DISINTEGRIN VARIANTS AND PHARMACEUTICAL USES THEREOF

CROSS-REFERENCE TO RELATED PATENT APPLICATION

[001] This application claims the benefit, pursuant to 35 U.S.C. §119(e), of U.S. provisional patent application No. 60/871,854 filed December 26, 2006, which is incorporated herein by reference in its entirety.

DESCRIPTION OF THE INVENTION

Field of the Invention

[002] The present invention relates generally to disintegrin variants, and more specifically to disintegrin variants as selective αvβ3 integrin antagonists for treatment and prevention of αvβ3 integrin-associated diseases.

Background of the Invention

[003] Bone is a complex tissue composed of several cell types which are continuously undergoing a process of renewal and repair termed "bone remodeling." The two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone. Bone remodeling has been known to be regulated by several systemic hormones (e.g., parathyroid hormone, 1,25-dihydroxy vitamin D_3, sex hormones, and calcitonin) and local factors (e.g., nitric oxide, prostaglandins, growth factors, and cytokines) [1].

[004] Integrins are heterodimeric matrix receptors that anchor cells to substrates and transmit externally derived signals across the plasma membrane [5]. Integrin αvβ3 is involved in the osteoclast-mediated bone resorption, both *in vivo* [6] and *in vitro* [7, 8]. This heterodimer molecule recognizes the amino acid motif Arg-Gly-Asp (RGD, SEQ ID NO: 2) contained in bone matrix proteins such as osteopontin and bone sialoprotein [7, 8]. Integrin αvβ3 is expressed in an osteoclast [9] and its expression is modulated by resorptive steroids and cytokines [10]. Based on blocking experiments, αvβ3 integrin has been identified as a major functional adhesion receptor on osteoclasts. Inhibitors of integrin αvβ3 reduce the capacity of osteoclasts to bind to and resorb bone [7, 11]. Integrin αvβ3 plays a major role in the function of osteoclasts [7, 11, 12] and inhibitors of this integrin are

being considered for preventing osteoporosis [11], osteolytic metastases [13] and malignancy-induced hypercalcemia.

[005] There are many bone diseases that are related to osteolysis that is mediated by osteoclasts. Osteoporosis is the most common one that is induced when resorption and formation of bone are not coordinated and bone breakdown overrides bone building. Osteoporosis is also caused by other conditions, such as hormonal imbalance, diseases, or medications (e.g., corticosteroids or anti-epileptic agents) [2]. Bone is one of the most common sites of metastasis by human breast, prostate, lung, and thyroid cancers, as well as other cancers [3, 4]. Osteoporosis may also result from post-menopausal estrogen deficiency. Secondary osteoporosis may be associated with rheumatoid arthritis. Bone metastasis shows a very unique step of osteoclastic bone resorption that is not seen in metastasis of other organs. It is widely accepted that osteolysis that is associated with cancer is essentially mediated by osteoclasts, which seem to be activated and may be indirectly activated through osteoblasts or directly by tumor products [4]. In addition, hypercalcemia (increased blood-calcium concentration) is an important complication of osteolytic bone diseases. It occurs relatively frequently in patients with an extensive bone destruction, and is particularly common in breast, lung, renal, ovarian, and pancreatic carcinomas as well as in myeloma [4].

[006] Disintegrins are a family of low-molecular-weight RGD-containing peptides that bind specifically to integrins αIIbβ3, α5β1, and αvβ3 expressed on platelets and other cells including vascular endothelial cells and some tumor cells [14, 15]. In addition to their potent antiplatelet activity, studies of disintegrins have revealed new uses in the diagnosis of cardiovascular diseases and the design of therapeutic agents in arterial thrombosis, osteoporosis, and angiogenesis-related tumor growth and metastasis [15]. Rhodostomin (Rho), a disintegrin derived from the venom of *Colloselasma rhodostoma*, has been found to inhibit platelet aggregation *in vivo* and *in vitro* [16, 17] through the blockade of platelet glycoprotein αIIbβ3. Furthermore, rhodostomin is reported to inhibit the adhesion of breast and prostate carcinoma cells to both unmineralized and mineralized bone extracellular matrices in a dose-dependent manner, without affecting the viability of tumor cells. In addition, rhodostomin inhibits the migration and invasion of breast

and prostate carcinoma cells [18]. Rhodostomin has also been shown to inhibit adipogenesis and obesity [19]. However, because rhodostomin non-specifically binds to integrins αIIbβ3, α5β1, and αvβ3, the pharmaceutical uses of rhodostomin cause serious side effects. For example, when applying rhodostomin in treating carcinomas, the inhibition of platelet aggregation is an undesirable side effect.

[007] Therefore, a need exists in the art to address these deficiencies and inadequacies, especially in connection with the making of a disintegrin variant that has a specific selectivity to integrin αvβ3.

SUMMARY OF THE INVENTION

[008] In accordance with the invention, one aspect of the invention is a polypeptide that is selective for αvβ3 integrin. The polypeptide exhibits reduced binding to αIIbβ3 and/or α5β1 integrin compared to a wild-type disintegrin. The polypeptide is encoded by a modified disintegrin nucleotide sequence that encodes a modified amino acid sequence, with reduced αIIbβ3 and/or α5β1 integrin binding activity. The polypeptide may be pegylated or conjugated with albumin.

[009] The disintegrin nucleotide sequence may be derived from snake venom. The disintegrin may be chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[010] Another aspect of the invention is a polypeptide that is a variant of rhodostomin, in which the rhodostomin comprises the amino acid sequence of SEQ ID NO: 1.

[011] Another aspect of the invention is a polypeptide comprises an amino acid chosen from SEQ ID NOs: 30-42.

[012] Another aspect of the invention is a polypeptide comprising the amino acid sequence of SEQ ID NO: 29, which further comprises, one, two, three of four amino acid substitutions in positions corresponding to amino acids 48, 50, 52, or 53 of SEQ ID NO: 1.

[013] Another aspect of the invention is a polypeptide comprising amino acid substitutions chosen from Ala at position 48, Leu, Ile, and His at position 50,

Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53 of SEQ ID NO: 1.

[014] Another aspect of the invention is a polypeptide that is encoded nucleotide sequence chosen from SEQ ID NOs: 43-56.

[015] Another aspect of the invention is polypeptide that exhibits at least about a 5, 50, or 100-fold decrease in affinity to αIIbβ3 and/or α5β1 as compared to rhodostomin. In one embodiment of the invention, the polypeptide exhibits at least about a 200-fold decrease in affinity to αIIbβ3 and/or α5β1 integrin as compared to rhodostomin. In another embodiment of the invention, the polypeptide exhibits at least about a 1000 or 2000-fold decrease in affinity to αIIbβ3 and/or α5β1 integrin as compared to rhodostomin. In another embodiment of the invention, the polypeptide exhibits at least about 5, 50, 100, 1000, or 2000-fold decrease in affinity to platelet as compared to rhodostomin. In still another embodiment of the invention, the polypeptide exhibits a substantially reduced activity in prolongation of blood clotting time as compared to rhodostomin and or a wild-type disintegrin.

[016] Yet another aspect of the invention is a physiologically acceptable composition comprising a polypeptide of the invention, and a pharmaceutically acceptable carrier.

[017] Another aspect of the invention is a polypeptide comprising an amino acid sequence chosen from SEQ ID NOs: 57-69.

[018] Yet another aspect of the invention is a method of using a disintegrin variant for treatment and/or prevention of an αvβ3 integrin-associated disease in a mammal including a human. The method includes the step of administering to the mammal in need thereof a therapeutically effective amount of a disintegrin variant. The polypeptide employed in the method may be pegylated or conjugated with albumin.

[019] In one aspect of the invention, the disintegrin may be derived from a snake venom, and may be chosen from one of rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[020] In one aspect of the invention, the disintegrin is rhodostomin.

[021] In another aspect of the invention, the rhodostomin comprises a variant of rhodostomin comprising the amino acid sequence of SEQ ID NO: 1.

[022] In another aspect, the rhodostomin comprises and RGD motif variant comprising an amino acid chosen from SEQ ID NOs: 30-42.

[023] In another aspect, the rhodostomin comprises an amino acid chosen from SEQ ID NOs: 57-69.

[024] In one aspect of the invention, the αvβ3 integrin-associated disease includes, but is not limited to, osteoporosis, bone tumor or cancer growth and symptoms related thereto, angiogenesis-related tumor growth and metastasis, tumor metastasis in bone, malignancy-induced hypercalcemia, angiogenesis-related eye diseases, Paget's disease, rheumatic arthritis and osteoarthritis.

[025] In another aspect of the invention, a polypeptide of the invention is used for treatment and/or prevention of an angiogenesis-related eye disease, which includes, but is not limited to, age-related macular degeneration, diabetic retinopathy, corneal neovascularizing diseases, ischaemia-induced neovascularizing retinopathy, high myopia, and retinopathy of prematurity.

[026] In still another aspect of the invention, a polypeptide of the invention is used for treatment and/or prevention of osteoporosis. The osteoporosis is may be associated with a pathological condition chosen from post-menopausal estrogen deficiency, secondary osteoporosis, rheumatoid arthritis, ovariectomy, Paget's disease, bone cancer, bone tumor, osteoarthritis, increased osteoclast formation, and increased osteoclast activity. Furthermore, the osteoporosis includes, but is not limited to, an ovariectomy-induced or post-menopausal osteoporosis or bone loss.

[027] Yet another aspect of the invention is a method of using a polypeptide of the invention for treatment and/or prevention of an ovariectomy-induced physiological change in a mammal including a human.

[028] Yet another aspect of the invention is a method of using a disintegrin variant for inhibition and/or prevention of tumor cell growth in bone and symptoms related thereto in a mammal including a human.

[029] Yet another aspect of the invention is a method for making a polypeptide of the invention, the method comprising the steps as follows: (a) transfecting a host cell with a polynucleotide encoding said polypeptide (b) growing said host cell in a culture medium; and isolating said polypeptide. The method of the invention may further comprise growing host cell in a culture medium free of amino acids; and collecting supernatant to obtain said polypeptide. The method may further comprise adding methanol to the culture medium to induce the polypeptide expression in the host cells. The method may further comprise the step of performing a column chromatography to obtain said polypeptide. In one embodiment, the method may further comprise the step of performing a HPLC to obtain the isolated polypeptide.

[030] Another aspect of the invention is a polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide may be a variant of a disintegrin isolated from snake venom.

[031] In another aspect of the invention, the disintegrin is chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[032] In another aspect of the invention, the disintegrin comprises rhodostomin.

[033] In another aspect of the invention, the disintegrin comprises a variant of rhodostomin comprising the amino acid sequence of SEQ ID NO: 1.

[034] In another aspect of the invention, the polypeptide comprises an RGD motif variant having an amino acid sequence chosen from SEQ ID NOs: 30-42.

[035] In another aspect of the invention, the polynucleotide comprises a sequence chosen from SEQ ID NOs: 43-56 and 78-135.

[036] In another aspect of the invention, the polynucleotide may encode the polypeptides with one, two, three of four amino acid substitutions in positions corresponding to amino acids 48, 50, 52, or 53 of SEQ ID NO: 1.

[037] Another aspect of the invention is a polynucleotide that hybridizes under stringent conditions to a polynucleotide of the invention.

[038] Another aspect of the invention is a polypeptide encoded by a polynucleotide that hybridizes to a polynucleotide sequence of the invention.

[039] These and other aspects will become apparent from the following description of the various embodiments taken in conjunction with the following drawings, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

[040] The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention.

[041] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[042] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[043] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[044] FIG. 1 is a graph showing less impact of RD and HSA-RD and ARLDDL than rhodostomin protein on bleeding time in mice.

[045] FIG. 2A are photographs of trabecular bones showing an inhibition of ovariectomy-induced trabecular bone loss in rats treated with PGP protein. Bar = 1 mm.

[046] FIG. 2B are photographs of Tartrate-resistant acid phosphatase (TRAP) stained trabecular bones showing inhibition of ovariectomy-induced increase in osteoclast number in rats treated with PGP protein. Bar = 100 mm.

[047] FIG. 3 is a graph showing an inhibition of ovariectomy-induced osteoclast activation in rats treated with RD protein or alendronate (drug treatment), rebound of the osteoclast marker concentration during the period of drug withdrawal, and reversal of ovariectomy-induced increase in osteoclast activity during re-administration with RD or alendronate.

[048] FIGs. 4A-4D are photographs of safranin-O and haematoxylin stained knee joint showing an inhibition of chondrocyte layer destruction in arthritic rats treated with RD protein. Arrows indicate the chondrocyte layer.

[049] FIGs. 5A-5C are graphs showing inhibition of blood cytokine elevation in osteoarthritis rat treated with RD protein.

[050] FIGs. 6A-6C are graphs showing inhibition of blood cytokine elevation in osteoarthritis mice treated with RD protein.

[051] FIG. 7 is a graph showing an inhibition of PC-3 bone tumor growth in SCID mice treated with RD protein, but not with alendronate.

[052] FIG. 8 is a graph showing an inhibition of tumor-induced decrease in body weight in SCID mice treated with RD protein.

[053] FIG. 9A are photographs showing a visible spherical tumor growth in the proximal tibia of each leg after the intratibial injection of PC-3 cells (shown by arrows in the middle panel) and an inhibition of the bone tumor growth in SCID mice treated with RD protein.

[054] FIG. 9B are radiographs of tibia bones showing an inhibition of PC-3 tumor cells-induced osteolytic bone lesion in SCID mice treated with RD protein.

[055] FIG. 9C is a graph of the quantitation of the data in FIG. 9B showing an inhibition of PC-3 tumor-induced osteolysis in SCID mice treated with RD protein.

[056] FIG. 9D is a graph showing an inhibition of PC-3 tumor-induced increase in C-terminal telopeptides of type-I collagen in SCID mice treated with RD protein.

[057] FIG. 9E is a graph showing an inhibition of PC-3 tumor-induced increase in the serum calcium concentration in SCID mice treated with RD protein or alendronate.

[058] FIG. 10A is a graph showing an inhibition of MDA-MB-231 bone tumor growth in nude mice treated with RD protein.

