(12) STANDARD PATENT (11) Application No. **AU 2008247637 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Compositions and methods for increasing vascularization

(51) International Patent Classification(s)
A61K 38/00 (2006.01)

(21) Application No: **2008247637** (22) Date of Filing: **2008.05.01**

(87) WIPO No: **WO08/137574**

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/915,293	**2007.05.01**	**US**

(43) Publication Date: **2008.11.13**
(44) Accepted Journal Date: **2013.12.05**

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 November 2008 (13.11.2008)

PCT

(10) International Publication Number
WO 2008/137574 A1

(51) International Patent Classification:
A61K 38/00 (2006.01)

(21) International Application Number:
PCT/US2008/062265

(22) International Filing Date: 1 May 2008 (01.05.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/915,293 1 May 2007 (01.05.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(54) Title: COMPOSITIONS AND METHODS FOR INCREASING VASCULARIZATION

(57) Abstract: Disclosed are methods of increasing vascularization in a tissue by administering a neublastin polypeptide to a mammal exhibiting impaired or inadequate blood flow in the tissue. The methods can be used to in the treatment or prevention of a disorder characterized by impaired or inadequate blood flow or to increase vascularization in an organ that has been transplanted into a subject.

WO 2008/137574 A1

COMPOSITIONS AND METHODS FOR INCREASING VASCULARIZATION

Technical Field

The invention relates to protein chemistry, molecular biology, and vascular biology.

Background

Neublastin, also known as artemin and enovin, is a 24 kDa homodimeric, secreted protein that promotes the outgrowth and survival of neurons of the peripheral and central nervous system (Baudet et al., 2000, *Development*, 127:4335; Masure et al., 1999, *Eur. J. Biochem.*, 266:892; Rosenblad et al., 2000, *Mol. Cell Neurosci.*, 15(2):199). Neublastin mRNA is expressed predominantly in embryonic kidney and lung, and in adults, is expressed highest in pituitary gland, trachea, and placenta (Baudet et al., 2000, *Development*, 127:4335).

Neublastin is a member of the glial cell line-derived neurotrophic factor (GDNF) ligand family. GDNF ligands activate both Ras and phosphatidylinositol-3-kinase signal transduction pathways by engaging the membrane-bound c-RET receptor tyrosine kinase. This c-RET-mediated signaling requires an additional co-receptor, a glycosylphosphatidyl inositol (GPI)-anchored GDNF family receptor alpha (GFRα) protein, which confers ligand specificity to c-RET. Four GFRα co-receptor proteins have been identified (GFRα1-4). Neublastin shows highest affinity for GFRα3 *in vitro*, however in studies using human fibroblasts, neublastin can stimulate c-RET-dependent signaling through either GFRα3 or GFRα1 (Baudet et al., 2000, Development, 127:4335; Masure et al., 1999, *Eur. J. Biochem.* 266:892; Rosenblad et al., 2000, Mol. Cell Neurosci., 15(2):199).

Neublastin and the other GDNF family members are members of the transforming growth factor beta (TGF beta) superfamily and thus, are characterized by the presence of seven conserved cysteine residues with similar spacing which form the structure of a cysteine knot (Saarma, 1999, *Microsc. Res. Tech.*, 45:292). Each monomer contains two disulfide bonds that form a closed loop structure encircling the third disulfide to form a tight knot structure. The seventh cysteine contained within each monomer forms an intermolecular disulfide bond, covalently linking the monomers to form the final dimer product (Rattenholl et al 2000, *J. Mol. Biol.*, 305:523).

Summary

The present invention is based, at least in part, on the discovery that administration of neublastin to a mammal promotes neovascularization and increased blood flow to ischemic muscle tissue following arterial occlusion.

In one aspect, the invention features a method of increasing vascularization in a tissue, which method includes the following steps: selecting a mammal exhibiting impaired or inadequate blood flow in a tissue (e.g., an ischemic tissue such as an ischemic muscle tissue); and administering to the mammal an amount of a polypeptide effective to increase vascularization in the tissue, wherein the polypeptide contains an amino acid sequence that is at least 80% identical to amino acids 15-113 of SEQ ID NO:1, and wherein the polypeptide, when dimerized, binds to a complex containing GFRalpha3 and RET. Also disclosed is the use of a polypeptide that contains an amino acid sequence that is at least 80% identical to amino acids 15-113 of SEQ ID NO:1, wherein the polypeptide, when dimerized, binds to a complex containing GFRalpha3 and RET for the preparation of a pharmaceutical composition for increasing vascularization in a tissue of a mammal exhibiting impaired or inadequate blood flow.

The amino acid sequence contained in the polypeptide of the methods and uses described herein can optionally be at least 90% identical (e.g., at least 95% or 98% identical) to amino acids 15-113 of SEQ ID NO:1. In some embodiments, the polypeptide contains or consists of amino acids 10-113 of SEQ ID NO:1, amino acids 15-113 of SEQ ID NO:1, amino acids 15-113 of SEQ ID NO:2, amino acids 15-113 of SEQ ID NO:3, amino acids 15-113 of SEQ ID NO:4, amino acids 15-113 of SEQ ID NO:5, amino acids 15-113 of SEQ ID NO:8, or amino acids 15-113 of SEQ ID NO:9. For example, the polypeptide can contain or consist of the amino acid sequence of SEQ ID NO:1, the amino acid sequence of SEQ ID NO:2, the amino acid sequence of SEQ ID NO:3, the amino acid sequence of SEQ ID NO:4, the amino acid sequence of SEQ ID NO:5, the amino acid sequence of SEQ ID NO:8, or the amino acid sequence of SEQ ID NO:9.

The polypeptide can be administered to the mammal, for example, via systemic administration (e.g., subcutaneous or intravenous administration) or local administration.

The tissue having impaired or inadequate blood flow can optionally be located in an extremity (e.g., hands or feet) of the mammal. In some embodiments, the tissue having impaired or inadequate blood flow contains a skin lesion (e.g., a skin lesion associated with a diabetic ulcer, such as a diabetic foot ulcer).

The mammal treated according to the methods and uses described herein can be, e.g., a human, a mouse, a rat, a cow, a pig, a dog, a cat, or a monkey.

In some embodiments of the methods and uses described herein, the heart of the mammal exhibits impaired blood flow and administration of the polypeptide increases vascularization of the heart, the mammal has suffered a stroke and exhibits impaired or inadequate blood flow in the tissue as a result of the stroke, the mammal has suffered a myocardial infarction and exhibits impaired or inadequate blood flow in the tissue as a result of the myocardial infarction, the mammal has a coronary artery disease and exhibits impaired or inadequate blood flow in the tissue as a result of the coronary artery disease, and/or the mammal has received a transplanted organ (e.g., a heart or dermis) and administration of the polypeptide increases vascularization in the transplanted organ.

A mammal treated according to the methods and uses described herein can have a disease or disorder such as an ischemic disease, a cardiovascular disease, and/or diabetes.

The methods and uses described herein can further include administering to the mammal one or more of an antithrombotic agent, a factor other than neublastin that increases vascularization, a cholesterol-lowering agent, a beta blocker, an anti-hypertensive agent, or an immunosuppressive agent.

The methods and uses described herein can further include determining whether increased vascularization has occurred subsequent to administration of the polypeptide.

In some embodiments of the methods and uses described herein, the mammal has not been diagnosed with a neurological disorder and/or has not been diagnosed with an ocular disorder.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is an alignment of wild type human (SEQ ID NO:10), mouse (SEQ ID NO:11), and rat (SEQ ID NO:12) pre pro neublastin polypeptides. The left and right vertical lines indicate, respectively, the start of the mature 113 amino and 104 amino acid forms. The RRXR heparin binding motif is boxed.

