TITLE OF THE INVENTION
Disintegrin Variants and Pharmaceutical Uses Thereof

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a Section 371 of International Application No. PCT/US2015/046322, filed August 21, 2015, which was published in the English language on February 25, 2016, under International Publication No. WO 2016/029131 A1, which claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62/040,503, filed August 22, 2014, and the disclosures of which are herein incorporated by reference in their entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] This application contains a sequence listing, which is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file name "688947-1PCT Sequence Listing.txt", creation date of August 20, 2015, and having a size of 157,219 bytes. The sequence listing submitted via EFS-Web is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The invention relates to disintegrin variants that bind specifically to one or more of α5β1 and αv integrins, such as αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8, but with reduced binding activity to αIIbβ3, and uses of the disintegrin variants for the treatment and prevention of a disease associated with an αv integrin or α5β1 integrin.

BACKGROUND OF THE INVENTION

[0004] Integrins are transmembrane receptors that bind extracellular matrix proteins or other adhesion receptors on neighboring cells. Heterodimeric pairing of integrin α and β subunits confers specificity of binding to one or more substrates (Weis et al., 2011, *Cold Spring Harb Perspect Med*;1:a006478). This family of adhesion molecules plays a pivotal role in broad contexts of biology, including inflammation, innate and antigen specific immunity, homeostasis, wound healing, tissue morphogenesis, and regulation of cell growth and differentiation. Dysregulation of integrins is involved in the pathogenesis of many disease states, from autoimmunity to thrombotic vascular diseases to cancer metastasis. Extensive efforts have been directed towards the discovery and development of integrin antagonists for clinical applications.

[0005] The αv integrins, each having an αv subunit paired with a β1, β3, β5, β6 or β8 subunit, appear to be particularly important during the tissue remodeling associated with wound repair, angiogenesis, and cancer (Weis et al., 2011, *supra)*. The αv integrins are being targeted for cancer, ophthalmological and orthopedic indications. Integrins αvβ3 and αvβ5 have

variant also has increased binding activity to at least one of αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrins as compared to a disintegrin not having the at least one selected from the group consisting of the mutant linker, the mutant RGD loop and the mutant C-terminus.

[0012] According to embodiments of the invention, the disintegrin variant can be a variant of any disintegrin, including, but not limited to a disintegrin selected from the group consisting 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. Preferably, the disintegrin variant is a variant of a disintegrin having the amino acid sequence selected from the group consisting of SEQ ID NOs: 1 to 6. More preferably, the disintegrin variant is a variant of rhodostomin having the amino acid sequence of SEQ ID NO: 1.

[0013] In a preferred embodiment, the disintegrin variant comprises a mutant RGD loop comprising at least one mutation at positions 1-3, 5, 7 and 8 of the amino acid sequence of SEQ ID NO: 333 (RIPRGDMP) and at least one of the mutant linker and the mutant C-terminus described herein.

[0014] In another preferred embodiment of the invention, the disintegrin variant comprises a mutant linker having the amino acid sequence selected from the group consisting of SEQ ID NO:306 to SEQ ID NO: 317.

[0015] In yet another preferred embodiment of the invention, the disintegrin variant comprises a mutant C-terminus having the amino acid sequence selected from the group consisting of SEQ ID NO: 319 to SEQ ID NO:328.

[0016] In a preferred embodiment of the invention, the disintegrin variant comprises a mutant RGD loop having the amino acid sequence selected from the group consisting of SEQ ID NO: 329 to SEQ ID NO: 331, and at least one of a mutant linker having the amino acid sequence selected from the group consisting of SEQ ID NO:306 to SEQ ID NO: 317, and a mutant C-terminus having the amino acid sequence selected from the group consisting of SEQ ID NO:319 to SEQ ID NO: 328. More preferably, the disintegrin variant comprises the mutant RGD loop, the mutant linker and the mutant C-terminus described herein.

[0017] According to embodiments of the invention, the disintegrin variant comprises the amino acid sequence selected from the group consisting of SEQ ID NO: 7 to SEQ ID NO:179. Preferably, the disintegrin variant according to an embodiment of the invention comprises the

comprising one or more mutations in the amino acid sequence that spans the linker region of a disintegrin. The linker region of a disintegrin is located immediately N-terminal to the RGD loop. For example, the linker region of Rho comprises the amino acid sequence of SEQ ID NO:332 (SRAGKIC). In preferred embodiments of the invention, a mutant linker comprises at least one mutation at positions 1 to 5 of the amino acid sequence of SEQ ID NO:332. More preferably, a mutant linker comprises the amino acid sequence selected from the group consisting of SEQ ID NO:306 to SEQ ID NO: 317.

[0052] In yet another embodiment, a disintegrin variant of the invention comprises a mutant C-terminus. As used herein, a "mutant C-terminus" or "mutant C-terminal region" refers to a peptide comprising one or more mutations in the amino acid sequence of the C-terminal region of a disintegrin. The C-terminal region of a disintegrin is located at the carboxyl end of the disintegrin. For example, the C-terminus of Rho comprises the amino acid sequence of SEQ ID NO: 334 (PRYH). In preferred embodiments of the invention, a mutant C-terminus comprises at least one mutation at positions 1-4 of the amino acid sequence of SEQ ID NO:334. More preferably, a mutant C-terminus comprises the amino acid sequence selected from the group consisting of SEQ ID NO:319 to SEQ ID NO: 328.

[0053] In preferred embodiments, a disintegrin variant of the invention comprises a mutant RGD loop and at least one of a mutant linker and a mutant C-terminus of a disintegrin.

[0054] In more preferred embodiments, a disintegrin variant of the invention comprises a mutant RGD loop, a mutant linker and a mutant C-terminus of a disintegrin described herein.

[0055] A disintegrin variant of invention can include naturally-occurring and non-naturally occurring amino acids. Examples of naturally-occurring amino acid include, but are not limited to, any of the twenty primary, naturally occurring amino acids which typically form peptides, polypeptides, and proteins. The following table 1 is a tabulation of 20 naturally occurring amino acids.

[0078] For example, it was found that a Rho mutation in the RGD loop can increase specificity for αVβ3 and/or α5β1 integrin, a mutation in the C-terminal region can cause less binding to αIIbβ3 (thus weaker inhibition on platelet aggregation and less bleeding side effect) and a mutation in the linker region can also reduce binding to αIIbβ3, wherein each of the three regions are identified by the amino acid residue numbers in the wild-type Rho, and the amino acid residues that can be modified, e.g., by an insertion, deletion, or substitution, according to embodiments of the invention are each independently marked as "X."

[0079] According to embodiments of the invention, a disintegrin variant comprises a mutation in the RGD loop, i.e., a mutant RGD loop.

[0080] In one embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{49}RXD^{51}. Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 7-24.

[0081] In another embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{48}XRGD^{51} (SEQ ID NO: 341). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 25-42.

[0082] In another embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{48}XRGDXP^{53} (SEQ ID NO: 342). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 44-61.

[0083] In another embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{48}XRGDMX^{53} (SEQ ID NO: 343). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 63-78.

[0084] In another embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{46}XXPRGD^{51}(SEQ ID NO: 344). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 79-94.