[059] FIG. 10B is a graph showing an inhibition of MDA-MB-231 bone tumor-induced increase in C-terminal telopeptides of type-I collagen in nude mice treated with RD protein.

[060] FIG. 10C is a graph showing an inhibition of MDA-MB-231 bone tumor-induced hypercalcemia in nude mice treated with RD protein.

[061] FIG. 10D is a graph showing no change in white blood cell counts in nude mice injected with MDA-MB-231 cells and treated with RD protein.

[062] FIG. 10E is a graph showing no change in red blood cell counts in nude mice injected with MDA-MB-231 cells and treated with RD protein.

[063] FIG. 10F is a graph showing no change in the platelet counts in nude mice injected with MDA-MB-231 cells and treated with RD protein.

[064] FIG. 11A are photographs showing a reduced blood vessel density in MATRIGEL™ plugs from C57BL/6 mice treated with RD or RD-albumin (HSA-RD) protein in comparison with untreated control mice.

[065] FIG. 11B is a graph showing a reduced hemoglobin content in MATRIGEL™ plugs from C57BL/6 mice treated with RD (every day-RD/1d or every other day-RD/2d) or RD-albumin (HSA-RD-every other day) protein in comparison with untreated control mice.

[066] FIG. 12A are photographs showing angiogenesis in a mouse model of retinopathy of prematurity (ROP), and reduced angiogenesis in a ROP mouse treated with RD protein (ROP+RD). Arrows indicate blood vessel profiles (BVPs).

[067] FIG. 12B is a graph showing reduced BVPs in a mouse model of retinopathy of prematurity (ROP) treated with RD protein.

[068] FIG. 13A is a graph showing an inhibition of ovariectomy-induced osteoclast activation in mice treated with RD or RD-albumin protein.

[069] FIG. 13B is a graph showing an inhibition of ovariectomy-induced alkaline phosphatase (ALP) inactivation in mice treated with RD or RD-albumin protein.

[070] FIG. 13C is a graph showing an inhibition of ovariectomy-induced decrease in BMD.

[071] FIG. 13D is a graph showing an inhibition of ovariectomy-induced decrease in BMC.

[072] FIGS. 14A-D show amino acid sequences SEQ ID NOs: 1, and 57-69 of rhodostomin variants.

[073] FIGS. 15A-C show nucleotide sequences SEQ ID NOs: 43-56 of rhodostomin variants.

[074] FIGS. 16A-H show amino acid and nucleotide sequences SEQ ID NOs: 78-135 of disintegrin variants.

DETAILED DESCRIPTION OF THE INVENTION

[075] The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art. Various embodiments of the invention are now described in detail. As used in the description and throughout the claims that follow, the meaning of "a", "an", and "the" includes plural reference unless the context clearly dictates otherwise. Also, as used in the description and throughout the claims that follow, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise. Additionally, some terms used in this specification are more specifically defined below. All references cited and discussed in this specification are incorporated herein by reference in their entireties and to the same extent as if each reference was individually incorporated by reference.

[076] Reference will now be made in detail to the present embodiments (exemplary embodiments) included in the invention, examples of which are illustrated in the accompanying drawings.

DEFINITIONS

[077] The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including

examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. The invention is not limited to various embodiments given in this specification.

[078] 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 pertains. In the case of conflict, the present document, including definitions will control.

[079] "Around," "about" or "approximately" shall generally mean within 20 percent, within 10 percent, within 5, 4, 3, 2, or 1 percent of a given value or range. Numerical quantities given are approximate, meaning that the term "around," "about" or "approximately" can be inferred if not expressly stated.

[080] The terms "polynucleotide," "nucleotide," "nucleic acid," "nucleic acid molecule," "nucleic acid sequence," "polynucleotide sequence," and "nucleotide sequence" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides can comprise deoxyribonucleotides, ribonucleotides, and/or their analogs or derivatives. The term includes variants. Variants may include insertions, additions, deletions, or substitutions. Nucleotide sequences are listed in the 5' to 3' direction.

[081] The terms "polypeptide," "peptide," and "protein," used interchangeably to refer to a polymeric form of amino acids of any length, which can include naturally-occurring amino acids, coded and non-coded amino acids, chemically or biochemically modified, derivatized, or designer amino acids, amino acid analogs, peptidomimetics, and depsipeptides, and polypeptides having modified, cyclic, bicyclic, depsicyclic, or depsibicyclic peptide backbones. The term includes single chain protein as well as multimers. The term also includes proteins conjugated to a label such as FITC, biotin, and radioisotopes, including, but not limited to ^{64}Cu, ^{67}Cu, ^{90}Y, ^{99}mTc, ^{111}In, ^{124}I, ^{125}I, ^{131}I, ^{137}Cs, ^{186}Re, ^{211}At, ^{212}Bi, ^{213}Bi, ^{223}Ra, ^{241}Am, and ^{244}Cm; enzymes having detectable products (for example, luciferase, peroxidase, alkaline phosphatase, β-galactosidase, and the like); fluorescers and fluorescent labels, fluorescence emitting metals, for example, ^{152}Eu, or others of the lanthanide series, electrochemiluniescent compounds, chemiluminescent compounds, for example, luminol, isoluminol, or acridinium salts;

specific binding molecules, for example, magnetic particles, microspheres, nanospheres, and the like. The term also includes peptides conjugated to therapeutic agents.

[082] The terms also include fusion proteins, including, but not limited to, glutathione S-transferase (GST) fusion proteins, fusion proteins with a heterologous amino acid sequence such as bioluminescent proteins, for example, luciferin, or aequorin (green fluorescent protein), with heterologous and homologous leader sequences, fusion proteins with or without N-terminal methionine residues, pegylated proteins, and immunologically tagged, or his-tagged proteins. Such fusion proteins also include fusions to epitopes. Such fusion proteins can comprise multimers of the peptides of the invention, e.g. homodimers or homomultimers, and heterodimers and heteromultimers. The term also includes peptide aptamers.

[083] The term "hybridizes specifically," in the context of a polynucleotide, refers to hybridization under stringent conditions. Conditions that increase stringency of both DNA/DNA and DNA/RNA hybridization reactions are widely known and published in the art. Examples of stringent hybridization conditions include hybridization in 4 X sodium chloride/sodium citrate (SSC), at about 65-70 °C, or hybridization in 4 X SSC plus 50% formamide at about 42-50 °C, followed by one or more washes in 1X SSC, at about 65-70 °C.

[084] The term "ligand" refers to a molecule that binds to another molecule, including a receptor.

[085] A "host cell" is an individual cell or cell culture which can be or has been a recipient of any recombinant vector(s) or polynucleotide. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected in vivo or in vitro with a recombinant vector or a polynucleotide of the invention. A host cell which comprises a recombinant vector of the invention may be called a "recombinant host cell."

[086] "Treatment," covers any administration or application of remedies for disease in a mammal, including a human, and includes inhibiting the disease, arresting its development, or relieving the disease, for example, by causing

regression, or restoring or repairing a lost, missing, or defective function; or stimulating an inefficient process. The term includes obtaining a desired pharmacologic and/or physiologic effect, covering any treatment of a pathological condition or disorder in a mammal, including a human. The effect may be prophylactic in terms of completely or partially preventing a disorder or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disorder and/or adverse affect attributable to the disorder. Thus, the invention provides both treatment and prophylaxis. It includes (1) preventing the disorder from occurring or recurring in a subject who may be predisposed to the disorder but is not yet symptomatic, (2) inhibiting the disorder, such as arresting its development, (3) stopping or terminating the disorder or at least its associated symptoms, so that the host no longer suffers from the disorder or its symptoms, such as causing regression of the disorder or its symptoms, for example, by restoring or repairing a lost, missing or defective function, or stimulating an inefficient process, or (4) relieving, alleviating, or ameliorating the disorder, or symptoms associated therewith, where ameliorating is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, such as inflammation, pain, and/or tumor size.

[087] A "pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material, formulation auxiliary, or excipient of any conventional type. A pharmaceutically acceptable carrier is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation.

[088] A "composition" refers to a mixture that usually contains a carrier, such as a pharmaceutically acceptable carrier or excipient that is conventional in the art and which is suitable for administration into a subject for therapeutic, diagnostic, or prophylactic purposes. It may include a cell culture in which the polypeptide or polynucleotide is present in the cells or in the culture medium. For example, compositions for oral administration can form solutions, suspensions, tablets, pills, capsules, sustained release formulations, oral rinses, or powders.

[089] "Disease" refers to any condition, infection, disorder, or syndrome that requires medical intervention or for which medical intervention is desirable. Such medical intervention can include treatment, diagnosis, and/or prevention.

Peptides

[090] The peptides of the invention can be expressed using methods known in the art. Cell-based methods and cell-free methods are suitable for producing peptides of the invention. Cell-based methods generally involve introducing a nucleic acid construct into a host cell in vitro and culturing the host cell under conditions suitable for expression, then harvesting the peptide, either from the culture medium or from the host cell, (for example, by disrupting the host cell), or both. The invention also provides methods of producing a peptide using cell-free in vitro transcription/translation methods, which are well known in the art.

[091] Suitable host cells include prokaryotic or eukaryotic cells, including, for example, bacterial, yeast, fungal, plant, insect, and mammalian cells.

[092] Typically, a heterologous peptide, whether modified or unmodified, may be expressed on its own, as described above, or as a fusion protein, and may include not only secretion signals, but also a secretory leader sequence. A secretory leader sequence of the invention may direct certain proteins to the endoplasmic reticulum (ER). The ER separates the membrane-bound proteins from other proteins. Once localized to the ER, proteins can be further directed to the Golgi apparatus for distribution to vesicles, including secretory vesicles, the plasma membrane, lysosomes, and other organelles.

[093] Additionally, peptide moieties and/or purification tags may be added to the peptides. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability, and to facilitate purification, among other reasons, are familiar and routine techniques in the art. Suitable purification tags include, for example, V5, polyhistidines, avidin, and biotin. Conjugation of peptides to compounds such as biotin can be accomplished using techniques well known in the art. (Hermanson ed. (1996) Bioconjugate Techniques; Academic Press). Peptides can also be conjugated with radioisotopes, toxins, enzymes, fluorescent labels, colloidal gold, nucleic acids, vinorelbine, and doxorubicin using techniques

known in the art. (Hermanson ed. (1996) Bioconjugate Techniques; Academic Press; Stefano et al. (2006).

[094] Fusion partners suitable for use in the invention include, for example, fetuin, human serum albumin, Fc, and/or one or more of their fragments. Conjugated proteins, such as polyethylene glycol conjugates, are also provided.

[095] The peptides of the invention can also be chemically synthesized using techniques known in the art (e.g., see Hunkapiller et al., Nature, 310:105 111 (1984); Grant ed. (1992) Synthetic Peptides, A Users Guide, W.H. Freeman and Co.; U.S. Patent No. 6,974,884)). For example, a polypeptide corresponding to a fragment of a polypeptide can be synthesized by use of a peptide synthesizer or through the use of solid-phase methods known in the art.

[096] Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-amino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

[097] The polypeptides of the invention can be recovered and purified from chemical synthesis and recombinant cell cultures by standard methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. In one embodiment, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

[098] A peptide or peptidomimetic of the invention can be modified with or covalently coupled to one or more of a variety of hydrophilic polymers to increase solubility and circulation half-life of the peptide. Suitable nonproteinaceous hydrophilic polymers for coupling to a peptide include, but are not limited to, polyalkylethers as exemplified by polyethylene glycol and polypropylene glycol, polylactic acid, polyglycolic acid, polyoxyalkenes, polyvinylalcohol, polyvinylpyrrolidone, cellulose and cellulose derivatives, dextran, and dextran derivatives. Generally, such hydrophilic polymers have an average molecular weight ranging from about 500 to about 100,000 daltons, from about 2,000 to about 40,000 daltons, or from about 5,000 to about 20,000 daltons. The peptide can be derivatized with or coupled to such polymers using any of the methods set forth in Zallipsky, S. (1995) Bioconjugate Chem., 6:150-165; Monfardini, C., et al. (1995) Bioconjugate Chem. 6:62-69; U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; 4,179,337, or WO 95/34326.

[099] In some embodiments, a peptide of the invention is provided in formulation with pharmaceutically acceptable carriers, excipients, and diluents, of which a wide variety are known in the art. These pharmaceutical carriers, excipients, and diluents include those listed in the USP pharmaceutical excipients listing. USP and NF Excipients, Listed by Categories, p. 2404-2406, USP 24 NF 19, United States Pharmacopeial Convention Inc., Rockville, Md. (ISBN 1-889788-03-1). Pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers, or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

[0100] Suitable carriers include, but are not limited to, water, dextrose, glycerol, saline, ethanol, and combinations thereof. The carrier can contain additional agents such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the formulation. Topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar

agents can be added as necessary. Percutaneous penetration enhancers such as Azone can also be included.

[0101] In pharmaceutical dosage forms, the compositions of the invention can be administered in the form of their pharmaceutically acceptable salts, or they can also be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. The subject compositions are formulated in accordance to the mode of potential administration.

Methods of Treatment

[0102] An αvβ3 integrin-associated disease, includes, but is not limited to, osteoporosis, bone tumor or cancer growth and symptoms related thereto, angiogenesis-related tumor growth and metastasis, tumor metastasis in bone, malignancy-induced hypercalcemia, angiogenesis-related eye diseases, Paget's disease, rheumatic arthritis and osteoarthritis.

[0103] Peptides of the invention may be administered to a subject in need of treatment by injection systemically, such as by intravenous injection; or by injection or application to the relevant site, such as by direct injection, or direct application to the site when the site is exposed in surgery; or by topical application, such as if the disorder is on the skin, for example.

[0104] Peptides of the invention can be used as monotherapy. Alternatively, the peptides of the invention can be used in combination with standard regimens to treat αvβ3 integrin associated diseases.

[0105] Administration of the agents can be achieved in various ways, including oral, buccal, nasal, rectal, parenteral, intraperitoneal, intradermal, transdermal, subcutaneous, intravenous, intra-arterial, intracardiac, intraventricular, intracranial, intratracheal, and intrathecal administration, etc., or otherwise by implantation or inhalation. Thus, the subject compositions can be formulated into preparations in solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols. The following methods and excipients are merely exemplary and are in no way limiting.