Fig. 2 is a bar graph depicting the effect of neublastin administration on post-ischemic vascularization in the mouse hindlimb. The Y-axis corresponds to capillary density, which is measured as the ratio of capillary number in the ischemic leg compared to a normal non-ischemic leg. "SEM" refers to standard error mean. P-values less than 0.05 (as compared to vehicle control) are indicated by "*."

Fig. 3 is a bar graph depicting the effect of neublastin administration on post ischemic cutaneous blood flow. The Y-axis indicates blood flow as measured by the ratio of perfusion in the ischemic leg as compared to a normal non-ischemic leg. "SEM" refers to standard error mean. P-values less than 0.01 are indicated by "**" and p-values less than 0.001 are indicated by "***."

Detailed Description

The present invention provides compositions and methods for increasing vascularization in tissues exhibiting impaired or inadequate blood flow. As disclosed in the accompanying examples, administration of neublastin was found to promote neovascularization and increase blood flow to ischemic muscle tissue in a mammal.

Neublastin Polypeptides

Mature wild type human neublastin is 113 amino acids in length and has the following amino acid sequence: AGGPGSRARAAGARGCRLRSQLVPVRALGLGHRSDELV RFRFCSGSCRRARSPHDLSLASLLGAGALRPPPGSRPVSQPCCRPTRYEAVSFMDVNSTW RTVDRLSATACGCLG (SEQ ID NO:1). Polypeptides having the amino acid sequence of SEQ ID NO:1 or biologically active variants of thereof can be used in the methods described herein. A variant neublastin polypeptide can contain one or more additions, substitutions, and/or deletions, as detailed in the following sections. Wild-type neublastin polypeptides and

biologically active variants thereof are collectively referred to herein as "neublastin polypeptides."

A variant neublastin polypeptide can vary in length from the corresponding wild-type polypeptide. Although the mature human neublastin polypeptide (SEQ ID NO:1) consists of the carboxy terminal 113 amino acids of pre pro neublastin (SEQ ID NO:10), not all of the 113 amino acids are required to achieve useful neublastin biological activity. Amino terminal truncation is permissible. Thus, a variant neublastin polypeptide can contain, for example, the carboxy terminal 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, or 113 amino acids of SEQ ID NO:1 (i.e., its length can be 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, or 113 amino acids).

A variant neublastin polypeptide can also vary in sequence from the corresponding wild-type polypeptide. In particular, certain amino acid substitutions can be introduced into the neublastin sequence without appreciable loss of a neublastin biological activity. In exemplary embodiments, a variant neublastin polypeptide (i) contains one or more amino acid substitutions, and (ii) is at least 70%, 80%, 85%, 90%, 95%, 98% or 99% identical to SEQ ID NO:1 (or 70%, 80%, 85%, 90%, 95%, 98% or 99% identical to amino acids 15-113 of SEQ ID NO:1). A variant neublastin polypeptide differing in sequence from SEQ ID NO:1 (or differing in sequence from amino acids 15-113 of SEQ ID NO:1) may include one or more amino acid substitutions (conservative or non-conservative), one or more deletions, and/or one or more insertions.

Fig. 1 is an alignment of the wild type human, mouse, and rat pre pro neublastin polypeptides. The vertical lines in Fig.1 indicate the start of the mature 113 amino acid form (left vertical line) and 104 amino acid form (right vertical line) of neublastin. The RRXR heparin binding motif is boxed. This alignment of naturally occurring, bioactive forms of neublastin indicates specific exemplary residues (i.e., those that are not conserved among the human, mouse, and rat forms) that can be substituted without eliminating bioactivity.

Percent identity between amino acid sequences can be determined using the BLAST 2.0 program. Sequence comparison can be performed using an ungapped alignment and using the default parameters (Blossom 62 matrix, gap existence cost of 11, per residue gap cost of 1, and a lambda ratio of 0.85). The mathematical algorithm used in BLAST programs is described in Altschul et al., 1997, *Nucleic Acids Research* 25:3389-3402.

A conservative substitution is the substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution.

Non-conservative substitutions include those in which (i) a residue having an electropositive side chain (e.g., Arg, His or Lys) is substituted for, or by, an electronegative residue (e.g., Glu or Asp), (ii) a hydrophilic residue (e.g., Ser or Thr) is substituted for, or by, a hydrophobic residue (e.g., Ala, Leu, Ile, Phe or Val), (iii) a cysteine or proline is substituted for, or by, any other residue, or (iv) a residue having a bulky hydrophobic or aromatic side chain (e.g., Val, Ile, Phe or Trp) is substituted for, or by, one having a smaller side chain (e.g., Ala, Ser) or no side chain (e.g., Gly).

A biologically active variant neublastin polypeptide, when dimerized, binds to a ternary complex containing GFRα3 and RET. Any method for detecting binding to this complex can be used to evaluate the biological activity a variant neublastin polypeptide. Exemplary assays for detecting the ternary complex-binding ability of a variant neublastin polypeptide are described in WO00/01815 (the content of which is incorporated herein by reference).

A variant neublastin polypeptide can also be assessed to evaluate its ability to trigger the neublastin signaling cascade. For example, the Kinase Receptor Activation (KIRA) assay can be used to assess the ability of a variant neublastin polypeptide to induce RET autophosphorylation (*See also,* Sadick et al., 1996, *Anal. Biochem.*, 235(2):207).

Substitutions at one or more of the following amino acid residues are expected to result in a variant neublastin polypeptide having reduced or absent heparin binding ability as compared to wild type neublastin: Arg 48, Arg 49, Arg 51, Ser 46, Ser 73, Gly 72, Arg 39, Gln 21, Ser 20, Arg 68, Arg 33, His 32, Val 94, Arg 7, Arg 9, or Arg 14. Reference to a neublastin amino acid

reside by position number refers to the numbering of residues relative to SEQ ID NO:1. A neublastin amino acid residue designated for substitution (e.g., an arginine residue at position 48, 49, and/or 51) can be substituted with a non-conservative amino acid residue (e.g., glutamic acid) or a conservative or amino acid residue. Exemplary amino acids that can be substituted at a residue identified herein (e.g., position 48, 49, and/or 51) include glutamic acid, aspartic acid, and alanine.

Examples of variant neublastin polypeptides that exhibit reduced or absent heparin binding are disclosed in Table 1 and in WO 2006/023781 (the content of which is incorporated herein by reference). Amino acid residues of the variant neublastin polypeptides that are mutated as compared to the corresponding wild type position are bolded and underlined in Table 1. In addition, the neublastin polypeptide (e.g., 113, 99, or 104 amino acids in length) used as the background for the substitution is depicted in Table 1.