[0085] In another embodiment, a disintegrin variant comprises a mutant RGD loop having a consensus sequence of ^{48}XRXDXP^{53} (SEQ ID NO: 345). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 95-101.

[0086] According to other embodiments of the invention, a disintegrin variant comprises a mutation in the C-terminal region, i.e., a mutant C-terminus.

[0087] In one embodiment, a disintegrin variant comprises a mutant C-terminus having a consensus sequence of ^{65}PRXXXXX^{71} (SEQ ID NO: 346). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 102-107.

[0088] In another embodiment, a disintegrin variant comprises a mutant C-terminus having a consensus sequence of ^{65}PRXXXXX^{71}(SEQ ID NO: 346), and further comprises a mutant RGD loop, such as those described herein. For example, the mutant RGD loop can have a consensus sequence of ^{48}ARGDMP^{53} (SEQ ID NO: 335). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 115-119.

[0089] According to yet other embodiments of the invention, a disintegrin variant comprises a mutation in the linker region, i.e., a mutant linker.

[0090] In one embodiment, a disintegrin variant comprises a mutant linker having a consensus sequence of ^{39}KKKRTIC^{47} (SEQ ID NO: 306). Preferably, the disintegrin variant further comprises a mutant RGD loop such as those described herein. For example, the mutant RGD loop can have a consensus sequence of ^{48}XRXDXP^{53} (SEQ ID NO: 345). Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs: 108-114.

[0091] According to further embodiments of the invention, a disintegrin variant comprises a mutation in the linker region, a mutation in the RGD loop and a mutation in the C-terminal region. Examples of such variants include, but are not limited to, those having the amino acid sequences selected from the group consisting of SEQ ID NOs:120-132 and 134-179.

[0092] A disintegrin variant of the invention can be made by any method suitable to the aims of the invention in view of the present disclosure. For example, a disintegrin variant can be constructed by a site-directed mutagenesis method. The disintegrin variant of the invention can be expressed using methods known in the art in view of the present disclosure. 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),

[0120] In some embodiments, a disintegrin variant of the invention is particularly effective against cancers which are associated with a highly expressed osteopontin. In preferred embodiments, the polypeptides of the invention can inhibit osteopontin-induced tumor invasion.

[0121] Administration of disintegrin variants 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.

[0122] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and that the scope of the invention is to be determined by the appended claims.

Examples

Example 1: Construction of Expression Vectors Expressing Wild-type Rho and Rho Mutants (Disintegrin Variants)

[0123] 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 CATGGTAAGGAATGTGACTGTTCT-3' (SEQ ID NO: 183) that had Eco R1 recognition site and encodes six histidine residues for facilitating purification. The antisense primer has the sequence of 5'-CCGCGGCCGCGGTCAGTGGTATCTTGGACAGTCAGC-3' (SEQ ID NO: 180) or 5'-CCGCGGCCGCGGTTAGTGGTATCTTGGACAGTCAGC-3' (SEQ ID NO: 184) 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, pPICZα A. 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 25 μg/ml antibiotic Zeocin.

[0124] The various DNA constructs encoding the mutants of Rho were synthesized and amplified by the PCR using an overlapping oligonucleotide strategy with primers containing Eco RI and Sac II restriction sites. For illustration purpose, Table 2 lists the consensus sequences of some distintegrin variants, and the primers used to construct these variants according to embodiments of the invention, wherein the primer sequences are presented from the 5' to 3' (left to the right).

[0125] Expression vectors encoding other variants encompassed by the invention have been or can be constructed in similar manner in view of the present disclosure. Various primers used for synthesizing or confirming distintegrin variants are listed in SEQ ID NOs: 180-305.

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 – [OD600 (Rho wild type or disintergrin-treated sample)/OD600 (untreated sample)] × 100.

[0132] IC50 was defined as the concentration (nM) of a disintegrin variant required for 50% inhibition of the cell adhesion mediated by a particular integrin. Therefore, lower IC50 indicates greater specificity or potency of the disintegrin variant in inhibiting the cell adhesion activity of the respective integrin, thus higher binding activity (or selectivity) of the disintegrin variant to the respective integrin. The IC50 results are summarized in Tables 3 to 14 below.

[0133] A series of Rho mutants involved in the RGD loop region ($R^{50}XD$, $^{48}XRGD$ (SEQ ID NO: 341), $XRGD^{52}XP$ (SEQ ID NO: 342), $XRGDM^{53}X$ (SEQ ID NO: 343), $^{46}X^{47}XPRGD$ (SEQ ID NO: 344), and $ARGD^{51}X^{52}X$ (SEQ ID NO: 340)), the linker region, and C-terminal region were recombinantly expressed and purified to homogeneity. The cell adhesion and platelet aggregation assays were used to determine their integrin-binding affinity. It was found that variants of rhodostomin or disintegrins with one or more modifications in these regions have different selective binding affinity to αVβ3, αVβ5, αVβ6, α5β1 and αIIbβ3 (Tables 3-14).

[0134] For example, it was found that Rho variants with certain mutations in the RXD motif, in which the "Gly50 (G)" residue was replaced by Leu (L), Val (V), Ile (I), Glu (E), Asp (D), Gln (Q), Phe (F), Trp (W), His (H), Lys (K), or Arg (R), had their highest effects on integrins in the following order: αIIbβ3 (~1686-fold) > α5β1 (~586-fold) > αvβ5 (~348-fold) > αvβ6 (~179-fold)> αvβ3 (~26-fold), showing their binding selectivity to αVβ3 (Table 3). Rho variants with the mutation in the XRGD (SEQ ID NO: 341) motif, in which the P48 residue was replaced by other amino acids, had their highest effects on integrins in the following order: α5β1 (~71-fold) > αIIbβ3 (~41-fold) > αvβ3 (~5-fold) (Table 4). Rho variants with the mutation in the XRGDXP (SEQ ID NO: 342) motif, in which the M52 residue was replaced by other amino acids, had their highest effects on integrins in the following order: αIIbβ3 (~209-fold) > α5β1 (~122-fold) >

αVβ3 (~14-fold) (Table 5). Rho variants with the mutation in the XRGDMX (SEQ ID NO: 343) motif, in which the P^{53} residue was replaced by other amino acids, had their highest effects on integrins in the following order: αIIbβ3 (~258-fold) > α5β1 (~45-fold) > αVβ3 (~40-fold) (Table 6). Rho variants with the mutation in the XXPRGD (SEQ ID NO: 344) motif, in which the R46 and I47 residues were replaced by other amino acids, had their highest effects on integrins in the following

order: αIIbβ3 (~73-fold) > α5β1 (~19-fold) > αVβ3 (~10-fold) (Table 7). These results showed that the mutations in the RGD loop exhibited significant effect on the inhibitory activity in integrins αIIbβ3 and α5β1, but not αVβ3 integrin.