[0106] Suitable excipient vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the

vehicle can contain minor amounts of auxiliary substances such as wetting or emulsifying agents or pH buffering agents. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. The composition or formulation to be administered will, in any event, contain a quantity of the agent adequate to achieve the desired state in the subject being treated.

[0107] A peptides of the invention can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers, and preservatives. Other formulations for oral or parenteral delivery can also be used, as conventional in the art.

[0108] "Disintegrins" refer to a family of polypeptides that may be purified from snake venoms, which contain the arginine-glycine-aspartic acid (RGD) sequence. Without being bound by any theory or mechanism, it is believed that the RGD tripeptide binds with high affinity to integrins to block the interaction of integrins with RGD-containing proteins. Disintegrins thus block adhesive functions and act as platelet aggregation inhibitors.

[0109] The abbreviation "Rho" means "rhodostomin," which is a disintegrin derived from the venom of *Colloselasma rhodostoma*. Rhodostomin non-specifically binds to integrins αIIbβ3, α5β1 and αvβ3, and prolongs blood clotting time by inhibiting platelet aggregation through the blockade of platelet glycoprotein αIIbβ3.

[0110] The "disintegrin variant" or "rhodostomin variant" refers to a functionally active protein, or a polypeptide or any derivatives thereof, that comprises an amino acid sequence derived or modified or mutated from a wild-type disintegrin such as rhodostomin. A functionally active disintegrin/rhodostomin variant can specifically bind to and inhibit integrin αvβ3 activity. The disintegrin or rhodostomin variant of the present invention can be constructed by any method suitable to the aims of the present invention, and in one embodiment by a site-directed mutagenesis method, and in another embodiment by a polymerase chain reaction method. Variants may include insertions, additions, deletions, or

substitutions compared with the subject peptides. Variants of polypeptide sequences include biologically active polymorphic variants.

[0111] Peptides of the invention can include naturally-occurring and non-naturally occurring amino acids. Peptides can comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" or "synthetic" amino acids (for example, β-methyl amino acids, Cα-methyl amino acids, and Nα-methyl amino acids, etc.) to convey special properties. Additionally, peptides can be cyclic. Peptides can include non-classical amino acids in order to introduce particular conformational motifs. Any known non-classical amino acid can be used. Amino acid analogs and peptidomimetics can be incorporated into a peptide to induce or favor specific secondary structures, including, but not limited to, LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid), a β-turn inducing dipeptide analog; β-sheet inducing analogs; β-turn inducing analogs; α-helix inducing analogs; γ-turn inducing analogs; Gly-Ala turn analogs; amide bond isostere; or tretrazol, and the like.

[0112] A desamino or descarboxy residue can be incorporated at the terminal ends of the peptide, so that there is no terminal amino or carboxyl group, to decrease susceptibility to proteases or to restrict conformation. C-terminal functional groups include amide, amide lower alkyl, amide di (lower alkyl), lower alkoxy, hydroxy, and carboxy, and the lower ester derivatives thereof, and the pharmaceutically acceptable salts thereof.

[0113] The term "IC_{50}," or "the half maximal inhibitory concentration" refers to the concentration of Rho or its variant that is required for 50% inhibition of its receptor. IC_{50} is a measure of how much of Rho or its variant is needed to inhibit a biological process by 50%,such as the variant's affinity to its receptor.

[0114] The term "therapeutically effective amount" as used refers to an amount which, when administered to a living subject, achieves a desired effect on the living subject. For example, an effective amount of the disintegrin or Rho variant of the invention for administration to the living subject is an amount that prevents and/or treats an integrin αvβ3-mediated disease. The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for systemic

versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction, and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

[0115] The term "receptor antagonist" refers to a binding ligand of a receptor that inhibits the function of a receptor by blocking the binding of an agonist to the receptor, or which allows agonist binding, but inhibits the ability of the agonist to activate the receptor.

[0116] The term "substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity" refers to a reduced activity of at least five fold in blocking integrin αIIbβ3 and/or α5β1 receptor compared to wild type rhodostomin or other disintegrins. For example, to calculate the reduction in αIIbβ3 and/or α5β1 receptor-blocking activity, the IC_{50} of a rhodostomin variant for inhibition of integrin αIIbβ3 and/or α5β1 binding to a matrix protein, such as fibrinogen, is compared to of the IC_{50} of Rho.

[0117] The term "RGD motif variant" refers to a peptide comprising a modification in the amino acid sequence that spans the RGD sequence of a corresponding wild-type sequence, such as the sequence comprising RGD in Rhodostomin. Examples of "RGD motif variants" include ^{48}ARGDDP^{53}, ^{48}PRLDMP^{53}, ^{48}PRIDMP^{53}, and ^{48}ARLDDL^{53}.

[0118] The term "RD" refers to a rhodostomin variant having a RGD motif variant ^{48}PRLDMP^{53}.

[0119] The term "PGP" refers to a rhodostomin variant having a RGD motif variant ^{48}PRGDGP^{53}.

[0120] The term ARLDDL refers to a rhodostomin variant having a RGD motif variant ^{48}ARLDDL^{53}.

[0121] The term "inhibitory selectivity for integrin αvβ3 relative to αIIbβ3 and/or α5β1 receptors" refers to a variant's binding selectivity toward integrin αvβ3 over αIIbβ3 and/or α5β1 receptors, which is expressed as a ratio of the IC_{50} of the variant for inhibition of αIIbβ3 and/or α5β1 receptors over that for inhibition of αvβ3 receptor.

[0122] The term "substantially reduced activity in prolongation of blood bleeding time" refers to a reduced ability to inhibit blood clotting in a statistically

significant manner as measured by the bleeding time experiment described in the specification.

[0123] The term "pegylated-RD" refers to a pegylated product of RD protein.

[0124] The term "albumin-RD, or HSA-RD" refers to a human albumin-conjugated product of RD protein.

OVERVIEW OF THE INVENTION

[0125] The present invention is related to disintegrin variants that are selective αvβ3 integrin antagonists. Disintegrin variants such as RD-related compounds potently inhibit osteoclast differentiation *in vitro*. They also inhibit osteoclast resorbing activity and ovariectomy-induced increase in osteoclast formation in animal studies. In addition, RD inhibits the tumor growth of human prostate and breast cancer cells in bone. Malignancy-induced hypercalcemia was also effectively blocked by RD-related proteins. Paget's disease (also known as osteitis deformans) is a chronic bone disorder that typically results in enlarged and deformed bones due to irregular breakdown and formation of bone tissues. Bisphosphonates have been approved for the treatment of Paget's disease. Osteoarthritis is also related to the increase in osteoclast activity. Based on the similar mechanism of action, RD derivatives should also be effective for treatment of these bone disorders. An intravenous injection of RD or PGP at a very large dose at 30 mg/kg did not affect the survival of mice (n=3). In addition, long term administration of PGP (I.V., 0.5 mg/kg/day) for 6 weeks did not affect serum level of creatinine, GOT, and GPT, suggesting lack of side effects on kidney and liver. Therefore, RD and its derivatives are potential drug candidates for treatment of osteoporosis, bone tumor, malignancy-induced hypercalcemia, Paget's disease, rheumatic arthritis, osteoarthritis and angiogenesis-related eye diseases.

[0126] Many kinds of snake venom contain proteins that comprise an RGD domain. These RGD domain-containing proteins are called disintegrins. A modification in the sequence spanning the RGD domain resulted in a very unique polypeptide variant with a reduced binding affinity to other kinds of integrins but an increased selectivity to αvβ3 integrin. The disintegrin variants including rhodostomin variants prove to be potential therapeutic candidates for, among

others, osteoporosis, suppression of tumor growth in bone, and angiogenesis-related eye diseases. Moreover, disintegrin variants including rhodostomin variants having a RGD-motif region with at least one amino acid substitution may be valuable tools for developing selective antagonists of αvβ3 integrin.

[0127] One aspect of the invention is a polypeptide that has integrin αvβ3 receptor-antagonist activity and reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity as compared to a wild-type disintegrin. The polypeptide is encoded by a modified disintegrin nucleotide sequence that encodes a modified amino acid sequence, which results in a polypeptide having substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity. The polypeptide may be pegylated or conjugated with albumin.

[0128] The disintegrin nucleotide sequence may be derived from snake venom. According to the invention, disintegrins include, but are not limited to, albolabrin (*Cryptelytrops albolabris*), applagin (*Agkistrodon piscivorus piscivorus*), basilicin (*Crotalus basiliscus*), batroxostatin (*Bothrops atrox*), bitistatin (*Bitis arietans*), cereberin (*Crotalus oreganus cerberus*), cerastin (*Crotalus cerastes cerastes*), crotatroxin (*Crotalus atrox*), durissin (*Crotalus durissus durissus*), elegantin (*Protobothrops elegans*), flavoridin (*Trimeresurus flavoviridis*), flavostatin (*Trimeresurus flavoviridis*), halysin (*Gloydius blomhoffi*), halystatin (*Gloydius halys*), jararacin (*Bothrops jararaca*), jarastatin (*Bothrops jararaca*), kistrin (*Calloselasma rhodostoma*), lachesin (*Lachesis muta muta*), lutosin (*Crotalus oreganus lutosus*), molossin (*Crotalus molossus molossus*), salmosin (*Gloydius blomhoffi brevicaudus*), saxatilin (*Gloydius halys*), tergeminin (*Sistrurus catenatus tergeminus*), trimestatin (*Trimeresurus flavoviridis*), trimucrin (*Protobothrops mucrosquamatus*), trimutase (*Protobothrops mucrosquamatus*), ussuristatin (*Gloydius ussuriensis*), viridin (*Crotalus viridis*).

[0129] Another aspect of the invention is an isolated polypeptide that is a variant of rhodostomin, in which the rhodostomin comprises an amino acid sequence defined by SEQ ID NO: 1, and the variant comprises an RGD motif variant.

[0130] In one embodiment of the invention, the RGD motif variant may contain an amino acid sequence chosen from SEQ ID NOs: 30-42.

[0131] Another aspect of the invention is a polypeptide comprising the amino acid sequence of SEQ ID NO: 29, which further comprises, one, two, three of four amino acid substitutions in positions corresponding to amino acids 48, 50, 52, or 53 of SEQ ID NO: 1.

[0132] Another aspect of the invention is a polypeptide comprising amino acid substitutions chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53 of SEQ ID NO: 1.

[0133] Another aspect of the invention is a polypeptide that is encoded nucleotide sequence chosen from SEQ ID NOs: 43-56.

[0134] For example, and specifically, in another embodiment of the invention, the RGD motif variant may comprise at least one amino acid substitution at a residue corresponding to Gly^{50} or Met^{52} of the wild-type RGD motif set forth by SEQ ID NO: 29. The at least one amino acid substitution occurs at a residue corresponding to Leu^{50} of SEQ ID NO: 36-37, Ile^{50} of SEQ ID NO: 39, His^{50} of SEQ ID NO: 40, Asn^{52} of SEQ ID NO: 41, or Gly^{52} of SEQ ID NO: 42.

[0135] In another embodiment of the invention, the RGD motif variant may comprise at least two amino acid substitutions at residues corresponding to Pro^{48} and Met^{52}, or Met^{52} and Pro^{53} of the wild-type RGD motif set forth by SEQ ID NO: 29. The at least two amino acid substitutions may be residues corresponding to Ala^{48} and Asp^{52} of SEQ ID NO: 30, or Asp^{52} and Met^{53} of SEQ ID NO: 35.

[0136] In still another embodiment of the invention, the RGD motif variant may comprise at least three amino acid substitutions at residues corresponding to Pro^{48}, Met^{52} and Pro^{53}, or Gly^{50}, Met^{52} and Pro^{53} of the wild-type RGD motif set forth by SEQ ID NO: 29. The at least three amino acid substitutions may be residues corresponding to Ala^{48}, Asp^{52} and Val^{53} of SEQ ID NO: 31, Ala^{48}, Asp^{52} and Leu^{53} of SEQ ID NO: 32, Ala^{48}, Asp^{52} and Met^{53} of SEQ ID NO: 34, Leu^{50}, Asp^{52} and Leu^{53} of SEQ ID NO: 37.

[0137] In still another embodiment of the invention, the RGD motif variant may comprise at least four amino acid substitutions at residues corresponding to Pro^{48}, Gly^{50}, Met^{52} and Pro^{53} of the wild-type RGD motif set forth by SEQ ID NO:

29. The at least four amino acid substitutions may be residues corresponding to Ala^{48}, Leu^{50}, Asp^{52} and Leu^{53} of SEQ ID NO: 38.

[0138] Another aspect of the invention is an isolated polypeptide that is encoded by a DNA having a modified rhodostomin nucleotide sequence chosen from SEQ ID NOs: 44-56. The polypeptide exhibits at least about a 5, 50, or 100-fold decrease in affinity to αIIbβ3 and/or α5β1 as compared to rhodostomin. In one embodiment of the invention, the polypeptide exhibits at least about a 200-fold decrease in affinity to αIIbβ3 and/or α5β1 integrin as compared to rhodostomin. In another embodiment of the invention, the polypeptide exhibits at least about a 1000 or 2000-fold decrease in affinity to αIIbβ3 and/or α5β1 integrin as compared to rhodostomin. In another embodiment of the invention, the polypeptide exhibits at least about 5, 50, 100, 1000, or 2000-fold decrease in affinity to platelet as compared to rhodostomin. In still another embodiment of the invention, the polypeptide exhibits a substantially reduced activity in prolongation of blood clotting time as compared to rhodostomin or wild-type disintegrin.

[0139] Yet another aspect of the invention is a physiologically acceptable composition comprising a polypeptide of the invention and a pharmaceutically acceptable carrier.