Table 1: Variant Neublastin Polypeptides

SEQ ID NO	Position Substituted	Length of Polypeptide	Amino Acid Sequence
2	Arg 48	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCERARSPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG
3	Arg 49	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCREARSPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG
4	Arg 51	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCRRAESPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG
5	Arg 48 and Arg 49	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCEEARSPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG
6	Arg 48 and Arg 49	99	GCRLRSQLVPVRALGLGHRSDELVRFRF CSGSCEEARSPHDLSLASLLGAGALRPPP GSRPVSQPCCRPTRYEAVSFMDVNSTW RTVDRLSATACGCLG
7	Arg 48 and Arg 49	104	AAGARGCRLRSQLVPVRALGLGHRSDE LVRFRFCSGSCEEARSPHDLSLASLLGA GALRPPPGSRPVSQPCCRPTRYEAVSFM DVNSTWRTVDRLSATACGCLG
8	Arg 49 and Arg 51	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCREAESPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG
9	Arg 48 and Arg 51	113	AGGPGSRARAAGARGCRLRSQLVPVRA LGLGHRSDELVRFRFCSGSCERAESPHD LSLASLLGAGALRPPPGSRPVSQPCCRPT RYEAVSFMDVNSTWRTVDRLSATACGC LG

A neublastin polypeptide can be optionally coupled to a polymer (e.g., a polyalkylene glycol moiety such as a polyethylene glycol moiety). In some embodiments, the polymer is coupled to the polypeptide at a site on the neublastin polypeptide that is an N terminus. In some embodiments, a variant neublastin polypeptide includes at least one amino acid substitution with respect to SEQ ID NO:1 (or with respect to amino acids 15-113 of SEQ ID NO:1), which provides an internal polymer conjugation site to which a polymer can be conjugated. In some embodiments, the polymer is coupled to a variant neublastin polypeptide at a residue (numbered in accordance with the sequence of SEQ ID NO:1) selected from the group consisting of position 14, position 39, position 68, and position 95. Exemplary neublastin variants that provide internal polymer conjugation sites are described in WO 02/060929 and WO 04/069176 (the contents of which are incorporated herein by reference).

A polypeptide can optionally contain heterologous amino acid sequences in addition to a neublastin polypeptide. "Heterologous," as used when referring to an amino acid sequence, refers to a sequence that originates from a source foreign to the particular host cell, or, if from the same host cell, is modified from its original form. Exemplary heterologous sequences include a heterologous signal sequence (e.g., native rat albumin signal sequence, a modified rat signal sequence, or a human growth hormone signal sequence) or a sequence used for purification of a neublastin polypeptide (e.g., a histidine tag).

Neublastin polypeptides can be isolated using methods known in the art. Naturally occurring or recombinantly produced neublastin polypeptides can be isolated from cells or tissue sources using standard protein purification techniques. Alternatively, mutated neublastin polypeptides can be synthesized chemically using standard peptide synthesis techniques. The synthesis of short amino acid sequences is well established in the peptide art. *See, e.g.*, Stewart, *et al.*, Solid Phase Peptide Synthesis (2d ed., 1984).

In some embodiments, neublastin polypeptides are produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding a neublastin polypeptide can be inserted into a vector, *e.g.*, an expression vector, and the nucleic acid can be introduced into a cell. Suitable cells include, *e.g.*, mammalian cells (such as human cells or CHO cells), fungal cells, yeast cells, insect cells, and bacterial cells (e.g., *E. coli*). When expressed in a recombinant cell, the cell is preferably cultured under conditions allowing for expression of a neublastin polypeptide. The neublastin polypeptide can be recovered from a cell suspension if desired. As used herein, "recovered" means that the mutated polypeptide is removed from those components

of a cell or culture medium in which it is present prior to the recovery process. The recovery process may include one or more refolding or purification steps. Buffers and methods for inducing folding of a denatured neublastin polypeptide are described in, e.g., WO 2006/023782.

Variant neublastin polypeptides can be constructed using any of several methods known in the art. One such method is site-directed mutagenesis, in which a specific nucleotide (or, if desired a small number of specific nucleotides) is changed in order to change a single amino acid (or, if desired, a small number of predetermined amino acid residues) in the encoded variant neublastin polypeptide. Many site-directed mutagenesis kits are commercially available. One such kit is the "Transformer Site Directed Mutagenesis Kit" sold by Clontech Laboratories (Palo Alto, CA).

Pharmaceutical Compositions

A neublastin polypeptide can be incorporated into a pharmaceutical composition containing a therapeutically effective amount of the polypeptide and one or more adjuvants, excipients, carriers, and/or diluents. Acceptable diluents, carriers and excipients typically do not adversely affect a recipient's homeostasis (e.g., electrolyte balance). Acceptable carriers include biocompatible, inert or bioabsorbable salts, buffering agents, oligo- or polysaccharides, polymers, viscosity-improving agents, preservatives and the like. One exemplary carrier is physiologic saline (0.15 M NaCl, pH 7.0 to 7.4). Another exemplary carrier is 50 mM sodium phosphate, 100 mM sodium chloride. Further details on techniques for formulation and administration of pharmaceutical compositions can be found in, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.).

Administration of a pharmaceutical composition containing a neublastin polypeptide can be systemic or local. Pharmaceutical compositions can be formulated such that they are suitable for parenteral and/or non-parenteral administration. Specific administration modalities include subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, intrathecal, oral, rectal, buccal, topical, nasal, ophthalmic, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic, vaginal, and intra-uterine administration.

Administration may be by periodic injections of a bolus of the pharmaceutical composition or may be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an IV bag) or internal (e.g., a bioerodable implant, a bioartificial organ, or a colony of implanted neublastin production cells). See, e.g., U.S. Pat. Nos.

4,407,957, 5,798,113, and 5,800,828, each incorporated herein by reference. Administration of a pharmaceutical composition may be achieved using suitable delivery means such as: a pump (see, e.g., Annals of Pharmacotherapy, 27:912 (1993); Cancer, 41:1270 (1993); Cancer Research, 44:1698 (1984), incorporated herein by reference); microencapsulation (see, e.g., U.S. Pat. Nos. 4,352,883; 4,353,888; and 5,084,350, herein incorporated by reference); continuous release polymer implants (see, e.g., Sabel, U.S. Pat. No. 4,883,666, incorporated herein by reference); macroencapsulation (see, e.g., U.S. Pat. Nos. 5,284,761, 5,158,881, 4,976,859 and 4,968,733 and published PCT patent applications WO92/19195, WO 95/05452, each incorporated herein by reference); injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation.

Examples of parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, pump delivery, encapsulated cell delivery, liposomal delivery, needle-delivered injection, needle-less injection, nebulizer, aeorosolizer, electroporation, and transdermal patch.

Formulations suitable for parenteral administration conveniently contain a sterile aqueous preparation of the neublastin polypeptide, which preferably is isotonic with the blood of the recipient (*e.g.*, physiological saline solution). Formulations may be presented in unit-dose or multi-dose form.

An exemplary formulation contains a neublastin polypeptide described herein and the following buffer components: sodium succinate (e.g., 10 mM); NaCl (e.g., 75 mM); and L-arginine (e.g., 100 mM).

Formulations suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the neublastin polypeptide; or a suspension in an aqueous liquor or a non-aqueous liquid, such as a syrup, an elixir, an emulsion, or a draught.

A neublastin polypeptide suitable for topical administration can be administered to a mammal (e.g., a human patient) as, e.g., a cream, a spray, a foam, a gel, an ointment, a salve, or a dry rub. A dry rub can be rehydrated at the site of administration. Neublastin polypeptides can also be in infused directly into (e.g., soaked into and dried) a bandage, gauze, or patch, which can then be applied topically. Neublastin polypeptides can also be maintained in a semi-liquid,

gelled, or fully-liquid state in a bandage, gauze, or patch for topical administration (see, e.g., U.S. Patent No. 4,307,717, the content of which is incorporated herein by reference).

