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(54) Title: CYSTINE KNOT SCAFFOLD PLATFORM

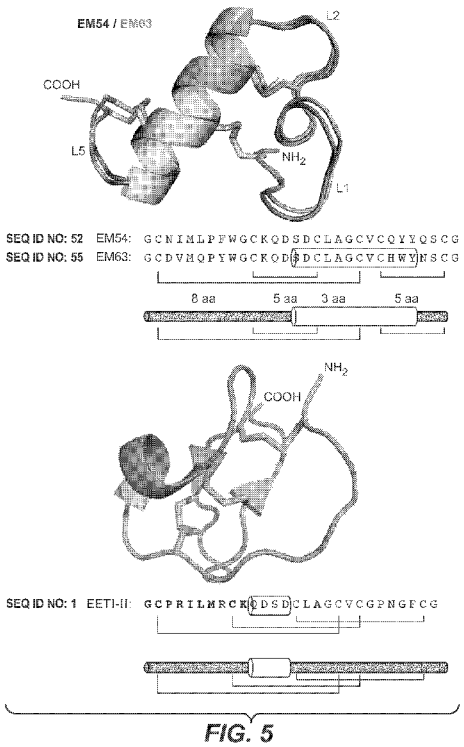

FIG. 5

(57) Abstract: Provided are non-naturally occurring cystine knot peptides (CKPs) that bind to VEGF-A. Additionally, provided are methods of using non-naturally occurring CKPs that bind to VEGF-A, including diagnostic and therapeutic compositions and methods. Non-naturally CKPs that bind low density lipoprotein receptor-related protein 6 (LRP6) are also provided.

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CYSTINE KNOT SCAFFOLD PLATFORM

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 62/219,063, filed September 15, 2015, which is incorporated herein by reference in its entirety.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 146392026840SEQLIST.txt, date recorded: September 15, 2016, size: 183 KB).

BACKGROUND OF THE INVENTION

[0003] The design and engineering of novel proteins from alternative protein scaffolds has been an emerging field in the last decade with a broad spectrum of applications ranging from structure biology and imaging tools to therapeutic reagents that are currently being tested in the clinic (HK Binz *et al.*, *Nat Biotechnol* 23, 1257-1268, 2005; HK Binz and A Pluckthun, *Curr Opin Biotechnol* 16, 459-469, 2005; SS Sidhu and S Koide, *Curr Opin Struct Biol* 17, 481-487, 2007; A Skerra, *Curr Opin Biotechnol* 18, 295-304, 2007; C Gronwall and S Stahl, *J Biotechnol* 140, 254-269, 2009; T Wurch *et al.*, *Trends Biotechnol* 30, 575-582, 2012; S Banta *et al.*, *Annu Rev Biomed Eng* 15, 93-113, 2013).

[0004] Desirable physical properties of potential alternative scaffold molecules include high thermal stability and reversibility of thermal folding and unfolding. Several methods have been applied to increase the apparent thermal stability of proteins and enzymes, including rational design based on comparison to highly similar thermostable sequences, design of stabilizing disulfide bridges, mutations to increase α-helix propensity, engineering of salt bridges, alteration of the surface charge of the protein, directed evolution, and composition of consensus sequences (Lehmann and Wyss, Cur Open Biotechnology 12, 371-375, 2001).

[0005] Cystine-knot peptides come from a wide range of sources and exhibit diverse pharmacological activities. They are roughly 30-50 amino acids in length and contain six conserved cysteine residues which form three disulfide bonds. One of the disulfides penetrates the macrocycle which is formed by the two other disulfides and their interconnecting backbones, thereby yielding a characteristic knotted topology with multiple

loops exposed on the surface. The loops are defined as the amino acid regions which flank the six conserved cysteine residues and are highly variable in nature. Furthermore, the unique arrangement of the disulfide bonds renders cystine-knot peptides highly stable to thermal, proteolytic and chemical degradation.

[0006] Thus, there is a need to develop small, stable, artificial antibody-like molecules for a variety of therapeutic and diagnostic applications, such as ocular diseases and disorders. The present invention meets this and other needs.

BRIEF SUMMARY OF THE INVENTION

[0007] In certain embodiments, provided herein is a non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A), wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6$ (SEQ ID NO: 2), $X_1X_2X_3X_4X_5X_6X_7$ (SEQ ID NO: 3), $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), $X_1X_2X_3X_4X_5X_6X_7X_8X_9$ (SEQ ID NO: 5), and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 6), wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$ (SEQ ID NO: 7), wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$, wherein each of X_1 – X_3 is any amino acid or an unnatural amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid or an unnatural amino acid;

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$ (SEQ ID NO: 7), wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

wherein the unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine; and

wherein the CKP binds to VEGF-A with an affinity of 500 pM or better.

[0008] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring (CKP) that binds to VEGF-A has an altered disulfide bond

connectivity-with reference to a wild-type *Ecballium elaterium* trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6.

[0009] In certain embodiments according to (or as applied to) any of the embodiments above, the unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0010] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and/or Z_2 is more than one amino acid, or an unnatural amino acid. In certain embodiments, Z_2 is two amino acids. In certain embodiments, Z_2 is three amino acids.

[0011] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and/or Z_2 is G.

[0012] In certain embodiments according to (or as applied to) any of the embodiments above, in L1, X_3 is not I; X_5 is not M; and/or X_6 is not R. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_1 is an amino acid selected from P, Q, R, T, V, D, N, K, L, and X; X_2 is an amino acid selected from T, D, L, V, I, R, P, N and X; X_3 is an amino acid selected from T, P, M, L, S, F, R, and X; X_4 is an amino acid selected from R, T, Q, D, W, L, E, S, K, and X; X_5 is an amino acid selected from F, P, V, E, K, L, I, and X; X_6 is an amino acid selected from K, N, F, P, L, Y, T, D, M, and X; X_7 is an amino

acid selected from Q, W, H and/X; and/or X_8 is an amino acid selected from Y, A, G, D, E, W, S, and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_9 is an amino acid selected from L, I, V, D, E and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_{10} is an amino acid selected from Y, T, M, N, F, and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0013] In certain embodiments according to (or as applied to) any of the embodiments above, X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0014] In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P).

In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G). In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is an amino acid selected from G, Q, H, R, L, and Q; X_2 is an amino acid selected from P, M, W, Y, F, L, and H; X_3 is an amino acid selected from N, F, H, and Y; X_4 is an amino acid selected from G, Q, D, N, K, H, E, and S; and/or X_5 is an amino acid selected from F, S, and T.

[0015] In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), wherein: X_1 is an amino acid selected from P, Q, and R; X_2 is an amino acid selected from T, L, and D; X_3 is an amino acid selected from T, M and L; X_4 is an amino acid selected from R, Q, and D; X_5 is an amino acid selected from F, P, and V; X_6 is an amino acid selected from K and F; X_7 is an amino acid selected from Q and W; and X_8 is an amino acid selected from Y, G, and D. In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9 X_{10} (SEQ ID NO: 6), wherein: X_1 is an amino acid selected from Q, R, T and V; X_2 is an amino acid selected from T and D; X_3 is P; X_4 is an amino acid selected from T and W; X_5 is an amino acid selected from F, E, P, and K; X_6 is an amino acid selected from N and P; X_7 is an amino acid selected from W and H; X_8 is an amino acid selected from A, D, E, and W; X_9 is an amino acid selected from L and I; and X_{10} is an amino acid selected from Y, T, M and N.

[0016] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is an amino acid selected from G, H, and Q; X_2 is an amino acid selected from P, M, W, and Y; X_3 is an amino acid selected from N and Y; X_4 is an amino acid selected from G, Q, and S; andX_5 is an amino acid selected from F and S.

[0017] In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), wherein: X_1 is an amino acid selected from D, Q, N, and K; X_2 is an amino acid selected from V, I, R, L, and P; X_3 is an amino acid selected from L, S, M, T, and F; X_4 is an amino acid selected from Q, L, and E; X_5 is P; X_6 is an amino acid selected from F, L, and Y; X_7 is W; and X_8 is G.

[0018] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_3 is Y; X_5 is S; and X_1, X_2 and X_4 are each any amino acid, with the exception that X_1 is not G, X_2 is not P, X_4 is not G, and/or X_5 is not F. In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is an amino acid

selected from H, L, R, and Q; X_2 is an amino acid selected from W, F, and Y; X_3 is Y; X_4 is an amino acid selected from Q, N, K, H, and E; and X_5 is S.

[0019] In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9 X_{10} (SEQ ID NO: 6), wherein: X_1 is an amino acid selected from K, Q, L, and R; X_2 is an amino acid selected from N and D; X_3 is an amino acid selected from P and L; X_4 is an amino acid selected from L, T, S and K; X_5 is an amino acid selected from F, V, I, and L; X_6 is an amino acid selected from N and D; X_7 is W; X_8 is an amino acid selected from A and S; X_9 is an amino acid selected from L, V, E and D; and X_{10} is an amino acid selected from Y and F.

[0020] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X1 is Q; X_2 is an amino acid selected from L, F, M, and H; X_3 is an amino acid selected from F, Y, and H; X_4 is an amino acid selected from D, Q, N, and K; and X_5 is an amino acid selected from S and T.

[0021] In certain embodiments according to (or as applied to) any of the embodiments above, in L2, X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D.

[0022] In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), wherein: X_5 is P; X_7 is W; X_8 is G; and wherein X_1, X_2, X_3, X_4 and X_6 are each any amino acid, with the exception that X1 is not P, X_2 is not R, X_3 is not I, and/or X_6 is not R. In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), wherein: X_1 is an amino acid selected from N and D; X_2 is an amino acid selected from I and V; X_3 is an amino acid selected from M and L; X_4 is an amino acid selected from L, Q, D and K; X_5 is P; X_6 is an amino acid selected from F, Y, T, L, and M; X_7 is W; and X_8 is G.

[0023] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is an amino acid selected from Q, H, L, and R; X_2 is an amino acid selected from Y and W; X_3 is Y; X_4 is an amino acid selected from Q and N; andX_5 is S. In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_3 is Y; X_5 is S; and X_1, X_2, and X_4 are each any amino acid, with the exception that: X_1 is not G, X_2 is not P, and/or X_4 is not G.

[0024] In certain embodiments according to (or as applied to) any of the embodiments above, in L2: X_1 is an amino acid selected from G or E; X_2 is an amino acid selected from Q, L, P, R, E, and M; X_3 is an amino acid selected from S, D, and N; X_4 is an amino acid selected from F, Y, L, M, and I; and/or X_5 is an amino acid selected from E, D, Q, L, and S,

[0025] In certain embodiments according to (or as applied to) any of the embodiments above, in L3, X_1 is L, X_2 is A, and X_3 is G.

[0026] In certain embodiments according to (or as applied to) any of the embodiments above, in L4, X1 is V or F.

[0027] In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P).

[0028] In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G).

[0029] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is any amino acid except G; X_2 is any amino acid except P; X_3 is any amino acid except N; X_4 is any amino acid except G; and/or X_5 is any amino acid except F.

[0030] In certain embodiments according to (or as applied to) any of the embodiments above, L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), wherein X_1 is an amino acid selected from N, D, and X; X_2 is an amino acid selected from I, V, and X; X_3 is M or X; X_4 is an amino acid selected from L, Q, and X; X_5 is P or X; X_6 is F, Y, or X; X_7 is W or X; and X_8 is G or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0031] In certain embodiments according to (or as applied to) any of the embodiments above, X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-

L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0032] In certain embodiments according to (or as applied to) any of the embodiments above, in L3, each of $X_1 - X_3$ is any amino acid or unnatural amino acid with the exception that X_1 is not Leucine (L), X_2 is not Alanine (A), and X_3 is not glycine (G), wherein the unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine. In certain embodiments according to (or as applied to) any of the embodiments above, in L3: X_1 is an amino acid selected from M, F, L V, and X; X_2 is an amino acid selected from S, N, Q, I, Y, E, V, T, and X; X_3 is an amino acid selected from D, Q, T, N, E, R, and X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0033] In certain embodiments according to (or as applied to) any of the embodiments above, in L4, X_1 is any amino acid except V or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine. In certain embodiments according to (or as applied to) any of the embodiments above, in L4, X_1 is I, L, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0034] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_3 is Y or X; X_5 is S or X; and X_1, X_2, and X_4 are each any amino acid or X, with the exception that X_1 is not G, X_2 is not P, and/or X_4 is not G, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine. In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P). In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G). In certain embodiments according to (or as applied to) any of the

embodiments above, in L5: X_1 is an amino acid selected from Q, H, and X; X_2 is an amino acid selected from Y, W, and X; X_3 is Y or X; X_4 is an amino acid selected from Q, N, or X; X_5 is S or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0035] In certain embodiments according to (or as applied to) any of the embodiments above, X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0036] In certain embodiments according to (or as applied to) any of the embodiments above, in L2: X_1 is G or X; X_2 is R, P, or X; X_3 is D or X; X_4 is F, I, or X; and X_5 is E, D, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, and L-4-fluorophenylalanine.