[0135] Mutants of rhodostomin or disintegrins with one or more modifications in addition to the RGD motif, e.g., in the linker region or the C-terminus, exhibited the capability to selectively binding to αVβ3, αVβ5, αVβ6, α5β1 or αIIbβ3 (Table 8-14). For example, Rho variants with the mutation in the linker region in which the SRAGKIC (SEQ ID NO: 332) was replaced by KKKRTIC (SEQ ID NO: 306), KKARTIC (SEQ ID NO: 313), MKKGTIC (SEQ ID NO: 307), IEEGTIC (SEQ ID NO: 308), LKEGTIC (SEQ ID NO: 310), AKKRTIC (SEQ ID NO: 311), KAKRTIC (SEQ ID NO: 312), KGAGKIC (SEQ ID NO: 309), KKKATIC (SEQ ID NO: 314), KKKRAIC (SEQ ID NO: 315), KAKRAIC (SEQ ID NO: 316), and SKAGTIC (SEQ ID NO: 317) amino acids, had their highest effects on integrins in the following order: αIIbβ3 (~2-fold) > α5β1 (~5-fold) > αVβ3 (~14-fold) (Table 8). These results showed that the mutations in the linker region of Rho exhibited significant effect on the inhibitory activity in integrin αVβ3.

[0136] Rho variants with the mutation in the C-terminal region, in which the PRYH(SEQ ID NO: 334) was replaced by PGLYG (SEQ ID NO: 324), PRNRFH (SEQ ID NO: 321), PRNRFHA (SEQ ID NO: 322), PRWNDL (SEQ ID NO: 319), PRNGLYG (SEQ ID NO: 323), and PRNPWNG (SEQ ID NO: 320) amino acids, had their highest effects on integrins in the following order: αIIbβ3 (~13-fold) > αVβ5 (~8-fold) = αVβ6 (~8-fold) > αVβ3 (~4-fold) > α5β1 (~2-fold) (Table 9). These results showed that the mutations on the C-terminal region of Rho exhibited significant effect on the inhibitory activity in integrins αIIbβ3, αVβ5, and αVβ6.

[0137] Rho variants with the mutation in the C-terminal region, in which PRYH (SEQ ID NO: 334) was replaced by PGLYG (SEQ ID NO: 324), PPLYG (SEQ ID NO: 327), PRLYG (SEQ ID NO: 328), PGLY (SEQ ID NO: 325), PYLYG (SEQ ID NO: 352) and PELYG (SEQ ID NO: 337) amino acids, had their highest effects on integrins in the following order: αIIbβ3 (~6493-fold) > α5β1 (~40-fold) > αvβ5 (~8-fold) > αvβ6 (~6-fold)> αvβ3 (~1-fold), showing its significant effect on integrins αIIbβ3 and α5β1 (Table 10).

[0138] Disintegrin (Rho) variants specific to integrins αvβ3 and α5β1 were successfully obtained by modifying the RGD loop region, the linker region, and C-terminal region of Rho. For example, the mutant ^{39}KKART-^{46}ARGRGDNP -^{66}DLYG (SEQ ID NO: 147) exhibited an excellent inhibitory activity to integrins αvβ3 and α5β1 but not to αIIbβ3, αvβ5, and αvβ6 (Table 11). The mutations in the RGD loop and linker region increased its activity in inhibiting integrins αvβ3 and α5β1 and significantly decreased its activity in

inhibiting integrin αIIbβ3. The mutations in the C-terminal region decreased its activity in inhibiting integrins αvβ5, and αvβ6.

[0139] Disintegrin (Rho) variants specific to integrins αvβ3, αvβ5, and α5β1 were successfully obtained by modifying the RGD loop region, the linker region, and C-terminal region of Rho. For example, the mutant ^{39}KKART-^{46}ARGRGDDL-^{66}GLYG (SEQ ID NO: 339) exhibited an excellent inhibitory activity to αvβ3, αvβ5, and α5β1 but not to αIIbβ3 and αvβ6 (Table 12). The mutations of SRAGKIC (SEQ ID NO: 332) into KKARTIC (SEQ ID NO: 313) in the linker region increased its activity in inhibiting RGD-binding integrins. The mutations of R46A, I47R, P48A, M52D, and P53L in the RGD loop decreased its activity in inhibiting integrins αIIbβ3 and αVβ6. The mutations of PRYH(SEQ ID NO: 334) into PGLYG(SEQ ID NO: 324) in the C-terminal region decreased its activity in inhibiting integrin αIIbβ3.

[0140] Disintegrin (Rho) variants specific to integrins αvβx and α5β1 were successfully obtained by modifying the RGD loop region, the linker region, and C-terminal region of Rho. For example, the mutant ^{39}KKART-^{46}ARGRGDNP -^{66}RYH (SEQ ID NO: 123) exhibit an excellent inhibitory to integrins αvβ3 and α5β1 but not to αIIbβ3, αvβ5, and αvβ6 (Table 13). The mutations in the RGD loop and linker region increased its activity in inhibiting integrins αVβ3 and α5β1 and significantly decreased its activity in inhibiting integrin αIIbβ3. The mutations on the C-terminal region decreased its activity in inhibiting integrins αvβ5, and αvβ6.

Example 4. Inhibition of Platelet Aggregation by Rho Mutants

[0141] Disintegrin (Rho) variants were also tested for their ability to inhibit platelet aggregation that is mediated by αIIbβ3. 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^{o}C. Ten microliters of 200 μM adenosine diphosphate (ADP) were further added to monitor the

[0142] **Table 3:** Integin Selectivity and Inhibition of Platelet Aggregation by the RXD Disintegrin Variants

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]			Platelet Aggregation (nM)
		α5β1	αVβ3	αIIbβ3	
^{49}RGD (Rho wild type) (SEQ ID NO: 1)		216.0	11.4	17.8	77.5
1.	RAD (SEQ ID NO: 7)	14425.3	74.6	299.1	176.5
2.	RPD (SEQ ID NO: 8)	104452.5	2275.0	6122.3	1236.0
3.	RVD (SEQ ID NO: 9)	3296.0	63.9	2554.3	523.8
4.	RLD (SEQ ID NO: 10)	3043.3	32.4	1998.8	480.3
5.	RID (SEQ ID NO: 11)	2604.3	119.9	4895.0	669.6
6.	RMD (SEQ ID NO: 12)	4915.6	188.7	185.4	414.4
7.	RFD (SEQ ID NO: 13)	3325.3	274.6	5486.6	652.6
8.	RYD (SEQ ID NO: 14)	1231.7	247.5	2001.6	508.3
9.	RWD (SEQ ID NO: 15)	1104.8	291.0	2425.8	476.5
10.	RSD (SEQ ID NO: 16)	6254.5	78.1	142.2	189.9
11.	RTD (SEQ ID NO: 17)	5420.6	54.1	1154.3	281.7
12.	RND (SEQ ID NO: 18)	43192.0	88.0	647.1	186.7
13.	RQD (SEQ ID NO: 19)	77335.0	132.3	2920.6	539.4
14.	RDD (SEQ ID NO: 20)	131702.0	123.5	10688.3	1216.5
15.	RED (SEQ ID NO: 21)	86954.5	89.9	35411.3	2162.3
16.	RHD (SEQ ID NO: 22)	6489.0	131.6	2109.0	465.7
17.	RKD (SEQ ID NO: 23)	150186.5	387.8	33915.7	1527.3
18.	RRD (SEQ ID NO: 24)	111949.0	404.2	4372.3	401.7