Therapeutically effective amounts of a pharmaceutical composition may be administered to a subject in need thereof in a dosage regimen ascertainable by one of skill in the art. For example, a composition can be administered to the subject, e.g., systemically at a dosage from 0.01μg/kg to 1000 μg/kg body weight of the subject, per dose. In another example, the dosage is from 1 μg/kg to 100 μg/kg body weight of the subject, per dose. In another example, the dosage is from 1 μg/kg to 30 μg/kg body weight of the subject, per dose, e.g., from 3 μg/kg to 10 μg/kg body weight of the subject, per dose.

In order to optimize therapeutic efficacy, a neublastin polypeptide can be first administered at different dosing regimens. The unit dose and regimen depend on factors that include, *e.g.*, the species of mammal, its immune status, the body weight of the mammal. Typically, protein levels in tissue are monitored using appropriate screening assays as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen.

The frequency of dosing for a neublastin polypeptide is within the skills and clinical judgement of physicians. Typically, the administration regime is established by clinical trials which may establish optimal administration parameters. However, the practitioner may vary such administration regimes according to the subject's age, health, weight, sex and medical status. The frequency of dosing may be varied depending on whether the treatment is prophylactic or therapeutic.

Methods of Treatment

The neublastin polypeptides described herein can be used for increasing vascularization in a mammal exhibiting impaired or inadequate blood flow in a tissue. For example, a neublastin polypeptide can be used to treat a mammal (e.g., a human) having, suspected of having, or at risk of developing, an ischemic disorder such as muscle ischemia, ischemic heart (e.g., resulting from myocardial infarction), a decubitus ulcer, ischemia resulting from varicose veins, ischemic complications of diabetes (e.g., a skin lesion such as a foot lesion), ischemic kidney, ischemic brain (e.g., resulting from a stroke), or ischemic liver. In addition, a neublastin polypeptide can be used to increase vascularization in a mammal that has received a transplanted organ and is in need of vascularization of the organ. Examples of specific medical conditions that can be treated

or prevented by administration of a neublastin polypeptide are reviewed in the following sections.

(i) Stroke

Stroke (e.g., ischemic stroke, thrombotic stroke, embolic stroke, systemic hypoperfusion stroke, hemorrhagic stroke, intracerebral hemorrhage stroke, or subarachnoid hemorrhage stroke) is a disorder characterized by impaired or inadequate blood flow to one or more regions of the brain. The disturbance in perfusion can be venous, but is most often arterial. Loss or reduction of blood flow to the brain results in damage to ischemic areas, which can seriously impair local or global brain function. A neublastin polypeptide described herein can be administered (e.g., intravenously, subcutaneously, intranasally, or by intracranial local delivery) to a subject so as to increase blood flow to one or more regions of the brain to thereby prevent or reduce damage caused by a stroke. Where the subject is at risk of having a stroke (e.g., a subject diagnosed as having a partial occlusion of an artery in the brain), neublastin can be administered to the subject to prevent the occurrence, or lessen the severity, of a stroke.

Risk factors for developing a stroke include, for example, a family history of stroke, older age, race, hypertension, elevated cholesterol levels (particularly elevated LDL), smoking, diabetes, and obesity.

Stroke can be diagnosed or evaluated by, e.g., the severity and/or number of symptoms presented by a subject. Symptoms of stroke can vary depending the specific region of the brain that is affected. Stroke symptoms include, e.g., weakness (hemiplegia), numbness, reduction in sensory or vibratory sensation, altered senses (e.g., smell, taste, hearing, or vision (total or partial)), drooping of an eyelid (ptosis), decreased reflexes (e.g., gag, swallow), decreased sensation and muscle weakness of the face, balance problems, altered breathing, altered heart rate, aphasia (inability to speak or understand language), apraxia (altered voluntary movements), vertigo, and/or disequilibrium.

Stroke can also be diagnosed and evaluated (e.g., the severity of the stroke or the extent of the brain affected) using a variety of quantitative techniques including computed axial tomography (CAT), computed tomography (CT), or magnetic resonance imaging (MRI) scans. A medical professional can also use more qualitative diagnoses to diagnose or evaluate a subject having a stroke, e.g., by evaluating a subject's ability to smile, elevate one or both limbs, speak

simple and/or complex sentences coherently, ability to walk or maintain balance, or any other symptoms of stroke described herein.

In addition to the administration of a neublastin polypeptide described herein, stroke can also be treated by a variety of techniques depending on the subject and the nature of the condition. Common treatments include mechanical thrombectomy or administration of tissue plasminogen activator (tPA)or other methods of thrombolysis.

(ii) Ischemic Heart Disease

Ischemic heart disease is characterized by impaired or insufficient blood flow to the heart muscle and can be caused by, e.g., atherosclerosis in one or more coronary arteries (coronary artery disease), cardiac arrhythmias, acute myocardial infarction, loss of heart muscle activity, or defective heart valves. Loss or reduction of blood flow to the heart results in damage to ischemic cardiac muscle tissue, which can cause permanent damage to the heart and/or death of the affected subject. A neublastin polypeptide described herein can be administered (e.g., intravenously, subcutaneously, or locally by myocardial or epicardial injection) to a subject so as to increase blood flow to one or more ischemic regions of the heart to thereby prevent or reduce damage caused by the ischemia. Where the subject is one at risk of developing ischemic heart disease, neublastin can be administered to prevent the occurrence, or lessen the severity, of cardiac ischemia.

Risk factors for developing ischemic heart disease include, e.g., poor diet, obesity, smoking, elevated and/or prolonged periods of stress, family history (e.g., a genetic predisposition), sedentary lifestyle, elevated cholesterol levels, and/or diabetes.

Ischemic heart disease can be diagnosed and/or evaluated by, e.g., the severity and/or number of symptoms of ischemic heart disease presented by the subject. Symptoms of ischemic heart disease vary and range in severity and include, but are not limited to, one or more of: chest pain, left arm pain, jaw pain, neck pain, back pain, sensation similar to heartburn, shortness of breath, pale skin, profuse sweating, weakness, light-headedness, nausea, vomiting, palpitations, and/or fatigue. Ischemic heart disease can be diagnosed or evaluated using a number of techniques known in the art, including electrocardiogram (ECG), coronary angiogram, chest radiograph, echocardiogram, and/or multiple gated acquisition (MUGA) scan. Ischemic heart disease can also be diagnosed or evaluated using biomarkers such as the level of one or more cardiac enzymes(e.g., creatine kinase, troponin I, and lactate dehydrogenase isozymes) in a

subject's blood. Additional methods of diagnosing or evaluating a subject having ischemic heart disease include exercise stress test, wherein a subject's heart is monitored while the subject is exercising. The subject's heart rate, breathing, and blood pressure can be monitored. An ECG (above) can also be performed.

In addition to the administration of a neublastin polypeptide described herein, treatment for a subject having or suspected of having an ischemic heart disease can include administration of oxygen, acetylsalicylic acid (aspirin), glyceryl trinitrate, and pain relievers. Patients at risk of developing ischemic heart disease can be administered one or more of cholesterol lowering agents (e.g., statins), beta blockers, or anti-hypertensives (e.g., diuretics, angiotensin-converting enzyme inhibitors, vasodilators, or alpha blockers).

(iii) Ulcers

Ulcers are cutaneous lesions resulting from impaired or inadequate blood flow to the affected area (e.g., a foot). Such ulcers can be the result of vascular complications of diabetes (e.g., diabetic ulcers of the foot), venous insufficiency (crural ulcers), or excessive pressure (e.g., decubitus ulcers or bed sores). Loss or reduction of blood flow to an area of skin results in damage and/or death of that area of skin and surrounding tissue. A neublastin polypeptide described herein can be administered (e.g., topically administered to a subject's ulcer) to a subject so as to increase blood flow at the site of the ulcer or surrounding tissue, thereby reducing the severity or duration of the ulcer. Where the subject is at risk of developing an ulcer (for example, a paralyzed subject in a prolonged prone or supine position or a subject having cardiovascular complications due to diabetes), neublastin can be administered to the subject (e.g., by topical administration to the legs and feet of diabetic patients) to prevent the occurrence, or lessen the severity, of an ulcer.

