resulting in tumor cell death.

Such treatment methods of the invention may be conducted in combination with one or more other treatment for angiogenesis. Non-limiting examples include therapies such as chemotherapy, gene therapy, and radiotherapy. In some embodiments, the administration of a peptide or mimetic of the invention is conducted during or after other therapeutic intervention, e.g., chemotherapy as a non-limiting example, although it may be preferable to inhibit angiogenesis after a regimen of chemotherapy at times where the treated tissue will be responding to the toxic assault by inducing angiogenesis to recover via the provision of a blood supply and nutrients to the tissue. In addition, the angiogenesis inhibition methods of the invention may be used after surgery, such as in the removal of a solid tumor as a non-limiting example. The methods of the invention may be viewed as a prophylactic, such as a prophylaxis against tumor metastases.

As discussed further below, the peptides of the present invention may be used to inhibit or reduce proliferation of cells expressing $\alpha 3\beta 1$ integrin. The inhibition may be after stimulation with a growth factor such as FGF-2 or VEGF-A as non-limiting examples. Means of measuring cell proliferation are known in the art.

Additional methods provided by the invention include those for identifying a compound as a modulator of $\alpha 3\beta 1$ integrin activation and/or function. Such a method may comprise assaying the compound for the ability to compete with a protein or mimetic of the invention for binding to $\alpha 3\beta 1$ integrin. Alternatively, such a method may comprise assaying the compound for the ability to stabilize or destabilize a complex comprising $\alpha 3\beta 1$ integrin and a peptide or mimetic of the invention.

A method based on competition may comprise contacting $\alpha 3\beta 1$ integrin with a protein or mimetic of the invention as well as a compound under conditions that a) permit formation of a ligand-receptor complex comprising $\alpha 3\beta 1$ integrin and the protein or mimetic and b) permit the compound to compete with said protein or mimetic for binding to

α3β1 integrin. Compounds that compete for binding may be identified as a modulator of α3β1 integrin. In some embodiments, the α3β1 integrin is human.

A method based on stabilizing or destabilizing may comprise forming a ligand-receptor complex comprising a peptide or mimetic of the invention as the ligand and α3β1 integrin as the receptor followed by contacting the complex with a compound. A compound may be identified as either stabilizing the complex or destabilizing the complex. Again, the α3β1 integrin may be human.

In such methods of the invention, the compounds may be all or part of a library of synthetic and/or naturally occurring compounds. The compounds may be used individually or in combinations of more than one. A compound may be optionally labeled to facilitate its detection, especially its detection in a complex with α3β1 integrin. In some embodiments, the compound may be considered a "test" compound, such that use of the compound in a method of the invention identifies it as a modulator, stabilizer, or destabilizer as described herein. Moreover, the α3β1 integrin may be present on the surface of a cell such that binding by a peptide, mimetic, or compound occurs on the surface of the cell. This may be used to advantageously permit identification of a compound as an agonist (full or partial), antagonist, or inverse agonist based on detection of the physiological activity of α3β1 integrin in the cell. In some embodiments, the activity is angiogenesis, or the induction thereof. In other embodiments, the activity is selected from RTK inactivation, Rap1 activation, RECK induction, inhibition of cell migration, induction of p27 synthesis, nuclear localization of p27, and/or G1 cell cycle arrest or inhibition of proliferation or growth in said cell.

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.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Identification of α3β1 binding sites in TIMP-2

Tryptic digests of TIMP-2 were prepared followed by isolation of peptides for screening in biological activity and binding assays. Three peptides, denoted P647, P663, and P665 (represented by SEQ ID NOS:6, 2, and 3, respectively) were identified.

Example 2: Peptide array analysis of α3β1 binding sites in TIMP-2

Peptide arrays were prepared in 96 well plates with various 18-mers derived from the TIMP-2 sequence. The 18-mers had 6 amino acid overlaps and were numbered 1 to 31. The arrays were used to assay binding to α3β1 integrin. The results are shown in Figure 1.

Peptides in the "number 9" region were observed to mediate TIMP-2 binding to α3β1 integrin. Peptide "9" has a sequence represented by SEQ ID NO:5.

Both the tryptic digest and peptide array assays identified a region of TIMP-2 represented by SEQ ID NO:7.