Table 4: Integin Selectivity and Inhibition of Platelet Aggregation by the XRGD Disintegrin Variants

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]			Platelet Aggregation (nM)
		αvβ3	α5β1	αIIbβ3	
^{48}PRGD (Rho wild type) (SEQ ID NO: 1)		13.0	256.8	21.0	83.2
1.	DRGD (SEQ ID NO: 26)	15.3	4188.3	860.2	631.3
2.	ERGD (SEQ ID NO: 27)	12.9	522.2	677.2	528.1
3.	GRGD (SEQ ID NO: 25)	19.6	92.2	68.4	370.2
4.	ARGD (SEQ ID NO: 32)	15.8	59.0	31.6	110.3
5.	TRGD (SEQ ID NO: 34)	20.4	139.4	38.6	214.6
6.	SRGD (SEQ ID NO: 33)	12.9	251.3	28.5	115.1
7.	YRGD (SEQ ID NO: 41)	11.4	310.6	20.4	68.9
8.	WRGD (SEQ ID NO: 42)	41.0	248.2	39.8	142.0
9.	FRGD (SEQ ID NO: 40)	14.9	282.1	36.1	124.0
10.	LRGD (SEQ ID NO: 29)	11.3	283.4	22.1	168.0
11.	IRGD (SEQ ID NO: 30)	17.6	281.7	47.9	183.9
12.	VRGD (SEQ ID NO: 28)	19.5	194.7	47.6	136.9
13.	MRGD (SEQ ID NO: 31)	11.3	222.7	26.4	137.4
14.	QRGD (SEQ ID NO: 36)	8.8	190.9	60.9	128.9

15.	NRGD (SEQ ID NO: 35)	8.0	264.6	28.5	274.4
16.	KRGD (SEQ ID NO: 38)	24.1	246.6	22.3	157.0
17.	RRGD (SEQ ID NO: 37)	15.5	214.4	30.0	179.4
18.	HRGD (SEQ ID NO: 39)	11.6	194.8	24.4	115.2

[0143] **Table 5:** Integin Selectivity and Inhibition of Platelet Aggregation by the ARGDXP Disintegrin Variants

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]			Platelet Aggregation (nM)
		αvβ3	α5β1	αIIbβ3	
1.	^{48}PRGDMP (wild type) (SEQ ID NO: 1)	13.0	256.8	21.0	83.2
2.	ARGDMP (SEQ ID NO: 32)	15.8	59.0	31.6	110.3
3.	ARGDDP (SEQ ID NO: 44)	45.3	5044.5	850.9	752.7
4.	ARGDEP (SEQ ID NO: 45)	156.3	2436.0	1063.0	518.8
5.	ARGDLP (SEQ ID NO: 46)	19.6	517.4	72.5	100.5
6.	ARGDVP (SEQ ID NO: 47)	21.5	368.2	36.2	145.7
7.	ARGDIP (SEQ ID NO: 48)	34.5	139.7	112.5	200.0
8.	ARGDKP (SEQ ID NO: 49)	18.8	199.7	70.5	106.9
9.	ARGDAP (SEQ ID NO: 50)	11.1	199.1	247.4	149.4
10.	ARGDSP (SEQ ID NO: 51)	36.9	138.8	71.6	146.2
11.	ARGDTP (SEQ ID NO: 52)	26.9	178.7	51.4	167.7
12.	ARGDNP (SEQ ID NO: 53)	18.3	44.1	262.7	213.6
13.	ARGDQP (SEQ ID NO: 54)	18.5	88.1	68.1	171.9
14.	ARGDWP (SEQ ID NO: 55)	29.6	52.0	12.4	129.8
15.	ARGDYP (SEQ ID NO: 56)	38.5	45.2	44.7	76.2

16.	ARGDFP (SEQ ID NO: 57)	17.5	51.1	16.6	99.4
17.	ARGDHP (SEQ ID NO: 58)	55.0	91.9	39.1	97.8
18.	ARGDRP (SEQ ID NO: 59)	3.0	49.1	51.2	77.1
19.	ARGDGP (SEQ ID NO: 60)	224.7	840.5	2643.3	359.6
20.	ARGDPP (SEQ ID NO: 61)	40940.0	62460.0	64665.0	49410.0

[0144] **Table 6:** Integin Selectivity and Inhibition of Platelet Aggregation by the ARGDMX Disintegrin Variants

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]			Platelet Aggregation (nM)
		αvβ3	α5β1	αIIbβ3	
^{48}PRGDMP (Rho wild type) (SEQ ID NO: 1)		13.0	256.8	21.0	83.2
1.	ARGDMP (SEQ ID NO: 32)	15.8	59.0	135.2	110.3
2.	ARGDMN (SEQ ID NO: 62)	97.2	4496.6	557.6	240.4
3.	ARGDMQ (SEQ ID NO: 63)	93.0	6212.3	2317.3	171.5
4.	ARGDMD (SEQ ID NO: 64)	144.6	11416.3	1198.3	196.1
5.	ARGDME (SEQ ID NO: 65)	196.0	5619.7	2792.0	200.8
6.	ARGDMH (SEQ ID NO: 66)	118.7	3329.3	3297.8	216.7
7.	ARGDMR (SEQ ID NO: 67)	213.8	9787.8	1142.4	86.5
8.	ARGDMK (SEQ ID NO: 68)	398.9	16794.7	1909.7	84.1
9.	ARGDMG (SEQ ID NO: 69)	83.5	4607.8	7057.7	224.5
10.	ARGDML (SEQ ID NO: 70)	57.4	785.6	1328.3	165.5
11.	ARGDMW (SEQ ID NO: 71)	18.6	1386.7	1002.0	130.2
12.	ARGDMF (SEQ ID NO: 72)	16.8	755.8	1280.0	187.5
13.	ARGDMY (SEQ ID NO: 73)	10.7	505.7	2212.7	160.1
14.	ARGDMM (SEQ ID NO: 74)	20.9	687.0	340.7	103.1

15.	ARGDMA (SEQ ID NO: 75)	11.3	1090.7	1237.3	138.6
16.	ARGDMI (SEQ ID NO: 76)	10.0	673.2	688.6	129.5
17.	ARGDMV (SEQ ID NO: 77)	12.9	139.1	181.6	139.3
18.	ARGDMT (SEQ ID NO: 78)	16.1	218.4	562.7	115.8

[0145] **Table 7:** Integin Selectivity and Inhibition of Platelet Aggregation by the XXPRGD Disintegrin Variants

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]			Platelet Aggregation (nM)
		αvβ3	α5β1	αIIbβ3	
^{46}RIPRGD (Rho wild type) (SEQ ID NO: 1)		13.0	256.8	21.7	83.2
1.	RR (SEQ ID NO: 79)	3.5	69.5	22.0	122.3
2.	RM (SEQ ID NO: 80)	8.3	393.3	31.5	198.4
3.	RV (SEQ ID NO: 81)	11.9	256.8	36.9	180.6
4.	RA (SEQ ID NO: 82)	15.5	383.0	94.1	178.4
5.	RQ (SEQ ID NO: 83)	15.9	768.1	63.6	200.3
6.	RE (SEQ ID NO: 84)	13.6	1292.3	51.1	189.7
7.	RF (SEQ ID NO: 85)	4.9	351.5	17.0	159.4
8.	RP (SEQ ID NO: 86)	18.8	460.7	89.3	397.7
9.	KR (SEQ ID NO: 87)	5.1	38.1	14.3	170.0
10.	KK (SEQ ID NO: 88)	14.2	76.6	36.7	235.5
11.	KM (SEQ ID NO: 89)	9.1	481.1	34.8	205.8
12.	KI (SEQ ID NO: 90)	12.1	449.8	70.3	152.7
13.					
14.	FI (SEQ ID NO: 91)	6.3	458.6	35.5	239.3
15.	QI	7.3	823.8	51.2	467.0