Risk factors for developing cutaneous ulcers include, e.g., prolonged periods of sitting or laying (e.g., supine or prone positions), diabetes, varicose veins (see below), infection, and/or poor hygiene.

Methods for diagnosing and/or evaluating an ulcer on a subject include visual inspection, e.g., the appearance of the ulcer itself, redness, soreness, or pain. The visual inspection can also be used to check for symptoms indicative of the development of ulcers including, e.g., decreased sweating, dry skin and fissure formation, and propensity to develop infections at the affected area. Symptoms of decreased blood flow to the foot (and risk of developing foot ulcers, e.g.,

often resulting from complications from diabetes) include brittle nails, calluses, and hammer toes. The visual inspection can also include evaluating the size of the ulcer and/or whether or not the ulcer is infected. A medical professional can administer one or more tests to determine the level of blood flow to an area suspected of having impaired or inadequate blood flow including transcutaneous oxygen measurement (TCOM) and a nylon monofilament test. The TCOM requires the placement of electrodes directly onto the suspected area of skin. Generally a measured oxygen pressure of less than 40 mm Hg is an indication that the area is deficient in blood flow. The nylon monofilament test is a sensation test that involves the use of a 10 gauge nylon monofilament to gently prick the affected skin. The test is abnormal if the subject cannot sense the touch of the monofilament when it is pressed against the foot with just enough pressure to bend the filament.

In addition to the administration of a neublastin polypeptide described herein, treatments for cutaneous ulcers can involve surgery to remove dead or infected tissue and administration of antibiotics, where required.

(iv) Varicose veins

Varicose veins (venous insufficiency) is a disorder characterized by an inability of the veins (generally of the legs) to transport deoxygenated blood back to the heart. Venous insufficiency can result from a thrombus (blood clot) or damage to, or loss of elasticity of, vein valves. A neublastin polypeptide described herein can be administered (e.g., topically to the subject's legs, subcutaneously, or intravenously to the affected veins) to a subject so as to increase blood flow to in the legs back to the heart, thereby reducing the severity of, or complications due to, varicose veins. Where the subject is at risk of developing varicose veins (e.g., a subject having one or more risk factors for varicose veins), neublastin can be administered to the subject to prevent the occurrence, or lessen the severity, of varicose veins.

Risk factors for developing varicose veins include, for example, old age, sex of the subject (women are more likely than men to develop varicose veins), family history (e.g., a genetic predisposition), obesity, and/or occupations that involve standing for prolonged periods of time.

Venous insufficiency can be diagnosed and/or evaluated in a subject by, e.g., the severity and/or number of symptoms presented by the subject including, e.g., pain or heaviness in the leg, feet and ankles, swelling, ulcers on the skin, or severe bleeding if the vein is injured. VI can be

diagnosed or evaluated in a subject using a variety of techniques including duplex or Doppler Ultrasound, a non-invasive technique that uses ultrasound to visualize clots or other abnormalities in the blood vessels. Other methods of diagnosing/evaluating Venous insufficiency include CT Scan, venography, angiography such as X-ray or magnetic resonance angiography (MRA).

In addition to the administration of a neublastin polypeptide described herein, treatments for venous insufficiency can include, for example, laser surgery, sclerotherapy/microsclerotherapy, surgical vein stripping, ambulatory phlembectomy, and endoscopic vein surgery. Non-surgical therapies include, where venous insufficiency occurs in the legs, leg elevation, compression therapy (compression socks or leggings), exercise, weight loss, and skin care.

(v) Transplanted Organs

An organ transplant is a process in which a whole or partial organ is transferred from one subject to another subject. Transplanted organs include, e.g., heart, lung, liver, kidney, small bowel, pancreas, hand, digit (finger or toe), or skin (e.g., a skin graft such as a face transplant; see below). For an organ transplant to be successful, vascularization must occur between the transplanted organ and the host. Thus, a neublastin polypeptide described herein can be administered to a subject so as to promote vascularization between the transplanted organ and the host and increased blood flow to the transplanted organ, thereby preventing graft failure.

A common organ transplant is a skin graft, in which a region of dermis is surgically removed from one area of the body and transplanted to another. Skin grafts can be autologous (from the same subject) or can be heterologous (from a different subject). In some instances, the skin tissue can be obtained from an animal of a different species than the recipient subject (e.g., xenotransplantation). Skin grafts can be performed on a subject that has, e.g., suffered extensive burns or had cutaneous infections with areas of skin loss. In these cases, skin grafts are often used to minimize bacterial concentration at the site of skin loss and/or prevent loss of fluids. Skin grafts are also used in cosmetic surgeries, such as elective surgeries or those accompanying surgical procedures such as a mastectomy or chest wall reconstruction. Skin grafts can be extensive such as a whole or partial face transplant. As discussed above, for a skin graft to be successful, vascularization must occur between the graft site and the grafted tissue. Thus, a neublastin polypeptide described herein can be administered to a subject so as to promote

vascularization between the grafted skin and the host to increase blood flow to the grafted skin, thereby preventing graft failure.

Monitoring the success of a skin graft can be performed a variety of ways including visual inspection, e.g., checking the color of the grafted skin, monitoring for a return of feeling to the grafted area, or monitoring the temperature of the grafted skin. Blood flow in a region or regions of the grafted skin can be measured directly, e.g., using laser Doppler perfusion monitoring (see below).

A subject suspected of having a disorder characterized by impaired or inadequate blood flow, as used herein, is a subject having one or more symptoms for a particular disorder characterized by impaired or inadequate blood flow (such as any of those described herein). For example, a subject suspected of having a stroke can be one having one or more symptoms of a stroke such as, but not limited to: weakness, numbness, drooping of an eyelid (ptosis), decreased reflexes (e.g., gag, swallow), decreased sensation and muscle weakness of the face, aphasia, apraxia, or any other symptoms described herein.

A subject at risk of developing a disorder characterized by impaired or inadequate blood flow, as used herein, is a subject having one or more risk factors of a particular disorder characterized by impaired or inadequate blood flow. For example, a subject at risk of developing ischemic heart disease can be subject with one or more risk factors for developing ischemic heart disease including, e.g., poor diet, obesity, smoking, elevated and/or prolonged periods of stress, sedentary lifestyle, elevated cholesterol levels, diabetes, or any other risk factors described herein.

A neublastin polypeptide can be administered to a subject in a variety of ways dependent, at least in part, on the type of disorder being treated and the location in the body of the impaired or inadequate blood flow. That is, in embodiments where a disorder is cutaneous in nature, such as a skin lesion, a decubitus ulcer, or a diabetic ulcer (e.g., diabetic foot ulcer), a neublastin polypeptide can be administered topically. For example, a neublastin polypeptide can be administered to the subject in a cream, salve, or ointment. Neublastin compositions described herein can also be infused into a bandage, gauze, or patch (see, e.g., U.S. Patent No. 4,307,717). In embodiments where a disorder characterized by impaired or inadequate blood flow in a subject is internal (e.g., a stroke, ischemic heart disease, or organ transplant), neublastin can be administered to the subject intravenously, subcutaneously, or locally at the site where increased

vascularization is needed. For example, neublastin can be administered to a transplanted kidney or heart and/or the surrounding host tissue during a transplant operation.