[0140] Yet another aspect of the invention is a method of using a disintegrin variant for treatment and/or prevention of an αvβ3 integrin-associated disease in a mammal including a human. The method includes the step of administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof. The polypeptide therein has integrin αvβ3 receptor-antagonist activity and substantially reduced integrin αIIbβ3 and/or integrin α5β1 receptor-blocking activity as compared to a wild-type disintegrin, and thereby results in treatment and/or prevention of the αvβ3 integrin-associated disease in the mammal. The polypeptide is encoded by a modified disintegrin nucleotide sequence that encodes a modified disintegrin amino acid sequence, which results in a polypeptide having substantially reduced integrin αIIbβ3 and/or integrin α5β1 receptor-blocking activity. The polypeptide employed in the method may be pegylated or conjugated with albumin.

[0141] As described above, the disintegrin nucleotide sequence may be derived from a snake venom, and may be chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[0142] In one embodiment of the invention, the method includes the step of administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide comprises the amino acid sequence of SEQ ID NO: 1, and the variant comprises an RGD motif variant.

[0143] In another embodiment of the invention, the method includes the step of administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide is a variant of rhodostomin comprising the amino acid sequence defined by SEQ ID NO: 1, and the variant comprises a RGD motif variant having an amino acid sequence chosen from SEQ ID NOs: 30-42.

[0144] In still another embodiment of the invention, the method includes the step of administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide comprises an amino acid sequence chosen from SEQ ID NOs: 57-69.

[0145] In one embodiment of the invention, a polypeptide of the invention is used for treatment and/or prevention of an αvβ3 integrin-associated disease, which includes, but is not limited to, osteoporosis, bone tumor or cancer growth and symptoms related thereto, angiogenesis-related tumor growth and metastasis, tumor metastasis in bone, malignancy-induced hypercalcemia, angiogenesis-related eye diseases, Paget's disease, rheumatic arthritis, and osteoarthritis.

[0146] In another embodiment of the invention, a polypeptide of the invention is used for treatment and/or prevention of an angiogenesis-related eye disease, which includes, but is not limited to, age-related macular degeneration,

diabetic retinopathy, corneal neovascularizing diseases, ischaemia-induced neovascularizing retinopathy, high myopia, and retinopathy of prematurity.

[0147] In still another embodiment of the invention, a polypeptide of the invention is used for treatment and/or prevention of osteoporosis. The osteoporosis is associated with a pathological condition chosen from post-menopausal estrogen deficiency, secondary osteoporosis, rheumatoid arthritis, ovariectomy, Paget's disease, bone cancer, bone tumor, osteoarthritis, increased osteoclast formation, and increased osteoclast activity. Furthermore, the osteoporosis includes, but is not limited to, an ovariectomy-induced osteoporosis or bone loss and post-menopausal osteoporosis or bone loss.

[0148] Yet another aspect of the invention is a method of using the a disintegrin variant for treatment and/or prevention of physiological changes in a mammal including a human induced by ovariectomy or post-menopausal osteoporosis. The method includes administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, which has integrin αvβ3 receptor-antagonist activity and substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity as compared to a wild-type disintegrin, and thereby resulting in treatment and/or prevention of the ovariectomy-induced physiological change in the mammal. The polypeptide is encoded by a modified disintegrin nucleotide sequence that encodes a modified amino acid sequence resulting in said polypeptide having substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity. The disintegrin nucleotide sequence may be derived from a snake venom, and may be chosen from one of rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[0149] The polypeptide variant used for treatment and/prevention of an ovariectomy-induced or post-menopausal physiological change in the mammal may contain an RGD motif variant that comprises an amino acid sequence chosen from SEQ ID NOs: 30-42.

[0150] In one embodiment of the invention, the method includes administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide variant comprises an amino acid sequence chosen from SEQ ID NOs: 57-69. In another embodiment, the polypeptide variant is pegylated or conjugated with albumin.

[0151] Yet another aspect of the invention is a method of using the a disintegrin variant for inhibition and/or prevention of tumor cell growth in bone and symptoms related thereto in a mammal including a human. The method includes administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, which has integrin αvβ3 receptor-antagonist activity and substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity as compared to a wild-type disintegrin, and thereby resulting in inhibition and/or prevention of tumor cell growth in bone and symptoms related thereto in the mammal. The polypeptide is encoded by a modified disintegrin nucleotide sequence that encodes a modified amino acid sequence and thereby results in said polypeptide having substantially reduced integrin αIIbβ3 and/or α5β1 receptor-blocking activity.

[0152] The disintegrin nucleotide sequence may be derived from a snake venom, and may be chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

[0153] The pathological symptoms related to tumor cell growth in bone include an increased osteoclast activity, an increased bone resorption, bone lesion, hypercalcemia, a body weight loss, and any combinations thereof. The tumor cell growth in bone includes bone cancer cells and metastasized cancer cells originating from prostate cancer, breast cancer, lung cancer, renal cancer, ovarian cancer, pancreatic cancer, or myeloma cancer.

[0154] In one embodiment of the invention, the method includes administering to the mammal in need thereof a therapeutically effective amount of

an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide is a variant of rhodostomin, in which rhodostomin comprises the amino acid sequence defined by SEQ ID NO: 1, and the variant comprises an RGD motif variant. The RGD motif variant may comprise an amino acid sequence chosen from SEQ ID NOs: 30-42.

[0155] In another embodiment of the invention, the method includes the step of administering to the mammal in need thereof a therapeutically effective amount of an isolated polypeptide, or a pharmaceutically acceptable salt thereof, in which the polypeptide comprises an amino acid sequence chosen from SEQ ID NOs: 57-69. The polypeptide variant may be pegylated or conjugated with albumin.

[0156] Yet another aspect of the invention is a method for making a polypeptide of the invention, the method comprising the steps as follows: (a) transforming a host cell with a polynucleotide encoding said polypeptide to obtain one or more than one transformant; (b) selecting a transformant having one or more than one copy of said DNA construct inserted into the tansformant; (c) growing the transformant in a culture medium to amplify cell population thereof; (d) harvesting transformant cells; (e) growing the harvested transformant cells in a culture medium free of amino acids; and (g) collecting supernatant to obtain said polypeptide.

[0157] The aforementioned step (e) may further comprise the step of adding methanol to the culture medium to induce the polypeptide expression in the transformant cells. In one embodiment, the step (g) may further comprise the step of performing a column chromatography to obtain said polypeptide. In a further embodiment, the aforementioned method may further comprise the step of performing a HPLC to obtain the purified, isolated polypeptide.

[0158] These and other aspects of the present invention are more specifically described below.

[0159] Human recombinant RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN). The C-terminal telopeptides of type-I collagen ELISA kit was obtained from Cross Laps (Herlev, Denmark). All other chemicals were obtained from Sigma.

EXAMPLE 1

Construction of DNAs Encoding Rhodostomin and Variants

[0160] Rhodostomin was cloned and expressed in the vector pGEX-2KS [20] as a template. The DNA encoding Rho was composed of codons preferentially used in *Pichia pastoris*. Rho DNA was amplified by the polymerase chain reaction (PCR) with the sense primer 5'-GAATTCGAATTCCATCATCATCATCATCAT CATGGTAAGGAATGTGACTGTTCTT-3' (Rho-Pic-1; SEQ ID NO: 3) that had *Eco R1* recognition and six histidine residues for facilitating purification. The antisense primer is 5'-CCGCGGCCGCGGTCAGTGGTATCTTGGACAGTCAGC-3' or 5'-CCGCGGCCGCGGTTAGTGGTATCTTGGACAGTCAGC-3' (Rho-Pic-2; SEQ ID NO: 4) with *Sac* II recognition and a TCA (or TTA) stop codon. The PCR product was purified and then ligated into the *Eco R1* and *Sac* II sites of the yeast recombination vector, pPICZαA. The recombinant plasmid was used to transform a *DH5α* strain, and colonies were selected on agar plates with low salt LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar at pH 7.0) and 25μg/ml antibiotic Zeocin.

[0161] Rhodostomin variants were synthesized and amplified by the polymerase chain reaction (PCR) using an overlapping oligonucleotide strategy with primers containing *Eco* RI and *Sac* II restriction sites. The nucleotide sequences of various primers used for synthesizing or confirming variants are listed in Table 1. RD-HSA fusion protein was constructed using similar procedures. The cDNA of human serum albumin was purchased from Invitrogen, and the structural gene of albumin was fused at N-terminus of Rho gene with a GSGSGS linker amino acid sequence and with six histidine residues at the N-terminus.

TABLE 1

Primer		Sequence	SEQ ID NO:
RLD-1	Sense	GAATCCCAAGA**CTT**GACATGCCAG	(SEQ ID NO: 5)
RLD-2	Antisense	CTGGCATGTC**AAG**TCTTGGGATTC	(SEQ ID NO: 6)
RHD-1	Sense	AGAATCCCAAGA**CAC**GACATGCCAGAC	(SEQ ID NO: 7)
RHD-2	Antisense	GTCTGGCATGTC**GTG**TCTTGGGATTCT	(SEQ ID NO: 8)
RID-1	Sense	AGAATCCCAAGA**ATC**GACATGCCAGAC	(SEQ ID NO: 9)
RID-2	Antisense	GTCTGGCATGTC**GAT**TCTTGGGATTCT	(SEQ ID NO: 10)
P48A-1	Sense	TGTAGAATC**GCT**AGAGGTGACATG	(SEQ ID NO: 11)
P48A-2	Antisense	CATGTCACCTCT**AGC**GATTCTACA	(SEQ ID NO: 12)
ARGD-2	Antisense	GTCACCTCT**TGC**GATTCTACAG	(SEQ ID NO: 13)
RGDNP-1	Sense	CAAGAGGTGAC**AAC**CCAGACGACAG	(SEQ ID NO: 14)
RGDNP-2	Antisense	CTGTCGTCTGG**GTT**GTCACCTCTTG	(SEQ ID NO: 15)
RGDDP-1	Sense	CAAGAGGTGAC**GAC**CCAGACGACAG	(SEQ ID NO: 16)
RGDDP-2	Antisense	CTGTCGTCTGG**GTC**GTCACCTCTTG	(SEQ ID NO: 17)
RGDGP-1	Sense	CAAGAGGTGAC**GGT**CCAGACGACAGATG	(SEQ ID NO: 18)
RGDGP-2	Antisense	CATCTGTCGTCTGG**ACC**GTCACCTCTTG	(SEQ ID NO: 19)
RGDDL-1	Sense	CAAGAGGTGAC**GACCTA**GACGACAGATG	(SEQ ID NO: 20)
RGDDL-2	Antisense	CATCTGTCGTC**TAGGTC**GTCACCTCTTG	(SEQ ID NO: 21)
RGDDM-1	Sense	CAAGAGGTGAC**GACATG**GACGACAGATG	(SEQ ID NO: 22)
RGDDM-2	Antisense	CATCTGTCGTC**CATGTC**GTCACCTCTTG	(SEQ ID NO: 23)
RGDDV-1	Sense	CAAGAGGTGAC**GACGTA**GACGACAGATG	(SEQ ID NO: 24)
RGDDV-2	Antisense	CATCTGTCGTC**TACGTC**GTCACCTCTTG	(SEQ ID NO: 25)
α-factor		TATTGCCAGCATTGCTGC	(SEQ ID NO: 26)
5'-AOX1	Sense	GACTGGTTCCAATTGACAAGC	(SEQ ID NO: 27)
3'-AOX1	Antisense	GCAAATGGCATTCTGACATCC	(SEQ ID NO: 28)
RLDDL-1	Sense	CAAGA**CTT**GAC**GACCTA**GAC	(SEQ ID NO: 74)
RLDD-2	Antisense	**GTC**GTC**AAG**TCTTGGGATTC	(SEQ ID NO: 75)
RGDDL-1	Sense	CAAGA**CTT**GAC**GACCTA**GAC	(SEQ ID NO: 76)
ARLDD-2	Antisense	**GTC**GTC**AAG**TCT**TGC**GATTC	(SEQ ID NO: 77)

[0162] Note: Primer α-factor was used as a sequencing primer; 5'-AOX1 & 3'-AOX1 primers were used for checking for the presence of the inserted DNA.

[0163] The polymerase chain reactions were carried out at 95° C for 1 min, 55° C for 1 min, then 72° C for 1 min for 25 cycles. A mixture of primers was also

used for generating multiple mutation sites. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The desired PCR products were purified and then ligated into the *Eco* RI and *Sac* II sites of the yeast transfer vector pPICZα A. The recombinant plasmid was used to transform an *Escherichia coli* XL1-blue strain and colonies selected on agar plates containing antibiotic Zeocin. The *E. coli* XL1-blue colonies were picked, plasmid DNA isolated, and the sequence confirmed by sequencing the insert. Table 2 lists primer sequence ID NOs. used for synthesizing DNAs encoding Rhodostomin and various variants.

TABLE 2

Primers for synthesizing DNAs encoding Rhodostomin and various variants

Sense Primer	Antisense Primer	Rhodostomin Variant
SEQ ID NO: 3	SEQ ID NO: 4	(Rho) ^{48}P**RGD**MP^{53}
SEQ ID NO: 16	SEQ ID NO: 13	Variant ^{48}A**RGD**DP^{53}
SEQ ID NO: 24	SEQ ID NO: 13	Variant ^{48}A**RGD**DV^{53}
SEQ ID NO: 70	SEQ ID NO: 71	Variant ^{48}A**RGD**DL^{53}
SEQ ID NO: 20	SEQ ID NO: 21	Variant ^{48}P**RGD**DL^{53}
SEQ ID NO: 72	SEQ ID NO: 73	Variant ^{48}A**RGD**DM^{53}
SEQ ID NO: 22	SEQ ID NO: 23	Variant ^{48}P**RGD**DM^{53}
SEQ ID NO: 5	SEQ ID NO: 6	Variant ^{48}PRLDMP^{53}
SEQ ID NO: 74	SEQ ID NO: 75	Variant ^{48}PRLDDL^{53}
SEQ ID NO: 76	SEQ ID NO: **77**	Variant ^{48}ARLDDL^{53}
SEQ ID NO: 9	SEQ ID NO: 10	Variant ^{48}PRIDMP^{53}
SEQ ID NO: 7	SEQ ID NO: 8	Variant ^{48}PRHDMP^{53}
SEQ ID NO: 14	SEQ ID NO: 15	Variant ^{48}P**RGD**NP^{53}
SEQ ID NO: 18	SEQ ID NO: 19	Variant ^{48}P**RGD**GP^{53}

EXAMPLE 2

Protein Expression and Purification of Rhodostomin and Variants

[0164] The protein expression of rhodostomin and its variants in *Pichia* was performed according to the protocols of the *Pichia* EasyComp™ Kit with minor modifications. Briefly, a total of 10 μg plasmids containing DNA encoding

rhodostomin or its variants were purified and digested with *Sac* I to linearize the plasmids. *Pichia* strain X33 was transformed with the linearized constructs by a heat shock method, using a *Pichia* EasyComp™ kit from Invitrogen®. The transformant integrated at the 5' AOX1 locus by a single crossover. PCR was used to analyze *Pichia* integrants to determine if the Rho gene had been integrated into the *Pichia* genome, and cells were lysed by Lyticase (Sigma). Colonies were selected on agar plates containing YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and 100 μg/ml Zeocin. A number of clones with multiple copies of Rho gene insertions were selected to pick the clone having the highest Rho protein expression.