[0037] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECLQQCICQYYQSCG (SEQ ID NO: 103). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally

occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECVERCICQYYQSCG (SEQ ID NO: 104). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 105). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECMNQCICQYYQSCG (SEQ ID NO: 106). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECMQTCICQYYQSCG (SEQ ID NO: 107). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECVYQCICQYYQSCG (SEQ ID NO: 108). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECFINCICQYYQSCG (SEQ ID NO: 109). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 110). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECVTECICQYYQSCG (SEQ ID NO: 111). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECFYECICQYYQSCG (SEQ ID NO: 112). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular

endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle). In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle).

[0038] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A) comprises the amino acid sequence

GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine.

[0039] In certain embodiments, provided is a non-naturally occurring cystine knot peptide (CKP) comprising the amino acid selected from the group consisting of: GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113), GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114), GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139), GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140), GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142), GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle), GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle), GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle), and GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in

GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle). In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP binds VEGF-A.

[0040] In certain embodiments, provided is a non-naturally occurring cystine knot peptide (CKP) comprising the amino acid selected from the group consisting of: GCDVX_1QPYWGCGPDI-D/E-CLS-N/K/X_2-CICHWYNSCG (SEQ ID NO: 534), GCDVX_1QPYWGCGPDI-N/K/X_2-CLS-D/E-CICHWYNSCG (SEQ ID NO: 535), GCNIX_1LPYWGCGRDF-D/E-CME-N/K/X_2-CICQYYQSCG (SEQ ID NO: 538), GCNIX_1LPYWGCGRDF-N/K/X_2-CME-D/E-CICQYYQSCG (SEQ ID NO: 539), GCNIX_1LPFWGCGRDF-D/E-CVS-N/K/X_2-CICQYYQSCG (SEQ ID NO: 540), and GCNIX_1LPFWGCGRDF-N/K/X_2-CVS-D/E-CICQYYQSCG (SEQ ID NO: 541), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVX_1QPYWGCGPDI-D/E-CLS-N/K/X_2-CICHWYNSCG (SEQ ID NO: 534), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVXQPYWGCGPDIDCLSKCICHWYNSCG (SEQ ID NO: 536), wherein X is norleucine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVX_1QPYWGCGPDIDCLSX_2CICHWYNSCG (SEQ ID NO: 537), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCDVX_1QPYWGCGPDI-N/K/X_2-CLS-D/E-CICHWYNSCG (SEQ ID NO: 535), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIX_1LPYWGCGRDF-D/E-CME-N/K/X_2-CICQYYQSCG (SEQ ID NO: 538), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in

GCNIXLPYWGCGRDFECMEKCICQYYQSCG (SEQ ID NO: 543), wherein X is norleucine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in $GCNIX_1LPYWGCGRDFECMEX_2CICQYYQSCG$ (SEQ ID NO: 544), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in $GCNIX_1LPYWGCGRDF$-N/K/X_2-CME-D/E-CICQYYQSCG (SEQ ID NO: 539), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in $GCNIX_1LPFWGCGRDF$-D/E-CVS-N/K/X_2-CICQYYQSCG (SEQ ID NO: 540), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in GCNIXLPFWGCGRDFECVSKCICQYYQSCG (SEQ ID NO: 545), wherein X is norleucine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in $GCNIX_1LPFWGCGRDFECVSX_2CICQYYQSCG$ (SEQ ID NO: 546),wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP comprises the amino acid sequence set forth in $GCNIX_1LPFWGCGRDF$-N/K/X_2-CVS-D/E-CICQYYQSCG (SEQ ID NO: 541), wherein X_1 is norleucine and X_2 is ornithine. In certain embodiments according to (or as applied to) any of the embodiments above, the CKP binds VEGF-A.

[0041] In certain embodiments according to (or as applied to) any of the embodiments above, provided is a non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A, wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of X_1 – X_3 is any amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid;

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid;

wherein the CKP has an altered disulfide bond connectivity with reference to a wild-type Ecballium elaterium trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6; and wherein the CKP has a percent alpha helix content of at least 20%.

[0042] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and Z_2 are any amino acid, more than one amino acid, or an unnatural amino acid. In certain embodiments according to (or as applied to) any of the embodiments above, each of X_1 - X_{10} in L1 is any amino acid or an unnatural amino acid. In certain embodiments according to (or as applied to) any of the embodiments above, each of $X_1 - X_5$ in L2 is any amino acid or an unnatural amino acid. In certain embodiments according to (or as applied to) any of the embodiments above, each of $X_1 - X_3$ in L3 is any amino acid or an unnatural amino acid. In certain embodiments according to (or as applied to) any of the embodiments above, X_1 in L4 is any amino acid or an unnatural amino acid. In certain embodiments according to (or as applied to) any of the embodiments above, each of $X_1 - X_5$ in L5 is any amino acid or an unnatural amino acid. In certain embodiments, the unnatural amino acid is selected from the group consisting of: L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine

ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0043] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring (CKP) that binds to VEGF-A binds to VEGF-A with an affinity of 500 pM or less. In certain embodiments according to (or as applied to) any of the embodiments above, the binding affinity is determined via surface plasmon resonance.

[0044] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and/or Z_2 is more than one amino acid, or an unnatural amino acid. In certain embodiments, Z_2 is two amino acids. In certain embodiments, Z_2 is three amino acids. In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P). In certain embodiments according to (or as applied to) any of the embodiments above, in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G).

[0045] In certain embodiments according to (or as applied to) any of the embodiments above, the C-terminal carboxyl group of the non-naturally occurring (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments according to (or as applied to) any of the embodiments above, the N-terminal amine group of the non-naturally occurring (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments according to (or as applied to) any of the embodiments above, the C-terminal carboxyl group of the non-naturally occurring (CKP) that binds to VEGF-A is capped and the N-terminal amine group of the non-naturally occurring (CKP) that binds to VEGF-A is modified (such as capped).

[0046] In certain embodiments according to (or as applied to) any of the embodiments above, the C-terminal carboxyl group of the non-naturally occurring (CKP) that binds to VEGF-A is amidated. In certain embodiments according to (or as applied to) any of the embodiments above, the N-terminal amine group of the non-naturally occurring (CKP) that binds to VEGF-A is acetylated. In certain embodiments according to (or as applied to) any of the embodiments above, the C-terminal carboxyl group of the non-naturally occurring (CKP) that binds to VEGF-A is amidated and the N-terminal amine group of the non-naturally occurring (CKP) that binds to VEGF-A is acetylated.

[0047] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring (CKP) that binds to VEGF-A inhibits VEGF-A activity. In certain embodiments according to (or as applied to) any of the embodiments above, CKP inhibits VEGF-A activity with and IC_{50} between about 0.5 nM and about 1.0 nM. In certain

embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring EETI-II scaffold protein binds human VEGF-A, mouse VEGF-A, and rat VEGF-A.

[0048] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring CKP competes with the antibody G6.31 for binding to VEGF-A. In certain embodiments according to (or as applied to) any of the embodiments above, provided is a non-naturally occurring CKP that competes with the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above for binding to VEGF-A.

[0049] In certain embodiments according to (or as applied to) any of the embodiments above, non-naturally occurring CKP that binds to an epitope on VEGF-A comprising at least one of the amino acid residues selected from the group consisting of: V14, V15, F17, D19, Y21, Q22, Y25, I46, K48, N62, D63, L66, M81, I83, K84, P85, H86, G88, Q89, I91, C104, R105, and P106.

[0050] In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: K48, N62, and D63. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: Y21, Y25, and P106. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: H86 and Q89. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: M81, D19, and Q22. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: F17, M81, and I91. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: V14, F17, D19, Q22, M81, and I91. In certain embodiments according to (or as applied to) any of the embodiments above, the residues are selected from the group consisting of: Y25.

[0051] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring CKP that binds to VEGF-A is conjugated to a therapeutic agent. In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring CKP that binds to VEGF-A is conjugated to a label. In certain embodiments according to (or as applied to) any of the embodiments above, the label is selected from the group consisting of a radioisotope, a fluorescent dye, and an enzyme.

[0052] In certain embodiments according to (or as applied to) any of the embodiments above, provided is an isolated nucleic acid encoding the non-naturally occurring (CKP) that

binds to VEGF-A of any one of embodiments above. Also provided is an expression vector encoding the nucleic acid molecule of any one of the embodiments above. Also provided is a cell comprising the expression vector of any one of the embodiments above. Also provided is a method of producing the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above, comprising culturing the cell of any one of the embodiments above, and recovering the non-naturally occurring (CKP) that binds to VEGF-A from the cell culture.

[0053] Also provided is a method of producing the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above, comprising chemically synthesizing the non-naturally occurring (CKP) that binds to VEGF-A.

[0054] Provided herein is a composition comprising the non-naturally occurring (CKP) that binds to VEGF-A of any one of the embodiments above and a pharmaceutically acceptable carrier. In certain embodiments according to (or as applied to) any of the embodiments above, the composition comprises one or more additional compounds. In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound binds to a second biological molecule selected from the group consisting of interleukin-6 (IL-6); interleukin-6 receptor (IL-6R); PDGF; angiopoietin; angiopoietin 2; Tie2; S1P; integrins αvβ3, αvβ5, and α5β1; betacellulin; apelin/APJ; erythropoietin; complement factor D; TNFα; HtrA1; a VEGF receptor; ST-2 receptor; and proteins genetically linked to age-related macular degeneration (AMD) risk such as complement pathway components C2, factor B, factor H, CFHR3, C3b, C5, C5a, C3a, HtrA1, ARMS2, TIMP3, HLA, interleukin-8 (IL-8), CX3CR1, TLR3, TLR4, CETP, LIPC, COL10A1, and TNFRSF10A. In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound is a non-naturally occurring CKP. In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound is an antibody or antigen-binding fragment thereof.

[0055] Provided herein is a method of treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject, comprising administering an effective amount of the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above to the subject. In certain embodiments according to (or as applied to) any of the embodiments above, the method further comprises administering one or more additional compounds. In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring CKP that binds to VEGF-A is administered simultaneously with the additional compound(s). In certain embodiments according to (or as

applied to) any of the embodiments above, the non-naturally occurring CKP that binds to VEGF-A is administered before or after the additional compound(s). In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound binds to a second biological molecule selected from the group consisting of interleukin-6 (IL-6); interleukin-6 receptor (IL-6R); PDGF; angiopoietin; angiopoietin 2; Tie2; S1P; integrins αvβ3, αvβ5, and α5β1; betacellulin; apelin/APJ; erythropoietin; complement factor D; TNFα; HtrA1; a VEGF receptor; ST-2 receptor; and proteins genetically linked to age-related macular degeneration (AMD) risk such as complement pathway components C2, factor B, factor H, CFHR3, C3b, C5, C5a, C3a, HtrA1, ARMS2, TIMP3, HLA, interleukin-8 (IL-8), CX3CR1, TLR3, TLR4, CETP, LIPC, COL10A1, and TNFRSF10A. In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound is a non-naturally occurring CKP. In certain embodiments according to (or as applied to) any of the embodiments above, the additional compound is an antibody or antigen-binding fragment thereof. In certain embodiments according to (or as applied to) any of the embodiments above, the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP).

[0056] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring (CKP) that binds to VEGF-A or the composition is administered to the subject via an implantable device. In certain embodiments according to (or as applied to) any of the embodiments above, the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

[0057] In certain embodiments according to (or as applied to) any of the embodiments above, provided is a composition comprising the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above for use in treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject. In certain embodiments according to (or as applied to) any of the embodiments above, the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD),

diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP). In certain embodiments according to (or as applied to) any of the embodiments above, the composition is administered to the subject via an implantable device. In certain embodiments according to (or as applied to) any of the embodiments above, the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

[0058] In certain embodiments according to (or as applied to) any of the embodiments above, provided is a composition comprising the non-naturally occurring (CKP) that binds to VEGF-A of any one of embodiments above for use in treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject. In certain embodiments according to (or as applied to) any of the embodiments above, the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP). In certain embodiments according to (or as applied to) any of the embodiments above, the medicament is administered to the subject via an implantable device. In certain embodiments according to (or as applied to) any of the embodiments above, the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

[0059] In certain embodiments according to (or as applied to) any of the embodiments above, the non-naturally occurring (CKP) that binds to VEGF-A is formulated for long acting delivery.