	(SEQ ID NO: 92)				
16.	AI (SEQ ID NO: 93)	62.1	1293.3	188.7	587.2
17.	EI (SEQ ID NO: 94)	18.7	913.4	477.1	1468.0

[0146] **Table 8:** Integin Selectivity and Inhibition of Platelet Aggregation by the Disintegrin Variants with Mutant RGD Loop, Mutant Linker and/or Mutant C-Terminus

	Consensus Sequence	IC_{50} (nM) [Interaction towards targets]			
		Platelet Aggregation	αIIbβ3	αVβ3	α5β1
	^{39}SRAGK-^{46}RIPRGDMP-^{67}YH (Rho wild type) (SEQ ID NO: 1)	64.2	33.8	18.8	223.4
1.	KKKRT RIPRGDMP YH (SEQ ID NO: 127)	104.5	25.6	1.3	76.1
2.	SRAGK RIARGDNP YH (SEQ ID NO: 128)	125.4	256.0	22.6	40.0
3.	KKKRT RIARGDNP YH (SEQ ID NO: 129)	88.2	133.4	5.9	10.0
4.	SRAGK RIARGDNP NGLYG (SEQ ID NO: 347)	195.0	146.1	23.0	53.0
5.	KKKRT RIARGDNP NGLYG (SEQ ID NO: 131)	160.2	31.7	3.7	11.5
6.	SRAGK RRARGDNP NGLYG (SEQ ID NO: 130)	199.8	85.0	9.6	10.9
7.	KKKRT RRARGDNP NGLYG (SEQ ID NO: 132)	147.6	31.4	3.6	2.7
8.	SRAGK RRARGDNP NGLYG (SEQ ID NO: 130)	233.7	98.7	2.5	2.2
9.	KKKRT RRARGDNP NGLYG (SEQ ID NO: 132)	153.5	44.3	3.2	1.6
10.	KKKRT RIARGDNP NGLYG (SEQ ID NO: 131)	160.2	31.7	3.7	11.5
11.	AKKRT RIARGDNP NGLYG (SEQ ID NO: 138)	132.2	27.6	6.9	64.0
12.	KAKRT RIARGDNP NGLYG (SEQ ID NO: 139)	190.2	65.5	16.0	47.6
13.	KKART RIARGDNP NGLYG (SEQ ID NO: 140)	157.5	34.2	3.5	15.5
14.	KKKAT RIARGDNP NGLYG (SEQ ID NO: 141)	140.9	26.7	6.9	21.0
15.	KKKRA RIARGDNP NGLYG (SEQ ID NO: 142)	192.2	78.4	19.2	58.2
16.	KAKRA RIARGDNP NGLYG (SEQ ID NO: 143)	156.6	25.0	1.9	68.4
17.	SKAGT RIARGDNP NGLYG (SEQ ID NO: 144)	174.6	124.4	23.7	71.3
18.	IEEGT RIARGDNP NGLYG (SEQ ID NO: 135)	206.1	401.0	15.7	50.6
19.	KGAGK RIARGDNP NGLYG (SEQ ID NO: 136)	176.0	43.8	9.8	116.1

20.	LKEGT RIARGDNP NGLYG (SEQ ID NO: 137)	187.9	80.6	8.2	118.2
21.	MKKGT RIARGDNP NGLYG (SEQ ID NO: 134)	178.5	151.8	4.0	113.4

[0147] **Table 9:** Integin Selectivity and Inhibition of Platelet Aggregation by the Disintegrin Variants with Mutant C-Terminus

	Consensus Sequence	IC_{50} (nM) [Interaction towards targets]					
		αvβ3	αvβ5	αvβ6	α5β1	αIIbβ3	Platelet Aggregation
	PRGDMP-^{65}PRYH (Rho wild type) (SEQ ID NO: 1)	13.0	94.4	176.2	256.8	52.2	83.2
1.	PRGDMP-^{65}PRWNDL (SEQ ID NO: 102)	9.8	88.5	133.7	365.9	53.7	100.9
2.	PRGDMP-^{65}PRNRFH (SEQ ID NO: 103)	15.0	162.9	140.8	590.8	81.8	107.9
3.	PRGDMP-^{65}PRNRFHA (SEQ ID NO: 104)	26.6	712.3	192.7	309.7	290.9	154.7
4.	PRGDMP-^{65}PRNPWNG (SEQ ID NO: 105)	40.7	681.1	160.2	260.0	235.2	121.9
5.	PRGDMP-^{65}PRNGLYG (SEQ ID NO: 106)	26.7	258.3	282.5	238.1	186.0	96.6
6.	PRGDMP-^{65}PGLYG (SEQ ID NO: 107)	30.1	274.8	1062.6	157.0	710.6	204.7

[0148] **Table 10:** Integin Selectivity and Inhibition of Platelet Aggregation by the Disintegrin Variants

	Consensus Sequence	IC_{50} (nM) [Interaction towards targets]					
		αvβ3	αvβ5	αvβ6	α5β1	αIIbβ3	Platelet Aggregation
	^{39}SRAGK-^{46}RIPRGDMP-^{65}PRYH (Rho wild type) (SEQ ID NO: 1)	13.0	94.4	176.2	256.8	52.2	83.2
1.	^{39}KKART-^{46}ARGRGDNP-^{65}PGLYG (SEQ ID NO: 124)	12.0	445.3	1081.8	27.8	133335.0	35253.2
2.	^{39}KKART-^{46}ARGRGDNP-	7.1	410.2	245.6	35.8	>95011.0	23790.5

	^{65}PPLYG (SEQ ID NO: 160)						
3.	^{39}KKART-^{46}ARGRGDNP-^{65}PRLYG (SEQ ID NO: 149)	6.8	363.5	417.0	6.5	43085.5	5208.5
4.	^{39}KKART-^{46}ARGRGDNP-^{65}PYLYG (SEQ ID NO: 120)	9.5	413.8	989.9	21.6	>136245.0	30285.5
5.	^{39}KKART-^{46}ARGRGDNP-^{65}PELYG (SEQ ID NO: 121)	13.6	720.2	986.8	15.3	>338956.0	46147.5