Combination Therapy

A neublastin polypeptide described herein can be administered to a subject as a monotherapy or as part of a multi-therapeutic regimen in conjunction with one or more additional agents that provide a therapeutic benefit to a subject having a disorder characterized by impaired or inadequate blood flow. For example, a neublastin polypeptide can be co-administered with an additional angiogenic factor such Angiogenin, Angiopoietin-1, Del-1, a fibroblast growth factor (e.g., aFGF, bFGF, or FGF2), Follistatin, Granulocyte Colony-Stimulating Factor (G-CSF), Hepatocyte Growth Factor (HGF), Interleukin-8 (IL-8), Leptin Midkine, Placental Growth Factor, Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF), Platelet-Derived Growth Factor-BB (PDGF-BB), Pleiotrophin (PTN), Progranulin, Proliferin, Transforming Growth Factor-Alpha (TGF-alpha), Transforming Growth Factor-Beta (TGF-beta), Tumor Necrosis Factor-Alpha (TNF-alpha), and/or Vascular Endothelial Growth Factor (VEGF). In addition, a neublastin polypeptide can be administered in combination with one or more therapeutic agents that do not increase vascularization but are otherwise beneficial to a subject having a disorder characterized by impaired or inadequate blood flow. For example, a neublastin polypeptide can be co-administered with any one of an antithrombotic agent (e.g., aspirin, streptokinase, urokinase, tissue plasminogen activator, heparin, or hirudin), a pain medication, an antibiotic, a cholesterol lowering agent (e.g., a statin), a beta blocker, and/or an anti-hypertensive (e.g., a diuretic, an angiotensin-converting enzyme inhibitor, a vasodilator, or an alpha blocker). Where a neublastin polypeptide is used to increase vascularization of an organ that has been transplanted into a recipient (e.g., a transplanted heart, liver, kidney, lung, limb such as a finger, or dermis), the neublastin polypeptide can optionally be co-administered with one or more immunosuppressive agents.

The neublastin polypeptide and the one or more additional agents can be administered at the same time, the neublastin polypeptide can be administered first in time and the one or more additional agents administered second in time, or the one or more additional agents can be administered first in time and the neublastin polypeptide administered second in time.

Neublastin can optionally replace or augment a previously or currently administered therapy. For example, upon treating with a neublastin polypeptide, administration of the one or

more additional agents can cease or diminish (e.g., be administered at lower levels). In some instances, a previous therapy can be maintained until the level of neublastin (e.g., the dosage or schedule) reaches a level sufficient to provide a therapeutic effect to the subject. In instances where a previous therapy is particularly toxic or poorly tolerated by a subject, administration of a neublastin polypeptide can be used to offset and/or lessen the amount of the previous therapy (e.g., an angiogenic therapy) to a level sufficient to give the same or improved therapeutic benefit, but without the toxicity.

In some instances where a subject is not responding to a first therapy, a subject can be administered neublastin. For example, if a subject (e.g., a human patient) is not responding to a first treatment such as VEGF (or another angiogenic factor described herein), a neublastin polypeptide can be administered to the subject. As used herein, a "subject not responsive to a treatment" refers to a patient in which treatment with one or more angiogenic therapies alone (i.e., not combined with neublastin) does not result in significant clinical improvement, more particularly does not result in a significant (and preferably long-term) improvement of the parameters used to measure angiogenic efficacy (such as but not limited to exercise treadmill testing (ETT or exercise stress test), angina time and angina frequency) (see, e.g., Fam et al. (2003) Circulation 108:2613). An example of such a group of subjects not responding to a VEGF angiogenic therapy is described by Henry et al. (2003) Circulation 107:1359 1365.

Evaluating Efficacy of Treatments

The efficacy of a neublastin treatment can be evaluated by any of the methods described herein (e.g., by directly monitoring the level of new blood vessel growth or evaluating a particular feature or symptom of a disorder characterized by impaired or inadequate blood flow). For example, the amount or density of vasculature in a subject's brain can be measured (e.g., before and after treatment) using MRI (see, e.g., Dunn et al. (2004) Magn Reson. Med. 51(1):55-61) or ultrasound techniques such as adaptations of those described by Fosberg et al. (2004) Ultrasonics 42(1):325-330. The effect of neublastin treatment in promoting neovascularization can also be evaluated by monitoring an increase in blood flow using, e.g., laser Doppler techniques as described in, e.g., Freccero et al. (2003) Microvasc Res. 66(3):183-9; and Rendell et al. (1989) Diabetes 38(7):819- 824. An exemplary device useful for measuring cutaneous blood flow by laser Doppler technique is the DRT4 (Moor Instruments, Devon, UK). Furthermore, the efficacy of neublastin to promote vascularization of a transplanted organ (e.g., a

transplanted kidney, heart, or skin) can be measured by as an increase in transplanted organ function or an increase in organ health (e.g., by biopsy) following treatment.

The efficacy of a treatment can be assessed by evaluating a subject before and after treatment (e.g., comparing the oxygen tension in an affected area before or after treatment). Where progression of improvement in a disorder following one or more neublastin treatments is to be assessed, a subject can be evaluated at multiple time points following neublastin treatment (e.g., a one day, two day, and one week evaluation; a one week, one month, and six month evaluation; a one month, six month, and one year evaluation).

Where administration of neublastin is used to prevent the occurrence of a disorder characterized by impaired or inadequate blood flow (e.g., a lesion such as a foot ulcer due to vascular complications of diabetes), efficacy can be assessed as a delay in presentation of, or a failure to present, one or more symptoms of the disorder. The efficacy of a treatment over time in ameliorating one or more symptoms of a disorder can be determined by assessing, e.g., the number or severity of one or more symptoms at multiple time points following treatment. For example, a subject can have an initial assessment of the severity of his or her disorder, be administered a treatment, and then be assessed two or more times subsequent to the treatment (e.g., at one week and one month; at one month at two months; at two weeks, one month, and six months; or six weeks, six months, and a year). Where one or more neublastin treatments are administered to a subject for a limited period of time (e.g., a predetermined duration) or number of administrations, the effect of treatment on ameliorating one or more symptoms of a disorder characterized by impaired or inadequate blood flow can be assessed at various time points after the final treatment. For example, following the last administration of a dose of neublastin, the number or severity of a patient's symptoms can be assessed at 1 month (e.g., at 2 months, at 6 months, at one year, at two years, at 5 years or more) subsequent to the final treatment.

Animal Models of Disorders Characterized by Impaired or Inadequate Blood Flow

The Examples below describe an *in vivo* animal model system useful for studying the effect of neublastin treatment on an ischemic disorder. The efficacy of such treatment can be evaluated by direct analysis of the ischemic tissue, for example, by measuring capillary density in an ischemic muscle using immunohistochemistry techniques and/or measuring blood flow in a ischemic muscle. To evaluate prevention or delayed onset of a disorder characterized by impaired or inadequate blood flow, a neublastin polypeptide can also be administered to an

animal before inducing the disorder. Additional animal models (e.g., mouse models) useful for evaluating the efficacy of neublastin treatments to increase vascularization include those described in, e.g., Couffinhal et al. (1988) Am J. Pathol. 152(6):1667-1679; Cao et al. (1998) Proc. Natl. Acad. Sci. USA 95(24):14389-14394; and Salven et al. (2002) FASEB J. 16:1471-1473. An animal model for impaired wound healing in diabetes is described in, e.g., Tsuboi et al. (1992) J. Dermatol. 19(11):673-75.