[0060] Provided herein is a formulation comprising the non-naturally occurring (CKP) that binds to VEGF-A of any of embodiments above and PLGA. In certain embodiments according to (or as applied to) any of the embodiments above, the PLGA is a PLGA rod.

[0061] Also provided herein is a non-naturally occurring cystine knot peptide (CKP) that binds to human low density lipoprotein receptor-related protein 6 (LRP6), wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2;$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6$, $X_1X_2X_3X_4X_5X_6X_7$, $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of X_1 – X_3 is any amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid; and

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid.

[0062] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and/or Z_2 is more than one amino acid, or an unnatural amino acid. In certain embodiments, Z_2 is two amino acids. In certain embodiments, Z_2 is three amino acids.

[0063] In certain embodiments according to (or as applied to) any of the embodiments above, Z_1 and/or Z_2 is G.

[0064] In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_1 is an amino acid selected from R, V, M, A, G, N, S, and E; X_2 is an amino acid selected from T, N, S, G, R, and A; X_3 is an amino acid selected from N, R, H, V, K, S, G, I, and Y; X_4 is an amino acid selected from R, V, N, I, K, S, and T; X_5 is an amino acid selected from V, R, K, I, T, S, L, and N; and X_6 is an amino acid selected from K, G, A, I, R, N, S, and V. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_7 is an amino acid selected from G, R, K, E, P, and T. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_8 is an amino acid selected from G, R, K, Q, A, and S. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_9 is an amino acid selected from R or G. In certain embodiments according to (or as applied to) any of the embodiments above, in L1: X_{10} is an amino acid selected from E, W, and G.

[0065] In certain embodiments according to (or as applied to) any of the embodiments above, in L5: X_1 is an amino acid selected from G, S, N, Y, A, and R; X_2 is an amino acid selected from P, G, S, V, E, R, F, and D; X_3 is an amino acid selected from N, G, S, E, P, K, H, and R; X_4 is an amino acid selected from G, R, H, S, Q, V, and D; and X_5 is an amino acid selected from F, D, N, R, G, Y, S, and T.

[0066] In certain embodiments according to (or as applied to) any of the embodiments above, in L2, X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments according to (or as applied to) any of the embodiments above, in L3, X_1 is L, X_2 is A, and X_3 is G. In certain embodiments according to (or as applied to) any of the embodiments above, in L4, X_1 is V.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067] **FIG. 1** depicts the structure of the EETI-II cystine knot protein.

[0068] **FIG. 2A** shows the results of experiments that were performed to determine whether EGF_CKP9.54.90 disrupts the interaction between VEGF-A(8-109) and KDR; VEGF-A(8-109) and Flt-1; VEGF-A 165 and KDR; VEGF-B and Flt-1; VEGF-C and Flt-4; VEGF-D and Flt-4; and PIGF-2 and FLT-1.

[0069] **FIG. 2B** shows the results of experiments that were performed to determine whether EGF_CKP9.54.90 disrupts the interaction between VEGF-A(8-109) and KDR; VEGF-A(8-109) and Flt-1; VEGF-A 165 and KDR; EGF and EGFR; PDGF and PDGFR; NGF and NGFR; and IGF and IGFR.

[0070] **FIG. 3** shows the results of experiments performed to determine whether VEGF_CKP9.54.90, VEGF_CKP9.54, and VEGF_CKP9.63.12 inhibit trypsin protease activity.

[0071] **FIG. 4** shows the results of experiments performed to determine whether VEGF_CKP9.54.90 and VEGF_CKP9.63.12 are resistant to trypsin digestion.

[0072] **FIG. 5** depicts the structure of VEGF_CKP9.54.90, the structure of wild type EETI-II, and provides schematics that compare the VEGF-binding CKP variant's disulfide bond connectivity pattern to that of wild type EETI-II.

[0073] **FIG. 6** depicts the co-crystal structure of VEGF_CKP9.54.90 in complex with VEGF-A.

[0074] **FIG. 7** depicts space filling models that show binding interfaces of VEGF_CKP9.54.90, antibody G6.31, and domain 2 of Flt-2 on VEGF-A.

[0075] **FIG 8** depicts ribbon diagram models that show binding interfaces of VEGF_CKP9.54.90, antibody G6.31, and domain 2 of Flt-2 on VEGF-A.

[0076] **FIG. 9** shows contact residues on VEGF-A at the interacting surface between VEGF-A and VEGF_CKP9.54.90.

[0077] **FIG. 10** shows the binding interfaces of bevacizumab Fab, Z-domain, and receptor-blocking peptide v108 on VEGF-A.

[0078] **FIG. 11** provides the results of an experiment that was performed to determine the effects of amino acid substitution mutations in VEGF-A on binding of VEGF_CKP9.54.90 to VEGF-A and on the binding of VEGF__CKP9.63.12 to VEGFA. The results of the experiment are shown against two different y axes.

[0079] **FIG. 12** provides the results of experiments that were performed to determine the effects of VEGF_CKP9.54.90 on CNV in rat eyes.

[0080] **FIG. 13** provides the results of experiments that were performed to determine the IC_{50} values of VEGF-binding CKP variants.

[0081] **FIG. 14** depicts the structures of VEGF_CKP9.54 and VEGF_CKP9.63. Also shown in FIG. 14 is portion of the co-crystal structure of VEGF_CKP9.63 in complex with VEGF-A that shows that residue at position 8 within loop1 of VEGF_CKP9.63 could form a hydrogen bond with the side chain of Gln22 of VEGF-A.

[0082] **FIG. 15** shows the results of phage competition ELISA experiments that were performed to assess the binding affinity of clones 9.54-28, 9.54, 9.54.1-2, 9.54.1-36, 9.54.1-42, 9.54.1-63, 9.54.1-90, and 9.54.1 for hVEGF(8-109).

[0083] **FIG. 16A** shows the results of phage competition ELISA experiments that were performed to assess the binding affinity of clones 9.63.44-1 to 9.63.44-7.

[0084] **FIG. 16B** shows the results of phage competition ELISA experiments that were performed to assess the binding affinity of clones 9.63.44-8 to 9.63.44-14.

DETAILED DESCRIPTION OF THE INVENTION

[0085] Provided are non-naturally occurring cystine knot peptides (CKPs) that specifically bind human VEGF-A. Such non-naturally occurring CKPs demonstrate one or more of the following characteristics: inhibition of VEGF-A activity with and IC_{50} between less than about 0.5 nM and less than about 1.0 nM; binding to human VEGF-A, mouse VEGF-A, and rat VEGF-A; resistance to trypsin digestion; a disulfide bond connectivity of C1-C4, C2-C3, and C5-C6; an alpha helix content of at least about at least about 15% to least

about 50%; binding to an epitope on VEGF-A that is different from the epitope bound by antibody G6.31, binding to an epitope on VEGF-A that is different from the epitope bound by bevacizumab, and/or binding to an epitope on VEGF-A that is different from the epitope bound by Flt-1.

[0086] Also provided are chimeric molecules and conjugates comprising non-naturally occurring cystine knot peptides that bind VEGF-A, nucleic acids encoding non-naturally occurring CKPs that bind VEGF-A, and compositions (such as pharmaceutical compositions). Also provided are methods of using non-naturally occurring CKPs that bind VEGF-A for treating ocular diseases and/or disorders (such as ocular vascular proliferative diseases and/or disorders) resulting from abnormal (such as excessive) angiogenesis and/or abnormal vascular permeability. Also provided are uses of non-naturally occurring CKPs that bind VEGF-A in the manufacture of a medicament for the treatment of ocular disease or disorders.

[0087] In a related aspect, non-naturally occurring CKPs that bind human low density lipoprotein receptor-related protein 6 (LRP6) are also provided.

[0088] Practice of the present disclosure employs, unless otherwise indicated, standard methods and conventional techniques in the fields of cell biology, toxicology, molecular biology, biochemistry, cell culture, immunology, oncology, recombinant DNA and related fields as are within the skill of the art. Such techniques are described in the literature and thereby available to those of skill in the art. See, for example, Alberts, B. *et al.*, "Molecular Biology of the Cell," 5th edition, Garland Science, New York, NY, 2008; Voet, D. *et al.* "Fundamentals of Biochemistry: Life at the Molecular Level," 3rd edition, John Wiley & Sons, Hoboken, NJ, 2008; Sambrook, J. *et al.*, "Molecular Cloning: A Laboratory Manual," 3rd edition, Cold Spring Harbor Laboratory Press, 2001; Ausubel, F. *et al.*, "Current Protocols in Molecular Biology," John Wiley & Sons, New York, 1987 and periodic updates; Freshney, R.I., "Culture of Animal Cells: A Manual of Basic Technique," 4th edition, John Wiley & Sons, Somerset, NJ, 2000; and the series "Methods in Enzymology," Academic Press, San Diego, CA.

Definitions

[0089] As used herein "non-naturally occurring" means, e.g., a polypeptide comprising an amino acid sequence that is not found in nature, or, e.g., a nucleic acid comprising a nucleotide sequence that is not found in nature. A "non-naturally occurring cystine knot

peptide" or "non-naturally occurring CKP" (or a nucleic acid encoding the same) provided herein does not have the amino acid sequence of a wild type EETI-II protein, i.e., G**C**PRILMR**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G (SEQ ID NO: 1), wherein Loop 1 (L1) is the amino acid sequence PRILMR (SEQ ID NO: 92), Loop 2 (L2) is the amino acid sequence KQDSD (SEQ ID NO: 93), Loop 3 (L3) is the amino acid sequence LAG, Loop 4 (L4) is the amino acid V, and Loop 5 (L5) is the amino acid sequence GPNGF (SEQ ID NO: 15). A non-naturally occurring CKP provided herein can be produced by genetic engineering methods or by chemical synthesis methods. Thus, a non-naturally occurring CKP described herein may be recombinant, i.e., produced by a cell, or nucleic acid, or vector, that has been modified by the introduction of a heterologous nucleic acid or the alteration of a native nucleic acid to a form not native to that cell, or that the cell is derived from a cell so modified. Alternatively, a non-naturally occurring CKP described herein can be produced via chemical peptide synthesis.

[0090] As used herein, the term "cystine-knot peptide" or "CKP" refers to a peptide between 26-50 amino acids in length, which contain six conserved cysteine residues that form three disulfide bonds. One of the disulfides penetrates the macrocycle which is formed by the two other disulfides and their interconnecting backbones, thereby yielding a characteristic knotted topology with multiple loops exposed on the surface. The loops are defined as the amino acid regions which flank the six conserved cysteine residues and are highly variable in nature.

[0091] As used herein, an "amino acid alteration" refers to the addition, deletion, or substitution of at least one amino acid in, e.g., a peptide sequence (such as in the WT EETI-II peptide sequence to generate a non-naturally occurring CKP, or in a non-naturally occurring CKP to generate another non-naturally occurring CKP).

[0092] An "isolated" non-naturally occurring CKP or composition is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the non-naturally occurring CKP, and can include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the non-naturally occurring CKP or composition will be purified (1) to greater than 95% by weight of non-naturally occurring CKP as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue

or, preferably, silver stain. Isolated non-naturally occurring CKP includes the CKP in situ within recombinant cells since at least one component of the CKP's natural environment will not be present. An isolated non-naturally occurring CKP will be prepared by at least one purification step.

[0093] "Percent (%) amino acid sequence identity" or "homology" with respect to the polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the polypeptide being compared, after aligning the sequences considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

[0094] As used herein the term "epitope" refers to a protein determinant capable of being specifically bound by a non-naturally occurring CKP provided herein. An epitope can comprise between about 3-10 amino acids in a spatial conformation, which is unique to the epitope. These amino acids can be linear within the protein (i.e., consecutive in the amino acid sequence) or they can be positioned in different parts of the protein (i.e., non-consecutive in the amino acid sequence). Methods of determining the spatial conformation of amino acids within a protein, or at the interface of two proteins, are known in the art, and include, for example, x-ray crystallography and 2- dimensional nuclear magnetic resonance.

[0095] The terms "disulfide bonding pattern (DBP)," "disulfide bond connectivity," and "disulfide linkage pattern" refers to the linking pattern of the cysteines relative to the WT EETI-II protein. The WT EETI-II protein comprises six conserved cysteine residues (numbered 1-6) that form three disulfide bonds with connectivities C1-C4, C2-C5, and C3-

C6. The disulfide bonding pattern is topologically constant, meaning the disulfide bonds can only be changed by unlinking one or more disulfides such as using redox conditions.

[0096] A "subject," "patient," or an "individual" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

[0097] An "effective amount" of a non-naturally occurring CKP (or a composition comprising such a non-naturally occurring CKP) as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and by known methods relating to the stated purpose.