[0149] **Table 11**. Disintegrin Variants Specific to Integrins αvβ3 and α5β1

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]					
		αvβ3	αvβ5	αvβ6	α5β1	αIIbβ3	Platelet Aggregation
	^{39}SRAGK-^{46}RIPRGDMP-^{66}RYH (Rho wild-type) (SEQ ID NO: 1)	13.0	94.4	176.2	256.8	52.2	83.2
1.	^{39}KKART-^{46} ARGRGDNP-^{66}GLYG (SEQ ID NO: 124)	12.0	445.3	1081.8	27.8	133335.0	35253.2
2.	^{39}KKART-^{46} ARGRGDNP-^{66}YLYG (SEQ ID NO: 120)	11.0	413.8	1072.7	7.5	136245.0	30285.5
3.	^{39}KKART-^{46} ARGRGDNP-^{66}ELYG (SEQ ID NO: 121)	13.6	720.2	979.4	16.9	643101.0	46147.5
4.	^{39}KKART-^{46} ARGRGDNP-^{66}DLYG (SEQ ID NO: 147)	6.6	833.7	1996.2	15.4	450958.0	84719.0

[0150] **Table 12:** Disintegrin Variants Specific to Integrins αvβ3, αvβ5, and α5β1

Consensus Sequence	IC_{50} (nM)

		[Interaction towards targets]					
		αvβ3	αvβ5	αvβ6	α5β1	αIIbβ3	Platelet Aggregation
	^{39}SRAGK-^{46}RIPRGDMP-^{66}RYH (Rho wild-type) (SEQ ID NO: 1)	13.0	94.4	176.2	256.8	52.2	83.2
1.	^{39}SRAGK-^{46}RIARGDMP-^{66}RYH (SEQ ID NO: 43)	15.8	70.6	217.4	59.0	126.2	110.3
2.	^{39}SRAGK-^{46}RIARGDDP-^{66}RYH (SEQ ID NO: 44)	45.3	6886.0	14980.5	5044.5	5117.2	752.7
3.	^{39}SRAGK-^{46}RIARGDDL-^{66}RYH (SEQ ID NO: 338)	41.3	226.6	15734.5	526.7	1518.1	454.6
4.	^{39}KKART-^{46}ARGRGDDL-^{66}GLYG (SEQ ID NO: 339)	42.5	242.5	17323.3	562.3	44232.2	47329.0

[0151] **Table 13:** Disintegrin Variants Specific to Integrins αvβx and α5β1

Consensus Sequence		IC_{50} (nM) [Interaction towards targets]					
		αvβ3	αvβ5	αvβ6	α5β1	αIIbβ3	Platelet Aggregation
	^{39}SRAGK-^{46}RIPRGDMP -^{66}RYH (Rho wild-type) (SEQ ID NO: 1)	13.0	94.4	176.2	256.8	52.2	83.2
1.	^{39}SRAGK-^{46}RIARLDDL -^{66}RYH (SEQ ID NO: 125)	42.0	941.4	20683.3	14539.0	23171.0	10380.0
2.	^{39}SRAGK-^{46}DDPRGDMP -^{66}RYH (SEQ ID NO: 126)	25.1	500.0	1251.5	8653.0	>44540.0	18922.3
3.	^{39}KKART-^{46} ARGRGDNP -^{66}RYH (KG, AR-NP) (SEQ ID NO: 123)	5.0	445.7	317.0	28.8	37925.0	4478.3
4.	^{39}KKART-^{46} ARGRGDNP -^{66}GLYG (KG–P) (SEQ ID NO: 124)	12.0	445.3	1081.8	27.8	133335.0	35253.2
5.	^{39}KKART-^{46} ARGRGDNP -^{66}RWNDL (KG–WN) (SEQ ID NO: 171)	1.8	43.7	108	25.6	27985	4533

[0152] **Table 14:** Integin Selectivity and Inhibition of Platelet Aggregation by Varying One or More of the Linker, RGD Loop and the C-Terminus

Consensus Sequence of Disintegrin Variant		IC_{50} (nM) [Interaction towards targets]			
		α5β1	αVβ3	αIIbβ3	Platelet Aggregation
1.	-----^{48}ARGD^{65}PR (SEQ ID NO: 115)	3636.5	479.6	1588.3	107.1
2.	-----^{48}ARGD ^{65}PR**NGL (**SEQ ID NO: 348**)**	5161.0	146.0	1086.3	69.9
3.	-----^{48}ARGD ^{65}PR**NGLYG (**SEQ ID NO: 119**)**	9529.3	1191.8	245.2	83.4
4.	-----^{48}ARGD ^{65}PR**NPWNG (**SEQ ID NO: 118**)**	2679.0	1259.0	184.7	132.7
5.	**^{39}KKKRT**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 131)	11.5± 1.3	3.7± 0.9	31.7± 4.7	160.2± 16.9
6.	**^{39}MKKGT**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO:349)	113.4± 2.3	4.0± 0.8	151.8± 23.7	178.5± 10.3
7.	**^{39}IEEGT**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 135)	50.6± 5.0	15.7± 2.2	401.0± 77.9	206.1± 17.9
8.	**^{39}SRAGK**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 347)	53.0± 7.8	23.0± 7.1	146.1± 30.9	195.0± 45.7
9.	**^{39}KGAGK**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 136)	116.1± 19.9	9.8± 1.8	43.8± 3.5	176.0± 35.9
10.	**^{39}LKEGT**-^{48}ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 137)	118.2± 6.2	8.2± 0.5	80.6± 2.5	187.9± 25.7
11.	**^{39}AKKR^{43}T-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 138)	64.0± 3.9	6.9± 1.0	27.6± 6.5	132.2± 27.1
12.	**K^{40}AKR^{43}T-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 139)	47.6± 9.5	16.0± 2.4	65.5± 3.5	190.2± 16.5
13.	**KK^{41}AR^{43}T-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 140)	15.5± 3.2	3.5± 0.4	34.2± 5.3	157.5± 10.

Consensus Sequence of Disintegrin Variant		IC_{50} (nM) [Interaction towards targets]			
		α5β1	αVβ3	αIIbβ3	Platelet Aggregation
14.	**KKK ^{42}A^{43}T-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 141)	21.0± 2.2	6.9± 1.3	26.7± 4.7	140.9± 10.6
15.	**KKKR^{43}A-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 142)	58.2± 6.7	19.2± 1.2	78.4± 11.5	192.2± 6.9
16.	**^{39}KAKRA^{43}-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 143)	68.4	1.9	25.0± 4.4	156.6± 28.2
17.	**^{39}SKAGT^{43}-^{48}ARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 144)	71.3	23.7	124.4± 0.9	174.6± 23.7
18.	**^{39}SRAGKICR-^{47}RARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 130)	2.2	2.5	98.7± 9.1	233.7± 22.3
19.	**^{39}KKKRTICR-^{47}RARGDN^{53}P-^{67}NGLY^{71}G** (SEQ ID NO: 132)	1.6	3.2	44.3± 10.1	153.5± 5.4
20.	^{9}KKKRT-**^{46}AR**ARGDN^{53}P-^{67}NGLY^{71}G (SEQ ID NO: 169)	2.7	3.6	31.4	147.6
21.	^{39}KKKR^{43}T -^{48}PRGDM^{53}P-^{67}Y^{68}H (SEQ ID NO: 127)	76.1± 22.3 (3)	1.3± 0.2 (2)	25.6± 7.0(3)	104.5± 23.0
22.	^{39}SRAG^{43}K-^{48}ARGDN^{53}P-^{67}Y^{68}H (SEQ ID NO: 128)	40.0± 5.8 (2)	22.6± 3.7 (2)	256.0± 8.5(2)	125.4± 25.4
23.	^{39}KKKR^{43}T-^{48}ARGDN^{53}P-^{67}Y^{68}H (SEQ ID NO: 166)	10.0± 2.4 (3)	5.9± 1.0 (3)	133.4± 15.1(3)	88.2± 17.2