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

Examples

Example1: Neublastin Promotes Post-Ischemic Neovascularization

A murine model of hindlimb ischemia was used to determine if neublastin administration increases vascularization in mammals. Right femoral arteries of mice hindlimbs were surgically ligated. Neublastin was administered subcutaneously three times a week at a dosage of 1 milligram per kilogram (mg/kg) or 0.1 mg/kg. Alternatively, a set of mice were administered vehicle alone (no neublastin) as a control. Ten mice were evaluated in each group. Twenty one days (3 weeks) later, the mice were sacrificed and the gastrocnemius muscles were removed.

Vessel density was evaluated by high definition microangiography at the end of the treatment period, as described in Silvestre et al. (2005) Nat. Med. 11(5):499-506. Briefly, mice were anesthetized (isoflurane inhalation) and a contrast medium (Barium sulfate, 1 g/ml) was injected through a catheter introduced into the abdominal aorta. Images (two per animal) acquired by a digital X-ray transducer were assembled to obtain a complete view of the hindlimbs. The vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area. Quantification zone was delineated by the place of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg. Both dosages of neublastin increased the angiographic score in muscles from ischemic hindlimbs as compared to the vehicle-only control (Fig. 2). These results indicate that neublastin administration induces vascularization in ischemic tissue.

2008247637 16 Oct 2013

Example 2: Neublastin Promotes Post-Ischemic Cutaneous Blood Flow

To determine if neublastin treatment increases blood flow in ischemic tissue (e.g., skin), mouse femoral arteries were ligated as described above. Rat neublastin (the mature 113 amino acid form of the protein) was administered subcutaneously three times a week for three weeks at 1 mg/kg or 0.1 mg/kg. As a control, a set of mice were treated with vehicle alone.

At 7, 14, and 21 days, hair was removed from a small surface of ischemic hindlimb skin and the exposed tissue was evaluated for blood flow using laser Doppler perfusion monitoring as described in, e.g., Hisaka et al. (2004) J. Am. Coll. Cardiol. 43(10): 1915-22. Measurements were performed in the paw only. An increase in cutaneous blood perfusion of ischemic limbs treated with neublastin was detected at 14 and 21 days (Fig. 3). These results indicate that neublastin administration results in increased blood flow in ischemic tissue.

The term 'comprise' and variants of the term such as 'comprises' or 'comprising' are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

Other Embodiments

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

2008247637 16 Oct 2013

Claims:

1. A method of increasing vascularization in a tissue, the method comprising:

selecting a mammal exhibiting impaired or inadequate blood flow in a tissue; and

administering to the mammal an amount of a polypeptide effective to increase vascularization in the tissue, wherein the polypeptide comprises an amino acid sequence that is at least 80% identical to amino acids 15-113 of SEQ ID NO:1, and wherein the polypeptide, when dimerized, binds to a complex containing GFRalpha3 and RET.

2. The method of claim 1, wherein the tissue is an ischemic tissue.

3. The method of claim 2, wherein the ischemic tissue is an ischemic muscle tissue.

4. The method of claim 1, wherein the heart of the mammal exhibits impaired blood flow and administration of the polypeptide increases vascularization of the heart.

5. The method of claim 1, wherein the mammal has suffered a stroke and exhibits impaired or inadequate blood flow in the tissue as a result of the stroke.

6. The method of claim 1, wherein the mammal has suffered a myocardial infarction and exhibits impaired or inadequate blood flow in the tissue as a result of the myocardial infarction.

7. The method of claim 1, wherein the mammal has a coronary artery disease and exhibits impaired or inadequate blood flow in the tissue as a result of the coronary artery disease.

8. The method of claim 1, wherein the mammal has received a transplanted organ and administration of the polypeptide increases vascularization in the transplanted organ.

9. The method of claim 8, wherein the transplanted organ is a heart.

10. The method of claim 8, wherein the transplanted organ is dermis.

11. The method of claim 1, wherein the mammal has an ischemic disease.

12. The method of claim 1, wherein the mammal has a cardiovascular disease.

2008247637 16 Oct 2013

13. The method of claim 1 , wherein the tissue is located in an extremity of the mammal.

14. The method of claim 12 or 13, wherein the mammal has diabetes.

15. The method of claim 1, wherein the tissue comprises a skin lesion.

16. The method of claim 15, wherein the skin lesion is associated with a diabetic ulcer.

17. The method of claim 16, wherein the diabetic ulcer is a diabetic foot ulcer.

18. The method of any one of claims 1-17, further comprising administering to the mammal one or more of an antithrombotic agent, a factor other than neublastin that increases vascularization, a cholesterol-lowering agent, a beta blocker, an anti-hypertensive agent, or an immunosuppressive agent.

19. The method of any one of claims 1-18, further comprising determining whether increased vascularization has occurred subsequent to administration of the polypeptide.

20. The method of any one of claims 1-19, wherein the polypeptide is administered to the mammal via systemic administration.

21. The method of any one of claims 1-19, wherein the polypeptide is administered to the mammal via subcutaneous administration.

22. The method of any one of claims 1-19, wherein the polypeptide is administered to the mammal via local administration.

23. The method of any one of claims 1-22, wherein the amino acid sequence is at least 90% identical to amino acids 15-113 of SEQ ID NO:1.

2008247637 16 Oct 2013

24. The method of any one of claims 1-22, wherein the amino acid sequence is at least 95% identical to amino acids 15-113 of SEQ ID NO: 1.

25. The method of any one of claims 1-22, wherein the amino acid sequence is at least 98% identical to amino acids 15-113 of SEQ ID NO:1.

26. The method of any one of claims 1-22, wherein the polypeptide comprises amino acids 15-113 of SEQ ID NO:1, amino acids 15-113 of SEQ ID NO:2, amino acids 15-113 of SEQ ID NO:3, amino acids 15-113 of SEQ ID NO :4, amino acids 15-113 of SEQ ID NO: 5, amino acids 15-113 of SEQ ID NO:8, or amino acids 15-113 of SEQ ID NO:9.

27. The method of any one of claims 1-22, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:1, the amino acid sequence of SEQ ID NO:2, the amino acid sequence of SEQ ID NO:3, the amino acid sequence of SEQ ID NO:4, the amino acid sequence of SEQ ID NO:5, the amino acid sequence of SEQ ID NO:8, or the amino acid sequence of SEQ ID NO:9.