[0098] The term "therapeutically effective amount" refers to an amount of a non-naturally occurring CKP or composition as disclosed herein, effective to "treat" a disease or disorder in a mammal (such as a human patient). In the case of ocular disease or ocular disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis), the therapeutically effective amount of a non-naturally occurring CKP that binds VEGF-A described herein (or a composition comprising such a non-naturally occurring VEGF-A-binding CKP) refers to the amount to reduce, stop or prevent at least one symptom of the ocular disease, such as a symptom or disorder of an ocular disease described in further detail elsewhere herein. For example, an effective amount would be considered as the amount sufficient to reduce or prevent a symptom of the ocular disease or ocular disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis), for example a complete or partial resolution and/or maintenance of the ocular disease as measured by optical coherence tomography (OCT) or an increase and/or maintenance in best corrected visual acuity (such as greater than 5 letters as assessed by EDTRS eye chart), or a reduction in the size of the neovascularization or neovascular permeability as assessed by fundus fluorescence angiography. An effective amount as used herein would also include an amount sufficient to prevent or delay the development of, e.g., macular edema, enhanced permeability (such as retinal vascular permeability), size of CNV lesion, and vision loss. An effective amount as used herein would also include an amount sufficient to prevent or delay the development of a symptom of the ocular disease, alter the course of a symptom disease (for example but not limited to, slow the progression of a symptom of the ocular disease), or reverse a symptom of the disease.

[0099] As used herein, "treatment" or "treating" is an approach for obtaining beneficial or desired results including clinical results. For purposes of this invention, beneficial or

desired clinical results include, but are not limited to, one or more of the following: alleviating one or more symptoms resulting from the ocular disease, diminishing the extent of the ocular disease, stabilizing the ocular disease (e.g., preventing or delaying the worsening of the disease), preventing or delaying the spread of the disease (such as to surrounding ocular tissues), preventing or delaying the recurrence of the ocular disease, delay or slowing the progression of the ocular disease, ameliorating the disease state, providing a remission or resolution (partial or total) of the ocular disease, decreasing the dose of one or more other medications required to treat the ocular disease, delaying the progression of the ocular disease, increasing or improving the quality of life, and/or preventing or delaying vision loss. Also encompassed by "treatment" is a reduction of pathological consequence of an ocular disease (such as, for example, vision loss). The methods provided herein contemplate any one or more of these aspects of treatment.

[0100] A "disorder" is any condition that would benefit from treatment with a non-naturally occurring CKP that binds VEGF-A described herein. Non-limiting examples of VEGF-A-related disorders to be treated herein include ocular diseases and disorders (such as ocular vascular proliferative diseases or ocular disorders characterized by excessive angiogenesis), as described elsewhere herein.

[0101] As used herein, by "pharmaceutically acceptable" or "pharmacologically compatible" is meant a material that is not biologically or otherwise undesirable, e.g., the material may be incorporated into a pharmaceutical composition administered to a patient without causing any significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. Pharmaceutically acceptable carriers or excipients have preferably met the required standards of toxicological and manufacturing testing and/or are included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug administration.

[0102] The term "detecting" is intended to include determining the presence or absence of a substance or quantifying the amount of a substance (such as a target ligand). The term thus refers to the use of the materials, compositions, and methods provided herein for qualitative and quantitative determinations. In general, the particular technique used for detection is not critical for practice of the invention.

[0103] For example, "detecting" according to the invention may include: observing the presence or absence of a target ligand (including, but not limited to, a human low density lipoprotein receptor-related protein 6 (LRP6) polypeptide or a human vascular endothelial growth factor A (VEGF-A) polypeptide); a change in the levels of a target ligand; and/or a

change in biological function/activity of a target ligand. In certain embodiments, "detecting" may include detecting levels of a target ligand (e.g., polypeptide levels of a human LRP6 or a human VEGF-A). Detecting may include quantifying a change (increase or decrease) of any value between 10% and 90%, or of any value between 30% and 60%, or over 100%, when compared to a control. Detecting may include quantifying a change of any value between 2-fold to 10-fold, inclusive, or more e.g., 100-fold.

[0104] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the non-naturally occurring CKP. The label may itself be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

[0105] With regard to the binding of a non-naturally occurring CKP to a target ligand, the term "specific binding" or "specifically binds to" or is "specific for" a particular target ligand means that binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. In certain embodiments, the extent of binding of the non-naturally occurring CKP to a "non-target" ligand will be less than about 10% of the binding of the non-naturally occurring CKP to its target ligand (such as LRP6 or VEGF-A) as determined by, e.g., fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). In certain embodiments, a non-naturally occurring CKP of the present disclosure specifically binds to a target ligand (such as human low density lipoprotein receptor-related protein 6 (LRP6) or human vascular endothelial growth factor A (VEGF-A)) with a dissociation constant (Kd) equal to or lower than 100 nM, optionally lower than 10 nM, optionally lower than 1 nM, optionally lower than 0.5 nM, optionally lower than 0.1 nM, optionally lower than 0.01 nM, or optionally lower than 0.005 nM; measured at a temperature of about 4 ºC, 25 ºC, 37 ºC, or 45 ºC.

[0106] Reference to "about" a value or parameter herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (and describes) aspects that are directed to that

value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0107] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

[0108] All references cited herein, including patent applications and publications, are hereby incorporated by reference in their entirety.

Non-Naturally Occurring Cystine Knot Peptides (CKPs) That Bind Human Vascular Endothelial Growth Factor A (VEGF-A)

[0109] In certain embodiments, provided herein is a non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A), wherein the CKP comprises the following cystine scaffold structure (i.e., scaffold structure I):

$$Z_1C_1\mathbf{L1}C_2\mathbf{L2}C_3\mathbf{L3}C_4\mathbf{L4}C_5\mathbf{L5}C_6Z_2 \qquad (I)$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, , t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine,

3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of $X_1 - X_3$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, , t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine,

benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, , t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide;

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide; and

wherein the CKP binds to VEGF-A with an affinity of 500 pM or less.

[0110] In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N-termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the

non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0111] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A has an altered disulfide bond connectivity-with reference to a wild-type *Ecballium elaterium* trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6.

[0112] In certain embodiments, Z_1 and/or Z_2 of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is G. In certain embodiments, Z_1 and/or Z_2 comprise more than one amino acid. In certain embodiments, Z_1 and/or Z_2 comprise 4 amino acids. In certain embodiments, Z_1 and/or Z_2 comprise 5 amino acids. In certain embodiments, Z_1 and/or Z_2 is an unnatural amino acid. In certain embodiments, the unnatural amino acid is N-acetylglycine or glycine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 wherein X_3 is not I; wherein X_5 is not M; and/or wherein X_6 is not R. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 wherein X_1 is an amino acid selected from P, Q, R, T, V, D, N, K, L, and X; wherein X_2 is an amino acid selected from T, D, L, V, I, R, P, N and X; wherein X_3 is an amino acid selected from T, P, M, L, S, F, R, and X; wherein X_4 is an amino acid selected from R, T, Q, D, W, L, E, S, K, and X; wherein X_5 is an amino acid selected from F, P, V, E, K, L, I, and X; wherein X_6 is an amino acid selected from K, N, F, P, L, Y, T, D, M, and X; wherein X_7 is an amino acid selected from Q, W, H and/X; and/or wherein X_8 is an amino acid selected from Y, A, G, D, E, W, S, and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-

L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 wherein X_9 is an amino acid selected from L, I, V, D, E and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 wherein X_{10} is an amino acid selected from Y, T, M, N, F, and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-

chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0113] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein each of $X_1 - X_5$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine,

L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide; with the exception that X_2 is not proline (P).

[0114] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein each of $X_1 - X_5$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide, with the exception that X_4 is not glycine (G). In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is an amino acid selected from G, Q, H, R, L, and Q; wherein X_2 is an amino acid selected from P, M, W, Y, F, L, and H; wherein X_3 is an amino acid selected from N, F, H, and Y; wherein X_4 is an amino acid selected from G, Q, D, N, K, H, E, and S; and/or wherein X_5 is an amino acid selected from F, S, and T. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is V or F.

[0115] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$, wherein: X_1 is an amino acid selected from P, Q, and R; X_2 is an amino acid selected from T, L, and D; X_3 is an amino acid selected from T, M and L; X_4 is an amino acid selected from R, Q, and D; X_5 is an amino acid selected from F, P, and V; X_6 is an amino acid selected from K and F; X_7 is an amino acid selected from Q and W; and X_8 is an amino acid selected from Y, G, and D. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9 X_{10}, wherein X_1 is an amino acid selected from Q, R, T and V; X_2 is an amino acid selected from T and D; X_3 is P; X_4 is an amino acid selected from T and W; X_5 is an amino acid selected from F, E, P, and K; X_6 is an amino acid selected from N and P; X_7 is an amino acid selected from W and H; X_8 is an amino acid selected from A, D, E, and W; X_9 is an amino acid selected from L and I; and X_{10} is an amino acid selected from Y, T, M and N. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is an amino acid selected from G, H, and Q; X_2 is an amino acid selected from P, M, W, and Y; X_3 is an amino acid selected from N and Y; X_4 is an amino acid selected from G, Q, and S; and X_5 is an amino acid selected from F and S. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is V or F.

[0116] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 having the structure $X_1X_2X_3X_4X_5X_6X_7X_8$, wherein: X_1 is an amino acid selected from D, Q, N, and K; X_2 is an amino acid selected from V, I, R, L, and P; X_3 is an amino acid selected from L, S, M, T, and F; X_4 is an amino acid selected from Q, L, and E; X_5 is P; X_6 is an amino acid selected from F, L, and Y; X_7 is W; and X_8 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_3 is Y; X_5 is S; and wherein X_1, X_2 and X_4 are each any amino acid, with the exception that X_1 is not G, X_2 is not P, X_4 is not G, and/or X_5 is not F. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is an amino acid selected from H, L, R, and Q; X_2 is an amino acid selected from W, F, and Y; X_3 is Y; X_4 is an amino acid selected from

Q, N, K, H, and E; and X_5 is S. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is V or F.

[0117] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9 X_{10}, wherein X_1 is an amino acid selected from K, Q, L, and R; X_2 is an amino acid selected from N and D; X_3 is an amino acid selected from P and L; X_4 is an amino acid selected from L, T, S and K; X_5 is an amino acid selected from F, V, I, and L; X_6 is an amino acid selected from N and D; X_7 is W; X_8 is an amino acid selected from A and S; X_9 is an amino acid selected from L, V, E and D; and X_{10} is an amino acid selected from Y and F. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is Q; X_2 is an amino acid selected from L, F, M, and H; X_3 is an amino acid selected from F, Y, and H; X_4 is an amino acid selected from D, Q, N, and K; and X_5 is an amino acid selected from S and T. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is V or F.

[0118] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$, wherein: X_5 is P; X_7 is W; X_8 is G; and wherein X_1, X_2, X_3, X_4 and X_6 are each any amino acid, with the exception that X_1 is not P, X_2 is not R, X_3 is not I, and/or X_6 is not R. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$, wherein X_1 is an amino acid selected from N and D; X_2 is an amino acid selected from I and V; X_3 is an amino acid selected from M and L; X_4 is an amino acid selected from L, Q, D and K; X_5 is P; X_6 is an amino acid selected from F, Y, T, L, and M; X_7 is W; and X_8 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is an amino acid selected from Q, H, L, and R; X_2 is an amino acid selected from Y and W; X_3 is Y; X_4 is an amino acid selected from Q and N; and X_5 is S.

In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_3 is Y; X_5 is S; and wherein X_1, X_2, and X_4 are each any amino acid, with the exception that X_1 is not G, X_2 is not P, and/or X_4 is not G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is an amino acid selected from G or E; X_2 is an amino acid selected from Q, L, P, R, E, and M; X_3 is an amino acid selected from S, D, and N; X_4 is an amino acid selected from F, Y, L, M, and I; and/or X_5 is an amino acid selected from E, D, Q, L, and S. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is V or F.

[0119] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5, wherein each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P). In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5, wherein each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G). In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5, wherein X_1 is any amino acid except G; X_2 is any amino acid except P; X_3 is any amino acid except N; X_4 is any amino acid except G; and/or X_5 is any amino acid except F.