Example 3: Peptides compete for TIMP-2 binding to α3β1

Radiolabeled α3β1 integrin was incubated with various peptides in the presence of TIMP-2 as a competitor. The results are shown in Figure 2 and indicate that Peptides "8" (represented by SEQ ID NO:4), "8-9" (represented by SEQ ID NO:1) and "9" as well as P647 effectively competed with TIMP-2 binding.

Example 4: Peptides inhibit cell growth

The effect of various peptides on cell proliferation was tested with dHMVEC cells that were first starved and then treated for 24 hours as described herein. Wells of a plate were seeded with 3 x 103 cells per well and then treated as described above for Figure 3 (10 ng/ml VEGF-A stimulated growth) and Figure 4 (50 ng/ml FGF-2 stimulated growth).

As shown in Figures 3 and 4, peptides 8, 8-9, and 9 as well as peptides P647, 663 and 665 all inhibited *in vitro* growth of the cells.

Example 5: Peptides inhibit angiogenesis *in vivo*

The effect of various peptides on angiogenesis was assessed generally as described by Guedez et al. ("Quantitative Assessment of Angiogenic Response by the Directed In Vivo Angiogenesis Assay." Am J Pathol. 2003; 162(5):1431-9). Briefly, the angiogenic response of TIMP-2 derived peptides 8, 8-9, and 9 as described herein was measured at concentrations ranging from 0.1 μM to 2.0 μM and in comparison to negative and positive (treatment with 500 ng/ml VEGF-A) controls. The results are shown in Figure 5.

All references cited herein, including patents, patent applications, and publications, are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

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 features hereinbefore set forth.

SEQUENCE LISTING

SEQ ID NO:1
QIKMFKGPEKDI

SEQ ID NO:2
DIEFIYTAPSSAV

SEQ ID NO:3
GVSLDVGGK

SEQ ID NO:4
IQYEIKQIKMFKGPEKDI

SEQ ID NO:5
QIKMFKGPEKDIEFIYTA

SEQ ID NO:6
DIEFIYTAPSSAVCGVSLDVGGK

SEQ ID NO:7
KRIQYEIKQIKMFKGPEKDIEFIYTAPSSAVCGVSLDVGGK

SEQUENCE LISTING

<110> The Government of the United States, As Represented By the Secretary of Health and Human Services
Stetler-Stevenson, William G.
Seo, Dong-Wan

<120> Angio-Inhibitory Peptides Derived From Human TIMP-2

<130> 015280-518001PC

<140> Unknown
<141> 2006-10-18

<150> US 60/728,146
<151> 2005-10-18

<160> 7

<170> PatentIn version 3.1

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<400> 1

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1 5 10

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<211> 13
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<400> 2

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1 5 10

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<400> 3

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1 5

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<400> 5

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Thr Ala

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1 5 10 15

Ser Leu Asp Val Gly Gly Lys
20

<210> 7
<211> 41
<212> PRT
<213> Homo sapiens

<400> 7

Lys Arg Ile Gln Tyr Glu Ile Lys Gln Ile Lys Met Phe Lys Gly Pro
1 5 10 15

Glu Lys Asp Ile Glu Phe Ile Tyr Thr Ala Pro Ser Ser Ala Val Cys
20 25 30

Gly Val Ser Leu Asp Val Gly Gly Lys
35 40

WHAT IS CLAIMED IS:

1. A protein comprising a sequence selected from
QIKMFKGPEKDI (SEQ ID NO:1);
DIEFIYTAPSSAV (SEQ ID NO:2); or
GVSLDVGGK (SEQ ID NO:3);
wherein said protein has a length of less than 48 amino acid residues.

2. The protein of claim 1, having a length of less than 35, less than 25, or less than 15 amino acid residues.

3. The protein of claim 1, comprising the sequence IQYEIKQIKMFKGPEKDI (SEQ ID NO:4) or QIKMFKGPEKDIEFIYTA (SEQ ID NO:5).

4. The protein of claim 1, comprising the sequence DIEFIYTAPSSAVCGVSLDVGGK (SEQ ID NO:6).

5. The protein of claim 1, comprising the sequence KRIQYEIKQIKMFKGPEKDIEFIYTAPSSAVCGVSLDVGGK (SEQ ID NO:7).

6. The protein of any one of claims 1-5, wherein at least one of the amino acid residues is a D-amino acid residue or
wherein said protein is cyclic.