28. The method of any one of claims 1-22, wherein the polypeptide comprises amino acids 10-113 of SEQ ID NO:l .

29. The method of any one of claims 1-22, wherein the polypeptide consists of amino acids 10-113 of SEQ ID NO:1.

30. The method of any one of claims 1-29, wherein the mammal has not been diagnosed with a neurological disorder.

31. The method of any one of claims 1-30, wherein the mammal has not been diagnosed with an ocular disorder.

32. The method of any one of claims 1-31, wherein the mammal is a human.

Date: 16 October 2013

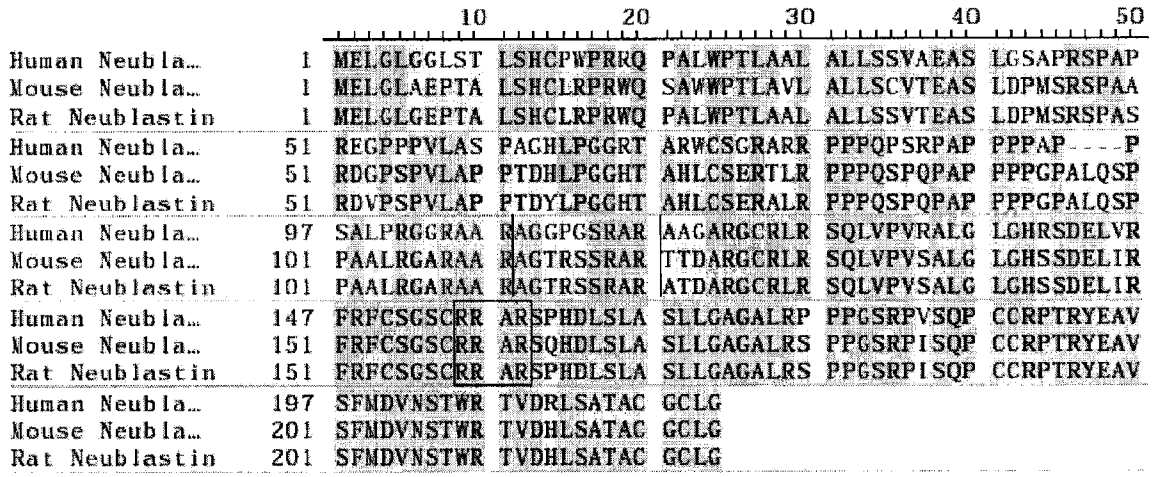

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Mouse Neubla… 1 MELGLAEPTA LSHCLRPRWQ SAWWPTLAVL ALLSCVTEAS LDPMSRSPAA
Rat Neublastin 1 MELGLGEPTA LSHCLRPRWQ PALWPTLAAL ALLSSVTEAS LDPMSRSPAS
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Mouse Neubla… 51 RDGPSPVLAP PTDHLPGGHT AHLCSERTLR PPPQSPQPAP PPPGPALQSP
Rat Neublastin 51 RDVPSPVLAP PTDYLPGGHT AHLCSERALR PPPQSPQPAP PPPGPALQSP
Human Neubla… 97 SALPRGGRAA RAGGPGSRAR AAGARGCRLR SQLVPVRALG LGHRSDELVR
Mouse Neubla… 101 PAALRGARAA RAGTRSSRAR TTDARGCRLR SQLVPVSALG LGHSSDELIR
Rat Neublastin 101 PAALRGARAA RAGTRSSRAR ATDARGCRLR SQLVPVSALG LGHSSDELIR
Human Neubla… 147 FRFCSGSCRR ARSPHDLSLA SLLGAGALRP PPGSRPVSQP CCRPTRYEAV
Mouse Neubla… 151 FRFCSGSCRR ARSQHDLSLA SLLGAGALRS PPGSRPISQP CCRPTRYEAV
Rat Neublastin 151 FRFCSGSCRR ARSPHDLSLA SLLGAGALRS PPGSRPISQP CCRPTRYEAV
Human Neubla… 197 SFNDVNSTWR TVDRLSATAC GCLG
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Rat Neublastin 201 SFNDVNSTWR TVDHLSATAC GCLG

Fig. 1

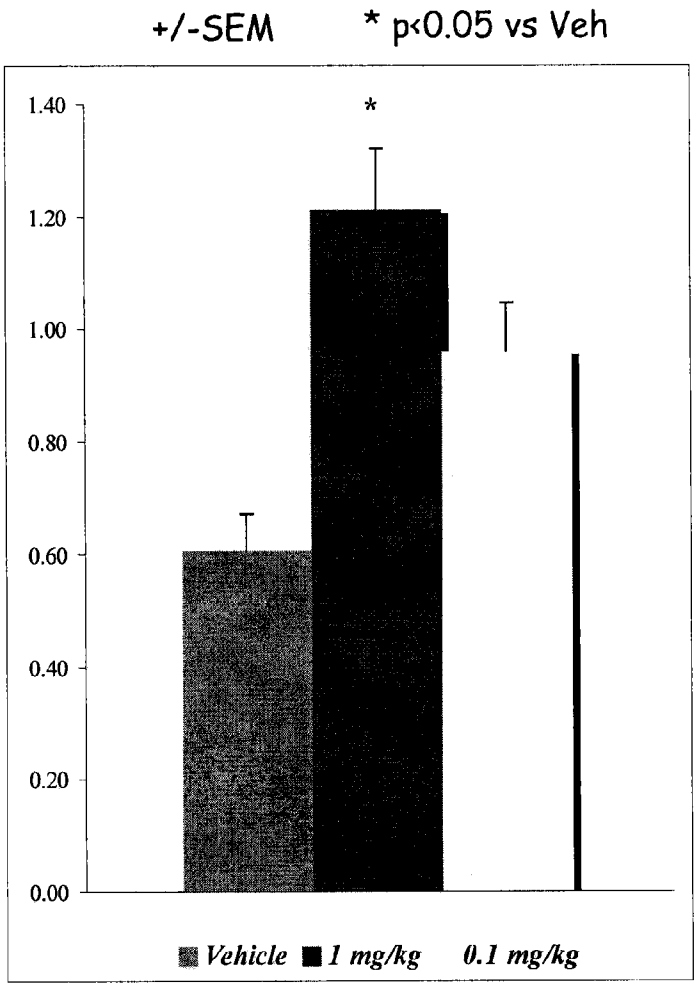

+/-SEM
* p<0.05 vs Veh
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
Vehicle
1 mg/kg
0.1 mg/kg

Fig. 2

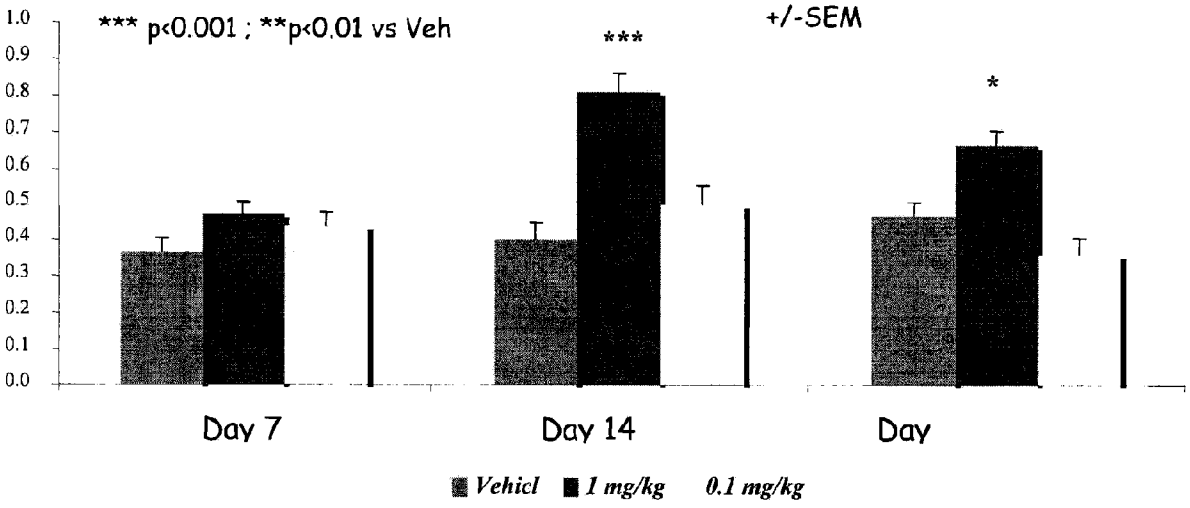

*** p<0.001 ; **p<0.01 vs Veh
+/-SEM
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
*
Day 7
Day 14
Day
Vehicl
1 mg/kg
0.1 mg/kg

Fig. 3