Example 5. Inhibition of Cell Migration by Disintegrin Variant and Wild Type of Rhodostomin

[0153] Transwell filters were equilibrated in serum containing DMEM for 2 h before use. DMEM containing 10% FBS was added to the lower compartments of the migration filters. In a volume of 100 ml serum-free DMEM, 2 x 10^4 A375 human melanoma cells were plated per transwell filter. Cells were allowed to migrate for 6 h at 37 ^oC in 5% CO_2, and were subsequently fixed by immersion of the filters in methanol for 15 min at room temperature. Rhodostomin, AR-NP (see Table 13 for consensus sequence), or PBS buffer were added into upper chamber. Filters were washed once with water, and were stained in 0.2% crystal violet in a 20% methanol/water solution for 10 min. Cells were removed from the upper surface of the membrane with a cotton swab. Cells that had migrated to the underside of the membrane were counted at 200x magnification from five random fields per membrane.

[0154] The inhibitory activity of Rho, AR-NP, or PBS buffer in this study as shown in Figure 2. Briefly, AR-NP markedly inhibited the migration of A375 human melanoma cells.

Example 6: Inhibition of Angiogenesis by Disintegrin Variants in Matrigel Angiogenesis Assay

[0155] The matrigel containing VEGF (100 ng/ml) and heparin (24–26 U/ml) is subcutaneously injected into B6 mice. After 5 days the gels are recovered, weighed and processed for hemoglobin quantification or histology as previously described. Hemoglobin content is measured with a Drabkin reagent kit 525 (Sigma). For histological analyses, the matrigel pellets are fixed in 4% paraformaldehyde and embedded in paraffin; four micron sections are stained with hematoxylin-eosin by standard procedures.

[0156] An aliquot (300 μl) of MATRIGEL™ (Becton Dickinson Lab.) containing VEGF (150 ng) and heparin (30 IU) was injected subcutaneously into the dorsal region of 6–8 week-old C57BL/6 mice. The MATRIGEL™ formed a plug rapidly. AR-NP (1mg/kg) or ARLDDL (SEQ ID NO: 351) (1mg/kg) (see Table 13 for consensus sequence) was administered once intravenously 24 hr later. After 5 days, plugs were taken and photographed (upper panel). 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) (B&C). The analysis showed that AR-NP was more effective than ARLDDL (SEQ ID NO: 351) when the drug was injected only once during 5 days angiogenesis period. See Figure 3.

Example 7: Inhibition of Angiogenesis by Hyperoxia/Normoxia-Driven Model of Retinopathy of Prematurity (ROP)

[0157] One-week-old C57BL/6j mice or ICR mice and their mothers are exposed to 75% ± 2% oxygen for 5 days (hyperoxia) and then returned to normoxic conditions for another 5 day (hyperoxic period, P7 to P12) for inducing relative hypoxic conditions, and were then housed in room air for a further 7 days (hypoxic-induced angiogenic period, P12 to P19). AR-NP (1 pg) was administered via intravitreous route on Day 12 and the mice were sacrificed on Day-19. Unexposed control animals are kept in room air. The animals are maintained at a constant temperature of 21 ± 1°C and on a 12-hour light–dark cycle. Oxygen concentration is measured with an oximeter. At the end of the oxygen exposure (day 12) and 5 days after return to normoxic conditions (day 17), the pups are killed, and retinal angiogenesis is evaluated by neovessels and endothelial cells.

[0158] The results of this study were shown in Figure 4. The analysis showed that AR-NP significantly reduced angiogenesis in a mouse model of retinopathy.

Example 8: Inhibition of Angiogenesis by Disintegrin Variants in Mice Aortic Ring Assays

[0159] . The thoracic aortas of mice 8-12 weeks of age are dissected out and cut into rings approximately 0.5 mm in width. The aortic rings are mounted in 200 ml matrigel covered with DMEM supplemented with 2.5% FCS and 30 ng/ml VEGF with or without the appropriate inhibitors or control agents. The experiment was conducted in CO2 incubator at 37℃. After 7 d in culture, the aortic rings are fixed with 4% formaldehyde and stained them with crystal violet. The number of sprouts grown from each ring by using inverted microscope was counted.

[0160] Mouse aortic rings were incubated with VEGF and 0.1 □M AR-NP in matrigel containing 100 mg/ml of fibronectin. The culture medium was changed every 3 days. Graphs showed the microvessel sprouting after 7 days culture. Note that AR-NP significantly reduced the vessel sprouting (see Fig. 5).

Example 9: Inhibition of Colony Formation by Disintegrin Variants on Breast Cancer Cells

[0161] The representative images in Figure 6 showed the results of colony formation assay. 4-T1 breast cancer cells were plated in 6-well dishes with a top layer of 0.35% agar

and a bottom layer of 0.7% agar in medium. 0.3 mL of medium is supplemented every 3 days. After 18 days, the number of cell clusters per dish is identified by crystal violet staining and counted. The analysis showed that both ARLDDL (SEQ ID NO: 351) (0.1 μM & 1 μM) and AR-NP (0.1 μM & 1 μM) inhibited colony formation of 4-T1 breast cancer cells.

Example 10: Inhibition of Osteoclastogenesis by Disintegrin Variants

[0162] Six- to eight-week-old SD rats are obtained from the Animal Center of National Laboratory and are kept under controlled conditions including a 22 ± 1°C room temperature and a 12-h light–dark cycle. Animals are fed with Purina Laboratory Rodent Diet and distilled water ad libitum. Bone marrow cells are prepared by removing from femurs and tibiae and flushing the bone marrow cavity with DMEM (Invitrogen, Carlsbad, California) which is supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 g/ml). The non-adherent cells (hematopoietic cells) are collected after 24 hr and used as osteoclast precursors. Cells are seeded at 1×10^6 cells/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) and M-CSF (20 ng/ml). The culture medium is replaced every 3 days. Osteoclast formation is measured on Day-8 by TRAP staining. In brief, adherent cells are fixed with 10% formaldehyde in PBS for 3 min and then stained with Naphthol AS-MX phosphate and tartrate solution for 1 hr at 37°C. Osteoclast-like cells in each well are scored by counting the number of TRAP-positive and multinucleated cells containing more than three nuclei.

[0163] The protein drugs were added on D1~D7. IC50 of osteoclastogenesis for α5β1 and αvβ3 dual integrin AR-NP is 3.61 nM. As shown in Figure 7, AR-NP protein or ARLDDL protein (SEQ ID NO: 351) inhibited RANKL-induced osteoclastogenesis in comparison with untreated control.

Example 11: Inhibition of Glioma Invasion by Disintegrin

[0164] Human glioma cells (U251) were cultured in upper chamber with matrigel containing 100 g/ml hyaluronan. Disintegrin variant was added in both upper and lower chambers. 24 hours later, the cells in the lower chamber were stained using crestal violet and counted. As shown in Figure 8, both ARLDDL (SEQ ID NO: 351) (0.1 μM) and AR-NP (0.1 μM) markedly inhibited glioma invasion.