[0120] In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L1 comprising the structure $X_1X_2X_3X_4X_5X_6X_7X_8$, wherein X_1 is an amino acid selected from N, D, and X; X_2 is an amino acid selected from I, V, and X; X_3 is M or X; X_4 is an amino acid selected from L, Q, and X; X_5 is P or X; X_6 is F, Y, or X; X_7 is W or X; and X_8 is G or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine,

benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein each of $X_1 - X_3$ is any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide, with the exception that X_1 is not Leucine (L), X_2 is not Alanine (A), and X_3 is not glycine (G), wherein the unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-

alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L3 wherein X_1 is an amino acid selected from M, F, L V, and X; X_2 is an amino acid selected from S, N, Q, I, Y, E, V, T, and X; and X_3 is an amino acid selected from D, Q, T, N, E, R, and X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A

comprises an L4 wherein X_1 is any amino acid except V, or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L4 wherein X_1 is I, L, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine,

3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_3 is Y or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide; X_5 is S or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-

isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide; and wherein X_1, X_2, and X_4 are each any amino acid or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide, with the exception that X_1 is not G, X_2 is not P, and/or X_4 is not G. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5, wherein each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P). In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G). In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L5 wherein X_1 is an amino acid selected from Q, H, and X; X_2 is an amino acid selected from Y, W, and X; X_3 is Y or X; X_4 is an amino acid selected from Q, N, or X; X_5 is S or X, wherein X is an unnatural amino acid

selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide. In certain embodiments, the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A comprises an L2 wherein X_1 is G or X; X_2 is R, P, or X; X_3 is D or X; X_4 is F, I, or X; and X_5 is E, D, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine,

2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

[0121] In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N-termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0122] Also provided herein is a non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A, wherein the CKP comprises the cystine scaffold structure provided below (i.e., scaffold structure I):

$$Z_1C_1\mathbf{L1}C_2\mathbf{L2}C_3\mathbf{L3}C_4\mathbf{L4}C_5\mathbf{L5}C_6Z_2 \text{ (I)}$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 – X_5$ is any amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of $X_1 – X_3$ is any amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid;

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 – X_5$ is any amino acid; wherein,

the CKP has an altered disulfide bond connectivity with reference to a wild-type *Ecballium elaterium* trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6; and wherein the CKP has a percent alpha helix content of at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%, including any range in between these values.

[0123] In certain embodiments, the non-naturally occurring CKP binds to VEGF-A with an affinity of about 500 pM or less.

[0124] In certain embodiments, the binding affinity of the non-naturally occurring CKP to VEGF-A is determined via, e.g., surface plasmon resonance or other assays detailed in the Examples below.

[0125] In certain embodiments, Z_1 and/or Z_2 of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is G. In certain embodiments, Z_1 and/or Z_2 comprise more than one amino acid. In certain embodiments, Z_1 and/or Z_2 comprise 4 amino acids. In certain embodiments, Z_1 and/or Z_2 comprise 5 amino acids. In certain embodiments, Z_1 and/or Z_2 is an unnatural amino acid. In certain embodiments, the unnatural amino acid is N-acetylglycine or glycine amide.

[0126] In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N-termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0127] In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L5 wherein each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P). In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G). In certain embodiments, the non-naturally occurring CKP that binds VEGF-A inhibits

VEGF-A activity with and IC_{50} of about 0.5 nM to about 1.0 nM. In certain embodiments, the degree of inhibition is determined via a cellular IC_{50} assay, as described in further detail in the Examples below.

[0128] In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L1 comprising the amino acid sequence HMMYDY (SEQ ID NO: 231) or K/P/Q/R-K/T/L/D-W/T/M/L-Q/R/D-W/F/P/V-W/K/F-Y/Q/W-M/Y/G/D (SEQ ID NO: 115) or E/G/P/Q/R/T/V-T/E/A/D-D/T/I/P-W/V/Q/T/W-Y/F/N/E/P/K-P/E/W/N/P-H/Q/K/W/H-Q/F/E/A/D/W-I/L/H-D/W/P/Y/T/M/N (SEQ ID NO: 232), with reference to scaffold structure I above. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments, the non-naturally occurring CKP that binds VEGF-A further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L4 comprising V or F. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L5 comprising the amino acid sequence G/E/Y/Q/H-P/M/W/Y-N/Y/W-G/D/T/Q/R/S-F/A/E/S (SEQ ID NO: 20) or SWWPSL (SEQ ID NO: 237).

[0129] In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 8-14 and 225-230, with reference to scaffold structure I above. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments, the non-naturally occurring CKP that binds VEGF-A further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A comprises an L4 comprising V or F. In certain embodiments, the non-naturally occurring CKP that binds VEGF-A further comprises an L5 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs 15-18 and 233-238). The amino acid sequences of SEQ ID NOs 8-18, 225-230, and 233-238 are provided in **Table 1** below.

Table 1

ETDWYPHQID (SEQ ID NO: 225)	GPNGF (SEQ ID NO: 233)
GETVFEQFLW (SEQ ID NO: 226)	GPNGF (SEQ ID NO: 234)
HMMYDY (SEQ ID NO: 227)	EMYDA (SEQ ID NO: 235)

KKWQWWYM (SEQ ID NO: 228)	YPWTE (SEQ ID NO: 236)
PAIQNWKEHP (SEQ ID NO: 229)	SWWPSL (SEQ ID NO: 237)
PTTRFKQY (SEQ ID NO: 8)	GPNGF (SEQ ID NO: 15)
QDPTFNWALY (SEQ ID NO: 9)	QMYQS (SEQ ID NO: 16)
QLMHPFWG (SEQ ID NO: 230)	HWYRS (SEQ ID NO: 238)
QLMQPFWG (SEQ ID NO: 10)	HWYQS (SEQ ID NO: 17)
RDLDVKWD (SEQ ID NO: 11)	QYYSS (SEQ ID NO: 18)
RTPWEPHDIT (SEQ ID NO: 12)	GPNGF (SEQ ID NO: 19)
TTPWPPHEIM (SEQ ID NO: 13)	
VTPWKPHWIN (SEQ ID NO: 14)	

[0130] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence on any one of SEQ ID NOs: 21-17 and 239-244. The amino acid sequences of SEQ ID NOs: 21-17 and 239-244 are provided in **Table 2** below.

Table 2

G**C**ETDWYPHQID**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 239)
G**C**GETVFEQFLW**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 240)
G**C**HMMYDY**C**KQDSD**C**LAG**C**V**C**EMYDA**C**G	(SEQ ID NO: 241)
G**C**KKWQWWYM**C**KQDSD**C**LAG**C**V**C**YPWTE**C**G	(SEQ ID NO: 242)
G**C**PAIQNWKEHP**C**KQDSD**C**LAG**C**V**C**SWWPSL**C**G	(SEQ ID NO: 243)
G**C**PTTRFKQY**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 21)
G**C**QDPTFNWALY**C**KQDSD**C**LAG**C**V**C**QMYQS**C**G	(SEQ ID NO: 22)
G**C**QLMHPFWG**C**KQDSD**C**LAG**C**V**C**HWYRS**C**G	(SEQ ID NO: 244)
G**C**QLMQPFWG**C**KQDSD**C**LAG**C**V**C**HWYQS**C**G	(SEQ ID NO: 23)
G**C**RDLDVKWD**C**KQDSD**C**LAG**C**F**C**QYYSS**C**G	(SEQ ID NO: 24)
G**C**RTPWEPHDIT**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 25)
G**C**TTPWPPHEIM**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 26)
G**C**VTPWKPHWIN**C**KQDSD**C**LAG**C**V**C**GPNGF**C**G	(SEQ ID NO: 27)

[0131] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence Q/H/E/N/D-L/V/R/P/I-M/F/L-Q/E/R/L-P-F/A/L/S-W-G (SEQ ID NO: 358), with reference to scaffold structure I above. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A

binding CKP further comprises an L4 comprising the amino acid V. IN certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYQS (SEQ ID NO: 17).

[0132] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs 33, 36, and 245-253, with reference to scaffold structure I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYQS (SEQ ID NO: 17). The amino acid sequences of SEQ ID NOs 33, 36, and 245-253 are provided in Table 3 below:

Table 3

HLFEPLWG (SEQ ID NO: 245)
QVMRPFWG (SEQ ID NO: 246)
QVMQPAWG (SEQ ID NO: 247)
HRLQPLWG (SEQ ID NO: 248)
ELLQPSWG (SEQ ID NO: 249)
NPMLPFWG (SEQ ID NO: 368)
NVLLPLWG (SEQ ID NO: 250)
DIMQPLWG (SEQ ID NO: 36)
DLMQPLWG (SEQ ID NO: 251)
NPMLPLWG (SEQ ID NO: 252)
QVLQPSWG (SEQ ID NO: 253)

[0133] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence on any one of SEQ ID NOs: 265-275. The amino acid sequences of SEQ ID NOs: 265-275 are provided in **Table 4** below.

Table 4

GCHLFEPLWGCKQDSDCLAGCVCHWYQSCG (SEQ ID NO: 265)

GCQVMRPFWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 266)
GCQVMQPAWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 267)
GCHRLQPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 268)
GCELLQPSWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 269)
GCNPMLPFWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 270)
GCNVLLPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 271)
GCDIMQPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 272)
GCDLMQPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 273)
GCNPMLPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 58)
GCQVLQPSWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 275)

[0134] In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L1 comprising the amino acid sequence QLMQPFWG (SEQ ID NO: 10), with reference to scaffold structure I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L5 comprising the amino acid sequence R/H-W-Y-N/Q/H-S (SEQ ID NO: 359).

[0135] In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L1 comprising the amino acid sequence QLMQPFWG (SEQ ID NO: 10), with reference to scaffold structure I above. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L5 comprising an amino acid sequence selected from the group consisting of HWYQS (SEQ ID NO: 17), RWYHS (SEQ ID NO: 43), and RWYNS (SEQ ID NO: 133).

[0136] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 23, 276, and 278. SEQ ID NOs 23, 276, and 278 are provided in Table 5 below.

Table 5

GCQLMQPFWGCKQDSDCLAGCVCRWYNSCG	(SEQ ID NO: 276)
GCQLMQPFWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 23)
GCQLMQPFWGCKQDSDCLAGCVCRWYHSCG	(SEQ ID NO: 278)

[0137] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence Q/D/K/N/A/R/H-L/V/I/R/P/V/-M/L/S/T/F-Q/E/L/H-P-F/L/M/Y/S-W-G (SEQ ID NO: 40), with reference to scaffold structure I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence H/L/R/Q/-W/F/Y-Y-Q/N/K/H/D/E-S (SEQ ID NO: 360).

[0138] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 28-39 and 254-261, with reference to scaffold structure I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 17, 41-46, 133, 262-264, and 567. The amino acid sequences of SEQ ID NOs: 17, 28-39, 41-46, 133, 254-264, and 567 are provided in **Table 6** below:

Table 6

DVLQPFWG (SEQ ID NO: 28)	HWYQS (SEQ ID NO: 17)
QISQPFWG (SEQ ID NO: 29)	HFYNS (SEQ ID NO: 41)
DRMQPLWG (SEQ ID NO: 30)	LWYKS (SEQ ID NO: 42)

QLLEPMWG (SEQ ID NO: 254)	HWYNS (SEQ ID NO: 46)
KLLQPMWG (SEQ ID NO: 255)	QWYKS (SEQ ID NO: 262)
DRMQPYWG (SEQ ID NO: 256)	RWYHS (SEQ ID NO: 43)
NLMLPFWG (SEQ ID NO: 31)	RWYQS (SEQ ID NO: 44)
QRTQPFWG (SEQ ID NO: 32)	LWYDS (SEQ ID NO: 263)
KIMQPLWG (SEQ ID NO: 257)	QYYQS (SEQ ID NO: 45)
NLMHPFWG (SEQ ID NO: 258)	RWYNS (SEQ ID NO: 133)
NIMLPFWG (SEQ ID NO: 33)	QWYQS (SEQ ID NO: 264)
DPMQPFWG (SEQ ID NO: 34)	NPMLPLWG (SEQ ID NO: 38)
DVMQPYWG (SEQ ID NO: 35)	KLFEPLWG (SEQ ID NO: 39)
DIMQPLWG (SEQ ID NO: 36)	RWYES (SEQ ID NO: 567)
ALLQPLWG (SEQ ID NO: 259)	
QLLQPLWG (SEQ ID NO: 37)	
RLLEPSWG (SEQ ID NO: 260)	
HLLLPLWG (SEQ ID NO: 261)	

[0139] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 47-59 and 279-286. The amino acid sequences of SEQ ID NOs: 47-59 and 279-286 are provided in **Table** 7 below.