7. A pharmaceutical composition comprising the protein of any one of claims 1-6.

8. The pharmaceutical composition of claim 7, wherein said composition is sterile.

9. A method of forming a ligand-receptor complex, said method comprising
contacting the protein of any one of claims 1-6 as the ligand with $\alpha 3\beta 1$ integrin as the receptor under conditions that allow the formation of a ligand-receptor complex comprising said protein and $\alpha 3\beta 1$ integrin.

10. The method of claim 9 wherein said $\alpha 3\beta 1$ integrin is present on a cell, optionally a human cell.

11. The method of claim 9 wherein said protein is conjugated to a heterologous polypeptide or a non-peptide substrate.

12. The method of claim 9 wherein formation of said complex inhibits or reduces $\alpha 3\beta 1$ integrin binding to another ligand.

13. The method of claim 9, 10, or 12 wherein said contacting occurs *in vivo*.

14. The method of claim 10 wherein formation of said complex mediates RTK inactivation, Rap1 activation, RECK induction, inhibition of cell migration, induction of p27 synthesis, nuclear localization of p27, or G1 cell cycle arrest or inhibition of proliferation or growth in said cell.

15. The method of claim 10 or 14 wherein said cell is an epithelial cell, an endothelial cell, a cancer or tumor cell, or a malignant cell.

16. A method of inhibiting or reducing angiogenesis in a subject, said method comprising administering the protein of any one of claims 1-6 to said subject.

17. A method of treating an angiogenesis-mediated disease in a subject, said method comprising administering the protein of any one of claims 1-6 to said subject.

18. The method of claim 17 wherein said disease is a chronic disease, an angioproliferative disease, macular degeneration (dry or wet form), or atherosclerosis plaque formation.

19. The method of claim 18 wherein said disease is a chronic disease selected from psoriasis, rheumatoid arthritis, or cancer that includes solid tumor formation and said administering induces tumor regression, or

wherein said disease is an angioproliferative disease selected from retinopathy, vasculopathy, hemangiomas, diabetic retinopathy, or retinopathy of prematurity.

20. The method of any one of claims 16-19 wherein said administering occurs in combination with another treatment of angiogenesis, such as chemotherapy, or radiotherapy.

21. A method of identifying a compound as a modulator of α3β1 integrin activation and/or function, said method comprising

contacting the protein of any one of claims 1-6, as the ligand, and α3β1 integrin, as the receptor, in the presence of a compound and under conditions that a) permit formation of a ligand-receptor complex comprising the protein and α3β1 integrin and b) permit the compound to compete with said protein for binding to α3β1 integrin; and

identifying a compound that competes with said protein as a modulator of α3β1 integrin, wherein said α3β1 integrin is optionally human α3β1 integrin.

22. The method of claim 21 wherein said compound is part of a library of synthetic compounds.

23. The method of claim 21 wherein said α3β1 integrin is present on the surface of a cell.

24. The method of claim 21 wherein said compound is identified as an agonist or antagonist of α3β1 integrin.

25. A protein comprising a sequence selected from

QIKMFKGPEKDI (SEQ ID NO:1), with a deletion of from 1 to 4 amino acid residues from the N or C terminus in the alternative;

DIEFIYTAPSSAV (SEQ ID NO:2), with a deletion of from 1 to 5 amino acid residues from the N or C terminus in the alternative; or

GVSLDVGGK (SEQ ID NO:3), with a deletion of from 1 to 3 amino acid residues from the N or C terminus in the alternative;

wherein said protein has a length of less than 48 amino acid residues.

26. A protein comprising a sequence selected from

a derivative of QIKMFKGPEKDI (SEQ ID NO:1) wherein at least one residue of SEQ ID NO:1 is conservatively substituted;

a derivative of DIEFIYTAPSSAV (SEQ ID NO:2) wherein at least one residue of SEQ ID NO:2 is conservatively substituted; or

a derivative of GVSLDVGGK (SEQ ID NO:3) wherein at least one residue of SEQ ID NO:3 is conservatively substituted; and

wherein said protein binds α3β1 integrin.