[0165] Recombinant Rho and its variants were produced as follows: Selected colonies were grown in the YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) containing 100 μg/ml Zeocin at 30° C. After 48 hours, cells were collected by centrifugation and grown in 1 liter of minimal methanol medium (containing 1.34% yeast nitrogen base with ammonium sulfate without amino acids and 4×10^{-5} % biotin). A total of 1% methanol was added once every 24 hours to induce Rho or variant expression for 2 days. The supernatant was collected by centrifugation and dialyzed twice against 5 liter buffer A (5 mM EDTA, 8M urea and 10 mM Na-phosphate buffer, pH 7.7). The final solution was loaded into a nickel-chelating column and eluted with a gradient of 200 mM imidazole. The recombinant rhodostomin and its variants were further purified by HPLC (reverse phase C18 HPLC). The purified recombinant Rho had a purity of greater than 95% as judged by tricine-SDS-PAGE.

[0166] Rho and its variants were subsequently subjected to electrospray mass spectrometry analyses for checking the molecular weight. The amino acid sequences of the RGD motifs of Rho and variants are shown in Table 3.

TABLE 3

SEQ ID NO.	RGD Motif and Variants thereof	Abbreviation
2	^{49}Arg-Gly-Asp^{51}	^{49}RGD^{51}
29	^{48}Pro-Arg-Gly-Asp-Met-Pro^{53} (Wild type)	^{48}P**RGD**MP^{53}
30	^{48}Ala-Arg-Gly-Asp-Asp-Pro^{53}	^{48}A**RGD**DP^{53}
31	^{48}Ala-Arg-Gly-Asp-Asp-Val^{53}	^{48}A**RGD**DV^{53}
32	^{48}Ala-Arg-Gly-Asp-Asp-Leu^{53}	^{48}A**RGD**DL^{53}
33	^{48}Pro-Arg-Gly-Asp-Asp-Leu^{53}	^{48}P**RGD**DL^{53}
34	^{48}Ala-Arg-Gly-Asp-Asp-Met^{53}	^{48}A**RGD**DM^{53}
35	^{48}Pro-Arg-Gly-Asp-Asp-Met^{53}	^{48}P**RGD**DM^{53}
36	^{48}Pro-Arg-Leu-Asp-Met-Pro^{53}	^{48}PRLDMP^{53} **(RD)(RLD)**
36	^{48}Pro-Arg-Leu-Asp-Met-Pro^{53} (pegylated)	*^{48}PRLDMP^{53}-5K
37	^{48}Pro-Arg-Leu-Asp-Asp-Leu^{53}	^{48}PRLDDL^{53}
38	^{48}Ala-Arg-Leu-Asp-Asp-Leu^{53}	^{48}ARLDDL^{53}
39	^{48}Pro-Arg-Ile-Asp-Met-Pro^{53}	^{48}PRIDMP^{53}**(RID)**
40	^{48}Pro-Arg-His-Asp-Met-Pro^{53}	^{48}PRHDMP^{53}
41	^{48}Pro-Arg-Gly-Asp-Asn-Pro^{53}	^{48}P**RGD**NP^{53}
42	^{48}Pro-Arg-Gly-Asp-Gly-Pro^{53}	^{48}P**RGD**GP^{53}**(PGP)**

*^{48}PRLDMP^{53}-5K refers to pegylated ^{48}PRLDMP^{53}.

EXAMPLE 3

Effects of RD and its Derivatives on Bleeding Time

[0167] Measurement of bleeding time was performed as follows: Mice were anaesthetized with trichloroacetaldehyde (200 mg/kg), and bleeding time was measured by a method described previously, with minor modifications [21]. Saline or proteins were injected intravenously through the tail vein of the mouse (ICR, male, with an average body weight of 23.5 ± 1.8 g). A sharp cut of 0.5 mm from the tail tip of the mouse was made 5 min after injection. The tail was then immediately immersed in a saline-filled beaker, kept at 37 °C, and the bleeding time was measured.

[0168] FIG. 1 shows the effects of RD and ARLDDL proteins on tail bleeding time in mice. Tail bleeding time was measured 5 min after intravenous

administration of saline, RD, ARLDDL, rhodostomin (0.6 mg/kg for each) of HSA-RD (5 mg/kg). An intravenous injection of rhodostomin (0.5 mg/kg) exerted a pronounced effect in prolonging the clotting time in mice. However, both RD and ARLDDL (0.5 mg/kg), which exerted selectivity for αvβ3 integrin, only slightly affected the clotting time in mice as compared to rhodostomin. Results are expressed as the mean ± S.E.M (n=6).

EXAMPLE 4

Platelet aggregation Assay

[0169] Venous blood (9 parts) samples from healthy donors who had not received any medication for at least two weeks were collected in 3.8 % sodium citrate (1 part). Blood samples were centrifuged at 150 x g for 10 min to obtain platelet-rich plasma (PRP) and allowed to stand for 5 min, and PRP was collected. The platelet-poor plasma (PPP) was prepared from the remaining blood by centrifuging at 2000x g for 25 min. The PPP platelet count was measured on a hematology analyzer and diluted to 250,000 platelets/μl. A solution of 190 μl of PRP and 10 μl of either Rho or PBS buffer were incubated for 5 min in a Hema Tracer 601 aggregometer at 37°C. Ten microliters of 200 μM adenosine diphosphate (ADP) were further added to monitor the response of platelet aggregation by light transmission.

EXAMPLE 5

Cell Adhesion Inhibition Assay

[0170] Cell adhesion inhibition assays were performed as described previously [27]. Briefly, wells of 96-well Immulon-2 microtiter plates (Costar, Corning, USA) were coated with 100 μl of phosphate-buffered saline (PBS: 10 mM phosphate buffer, 0.15M NaCl, pH 7.4) containing substrates at a concentration of 50-500 nM, and incubated overnight at 4 °C. The substrates and their coating concentrations were fibrinogen (Fg) 200 μg/ml, vitronectin (Vn) 50 μg/ml, and fibronectin (Fn) 25 μg/ml. Non-specific protein binding sites were blocked by incubating each well with 200 μl of heat-denatured 1% bovine serum albumin (BSA, Calbiochem) at room temperature (25° C) for 1.5 hr The heat-denatured BSA was discarded and each well was washed twice with 200 μl of PBS.

[0171] Chinese hamster ovary (CHO) cells expressing αvβ3 (CHO-αvβ3) and αIIbβ3 (CHO-αIIbβ3) integrins were maintained in 100 µl of Dulbecco's Modified Eagle's Medium (DMEM) medium. Chinese hamster ovary (CHO) cells expressing integrins αvβ3 (CHO-αvβ3) and αIIbβ3 (CHO-αIIbβ3) were kindly provided by Dr. Y. Takada (Scripps Research Institute). Human erythroleukemia K562 cells were purchased from ATCC and cultured in the RPMI-1640 medium containing 5% fetal calf serum. CHO and K562 cells growing in log phase were detached by trypsinization and used in the assay at 3×10^5 and 2.5×10^5 cells/ml, respectively. Rho and its variants were added to the cultured cells and incubated at 37° C, 5% CO_2 for 15 minutes. Rho and its variants were used as inhibitors at the concentrations of 0.001-500 µM. The treated cells were then added into the coated plate and reacted at 37° C, 5% CO_2 for 1 hour. The incubation solution was then discarded and non-adhered cells were removed by washing twice with 200 µl PBS. Bound cells were quantified by crystal violet staining. Briefly, the well was fixed with 100 µl of 10% formalin for 10 minutes and dried. Fifty microliters of 0.05% crystal violet were then added into the well at room temperature for 20 minutes. Each well was washed with 200 µl of distilled water four times and dried. Colorization was carried out by adding 150 µl of colorizing solution (50% alcohol and 0.1% acetic acid). The resulting absorbance was read at 600 nm and the readings were correlated with the number of adhering cells. Inhibition was defined as % inhibition = 100 – [OD_{600} (rhodostomin variant-treated sample)/ OD_{600} (untreated sample)] × 100.

EXAMPLE 6

Inhibitory Effects of RD and its Derivatives on Integrins αvβ3, αIIbβ3, and α5β1

[0172] The IC_{50} of RD and its derivatives on integrin binding was obtained by the cell adhesion assay as described in Example 5 above. Briefly, matrix proteins such as fibronectin, vitronectin, or fibrinogen, were coated at a fixed concentration on microtiter plates, as described in Example 5, and Rho and its variants were added to the cells expressing integrin at different concentrations ranging from 0.001~500 µM to obtain an IC_{50}. The lower the IC_{50}, the greater the specificity or potency of the variant.

[0173] The modification of the RGD motif of Rho had unique effects on Rho's biological activities: the activity in inhibiting the integrin αIIbβ3 or α5β1's binding to the matrix proteins was reduced and the selectivity to the integrin αvβ3 was increased as a result of the sequence modification. Table 4 shows the result of IC_{50} for inhibition of cell adherence by Rho and its derivatives.

TABLE 4

Inhibition of Cell adhesion to ECM by RD and its Derivatives

	IC50 (nM)		
RGD motif variant & SEQ ID NO:	αIIbβ3 / Fg	αvβ3 / Fg	α5β1 / Fn
^{48}PRGDMP^{53} (29)	21.0 ± 11.2 (17[a])	13.0 ± 5.7 (20)	256.8 ± 87.5 (21)
^{48}ARGDDP^{53} (30)	850.9 ± 322.6 (3)	45.3 ± 17.4 (3)	5044.5 ± 1554.5 (3)
^{48}ARGDDV^{53} (31)	255.2 ± 107.2 (3)	15.8 ± 5.5 (3)	213.1 ± 74.4 (3)
^{48}ARGDDL^{53} (32)	1518.1 ± 740.4 (4)	41.3 ± 16.0 (5)	526.7 ± 200.3 (5)
^{48}PRGDDL^{53} (33)	1224.0 ± 231.2 (4)	76.0 ± 16.0 (4)	3017.0 ± 801.5 (3)
^{48}ARGDDM^{53} (34)	627.2 ± 317.6 (3)	49.0 ± 19.2 (6)	350.8 ± 81.0 (4)
^{48}PRGDDM^{53} (35)	1117.8 ± 379.7 (3)	211.0 ± 91.3 (5)	4047.3 ± 1784.3 (4)
^{48}PRLDMP^{53} (36)	2219.8 ± 996.9 (6)	35.0 ± 14.1 (5)	3043.3 ± 1117.6 (4)
*^{48}PRLDMP^{53}-5K	2408.0 ± 1090.0 (2)	114.8 ± 51.1 (4)	>3730 (3)
^{48}PRLDDL^{53} (37)	>5880 (3)	342.3 ± 110.1(3)	>5880 (4)
^{48}ARLDDL^{53} (38)	59217.8 ± 966.4 (3)	36.8 ± 12.8 (3)	23171.0 ± 925.5 (3)
^{48}PRIDMP^{53} (39)	>5880 (2)	119.9 ± 19.7(3)	>5880 (3)
^{48}PRHDMP^{53} (40)	2109.0 ± 810.0 (3)	131.6 ± 35.3(3)	>5880 (3)
^{48}PRGDNP^{53} (41)	185.0 ± 61.7 (4)	24.8 ± 13.1 (5)	357.0 ± 80.6 (3)
^{48}PRGDGP^{53} (42)	2591.0 ± 572.2 (3)	204.1 ± 87.0 (3)	3462.0 ± 1525.5 (4)

*: ^{48}PRGDMP^{53} (29) refers to the RGD motif of Rho and comprises an amino acid sequence set forth by SEQ ID NO: 29; ^{48}PRLDMP^{53} (36) refers to the RGD motif variant of RD and comprises an amino acid sequence set forth by SEQ ID NO: 36.
[a]The numbers represents the numbers of experiments.

[0174] The rhodostomin variants have a much lower affinity to αIIbβ3 and/or α5β1 as compared to the Rho (Table 4). As shown in Table 4, for example,

the IC_{50} of RD (i.e., PRLDMP) in inhibition of integrins αIIbβ3 and α5β1 increased more than 104 and 10-fold, respectively, as compared to that of the Rho. Moreover, the IC_{50} of ARLDDL in inhibition of integrins αIIbβ3 and α5β1 increased more than 2000-fold compared to that of the Rho. The IC_{50} of pegylated RD and human albumin-conjugated RD on αIIbβ3 binding increased 113.7- and 129.9-fold, respectively, as compared to that of the Rho (Table 5). Therefore, the variants' affinities to platelets were markedly reduced compared to that of Rho (Table 5).

EXAMPLE 7

Effects of RD and its Derivatives on Osteoclastogenesis

[0175] Osteoclasts are specialized monocyte/macrophage family members that differentiate from bone marrow hematopoietic precursors. Cultures of osteoclast precursors in the presence of M-CSF (20 ng/ml) and sRANKL (50 ng/ml) for 8 days induced the formation of large mature osteoclasts with multi-nuclei, which were characterized by the acquisition of mature phenotypic markers, such as TRAP. The method of osteoclastogenesis from cultured hematopoietic cells of bone marrow and the effects of RD and its derivatives on osteoclastogenesis were investigated as follows.