Table 7

GCDVLQPFWGCKQDSDCLAGCVCHWYQSCG (SEQ ID NO: 47)
GCQISQPFWGCKQDSDCLAGCVCHFYNSCG (SEQ ID NO: 48)
GCDRMQPLWGCKQDSDCLAGCVCLWYKSCG (SEQ ID NO: 49)
GCQLLEPMWGCKQDSDCLAGCVCHWYNSCG (SEQ ID NO: 279)
GCKLLQPMWGCKQDSDCLAGCVCRWYQSCG (SEQ ID NO: 280)
GCDRMQPYWGCKQDSDCLAGCVCQWYKSCG (SEQ ID NO: 281)
GCNLMLPFWGCKQDSDCLAGCVCRWYHSCG (SEQ ID NO: 50)
GCQRTQPFWGCKQDSDCLAGCVCRWYQSCG (SEQ ID NO: 51)
GCKIMQPLWGCKQDSDCLAGCVCLWYDSCG (SEQ ID NO: 282)
GCNLMHPFWGCKQDSDCLAGCVCHWYQSCG (SEQ ID NO: 283)
GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG (SEQ ID NO: 52)
GCNPMLPFWGCKQDSDCLAGCVCHWYQSCG (SEQ ID NO: 53)
GCDPMQPFWGCKQDSDCLAGCVCRWYQSCG (SEQ ID NO: 54)
GCDVMQPYWGCKQDSDCLAGCVCHWYNSCG (SEQ ID NO: 55)
GCDIMQPLWGCKQDSDCLAGCVCHWYQSCG (SEQ ID NO: 56)

GCALLQPLWGCKQDSDCLAGCVCRWYNSCG	(SEQ ID NO: 284)
GCQLLQPLWGCKQDSDCLAGCVCRWYQSCG	(SEQ ID NO: 57)
GCRLLEPSWGCKQDSDCLAGCVCQWYQSCG	(SEQ ID NO: 285)
GCHLLLPLWGCKQDSDCLAGCVCRWYHSCG	(SEQ ID NO: 286)
GCNPMLPLWGCKQDSDCLAGCVCHWYQSCG	(SEQ ID NO: 58)
GCKLFEPLWGCKQDSDCLAGCVCRWYESCG	(SEQ ID NO: 59)

[0140] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence Q/D/K/W/E/L/R-D/N-P/R/L/T-T/S/L/K-F/V/L/I-N/D-W-A/S/G-L/V/E/T/Q/D-F/Y(SEQ ID NO: 70), with reference to scaffold structure I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence Q/R-M/L/F/H-Y/F/H-D/Q/N/K-S/T (SEQ ID NO: 80).

[0141] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 60-69 and 287-291. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 71-79, 274, and 292. The amino acid sequences of SEQ ID NOs: 16, 60-69, 71-79, 274, and 287-292 are provided in Table 8 below.

Table 8

DDPSFDWSVY (SEQ ID NO: 287)	RMYDS (SEQ ID NO: 292)
KNPLFNWALY (SEQ ID NO: 60)	QLFDS (SEQ ID NO: 71)
QDPTVNWAVY (SEQ ID NO: 61)	QFYQS (SEQ ID NO: 72)

QDPTFNWAEY (SEQ ID NO: 62)	QLYQS (SEQ ID NO: 73)
WDPTFNWALY (SEQ ID NO: 288)	QMYDS (SEQ ID NO: 76)
QDPTLNWATY (SEQ ID NO: 289)	QMYQ**S** (SEQ ID NO: 16)
EDPTVDWAQY (SEQ ID NO: 290)	QMHQS (SEQ ID NO: 74)
QDPSLNWADY (SEQ ID NO: 63)	QMYNS (SEQ ID NO: 75)
LDRTLNWALY (SEQ ID NO: 64)	QLYQS (SEQ ID NO: 73)
LDPSFNWSLY (SEQ ID NO: 65)	QHYKT (SEQ ID NO: 77)
RDLTINWALF (SEQ ID NO: 66)	QLFNS (SEQ ID NO: 78)
KDTTFNWGLF (SEQ ID NO: 291)	QLYNS (SEQ ID NO: 79)
LDPTVNWALF (SEQ ID NO: 67)	QMFNS (SEQ ID NO: 274)
QDPKLNWAVY (SEQ ID NO: 68)	
LDPSFDWALY (SEQ ID NO: 69)	

[0142] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 81-91 and 293-297. The amino acid sequences of SEQ ID NOs: 81-91 and 293-297 are provided in **Table 9** below.

Table 9

GCDDPSFDWSVYCKQDSDCLAGCVCRMYDSCG (SEQ ID NO: 293)
GCKNPLFNWALYCKQDSDCLAGCVCQLFDSCG (SEQ ID NO: 81)
GCQDPTVNWAVYCKQDSDCLAGCVCQFYQSCG (SEQ ID NO: 82)
GCQDPTFNWAEYCKQDSDCLAGCVCQLYQSCG (SEQ ID NO: 83)
GCWDPTFNWALYCKQDSDCLAGCVCQMYDSCG (SEQ ID NO: 294)
GCQDPTFNWAEYCKQDSDCLAGCVCQMYQSCG (SEQ ID NO: 84)
GCQDPSLNWADYCKQDSDCLAGCVCQMHQSCG (SEQ ID NO: 85)
GCQDPTLNWATYCKQDSDCLAGCVCQMYQSCG (SEQ ID NO: 295)
GCEDPTVDWAQYCKQDSDCLAGCVCQMYQSCG (SEQ ID NO: 296)
GCLDRTLNWALYCKQDSDCLAGCVCQMYNSCG (SEQ ID NO: 86)
GCLDPSFNWSLYCKQDSDCLAGCVCQMYDSCG (SEQ ID NO: 87)
GCRDLTINWALFCKQDSDCLAGCVCQMFNSCG (SEQ ID NO: 88)
GCKDTTFNWGLFCKQDSDCLAGCVCQLYQSCG (SEQ ID NO: 297)
GCLDPTVNWALFCKQDSDCLAGCVCQHYKTCG (SEQ ID NO: 89)
GCQDPKLNWAVYCKQDSDCLAGCVCQLFNSCG (SEQ ID NO: 90)
GCLDPSFDWALYCKQDSDCLAGCVCQLYNSCG (SEQ ID NO: 91)

[0143] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence K/G/Q/S/N-Q/L/P/A/V/T/R/W/K/G/Y-D/S/E/N-S/F/Y/L/F/Q/M-D/E/N/A/L/F/H/Q (SEQ ID NO: 98). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45).

[0144] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 94-97 and 298-309. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45). The amino acid sequences of SEQ ID NOs: 94-97 and 298-309 are provided in **Table 10** below.

Table 10

GQSFE (SEQ ID NO: 94)	GWDQF (SEQ ID NO: 304)
GLDYD (SEQ ID NO: 95)	GKDFH (SEQ ID NO: 305)
GPELN (SEQ ID NO: 298)	GPDLQ (SEQ ID NO: 96)
QADYA (SEQ ID NO: 299)	SGDFA (SEQ ID NO: 306)
GVDYL (SEQ ID NO: 300)	GKELN (SEQ ID NO: 307)
GTNFL (SEQ ID NO: 301)	GWSMD (SEQ ID NO: 308)
SRDFD (SEQ ID NO: 302)	GYDLQ (SEQ ID NO: 309)
NRDFL (SEQ ID NO: 303)	GRDFE (SEQ ID NO: 97)

[0145] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 99-102 and 310-321. The amino acid sequences of SEQ ID NOs: 99-102 and 310-321 are provided in **Table 11** below.

Table 11

GCNIMLPFWGCGQSFECLAGCVCQYYQSCG (SEQ ID NO: 99)
GCNIMLPFWGCGLDYDCLAGCVCQYYQSCG (SEQ ID NO: 100)
GCNIMLPFWGCGPELNCLAGCVCQYYQSCG (SEQ ID NO: 310)
GCNIMLPFWGCQADYACLAGCVCQYYQSCG (SEQ ID NO: 311)
GCNIMLPFWGCGVDYLCLAGCVCQYYQSCG (SEQ ID NO: 312)
GCNIMLPFWGCGTNFLCLAGCVCQYYQSCG (SEQ ID NO: 313)
GCNIMLPFWGCSRDFDCLAGCVCQYYQSCG (SEQ ID NO: 314)
GCNIMLPFWGCNRDFLCLAGCVCQYYQSCG (SEQ ID NO: 315)
GCNIMLPFWGCGWDQFCLAGCVCQYYQSCG (SEQ ID NO: 316)
GCNIMLPFWGCGKDFHCLAGCVCQYYQSCG (SEQ ID NO: 317)
GCNIMLPFWGCGPDLQCLAGCVCQYYQSCG (SEQ ID NO: 101)
GCNIMLPFWGCSGDFACLAGCVCQYYQSCG (SEQ ID NO: 318)
GCNIMLPFWGCGKELNCLAGCVCQYYQSCG (SEQ ID NO: 319)
GCNIMLPFWGCGWSMDCLAGCVCQYYQSCG (SEQ ID NO: 320)
GCNIMLPFWGCGYDLQCLAGCVCQYYQSCG (SEQ ID NO: 321)
GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102)

[0146] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence N-I-M/L-L/S/T/Q/N/E/D-P-F/Y/S-WG (SEQ ID NO: 454), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence GRDFE (SEQ ID NO: 97). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the

amino acid sequence L/V/M/F-A/Q/E/S/N/Y/I/T-G/Q/R/D/T/N/E. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V or I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45).

[0147] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence selected from the group consisting of NIMLPFWG (SEQ ID NO: 33), NILLPFWG (SEQ ID NO: 396), NILLPYWG (SEQ ID NO: 397), NIMSPFWG (SEQ ID NO: 398), NIMTPFWG (SEQ ID NO: 399), NIMQPFWG (SEQ ID NO: 400), NIMNPFWG (SEQ ID NO: 401), NIMEPFWG (SEQ ID NO: 402), NIMDPFWG (SEQ ID NO: 403), NIMLPSWG (SEQ ID NO: 414), and NIMLPYWG (SEQ ID NO: 141) with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33), with reference to scaffold structure V or I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence GRDFE (SEQ ID NO: 97). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising an amino acid sequence selected from the group consisting of: LQQ, VER, MSD, MNQ, MQT, VYQ, FIN, VSQ, VTE, FYE, MEQ, and VYR. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid I. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45).

[0148] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 103-114. The amino acid sequences of SEQ ID NOs: 103-114 are provided in **Table 12** below.

Table 12

GCNIMLPFWGCGRDFECLQQCICQYYQSCG (SEQ ID NO: 103)
GCNIMLPFWGCGRDFECVERCICQYYQSCG (SEQ ID NO: 104)
GCNIMLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 105)
GCNIMLPFWGCGRDFECMNQCICQYYQSCG (SEQ ID NO: 106)
GCNIMLPFWGCGRDFECMQTCICQYYQSCG (SEQ ID NO: 107)

GCNIMLPFWGCGRDFECVYQCICQYYQSCG (SEQ ID NO: 108)
GCNIMLPFWGCGRDFECFINCICQYYQSCG (SEQ ID NO: 109)
GCNIMLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 110)
GCNIMLPFWGCGRDFECVTECICQYYQSCG (SEQ ID NO: 111)
GCNIMLPFWGCGRDFECFYECICQYYQSCG (SEQ ID NO: 112)
GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113)
GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114)
G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**G (SEQ ID NO: 386)
G**C**NILLPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 405)
G**C**NILLPYWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 406)
G**C**NIMSPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 407)
G**C**NIMTPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 408)
G**C**NIMQPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 409)
G**C**NIMNPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 410)
G**C**NIMEPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 411)
G**C**NIMDPFWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 412)
G**C**NIMLPSWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**G (SEQ ID NO: 415)
G**C**NIMLPFWG**C**GRDFE**C**LSG**C**V**C**QYYQS**C**G (SEQ ID NO: 421)
G**C**NIMLPFWG**C**GRDFE**C**LTG**C**V**C**QYYQS**C**G (SEQ ID NO: 422)
G**C**NIMLPFWG**C**GRDFE**C**LEG**C**V**C**QYYQS**C**G (SEQ ID NO: 423)
G**C**NIMLPYWG**C**GRDFE**C**LAG**C**L**C**QYYQS**C**G (SEQ ID NO: 424)
G**C**NIMLPYWG**C**GRDFE**C**LAG**C**I**C**QYYQS**C**G (SEQ ID NO: 425)
G**C**NIMLPYWG**C**GRDFE**C**LAG**C**V**C**QYYQS**C**S (SEQ ID NO: 431)
G**C**NILLPYWG**C**GRDFE**C**MEQ**C**I**C**QYYQS**C**G (SEQ ID NO: 435)

[0149] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence K/G/D/A/E-Q/E/R/V/P/D/M/G/N/L/A/F-D/N/Y/S-S/F/L/I/M/Y/V/N/E-D/L/Q/S/E/T/L/A/N (SEQ ID NO: 121). In certain embodiments the non-naturally occurring VEGF-A binding CKP further

comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46).

[0150] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 117-120, 211, and 322-339. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46). The amino acid sequences of SEQ ID NOs: 117-120, 211, and 322-339 are provided in **Table 13** below.