Figure 1

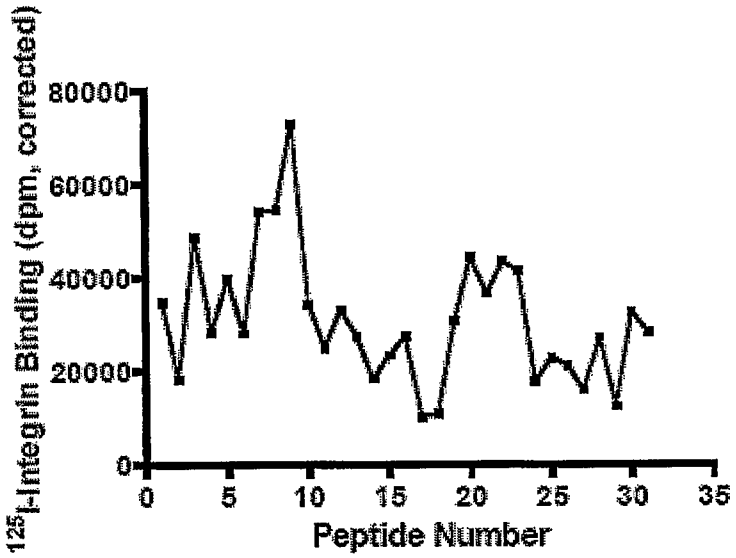


Figure 2

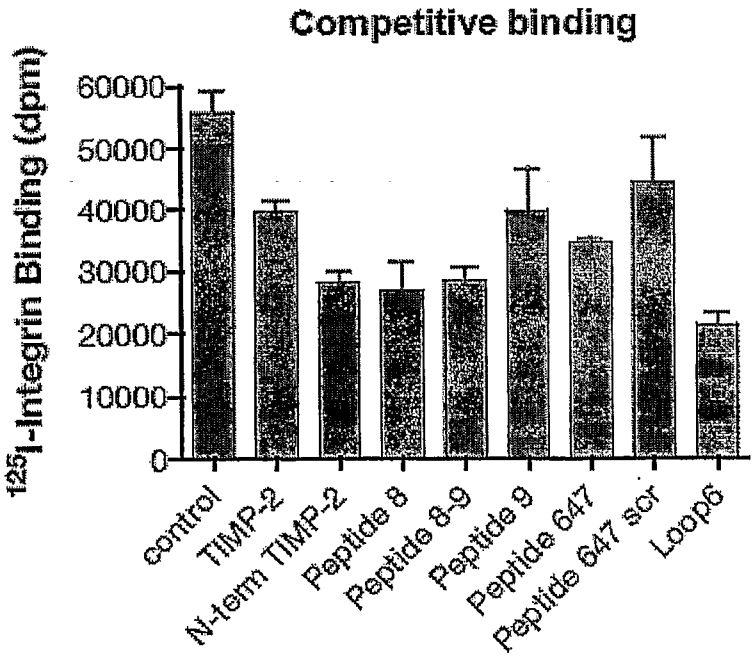


Figure 3

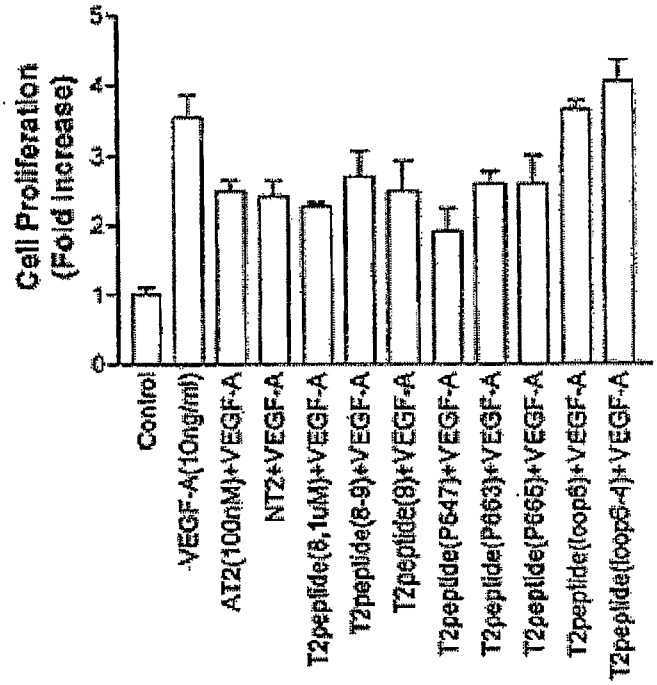


Figure 4

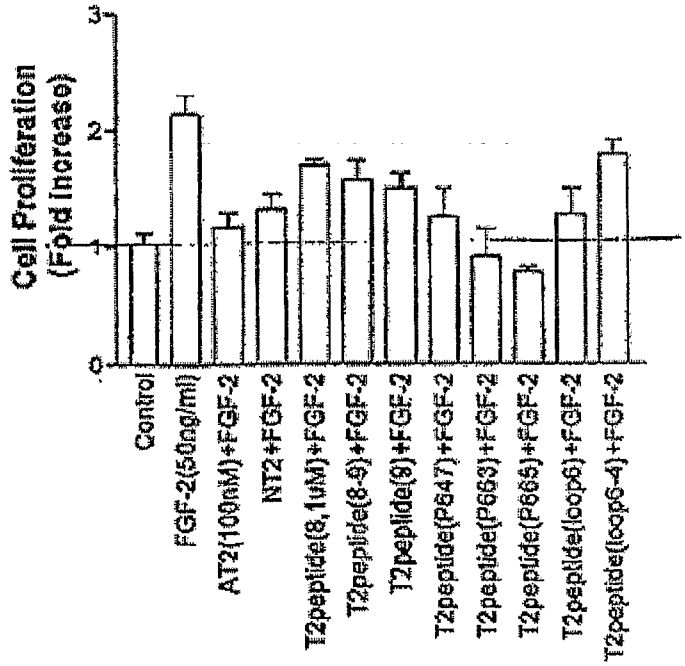
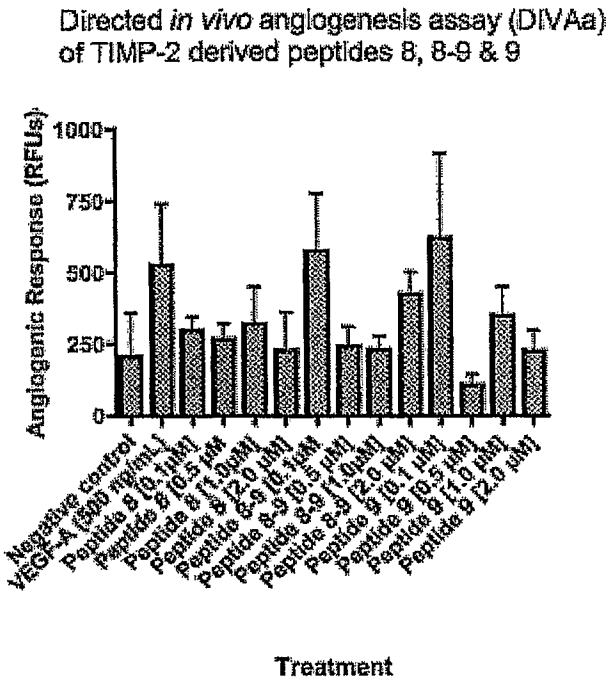


Figure 5


Directed in vivo angiogenesis assay (DIVAa)
of TIMP-2 derived peptides 8, 8-9 & 9
Angiogenic Response (RFUs)
1000
750
500
250
0
Negative control
VEGF-A (500 ng/mL)
Peptide 8 [0.1 µM]
Peptide 8 [0.5 µM]
Peptide 8 [1.0µM]
Peptide 8 [2.0 µM]
Peptide 8-9 [0.1µM]
Peptide 8-9 [0.5 µM]
Peptide 8-9 [1.0µM]
Peptide 8-9 [2.0 µM]
Peptide 9 [0.1 µM]
Peptide 9 [0.5 µM]
Peptide 9 [1.0 µM]
Peptide 9 [2.0 µM]
Treatment