[0176] Bone marrow cells were prepared by removing femurs from 6~8-week-old SD rats and flushing the bone marrow cavity with a-MEM which was supplemented with 20 mM HEPES and 10% heat-inactivated FCS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent cells (hematopoietic cells) were collected and used as osteoclast precursors after 24 hr. Cells were seeded at 1×10^6 cells/well (0.5 ml) in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) and murine M-CSF (20 ng/ml). The culture medium was replaced every 3 days. Osteoclast formation was confirmed by an assay of tartrate-resistant acid phosphatase (TRAP) on day-8. In brief, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline for 3 min. After treatment with ethanol/acetone (50:50 v/v) for 1 min, the cell surface was air-dried and incubated for 10 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma) and 0.03% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate. Osteoclast-like TRAP-positive cells in each well were scored

by counting the number of TRAP-positive and multinucleated cells containing more than three nuclei.

[0177] RD derivatives markedly inhibited the differentiation of osteoclasts, which was correlated with their inhibitory activities on αvβ3 (Table 5). On the other hand, AKGDWN and PRGEMP were less effective in inhibiting integrin αvβ3 and differentiation of osteoclasts (Table 5).

TABLE 5

Inhibition of Platelet Aggregation, Cell adhesion, and Osteoclastogenesis by RD and its Derivatives

	IC50 (nM)				
	Platelet aggregation	αvβ3	α5β1	αIIbβ3	Osteoclastogenesis
Rhodostomin	83	13	257	21	5.52
ARGDDL	455	41	527	1518	8.02
PGP	283	24	4000	500	5.76
RD	433	35	3043	2220	3.32
RID	448	120	>5850	>5850	5.82
PRGDDL	396	76	3017	1224	3.15
ARLDDL	**10380**	**37**	**23171**	**59217**	--
PRPDDL	3530	188	21381	13590	
AKGDWN	138	96637	>119000	69	78400
PRGEMP	4908	>17850	>5950	>59500	68200
Pegylated-RD	398	115	>5880	2408	3.88
HSA-RD	**438**	45	>5880	2750	6.68

EXAMPLE 8

Ovariectomy-Induced Osteoporosis

[0178] Female Sprague–Dawley rats (3 months old, 270 ~ 290 g) or ICR mice (4 weeks old, 22 ~ 28 g) were used for this study. Rats or mice were ovariectomized (OVX) bilaterally under trichloroacetaldehyde anesthesia and control rats were sham-operated (Sham) for comparison. The animals were all kept

under controlled conditions at the room temperature (22 ± 1 °C) and a 12-hr light-dark cycle. Animals were fed with Purina Laboratory Rodent Diet (PMI; St. Louis, MO) (0.95% calcium) and water ad libitum. The body weights of the rats were recorded weekly.

EXAMPLE 9

Analysis of Bone Mineral Density (BMD) and Content (BMC)

[0179] At the end of the experiment, rats or mice were sacrificed by decapitation. The tibia and femur were removed, cleaned of soft tissue, and the length and weight of the tibia and femur were measured with a precision caliper (± 0.05 mm) as described by Weinreb et al. [26]. BMD and BMC of the tibia were measured with a dual-energy X-ray absorptiometer (DEX, XR-26; Norland, Fort Atkinson, WI). The mode adapted to the measurements of small subjects was adopted. See Chih-Hsin *et al.*, "Enhancement of Fibronectin Fibrillogenesis and Bone Formation by Basic Fibroblast Growth Factor via Protein Kinase C-Dependent Pathway in Rat Osteoblasts," Mol. Pharmacol: 66: 440-449, (2004). A coefficient of variation of 0.7% was calculated from daily measurements of BMD on a lumbar phantom for more than 1 year [22, 23]. The whole tibia and femur were scanned and BMD and BMC were measured by an absorptiometer.

EXAMPLE 10

Histomorphometry of Bone

[0180] Tibiae were fixed by 4% formaldehyde and then decalcified with 12% EDTA and dehydrated in an ascending series of ethanol solution and acetone, and embedded in paraffin. Serial sections (5 mm) were cut longitudinally and stained with Mayer's hematoxylin-eosin solution. Images of the growth plate and proximal tibia were photographed using an Olympus microscope. The bone volume was measured using an image analysis software (Image-pro plus 3.0) in the secondary spongiosa, which was located under the primary spongiosa and characterized by a network of larger trabeculae. To measure the number of osteoclasts, the sections were stained with tartrate-resistant acid phosphatase (TRAP).

EXAMPLE 11

Biomechanical Three-Point Bending Test

[0181] Mechanical properties of bone tissues were measured by performing three-point bending test in a material testing system (MTS-858, MTS System Inc., Minneapolis, MN). The span of the two support points was 20 millimeters and the deformation rate was 1 mm/min. Load/deformation curves were input to a computer and analyzed by Team 490 software (version 4.10, Nicolet Instrument Technologies Inc., Madison, WI). Cross-sectional parameters were measured from the photographs and used in the calculation of the cross-sectional moment of inertia. The cross-sectional moment of inertia was calculated under the assumption that the cross-sections were elliptically shaped [24]:

[0182] $I = \pi [(ab3 \times (a \times 2t)(b \times 2t)3) / 64$

[0183] where *a* is the width of the cross section in the mediolateral direction, *b* is the width of the cross section in the anteroposterior direction, and *t* is the average of the cortical thickness. All of these parameters were obtained using the image software Image Pro Plus 3.0 for Windows (Media Cybernetics, Silver Spring, MD). The maximal stress, ultimate stress, and elastic modulus (Young's modulus) were calculated using the following equations [25]:

$$\sigma = FLc / 4I$$

$$E = F/d\ э L3/48I$$

where σ is ultimate stress, c is the distance from the center of mass (equal to ½ *b* as described above), F is the applied load (N), d is the displacement (mm), and L is the span between the two support points of the bending fixture (mm). In addition, the energies in the ultimate stress were measured by computing the respective areas under the stress-strain curve.

EXAMPLE 12

Inhibition of OVX-induced bone loss by RD derivatives in mice

[0184] To examine the effects of RD derivatives on bone loss, osteoporosis was induced in female mice by ovariectomy (OVX), as described in Example 8. OVX mice showed a decrease in BMD and BMC of total body. Treatment with RD derivatives (I.M., 1.5 mg/kg/alternate day) or alendronate (p.o., 1.5 mg/kg/alternate day) for 2 weeks inhibited the loss of BMD and BMC (Table 6). The blood

concentration of the C-terminal telopeptide of collagen can reflect the osteoclastic activity. As shown in Table 6, RD derivatives or alendronate also inhibited ovariectomy-induced increase in osteoclast activity (Table 6). It appeared that some of the RD derivatives were much more effective than alendronate. In addition, treatment with RD (I.M., 1.5 mg/kg) once per week for 2 weeks also inhibited the loss of BMD and BMC (Table 6). These data indicate that RD and its derivatives may inhibit osteoporosis at longer dosing intervals.

TABLE 6

Inhibition of OVX-induced bone loss by RD and its derivatives in mice

	BMD (g/cm^2)	BMC (g)	C-terminal telopeptide of COL(1)α1 chain (ng/ml)
(Alternate day)			
Sham	0.095±0.001	0.649±0.011	395±11.9
OVX	0.075±0.002*	0.517±0.007*	686±12.1*
OVX+RD	0.091±0.001§	0.627±0.008§	391±8.3§
OVX+PGP	0.092±0.001§	0.621±0.006§	372±24.2§
OVX+ARGDDL	0.093±0.001§	0.624±0.007§	389±16.4§
OVX+RID	0.091±0.001§	0.622±0.004§	391±12§
OVX+Rhodostomin	0.090±0.001§	0.619±0.006§	397±13.6§
OVX+PRLDDL	0.091±0.002§	0.563±0.056§	410±25.1§
OVX+Alendronate	0.086±0.002§	0.607±0.051§	504±19.6§
OVX+pegylated-RD	0.092±0.002§	0.629±0.008§	398±9.2§
(Once/week)			
OVX+RD	0.085±0.002§	0.582±0.009§	533±27.4§
OVX+pegylated-RD	0.087±0.005§	0.595±0.009§	538±19.8§

Values are means ± SE.

*Compared with Sham-group, $p<0.05$

§Compared with OVX-group, $p<0.05$

EXAMPLE 13

Inhibition of ovariectomy-induced bone loss by PG0 P and RD derivatives in rats

[0185] PGP (a RD derivative) was chosen to examine in more detail the protection from ovariectomy (OVX)-induced bone loss in rats. Adult female rats (3 month-old) were ovariectomized, as in Example 8, and bone volumes measured 6 weeks after the ovariectomy, as in Examples 9-11. It was shown that PGP protein inhibited both ovariectomy-induced bone volume decrease and osteoclast number increase.

[0186] As shown in FIG. 2A, compared to the sham-operated rats (Sham), ovariectomy (OVX) caused a significant loss of the trabecular bone. However, treatment with PGP (IV, 0.3 mg/kg/day or IM, 1.5 mg/kg/alternate day) significantly inhibited the ovariectomy-induced loss of the trabecular bone in secondary spongiosa.

[0187] In FIG. 2B, tartrate-resistant acid phosphatase (TRAP) staining showed that osteoclasts were predominantly localized around the trabecular bone, and that PGP inhibited the OVX-induced increase in osteoclast formation.

[0188] OVX rats also showed an increase in body weight at the end of the experiment. Treatment with PGP (I.V., 0.3 mg/kg/day or I.M., 1.5 mg/kg/alternate day) significantly inhibited the OVX-induced increase in body weight.

[0189] Rats also showed a decrease in wet weight, BMD and BMC in both femur and tibia 6 weeks after the ovariectomy (Table 7). Treatment with PGP (either I.V. or I.M.) inhibited the reduction in wet weight, BMD and BMC of both tibia and femur in OVX rats. Histomorphometry demonstrated that ovariectomy caused the loss of trabecular bone in secondary spongiosa (FIG. 2). Treatment with PGP markedly reversed the loss of bone volume (FIG. 2 and Table 7). In addition, TRAP staining demonstrated that osteoclasts were predominantly localized around the trabecular bone and OVX increased the osteoclast number. A long-term administration of PGP antagonized the OVX-induced osteoclastic formation (FIG. 2 and Table 7). The serum level of the C-terminal telopeptide of collagen reflects osteoclast activity. It was found that the value was markedly increased in the OVX-

group compared with that of the Sham-group, whereas treatment with PGP effectively antagonized the OVX-induced increase in osteoclast activity (Table 7).

TABLE 7

Inhibition of Ovariectomy-induced Bone Loss by PGP Protein in Rats

	Sham (n=22)	OVX (n=22)	OVX+PGP(IV) (n=13)	OVX+PGP(IM) (n=12)
Bone length, mm				
Tibia	4.04±0.01	4.04±0.01	4.05±0.01	4.04±0.01
Femur	3.62±0.01	3.62±0.02	3.60±0.02	3.64±0.02
Wet weight, mg				
Tibia	755.5±6.3	647.5±8.5*	735.8±8.5§	740.6±8.7§
Femur	946.0±10.9	850±9.9*	904.9±10.2§	904.0±13.5§
BMD, g/cm^2				
Tibia	0.108±0.002	0.099±0.015*	0.109±0.002§	0.111±0.002§
Femur	0.131±0.012	0.126±0.003*	0.130±0.002§	0.136±0.002§
BMC, g				
Tibia	0.301±0.011	0.269±0.011*	0.286±0.010§	0.316±0.009§
Femur	0.420±0.005	0.369±0.011*	0.422±0.013§	0.437±0.009§
Bone volume, %	18.1±1.5	9.4±1.5*	14.3±1.5§	16.8±1.8§
N.Oc/BS (No/mm)	1.77±0.09	2.06±0.17*	1.83±0.10§	1.81±0.09§
C-terminal telopeptide of collagen (n=5 for each)	230±5.1	459±18.4*	259±22.8§	221±15.1§

ALP, alkaline phosphatase; N.Oc/BS, osteoclast number/mm bone surface.

Values are means ± SE.

*Compared with Sham-group, $p<0.05$

§Compared with OVX-group OVX, $p<0.05$

[0190] A three-point bending test was performed in the femur to examine the mechanical activity of the bone. Compared with the Sham-operated group, the maximal load, ultimate load, Young's modulus and ultimate stress decreased in OVX rats. Treatment with PGP exhibited a protection against the OVX-induced decrease in bone strength (Table 8). These results suggest that PGP-like protein drugs may markedly inhibit the bone loss caused by ovariectomy.

TABLE 8

Increase in biomechanical properties by PGP protein in OVX rats

	Sham (n=22)	OVX (n=22)	OVX+PGP (IV) (n=13)	OVX+PGP (IM) (n=12)
Maximal load, N	132.7±4.8	112.1±4.3*	123.9±4.9§	129.8±7.9§
Ultimate loading, N	85.5±3.7	70.4±3.1*	81.9±2.2§	83.2±2.5§
Young's modulus, GPa	203.7±3.6	185.5±3.3*	194.0±3.0§	199.9±6.2§
Energy to ultimate stress, mJ/mm^3	5.9±0.4	3.5±0.3*	4.9±0.8§	5.1±0.9§

Values are means ± SE.

*Compared with Sham-group, $p<0.05$

§Compared with OVX-group OVX, $p<0.05$

EXAMPLE 14

Post-treatment of RD protein inhibited OVX-induced osteoclast activation

[0191] To examine the dynamic therapeutic effect of RD derivatives on osteoclast activity *in vivo*, osteoporosis was induced in female rats by ovariectomy and the C-terminal telopeptides of type-I collagen were measured from blood at different intervals. Compared with sham-operated rats (Sham), ovariectomy (OVX) caused a significant increase in the osteoclast activity. Measurement of the serum level of the C-terminal telopeptide of COL(1)α1 chain showed an increase in the osteoclast activity in OVX rats. The C-terminal telopeptide of type-I collagen increased from a basal level of 361 ± 25.6 (n=15) to 708 ± 50.7 ng/ml (n=15) 28 days after the ovariectomy.