Example 12: **Effect of AR-NP on Blood Pressure and Heart Rate**

[0165] Blood pressure and heart rate were recorded from tail using non-invasive method under isoflurane anesthesia in Wistar rat. AR-NP was administered from

tail vein after getting a stable measurement. Note that AR-NP at 5 mg/kg did not significantly affect blood pressure and heart rate (Figure 9). The usual dose of AR-NP for pharmacological effect is 1 mg/kg.

Example 13: Inhibition of A375 Melanoma Growth by AR-NP

[0166] A375 tumor cells (at $5x10^6$) were injected subcutaneously in the flank of 4-5 week-old male SCID mice. One week after cell implantation, mice were injected with AR-NP (KKART-ARGRGDNP; SEQ ID NO: 350) (2 mg/kg, 5 days/week, i.p.). Tumor volume was measured every two days. Tumors were excised and weighed after 18 days of drug treatment. Scale bar: 1 cm. Note that AR-NP treatment markedly inhibited tumor growth (see Fig. 10).

Example 14: **Inhibition of Tumor Growth by KG (AR-NP) in K-rasG12D transgenic mice**

[0167] $K\text{-}Ras^{G12D}$ transgenic mice was fed with 400 mg/L doxycycline to induce lung cancer. After 3.5 months, TG mice were intraperitoneally (IP) injected with 2mg/kg of AR-NP at two days interval for one month. Two weeks after fifteenth treatment, mice were sacrificed. Lungs were injected with India ink and fixated in Fekete's solution. The number of tumor nodules on lung were counted. **, *P* value < 0.001. As shown in Figure 11, the disintegrin variant (AR-NP) inhibited tumor growth in K-rasG12D transgenic mice.

Example 15: Inhibition of Brain Tumor Growth in U87-bearing Mice by KG

[0168] NOD-SCID mice were originally purchased and bred/maintained in a specific-pathogen-free vivarium with a well-controlled environment with a 12-h/12-h light/dark cycle and controlled humidity and temperature. 8-10 week-old mice weighing approximately 22-25 g were used. The mice were intraperitoneally anesthetized with a mixture of Dexdomitor/Zoletil (20μg/kg/2mg/kg), then placed in a stereotactic frame, and the skull was exposed by incision. U87-MG cells were harvested and adjusted to a density of $2.5x10^5$ cells/μL in phosphate buffered saline (PBS) before intracranial injection. 2 μL of U87-MG cells were injected into the striatum at the designated coordinates from the Bregma using a micro-infusion pump and 10-ml Hamilton syringe with a 30S-gage needle. The skull was then cleaned, the hole was sealed with bone wax, and the incision was sutured.

[0169] MRI was performed in a horizontal 7.0-T spectrometer with an active shielding gradient of 300 mT/m in 80 μs. The outlines of the tumors were delineated based on the contrast provided by the T2WIs between the tumor and the brain tissues. The total tumor

What is claimed is:

1. A disintegrin variant of rhodostomin comprising the amino acid sequence of SEQ ID NO: 1, comprising a mutant RGD loop, wherein the amino acid sequence at positions 46 to 53 of SEQ ID NO: 1 is substituted with an amino acid sequence selected from the group consisting of SEQ ID NOs: 329, 330, 331, 353 and 354, and at least one selected from the group consisting of:

   (a) a mutant linker, wherein the amino acid sequence at positions 39 to 45 of SEQ ID NO: 1 is substituted with an amino acid sequence selected from the group consisting of SEQ ID NO: 306 to SEQ ID NO: 317; and

   (b) a mutant C-terminus, wherein the amino acid sequence at positions 65 to 68 of SEQ ID NO: 1 is substituted with an amino acid sequence selected from the group consisting of SEQ ID NOs: 319, 323, 324, 326, 327, 328, 337 and 352,

   wherein the disintegrin variant has an increased selectivity for at least one of αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrins over αIIbβ3 integrin as compared to wild-type rhodostomin having the amino acid sequence of SEQ ID NO: 1.

2. The disintegrin variant of claim 1, comprising the mutant RGD loop, the mutant linker and the mutant C-terminus.

3. The disintegrin variant of claim 1, comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 123, 124, 147, 149 and 171.

4. The disintegrin variant of any one of claims 1 to 3, wherein the disintegrin variant is pegylated or conjugated with albumin or Fc.

5. A polynucleotide encoding the disintegrin variant of any one of claims 1 to 4.

6. A recombinant host cell comprising a polynucleotide encoding the disintegrin

variant of any one of claims 1 to 4.

7. A pharmaceutical composition comprising the disintegrin variant of any one of claims 1 to 4 and a pharmaceutically acceptable carrier.
8. The pharmaceutical composition of claim 7, which is for treating a disease associated with at least one integrin selected from the group consisting of αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrins in a subject in need thereof, wherein the disease is selected from the group consisting of 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.
9. The pharmaceutical composition of claim 8, wherein the integrin-associated disease is an angiogenesis-related eye disease selected from the group consisting of age-related macular degeneration, diabetic retinopathy, corneal neovascularizing diseases, ischaemia-induced neovascularizing retinopathy, high myopia, and retinopathy of prematurity.
10. The pharmaceutical composition of claim 8, wherein the integrin-associated disease is a cancer selected from the group consisting of metastatic melanoma, metastatic prostate cancer, metastatic breast cancer, colorectal carcinoma, liver cancer, ovarian cancer, cervical cancer, pancreatic cancer, non-small-cell lung cancer, and glioblastoma multiforme.
11. Use of a disintegrin variant of any one of claims 1 to 4 in the manufacture of a medicament for the treatment of a disease associated with at least one integrin selected from the group consisting of αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrins in a subject in need thereof, wherein the disease is selected from the

group consisting of 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.

12. The disintegrin variant of claim 1, comprising a mutant RGD loop of SEQ ID NO: 331 and a mutant linker of SEQ ID NO: 313.
13. A disintegrin variant comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 120, 121, 123, 124, 129-132, 134-144, 147, 149, 160, 166, 169, 171, 339, 347, and 349.
14. A pharmaceutical composition comprising the disintegrin variant of claim 13 and a pharmaceutically acceptable carrier.
15. The pharmaceutical composition of claim 14, which is for treating a disease associated with at least one integrin selected from the group consisting of αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrins in a subject in need thereof, wherein the disease is selected from the group consisting of 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.
16. The pharmaceutical composition of claim 14, wherein the integrin-associated disease is an angiogenesis-related eye disease selected from the group consisting of age-related macular degeneration, diabetic retinopathy, corneal neovascularizing diseases, ischaemia-induced neovascularizing retinopathy, high myopia, and retinopathy of prematurity.
17. The pharmaceutical composition of claim 14, wherein the integrin-associated

disease is a cancer selected from the group consisting of metastatic melanoma, metastatic prostate cancer, metastatic breast cancer, colorectal carcinoma, liver cancer, ovarian cancer, cervical cancer, pancreatic cancer, non-small-cell lung cancer, and glioblastoma multiforme.