Table 13

GENFL (SEQ ID NO: 117)	DGDFD (SEQ ID NO: 331)
GRDLQ (SEQ ID NO: 322)	AGDFE (SEQ ID NO: 332)
GVDLS (SEQ ID NO: 323)	EMDFD (SEQ ID NO: 120)
GPDID (SEQ ID NO: 118)	GNSFE (SEQ ID NO: 333)
GDDLE (SEQ ID NO: 324)	GQDLT (SEQ ID NO: 334)
GVDMT (SEQ ID NO: 325)	GENLA (SEQ ID NO: 335)
GMDIE (SEQ ID NO: 326)	GQDYN (SEQ ID NO: 336)
DGDYQ (SEQ ID NO: 327)	GADLS (SEQ ID NO: 337)
GNDVS (SEQ ID NO: 328)	GFDMD (SEQ ID NO: 338)
GRDMD (SEQ ID NO: 119)	GESLS (SEQ ID NO: 211)
AGDEL (SEQ ID NO: 329)	DLNYE (SEQ ID NO: 339)
GLDEE (SEQ ID NO: 330)	

[0151] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 122-

126 and 340-357. The amino acid sequences of SEQ ID NOs: SEQ ID NOs: 122-126 and 340-357 are provided in **Table 14** below.

Table 14

GCDVMQPYWGCGENFLCLAGCVCHWYNSCG (SEQ ID NO: 122)
GCDVMQPYWGCGRDLQCLAGCVCHWYNSCG (SEQ ID NO: 340)
GCDVMQPYWGCGVDLSCLAGCVCHWYNSCG (SEQ ID NO: 341)
GCDVMQPYWGCGPDIDCLAGCVCHWYNSCG (SEQ ID NO: 123)
GCDVMQPYWGCGDDLECLAGCVCHWYNSCG (SEQ ID NO: 342)
GCDVMQPYWGCGVDMTCLAGCVCHWYNSCG (SEQ ID NO: 343)
GCDVMQPYWGCGMDIECLAGCVCHWYNSCG (SEQ ID NO: 344)
GCDVMQPYWGCDGDYQCLAGCVCHWYNSCG (SEQ ID NO: 345)
GCDVMQPYWGCGNDVSCLAGCVCHWYNSCG (SEQ ID NO: 346)
GCDVMQPYWGCGRDMDCLAGCVCHWYNSCG (SEQ ID NO: 124)
GCDVMQPYWGCAGDELCLAGCVCHWYNSCG (SEQ ID NO: 347)
GCDVMQPYWGCGLDEECLAGCVCHWYNSCG (SEQ ID NO: 348)
GCDVMQPYWGCDGDFDCLAGCVCHWYNSCG (SEQ ID NO: 349)
GCDVMQPYWGCAGDFECLAGCVCHWYNSCG (SEQ ID NO: 350)
GCDVMQPYWGCEMDFDCLAGCVCHWYNSCG (SEQ ID NO: 125)
GCDVMQPYWGCGNSFECLAGCVCHWYNSCG (SEQ ID NO: 351)
GCDVMQPYWGCGQDLTCLAGCVCHWYNSCG (SEQ ID NO: 352)
GCDVMQPYWGCGENLACLAGCVCHWYNSCG (SEQ ID NO: 353)
GCDVMQPYWGCGQDYNCLAGCVCHWYNSCG (SEQ ID NO: 354)
GCDVMQPYWGCGADLSCLAGCVCHWYNSCG (SEQ ID NO: 355)
GCDVMQPYWGCGFDMDCLAGCVCHWYNSCG (SEQ ID NO: 356)
GCDVMQPYWGCGESLSCLAGCVCHWYNSCG (SEQ ID NO: 126)
GCDVMQPYWGCDLNYECLAGCVCHWYNSCG (SEQ ID NO: 357)

[0152] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence D-V-M/L-Q/K/D-P-Y/M/T/L-W-G (SEQ ID NO: 130), with reference to scaffold structure I. In certain embodiments, the non-

naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence

[0153] KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence H/L/Q/R-W-Y-N-S (SEQ ID NO: 134).

[0154] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising an amino acid sequence selected from SEQ ID NOs: 127-129, with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence LAG. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 131-133. The amino acid sequences of SEQ ID NOs: 127-129 and 131-133 are provided in **Table 15** below:

Table 15

DVMKPMWG (SEQ ID NO: 127)	QWYNS (SEQ ID NO: 131)
DVLDPTWG (SEQ ID NO: 128)	LWYNS (SEQ ID NO: 132)
DVLQPLWG (SEQ ID NO: 129)	RWYNS (SEQ ID NO: 133)

[0155] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 135-137. The amino acid sequences of SEQ ID NOs: 135-127 are provided in **Table 16** below:

Table 16

GCDVMKPMWGCKQDSDCLAGCVCQWYNSCG (SEQ ID NO: 135)

GCDVLDPTWGCKQDSDCLAGCVCLWYNSCG (SEQ ID NO: 136)
GCDVLQPLWGCKQDSDCLAGCVCRWYNSCG (SEQ ID NO: 137)

[0156] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence GPDID (SEQ ID NO: 118). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising the amino acid sequence L/F-A/V/S-G/R/N. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising an amino acid selected from V, I, and L. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46).

[0157] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising the amino acid sequence GPDID (SEQ ID NO: 118). In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising an amino acid sequence FVR and LSN. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising an amino acid selected from V, I, and L. In certain embodiments the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46).

[0158] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140).

[0159] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1, L2, L3, L4 and/or L5 of any one of the non-naturally occurring VEGF-A binding CKPs disclosed herein. Thus, in certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an L1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 8-14, 28-39, 60-69, 127-129, 141, 225-230, 245-261, 287-291, 396-403, and 414 with reference to scaffold structure I. In certain embodiments, the

non-naturally occurring VEGF-A binding CKP further comprises an L2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 93-97, 117-120, 211, 298-309, and 322-339. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L3 comprising an amino acid sequence selected from the group consisting of LAG, LQQ, VER, MSD, MNQ, MQT, VYQ, FIN, VSQ, VTE, FYE, MEQ, and VYR, FVR and LSN. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L4 comprising the amino acid V, F, I, or L. In certain embodiments, the non-naturally occurring VEGF-A binding CKP further comprises an L5 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 15-18, 41-46, 71-79, 131-133, 233-238, 262-264, and 292. In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N- termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0160] In certain embodiments, at least one amino acid is deleted from a VEGF-A binding CKP provided herein. In certain embodiments, at least one amino acid is deleted from the N-terminus. In certain embodiments, at least one amino acid is deleted from the C-terminus. In certain embodiments, at least one amino acid is deleted from the N-terminus and the C-terminus. In certain embodiments, at least one internal amino acid is deleted. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence CNIMLPYWGCGRDFECLAGCVCQYYQSC (SEQ ID NO: 217). In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N- termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally

occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0161] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises at least one amino acid addition. In certain embodiments, at least one amino acid is added to the N-terminus. In certain embodiments, at least one amino acid is added to the C-terminus. In certain embodiments, at least one amino acid is added to the N-terminus and the C-terminus.

[0162] In certain embodiments, two amino acids are added to the N-terminus of a non-naturally occurring VEGF-A binding CKP provided herein. In certain embodiments, two amino acids are added to the N-terminus of the non-naturally occurring VEGF-A binding CKP set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102). In certain embodiments, the two amino acids added to the N-terminus of SEQ ID NO: 102 are F/I/G/T/V/L-H/A/S/R. In certain embodiments, the two amino acids added to the N-terminus of SEQ ID NO: 102 are selected from the group consisting of: FH, IA, GS, TR, VH, and LS. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGFH (SEQ ID NO: 379). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGIA (SEQ ID NO: 380). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGGS (SEQ ID NO: 381). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGTR (SEQ ID NO: 382). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGVH (SEQ ID NO: 383). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGLS (SEQ ID NO: 384).

[0163] In certain embodiments, two amino acids are added to the N-terminus of the non-naturally occurring VEGF-A binding CKP set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 386). In certain embodiments, the two amino acids added to the N-terminus of SEQ ID NO: 102 are R/W/P/D/Q/E/S-T/K/E/F/Q/L/S. In certain embodiments, the two amino acids added to the N-terminus of SEQ ID NO: 102 are selected from the group consisting of: RT, WK, PL, DE, QF, EQ, PT, RL, and SL. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGRT (SEQ ID NO: 387). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGWK (SEQ ID NO: 388). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGPL (SEQ ID NO: 389). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGDE (SEQ ID NO: 390). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGQF (SEQ ID NO: 391). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGEQ (SEQ ID NO: 392). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGPT (SEQ ID NO: 393). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGRL (SEQ ID NO: 394). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCDVLQPYWGCGPDIDCLSNCICHWYNSCGSL (SEQ ID NO: 395).

[0164] In certain embodiments, three amino acids are added to the N-terminus of a non-naturally occurring VEGF-A binding CKP provided herein. In certain embodiments, two amino acids are added to the N-terminus of the non-naturally occurring VEGF-A binding CKP set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102). In certain embodiments, the three amino acids added to the N-terminus of SEQ ID NO: 102 are P/N/T/D/E/Y/W-L/Y/F/H/D/P-I/Q/V/K/S/Y/H. In certain embodiments, the three amino

acids added to the N-terminus of SEQ ID NO: 102 are selected from the group consisting of: PLI, NYQ, PLQ, TFQ, DLV, EHK, YLS, WDY, WPH, and PHQ. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPLI (SEQ ID NO: 369). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGNYQ (SEQ ID NO: 370). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPLQ (SEQ ID NO: 371). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGTFQ (SEQ ID NO: 372). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGDLV (SEQ ID NO: 373). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGEHK (SEQ ID NO: 374). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGYLS (SEQ ID NO: 375). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGWDY (SEQ ID NO: 376). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGWPH (SEQ ID NO: 377). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an amino acid sequence set forth in GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPHQ (SEQ ID NO: 378).

[0165] In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N-termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal

carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

[0166] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP is a variant of a non-naturally occurring VEGF-A-binding CKP described herein. In certain embodiments, such a variant comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 amino acid substitutions in one or more of the sequences set forth in SEQ ID NOs: 8-14, 28-39, 60-69, 127-129, 141, 225-230, 245-261, 287-291, 396-403, and 414; SEQ ID NOs: 93-97, 117-120, 211, 298-309, and 322-339; amino acid sequences LAG, LQQ, VER, MSD, MNQ, MQT, VYQ, FIN, VSQ, VTE, FYE, MEQ, and VYR, FVR and LSN; and/or 15-18, 41-46, 71-79, 131-133, 233-238, 262-264, and 292. In certain embodiments, the amino acid substitution(s) are conservative amino acid substitution(s). In certain embodiments, the amino acid substitutions do not substantially reduce the ability of the non-naturally occurring VEGF-A-binding CKP to bind human VEGF-A. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce VEGF-A binding affinity may be made. The binding affinity of a variant of a non-naturally occurring VEGF-A -binding CKP can be assessed using a method described in the Examples below.

[0167] Conservative substitutions are shown in **Table 17** below under the heading of "conservative substitutions." More substantial changes are provided in **Table 17** under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into a variant of a non-naturally occurring VEGF-A-binding CKP and the products screened for a desired activity, e.g., retained/improved VEGF-A binding.

Table 17: Conservative Substitutions

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp

Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

[0168] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0169] An exemplary substitutional variant is an affinity matured non-naturally occurring VEGF-A-binding CKP, which may be conveniently generated, e.g., using phage display based affinity maturation techniques such as those described herein. Briefly, one or more residues in L1, L2, L3, L4, and/or L5 is altered (i.e., added, deleted, or substituted) and the variant VEGF-A-binding CKP is displayed on phage and screened for VEGF-A binding affinity. In certain embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, loop shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any non-naturally occurring CKP variants with the desired affinity for VEGF-A. In certain embodiments, introducing diversity involves loop-directed approaches, in which several residues in L1, L2, L3, L4, and/or L5 (e.g., about 5, about 4-6, or about 6-10 residues at a time) are randomized. L1, L2, L3, L4, and/or L5 residues involved in binding a target ligand may be identified, e.g., using alanine scanning mutagenesis or modeling.

[0170] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGQSFECLAGCVCQYYQSCG (SEQ ID NO: 215).

[0171] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 216).

[0172] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAKCVCQYYQSCG (SEQ ID NO: 542).

[0173] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 363).

[0174] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 364).

[0175] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECMNQCICQYYQSCG (SEQ ID NO: 222).

[0176] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECFYECICQYYQSCG (SEQ ID NO: 223).

[0177] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142).

[0178] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 405).