[0192] RD or alendronate was then post-treated one month after the ovariectomy. The post-treatment with RD (I.M., 1.5 mg/kg/alternate day) or

alendronate (p.o., 1.5 mg/kg/alternate day) reversed the ovariectomy-induced increase in osteoclast activity, and RD was much more effective than alendronate (FIG. 3). Osteoclast activity recovered after the withdrawal of the RD or alendronate treatment. The recovery of osteoclast activity was much faster in the alendronate-treated group than the RD-treated group. However, re-application of RD effectively inhibited the ovariectomy-induced increase in osteoclast activity. The value of the osteoclast in response to the RD treatment was much lower than that in response to the alendronate treatment (FIG. 3). These results indicate that RD-related proteins have therapeutic effects on the bone loss caused by ovariectomy-induced osteoclastic activation.

[0193] After the second drug treatment period, rats were sacrificed, and BMD and BMC of the tibia and femur were measured. As shown in Table 9, posttreatment of RD effectively reversed the bone loss effect of ovariectomy. With regard to the preservation of the bone volume, RD was more effective than alendronate.

TABLE 9

Post-treatment of RD inhibits OVX-induced bone loss in rats

	Sham	OVX	RD	Alendronate
Bone length, mm				
Tibia	4.04±0.03	4.05±0.03	4.01±0.03	4.02±0.05
Femur	3.69±0.03	3.68±0.02	3.71±0.06	3.68±0.05
Wet weight, mg				
Tibia	766.6±19.9	661±18.2*	749±20.1§	712.1±10.1§
Femur	1002.6±17.4	861±10.7*	985.3±9.8§	912.4±10.9§
BMD, g/cm^2				
Tibia	0.114±0.003	0.101±0.002*	0.113±0.009§	0.108±0.002§
Femur	0.137±0.002	0.121±0.002*	0.135±0.004§	0.131±0.003§
BMC, g				
Tibia	0.315±0.013	0.272±0.006*	0.299±0.011§	0.289±0.009§
Femur	0.442±0.012	0.391±0.008*	0.441±0.018§	0.401±0.017§
Bone volume, %	18.1±1.2	7.9±2.1*	16.3±2.7§	12.1±1.3§

BMD is the abbreviation of bone mineral density; BMC is the abbreviation of bone mineral content.

Values are means ± SD.

*Compared with Sham-group, P<0.05

§ Compared with OVX-group OVX, P<0.05

RD: I.M. injection (1.5/mg/kg/alternate day)

Alendronate: p.o. (1.5 mg/kg/alternate day)

n=5 for each group

EXAMPLE 15

Inhibition of chondrocyte damage by RD in osteoarthritis animals

[0194] An osteoarthritis animal model was obtained by surgery only on the right knees of male Sprague-Dawley rats. Surgery involved anterior cruciate ligation transaction and partial medial meniscectomy. After the surgery, RD protein was administered via intramuscular route (1.5 mg/kg/alternate day) or local joint injection (once/week) until the last day when the rats were sacrificed 6 weeks after the surgery. Each joint was embedded in paraffin wax, sectioned and stained with 0.1% safranin-O and haematoxylin. As shown in FIG. 4B, the chondrocyte layer in the right knee joint was damaged by arthritis and RD in either intramuscular injection (FIG. 4D) or local application (FIG. 4C) inhibited the chondrocyte layer destruction.

EXAMPLE 16

Inhibition of blood cytokine elevation by RD in osteoarthritis rat

[0195] Osteoarthritis rats were generated by surgery as described above. Six weeks after surgery, serum was obtained to measure the blood level of cytokines. As shown in FIGs. 5A-5C, the serum levels of cytokines, such as c-reactive protein, IL-1β and IL-6, increased in osteoarthritic rats, and RD administration markedly inhibited the osteoarthritis-induced elevation of cytokines.

EXAMPLE 17

Inhibition of blood cytokine elevation by RD in osteoarthritis mice

[0196] Osteoarthritis mice were generated by surgery as described above. Six weeks after the surgery, the serum was obtained to measure the level of cytokines. As shown in FIG. 6A-6C, the serum levels of cytokines, such as c-

reactive protein, IL-1□ ?and IL-6, increased in osteoarthritic mice, and RD administration markedly inhibited the osteoarthritis-induced elevation of cytokines.

EXAMPLE 18

Intra-tibia Injection of prostate or breast cancer cells in mice

[0197] Severe Combined Immune Deficiency (SCID) male mice or BALB/c-nu/nu male mice weighing 20-22 g (6 weeks old) bred in an animal isolator (IVC racks) under a specific pathogen-free (SPF) condition at 22 ± 2°C were used. Human prostate adenocarcinoma PC-3 cells or human breast cancer MDA-MB-231 cells (1×10^6 cells in 15 ml sterile PBS) were injected into the bone marrow of both the left and right tibia of the SCID mice or nude mice on day-1, respectively. On day-11, the animals were randomly assigned into three groups and the administration of test substances was initiated. RD (1.5 mg/kg) was administered by intramuscular (IM) injection and alendronate (1.5 mg/kg) was given by subcutaneous (SC) injection, once daily for a total of 15 doses (5 days on, 2 days off for 3 weeks). The body weight and tumor growth condition were observed and recorded every week during the experimental period. After one month, the mice were sacrificed and the weight of the hind legs was measured. The weight of the control leg was subtracted to reflect the relative tumor weight. In addition, blood samples were collected at the end of the experiment for the blood count of red blood cells (RBC), white blood cell (WBC) and platelet, and for the measurement of the C-terminal telopeptides of type I collagen (CTX) and serum calcium concentration.

[0198] In order to determine bone osteolysis, radiographs were taken by a soft X-ray generating unit (Young-kid Enterprise Co., Ltd., Taipei, Taiwan). Animals were deeply anesthetized with trichloroacetaldehyde monohydrate, laid down in a prone position on a Kodak Scientific Imaging film (13 × 18 cm), and X-ray exposure was performed at 45 kV for 5 seconds. The degree of osteolysis was measured using image analysis software (Image-pro plus 3.0).

EXAMPLE 19

Inhibition of tumor growth in bone and hypercalcemia

[0199] The tumor cell growth in bone is related to the bone resorption activity. The effect of RD protein on the tumor growth of prostate cancer cells in

bone was thus examined. PC-3 cells (1×10^6) were locally injected into the bone marrow cavities of both tibia in SCID mice. RD or alendronate was administered 10 days after the implantation of tumor cells. RD at 1.5 mg/kg was given by the intramuscular (IM) route while alendronate was given at 1.5 mg/kg by the subcutaneous (SC) injection, once daily for a total of 15 doses (5 days on, 2 days off for 3 weeks). The swelling of hind legs was calculated to reflect the tumor growth on day 33. The body weight was measured throughout the experimental period. The results indicated that RD at 1.5 mg/kg caused a significant inhibition in tumor cell-induced swelling in hind legs on day-33 (43.8 ± 4.1%, n=21-22) (FIG. 7). However, the subcutaneous administration of alendronate at 1.5 mg/kg did not inhibit the tumor growth in legs (FIG. 7).

[0200] FIG. 8 shows the effect of RD on the decrease in body weight in response to the tumor growth in SCID mice. Untreated control mice showed a decrease in body weight at the end of the experiment. Treatment with RD prevented the loss of body weight caused by the tumor growth. Alendronate was used as a positive control.

[0201] FIG. 9 shows the inhibition of tumor growth and osteolytic bone lesion by RD in SCID mice. In order to determine bone osteolysis, radiographs were taken by a soft X-ray generating unit. In FIG. 9A, photographs were taken 33 days after the intratibial injection of PC-3 cells. A visible spherical tumor grew up from the proximal tibia. Treatment with RD (I.M., 1.5 mg/kg/alternate day) inhibited tumor growth. In FIG. 9B, radiographs taken on day-33 revealed that osteolytic lesions appeared in the cancer cell-injected tibia and treatment of RD inhibited osteolysis. FIG. 9C shows the quantitation of the data. FIG. 9D shows that RD inhibited a tumor-induced increase in C-terminal telopeptides of type-collagen (a marker for the osteoclast activity) using an ELISA method. FIG. 9E demonstrates that RD and alendronate (1.5 mg/kg/alternate day) also inhibited a tumor-induced increase in the serum calcium concentration (i.e., hypercalcemia). *: $p<0.05$, as compared with control. #: $p<0.05$, as compared with the PC-3-injected group.

[0202] Breast cancer has a strong predilection for metastasizing to bone. FIG. 10 shows an inhibition of tumor growth by RD in nude mice. Human breast cancer cells MDA-MB-231 (1×10^6) were locally injected into the bone marrow

cavities of both tibia in nude mice. RD (I.M., 1 mg/kg/day) was administered 10 days after the implantation of tumor cells for a total of 14 days. Treatment with RD (IM, 1.5 mg/kg/day) for 2 weeks inhibited MDA-MB-231-induced increase in tumor growth in bone (FIG. 10A). Furthermore, RD also prevented the tumor-induced increase in osteoclast activity (FIG. 10B) and hypercalcemia (FIG. 10C), but did not affect the blood counts of RBC, WBC and platelet (FIGs. 10D-10F). *: $p<0.05$, as compared with the control. #: $p<0.05$, as compared with the MDA-MB-231-injected group.

EXAMPLE 20

Pegylation of RD protein

[0203] A pegylated product or a conjugation with albumin can prolong the duration and decrease the antigenecity of a protein drug. In order to minimize the antigenecity and prolong the duration of RD protein, pegylated RD protein was prepared as follows: RD protein (4 mg) in 20 mM $NaCNBH_3$ at pH 5 was reacted with 5 mM PEGk5-propionaldehyde (O-Methyl-O'-[2-(6-oxocaproylamino)ethyl]polyethylene glycol 5,000)(Sigma) at 4 °C for 12 h. The pegylated RD protein was purified by reverse phase C18 HPLC. The final yield of pegylated RD protein after purification was greater than 60%.

[0204] As shown in Table 6, pegylated-RD inhibited the differentiation of osteoclasts. In addition, treatment with pegylated-RD (I.M., 1.5 mg/kg, once/week) for 2 weeks inhibited the loss of BMD and BMC (Table 6). These data indicate that pegylated-RD did not lose its activity *in vivo*.

EXAMPLE 21

MATRIGEL™ Plug Antiangiogenesis Assays

[0205] It has been reported that integrin $\alpha v\beta 3$ is related to angiogenesis. Whether RD protein can inhibit angiogenesis was thus investigated using MATRIGEL™ plug angiogenesis assays as described previously with minor modifications [32]. Briefly, an aliquot (500μl) of MATRIGEL™ (Becton Dickinson Lab.) containing 200ng/ml VEGF was injected subcutaneously into the dorsal region of 6–8 week-old C57BL/6 mice. The MATRIGEL™ formed a plug rapidly. RD was administered intramuscularly (3 mg/kg) daily (RD/1d) or the other day (RD/2d or HSA-RD) before sacrifice. After 8 days, plugs were taken and photographed

(FIG. 11A). Neovessels were quantified by measuring the hemoglobin of the plugs as an indication of blood vessel formation with the Drabkin method and Drabkin reagent kit 525 (Sigma) (FIG. 11B). As shown in FIGs. 11A and 11B, RD protein was effective in inhibiting angiogenesis using MATRIGEL™ plug assays. *: $P < 0.05$ versus control group.

EXAMPLE 22

Radioligand-Binding Assay: Specificity of RD-related proteins

[0206] To determine whether RD and its derivative PGP bind to other receptors besides αvβ3 integrin, RD and its derivative PGP were used to analyze the target specificity toward receptors whose ligands are proteins (assayed by MDS Pharma services, Taipei, Taiwan).

[0207] As shown in Table 10, RD and PGP did not affect the binding activity of calcitonin, endothelin ETA, endothelin ETB, insulin, leptin, sodium channel, transforming growth factor-ß (TGF-ß), tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) to their respective receptors. This indicates that RD-related proteins exert selective binding activity toward target protein αvβ3 *in vivo*.

TABLE 10

The binding assay of RD and PGP protein

Target	Inhibition	
	RD	PGP
Calcitonin	4%	-6%
Endothelin ET_A	26%	-3%
Endothelin ET_B	10%	-7%
Insulin	-6%	-8%
Leptin	1%	2%
Sodium Channel (Site 2)	2%	-14%
TGF-ß	14%	-10%
TNF	4%	20%
VEGF	-14%	19%

TGF-ß: transforming growth factor-ß

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

EXAMPLE 23

Inhibition of angiogenesis by RD in a mouse model of retinopathy of prematurity

[0208] An animal model for retinopathy of prematurity in mice was generated by using hypoxic-induced angiogenesis as described in Wilkinson-Berka *et al.* [28]. Briefly, seven-day-old pups and their mother were housed in sealed chambers containing 75 % O_2 and air. Mice remained in the chamber for five days (hyperoxic period, P7 to P12) and were then housed in room air for a further seven days (hypoxic-induced angiogenic period, postnatal 12 days to postnatal 19 days, or P12 to P19). RD (2 μg) was administered via an intravitreous route on day-12 and the mice were sacrificed on day-19.

[0209] Three sections from one of the eyes of each animal were made, deparaffinized, and stained with hematxylin and eosin. Blood vessel profiles (BVPs) were counted in the inner retina, and included vessels adherent to the inner limiting

membrane. Counting was performed on a photomicroscope (Leica) at a magnification of 100x.

[0210] As shown in FIG. 12A, RD protein inhibited angiogenesis in a mouse model of retinopathy of prematurity (ROP). FIG. 12B shows reduced BVPs in a mouse model of retinopathy of prematurity (ROP) treated with RD protein. Angiogenesis was quantitated by counting blood vessel profiles (BVPs) in the inner retina and extending into the vitreous cavity from three hematoxylin-and-eosin-stained sections. The ROP group treated with RD (2 μg) (i.e., ROP+ RD) reduced about 46% of angiogenesis compared to the ROP group treated with vehicle (ROP). (n=7 for each ROP group; n=2 for sham group.) Data are presented as Mean±SE. #: $p< 0.01$, as compared to the sham group. **: $p< 0.001$, as compared to the ROP group.

EXAMPLE 24

Inhibition of ovariectomy-induced osteoporosis by albumin-conjugated RD

[0211] The effect of albumin-conjugated RD on osteoporosis was examined in ovariectomized female mice. Human serum albumin-conjugated RD (i.e., RD-albumin) was administered as indicated by arrows shown in FIGs. 13A and 13B. The data for RD was incorporated into FIGs. 13A-13D for comparison. Serum levels of the c-terminal peptide of type I collagen and alkaline phosphatase (ALP) were measured as indicators of osteoclast and osteoblast activity, respectively. The BMD and BMC were also measured every 2 weeks, as shown in FIGs. 13C and 13D. Treatment of RD-albumin (15 mg/kg/week) markedly reduced osteoclast, but increased ALP, activities in a reversible manner.

EXAMPLE 25

Inhibition of rheumatoid arthritis by RD

[0212] Rheumatoid arthritis is a chronic systemic inflammatory disorder of unknown etiology characterized by invasive synovial hyperplasia that leads to progressive joint destruction. Osteoclasts, derived from the monocyte/macrophage lineage, play a crucial role in subchondral bone destructions in rheumatoid arthritis. Radiographic studies have shown that in rheumatoid arthritis, osteopenia of the subchondral bone and bone erosion begin at an early stage of the disease, and are gradually exacerbated [29]. Bone-resorbing osteoclasts are observed at the

erosive synovium/bone interface [30]. A recent review discusses the role of osteoclasts in rheumatoid arthritis [31]. Therefore, RD-related proteins, which markedly inhibit osteoclast functions, may be useful for treatment of rheumatoid arthritis.

[0213] Lewis rats are given intradermal/subcutaneous (SC) injections of bovine type II collagen (2 mg/ml in Freund's incomplete adjuvant). As rats develop the rheumatoid disease, they are randomly divided into several study groups. Treatment is initiated on the first day when the clinical signs of arthritis are clearly visible, as evidenced, for example, by ankle joint swelling. After measuring the paw volume, rats are sacrificed, and ankle and knee joints are collected for the examination of histopathological change.

[0214] FIGS. 14A-D show amino acid sequences of Rho and its variants, SEQ ID NOs: 1, and 57-69, respectively. FIGS. 15A-C show nucleotide sequences of rhodostomin variants, SEQ ID NOs: 43-56. FIGS. 16A-H show amino acid and nucleotide sequences of disintegrin variants, SEQ ID NOs: 78-135.

[0215] The foregoing description of the exemplary embodiments of the invention has been presented only for the purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in light of the above teaching.

[0216] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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CLAIMS

WHAT IS CLAIMED IS:

1. A method for treatment and/or prevention of an αvβ3 integrin-associated disease comprising:

administering to a mammal in need thereof a therapeutically effective amount of a polypeptide, or a pharmaceutically acceptable salt thereof, wherein the polypeptide comprises a disintegrin variant that is selective for αvβ3 integrin.

2. The method of claim 1, wherein the disintegrin is a variant of a disintegrin chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

3. The method of claim 2, wherein the disintegrin variant comprises a variant of rhodostomin.

4. The method of claim 3, wherein said rhodostomin variant comprises a variant of the amino acid sequence of SEQ ID NO: 1.

5. The method of claim 4, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 30-42.

6. The method of claim 4, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 57-69.

7. The method of claim 1, wherein said polypeptide is pegylated or conjugated with albumin.

8. The method of claim 1, wherein the αvβ3 integrin-associated disease is chosen from osteoporosis, bone tumor or cancer growth and symptoms related thereto, angiogenesis-related tumor growth and metastasis, tumor metastasis in bone, malignancy-induced hypercalcemia, angiogenesis-related eye disease, Paget's disease, rheumatic arthritis, and osteoarthritis.

9. The method of claim 8, wherein the angiogenesis-related eye disease is chosen from age-related macular degeneration, diabetic retinopathy, corneal neovascularizing diseases, ischaemia-induced neovascularizing retinopathy, high myopia, and retinopathy of prematurity.

10. The method of claim 8, wherein the αvβ3 integrin-associated disease comprises osteoporosis.

11. The method of claim 10, wherein said osteoporosis is associated with a pathological condition chosen from post-menopausal estrogen deficiency, secondary osteoporosis, ovariectomy, Paget's disease, bone cancer, bone tumor, osteoarthritis, increased osteoclast formation, and increased osteoclast activity.

12. The method of claim 11, wherein the osteoporosis comprises post-menopausal osteoporosis or bone loss.

13. The method of claim 1, wherein the mammal is a human.

14. A method for treatment and/or prevention of an ovariectomy-induced physiological change comprising:

administering to a mammal in need thereof a therapeutically effective amount of a polypeptide, or a pharmaceutically acceptable salt thereof, wherein the polypeptide comprises a disintegrin variant that is selective for αvβ3 integrin.

15. The method of claim 14, wherein the disintegrin is a variant of a disintegrin chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

16. The method of claim 15, wherein the disintegrin variant comprises a variant of rhodostomin.

17. The method of claim 16, wherein said rhodostomin comprises a variant of the amino acid sequence of SEQ ID NO: 1.

18. The method of claim 17, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 30-42.

19. The method of claim 17, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 57-69.

20. The method of claim 14, wherein said polypeptide is pegylated or conjugated with albumin.

21. The method of claim 14, wherein the mammal is a human.

22. A method for inhibition and/or prevention of tumor cell growth in bone and symptoms related thereto comprising:

administering to a mammal in need thereof a therapeutically effective amount of a polypeptide, or a pharmaceutically acceptable salt thereof, wherein the polypeptide comprises a disintegrin variant that is selective for αvβ3 integrin.

23. The method of claim 22, wherein the disintegrin is a variant of a disintegrin chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

24. The method of claim 23, wherein the disintegrin variant comprises a variant of rhodostomin.

25. The method of claim 24, wherein said rhodostomin variant comprises a variant of SEQ ID NO: 1.

26. The method of claim 25, wherein said rhodostomin variant comprises amino acid sequence chosen from SEQ ID NOs: 30-42.

27. The method of claim 25, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 57-69.

28. The method of claim 22, wherein said polypeptide is pegylated or conjugated with albumin.

29. The method of claim 22, wherein said symptoms comprise a pathological symptom chosen from an increased osteoclast activity, increased bone resorption, bone lesion, hypercalcemia, a body weight loss, and any combinations thereof.

30. The method of claim 22, wherein the tumor cell growth in bone includes bone cancer cells, and metastasized cancer cells originating from one or more of prostate cancer, breast cancer, lung cancer, thyroid cancer, renal cancer, ovarian cancer, pancreatic cancer, and myeloma cancer.

31. The method of claim 22, wherein the mammal is a human.

32. A polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of a disintegrin.

33. The polypeptide of claim 32, wherein the disintegrin is a variant of a disintegrin chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin,

halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

34. The polypeptide of claim 32, wherein the disintegrin comprises a variant of rhodostomin.

35. The polypeptide of claim 32, wherein said rhodostomin variant comprises a variant of SEQ ID NO: 1.

36. The polypeptide of claim 35, wherein said rhodostomin variant comprises an amino acid sequence chosen from SEQ ID NOs: 30-42.

37. A polypeptide of claim 32, wherein said rhodostomin variant comprises the amino acids sequence of SEQ ID NO: 29 and comprises at least one amino acid substitution in a position chosen from positions 48, 50, 52, or 53 of SEQ ID NO: 1.

38. The polypeptide of claim 37, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

39. The polypeptide of claim 38, wherein the amino acid substitution is at position 50 or 52.

40. The polypeptide of claim 37, wherein the polypeptide comprises amino acid substitutions in at least two positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

41. The polypeptide of claim 40, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

42. The polypeptide of claim 41, wherein the amino acid substitutions are at positions 48 and 52.

43. The polypeptide of claim 41, wherein the amino acid substitutions are at positions 52 and 53.

44. The polypeptide of claim 37, wherein the polypeptide comprises amino acid substitutions in at least three positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

45. The polypeptide of claim 44, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

46. The polypeptide of claim 45, wherein the amino acid substitutions are at positions 48, 52, and 53.

47. The polypeptide of claim 45, wherein the amino acid substitutions are at positions 50, 52, and 53.

48. The polypeptide of claim 37, wherein the polypeptide comprises amino acid substitutions in at least four positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

49. The polypeptide of claim 48, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

50. The polypeptide of claim 32, wherein said polypeptide is encoded by a polynucleotide comprising a nucleotide sequence chosen from SEQ ID NOs: 43-56.

51. The polypeptide of claim 32, wherein said polypeptide exhibits at least about a 5, 50, or 100-fold decrease in affinity to αIIbβ3 and/or α5β1 as compared to rhodostomin.

52. The polypeptide of claim 32, wherein said polypeptide exhibits at least about a 200-fold decrease in affinity to αIIbβ3 and/or α5β1 as compared to rhodostomin.

53. The polypeptide of claim 32, wherein said polypeptide exhibits at least about a 1000, or 2000-fold decrease in affinity to αIIbβ3 and/or α5β1 as compared to rhodostomin.

54. The polypeptide of claim 32, wherein said polypeptide exhibits at least about 5, 50, 100, 1000, or 2000-fold decrease in affinity to platelet as compared to rhodostomin.

55. The polypeptide of claim 32, wherein said polypeptide exhibits reduced activity in prolongation of blood clotting time as compared to rhodostomin.

56. The polypeptide of claim 32, wherein said polypeptide is pegylated or conjugated with albumin.

57. A physiologically acceptable composition comprising the polypeptide of claim 37 and a pharmaceutically acceptable carrier.

58. A physiologically acceptable composition comprising the polypeptide of claim 40 and a pharmaceutically acceptable carrier.

59. A physiologically acceptable composition comprising the polypeptide of claim 44 and a pharmaceutically acceptable carrier.

60. A physiologically acceptable composition comprising the polypeptide of claim 48 and a pharmaceutically acceptable carrier.

61. A physiologically acceptable composition comprising the polypeptide of claim 50 and a pharmaceutically acceptable carrier.

62. The physiologically acceptable composition of claim 57, wherein said polypeptide is pegylated or conjugated with albumin.

63. A polypeptide comprising an amino acid sequence chosen from SEQ ID NOs: 57-69.

64. A method for making a polypeptide according to claim 32, comprising the steps of:

a. transfecting a host cell with a polynucleotide encoding said polypeptide;

b. growing said host cell in a culture medium; and

c. isolating said polypeptide.

65. The method of claim 64, wherein (b) further comprises adding methanol to said culture medium.

66. The method of claim 64, further comprising growing the host cell in a culture medium free of amino acids.

67. The method of claim 64, wherein (c) further comprises performing a column chromatography to obtain said polypeptide.

68. The method of claim 64, further comprising performing a HPLC to obtain said polypeptide.

69. The method of claim 64, wherein said host cell is a yeast cell.

70. A polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of a disintegrin.

71. The polynucleotide of claim 70, wherein the disintegrin is a variant of a disintegrin chosen from rhodostomin, albolabrin, applagin, basilicin, batroxostatin, bitistatin, cereberin, cerastin, crotatroxin, durissin, elegantin, flavoridin, flavostatin, halysin, halystatin, jararacin, jarastatin, kistrin, lachesin, lutosin, molossin, salmosin, saxatilin, tergeminin, trimestatin, trimucrin, trimutase, ussuristatin, and viridin.

72. The polynucleotide of claim 70, wherein the disintegrin comprises a variant of rhodostomin.

73. The polynucleotide of claim 72, wherein said rhodostomin variant comprises a variant of SEQ ID NO: 1.

74. The polynucleotide of claim 73, wherein said disintegrin variant comprises an amino acid sequence chosen from SEQ ID NOs: 30-42.

75. The polynucleotide of claim 70, wherein said rhodostomin variant comprises the amino acids sequence of SEQ ID NO: 29 and comprises at least one amino acid substitution in a position chosen from positions 48, 50, 52, or 53 of SEQ ID NO: 1.

76. The polynucleotide of claim 75, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

77. The polynucleotide of claim 76, wherein the amino acid substitution is at position 50 or 52.

78. The polynucleotide of claim 75, wherein the polypeptide comprises amino acid substitutions in at least two positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

79. The polynucleotide of claim 78, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 52, and Val, Leu, and Met at position 53.

80. The polynucleotide of claim 79, wherein the amino acid substitutions are at positions 48 and 52.

81. The polynucleotide of claim 79, wherein the amino acid substitutions are at positions 52 and 53.

82. The polynucleotide of claim 75, wherein the polypeptide comprises amino acid substitutions in at least three positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

83. The polynucleotide of claim 82, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 42, and Val, Leu, and Met at position 53.

84. The polynucleotide of claim 83, wherein the amino acid substitutions are at positions 48, 52, and 53.

85. The polynucleotide of claim 83, wherein the amino acid substitutions are at positions 50, 52, and 53.

86. The polynucleotide of claim 75, wherein the polypeptide comprises amino acid substitutions in at least four positions chosen from positions 48, 50, 52, and 53 of SEQ ID NO: 1.

87. The polynucleotide of claim 86, wherein the amino acid substitutions are chosen from Ala at position 48, Leu, Ile, and His at position 50, Asp, Met, and Asn at position 42, and Val, Leu, and Met at position 53.

88. The polynucleotide of claim 70, wherein the polynucleotide comprises a sequence chosen from SEQ ID NOs: 43-56 and 78-135.

89. The polynucleotide of claim 70, wherein the polynucleotide encodes the polypeptide of claim 37.

90. The polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of integrin and wherein the polynucleotide encodes the polypeptide of claim 40.

91. The polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of integrin and wherein the polynucleotide encodes the polypeptide of claim 44.

92. The polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of integrin and wherein the polynucleotide encodes the polypeptide of claim 48.

93. The polynucleotide encoding a polypeptide selective for αvβ3 integrin, wherein the polypeptide is a variant of integrin and wherein the polynucleotide encodes the polypeptide of claim 50.

94. A polynucleotide that hybridizes under stringent conditions to the polynucleotide of claim 73.

95. A polypeptide encoded by the nucleotide of claim 94.