[0179] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPYWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 406).

[0180] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMSPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 407).

[0181] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMTPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 408).

[0182] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMQPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 409).

[0183] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMNPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 410).

[0184] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMEPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 411).

[0185] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMDPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 412).

[0186] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPSWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 415).

[0187] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCGRDFECLSGCVCQYYQSCG (SEQ ID NO: 421).

[0188] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCGRDFECLTGCVCQYYQSCG (SEQ ID NO: 422).

[0189] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCGRDFECLEGCVCQYYQSCG (SEQ ID NO: 423).

[0190] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCLCQYYQSCG (SEQ ID NO: 424).

[0191] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCICQYYQSCG (SEQ ID NO: 425).

[0192] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYYQSCS (SEQ ID NO: 431).

[0193] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 435).

[0194] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVLQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 386).

[0195] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 547).

[0196] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILQPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 548).

[0197] In certain embodiments, one or more amino acids in the sequence of a non-naturally occurring VEGF-A binding CKP provided herein are substituted with unnatural amino acids. In certain embodiments, the one or more amino acids are substituted with the same unnatural amino acid. In certain embodiments, the one or more amino acids are each substituted with a different unnatural amino acid. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises an unnatural amino acid at any amino acid position in L1, L2, L3, L4, and/or L5, with respect to scaffold structure I.

[0198] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 216), wherein the N-terminal glycine is capped with C(=O)-oxetane-3yl.

[0199] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAXCVCQYYQSCG (SEQ ID NO: 568, wherein X is ornithine.

[0200] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence XCNIMLPYWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 361), wherein X is N-acetylglycine.

[0201] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is sulfotyrosine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is 3,4-difluoro-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A

binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is 3,4-dichloro-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A -binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is 4-chloro-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is 3-F,4-Cl-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is 2-pyridone (NH para)-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPXWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 362), wherein X is pyridone (NH meta)-L-alanine.

[0202] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 218), wherein X is norleucine.

[0203] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 219), wherein X is 1-naphthylalanine.

[0204] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 219), wherein X is 2-naphthylalanine.

[0205] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence XCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 221), wherein X is PEG6-propargylglycine.

[0206] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 365), wherein X is norleucine.

[0207] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine.

[0208] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine.

[0209] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX$_1$LPFWGCGRDF-D/E-CVS-N/K/X$_2$-CICQYYQSCG (SEQ ID NO: 540) wherein X$_1$ is norleucine and X$_2$ is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPFWGCGRDFECVSKCICQYYQSCG (SEQ ID NO: 545) wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX$_1$LPFWGCGRDFECVSX$_2$CICQYYQSCG (SEQ ID NO: 546) wherein X$_1$ is norleucine and X$_2$ is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX$_1$LPFWGCGRDF-N/K/X$_2$-CVS-D/E-CICQYYQSCG (SEQ ID NO: 541), wherein X$_1$ is norleucine and X$_2$ is ornithine.

[0210] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPFWGCGRDFKCVS-D/E-CICQYYQSCG (SEQ ID NO: 561, herein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence CNIXLPFWGCGRDFKCVSDCICQYYQSCG (SEQ ID NO: 562, herein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence CNIXLPFWGCGRDFKCVSECICQYYQSCG (SEQ ID NO: 563, herein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPFWGCGRDFX2CVS-D/E-CICQYYQSCG (SEQ ID NO: 564, herein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPFWGCGRDFX2CVSDCICQYYQSCG (SEQ ID NO: 565, herein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPFWGCGRDFX2CVSECICQYYQSCG (SEQ ID NO: 566, herein X1 is norleucine and X2 is ornithine.

[0211] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine.

[0212] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDF-D/E-CME-N/K/X2-CICQYYQSCG (SEQ ID NO: 538) wherein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFECMEKCICQYYQSCG (SEQ ID NO: 543), wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDFECMEX2CICQYYQSCG (SEQ ID NO: 544), wherein X1 is norleucine and X2 is ornithine.

[0213] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDF-N/K/X2-CME-D/E-CICQYYQSCG (SEQ ID NO: 539) wherein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFKCME-D/E-CICQYYQSCG (SEQ ID NO: 555) wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFKCMEDCICQYYQSCG (SEQ ID NO: 556) wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFKCMEECICQYYQSCG (SEQ ID NO: 557) wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDFX2CME-D/E-CICQYYQSCG (SEQ ID NO: 558) wherein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDFX2CMEDCICQYYQSCG (SEQ ID NO: 559) wherein X1 is norleucine and X2 is ornithine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX1LPYWGCGRDFX2CMEECICQYYQSCG (SEQ ID NO: 560) wherein X1 is norleucine and X2 is ornithine.

[0214] In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDI-D/E-CLS-N/K/X2-CICHWYNSCG (SEQ ID NO: 534), wherein X1 is norleucine and X2 is ornithine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVXQPYWGCGPDIDCLSKCICHWYNSCG (SEQ ID NO: 536), wherein X is norleucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDIDCLSX2CICHWYNSCG

(SEQ ID NO: 537), wherein X1 is norleucine and X2 is ornithine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDI-N/K/X2-CLS-D/E-CICHWYNSCG (SEQ ID NO: 535), wherein X1 is norleucine and X2 is ornithine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVXQPYWGCGPDIKCLS-D/E-CICHWYNSCG (SEQ ID NO: 549), wherein X is norleucine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVXQPYWGCGPDIKCLSDCICHWYNSCG (SEQ ID NO: 550), wherein X is norleucine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVXQPYWGCGPDIKCLSECICHWYNSCG (SEQ ID NO: 551), wherein X is norleucine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDIX2CLS-D/E-CICHWYNSCG (SEQ ID NO: 552), wherein X1 is norleucine and X2 is ornithine. In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDIX2CLSDCICHWYNSCG (SEQ ID NO: 553), wherein X1 is norleucine and X2 is ornithine In certain embodiments the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX1QPYWGCGPDIX2CLSECICHWYNSCG (SEQ ID NO: 554), wherein X1 is norleucine and X2 is ornithine.

[0215] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLXFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 413), wherein X is gamma-benzyl-L-proline. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLXFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 413), wherein X is gamma-(4-fluoro-benzyl)-L-proline. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLXFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 413), wherein X is 4-OH-L-proline. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLXFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 413), wherein X is 4-fluoro-L-proline. In certain embodiments, the non-naturally

occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLXFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 413), wherein X is 4-[4-(trifluoromethyl)benzyl]-L-proline.

[0216] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 417), wherein X is N-methyl indole. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 417), wherein X is N-ethyl indole. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 417), wherein X is N-isopropyl indole. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYXGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 417), wherein X is 5-aza-indole.

[0217] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 4-methyl-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 2-naphthyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 2-quinolyl-Alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 4,4'-biphenyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 3-(3-quinolinyl)-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 3-(2-quinolinyl)-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 3-(2-quinoxalinyl)-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 4-

methyl-2-pyridyl-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 4-ethyl-2-pyridyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is benzothiazole-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is benzothiophene-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDXECLAGCVCQYYQSCG (SED ID NO: 419), wherein X is 3-(3-isoquinolinyl)-L-alanine.

[0218] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECXAGCVCQYYQSCG (SEQ ID NO: 420), wherein X is t-butyl-L-alanine (also known as L-Nepentylglycine). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECXAGCVCQYYQSCG (SEQ ID NO: 420), wherein X is 3-cyclobutyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECXAGCVCQYYQSCG (SEQ ID NO: 420), wherein X is 3-cyclopentyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECXAGCVCQYYQSCG (SEQ ID NO: 420), wherein X is 5,5,5-Trifluoro-L-leucine.

[0219] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is L-tert-Leucine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is t-butyl-L-alanine (also known as L-Nepentylglycine). In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is L-cyclopentylglycine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG

(SEQ ID NO: 426), wherein X is 3-cyclopentyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is L-cyclobutyl-L-glycine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is 3-cyclobutyl-L-alanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCXCQYYQSCG (SEQ ID NO: 426), wherein X is 5,5,5-Trifluoro-L-leucine.

[0220] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQXYQSCG (SEQ ID NO: 428), wherein X is 2-pyridone. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQXYQSCG (SEQ ID NO: 428), wherein X is 3,4-hydroxy-L-phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQXYQSCG (SEQ ID NO: 428), wherein X is 3,4-fluoro phenylalanine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQXYQSCG (SEQ ID NO: 428), wherein X is 3-fluoro,4-OH-L-phenylalanine.

[0221] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYXQSCG (SEQ ID NO: 430), wherein X is 2-Chloro-L-Tyrosine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYXQSCG (SEQ ID NO: 430), wherein X is 2-methyl-L- tyrosine. In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYXQSCG (SEQ ID NO: 430), wherein X is 2-ethyl-L-tyrosine, or 4-(naphthalen-1-ol-)-L-alanine.

[0222] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPYWGCGRDFECLAGCVCQYYQSCX (SEQ ID NO: 432), wherein X is D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methylglycine, glycine with its carboxy terminus converted to an ester of glycerol, glycine with its carboxy terminus converted to an ester of glycol, glycine with its carboxy terminus converted to an ester of oxetanyl alcohol, or glycine morpholine amide.

[0223] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXQPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 436), wherein X is norleucine.

[0224] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX$_1$LPYWGCGRDFECX$_2$EQCICQYYQSCG (SEQ ID NO: 437). In certain embodiments, X$_1$ and X$_2$ are the same unnatural amino acid. In certain embodiments, X$_1$ and X$_2$ are different unnatural amino acids. In certain embodiments, X$_1$ and X$_2$ are norleucine. In certain embodiments, X$_1$ is norleucine and X$_2$ is 3-cyclobutyl-L-alanine.

[0225] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIXLPYWGCGRDFECLEQCICQYYQSCG (SEQ ID NO: 438), wherein X is norleucine.

[0226] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIX$_1$LPYWGCGRDFECX$_2$EQCX$_3$CQYYQSCG (SEQ ID NO: 439). In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are the same unnatural amino acid. In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are not the same unnatural amino acid. In certain embodiments, X$_1$ is norleucine, X$_2$ is 3-cyclobutyl-L-alanine, and X$_3$ is cyclobutyl-L-glycine. In certain embodiments, X$_1$ is norleucine, X$_2$ is 3-cyclobutyl-L-alanine, and X$_3$ is 3-cyclobutyl-L-alanine. In certain embodiments, X$_1$ is norleucine, X$_2$ is 3-cyclobutyl-L-alanine, and X$_3$ is norleucine.

[0227] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence CNIX$_1$QPYWGCGRDFECX$_2$EQCX$_3$CQYYQSCG (SEQ ID NO: 440). In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are the same unnatural amino acid. In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are not the same unnatural amino acid. In certain embodiments, X$_1$ is norleucine, X$_2$ is 3-cyclobutyl-L-alanine, and X$_3$ is cyclobutyl-L-glycine. In certain embodiments, X$_1$ is norleucine, X$_2$ is 3-cyclobutyl-L-alanine, and X$_3$ is 3-cyclobutyl-L-alanine.

[0228] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPYWGCGRDFECXEQCICQYYQSCG (SEQ ID NO: 441), wherein X is 3-cyclobutyl-L-alanine or t-butyl-L-alanine (also known as L-Nepentylglycine).

[0229] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNILLPYWGCGRDFECMEQCXCQYYQSCG (SEQ ID NO: 442), wherein X is cyclobutyl-L-glycine or 3-cyclobutyl-L-alanine.

[0230] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X_1CNIX_2LPYWGCGRDFECMEQCICQYYQSCX_3 (SEQ ID NO: 443). In certain embodiments, X_1, X_2, and/or X_3 are the same unnatural amino acid. In certain embodiments, X_1, X_2, and/or X_3 are not the same unnatural amino acid. In certain embodiments, X_1 is N-acetylglycine, X_2 is norleucine, and X_3 is glycine amide.

[0231] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X_1CNILLPYWGCGRDFECMEQCICQYYQSCX_2 (SEQ ID NO: 444). In certain embodiments, X_1 and X_2 are the same unnatural amino acid. In certain embodiments, X_1 and X_2 are different unnatural amino acids. In certain embodiments, X_1 is N-acetylglycine and X_2 is glycine amide.

[0232] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X_1CNILQPYWGCGRDFECMEQCICQYYQSCX_2 (SEQ ID NO: 445). In certain embodiments, X_1 and X_2 are the same unnatural amino acid. In certain embodiments, X_1 and X_2 are different unnatural amino acids. In certain embodiments, X_1 is N-acetylglycine and X_2 is glycine amide.

[0233] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X_1CNILQPYWGCGRDFECLEQCICQYYQSCX_2 (SEQ ID NO: 446). In certain embodiments, X_1 and X_2 are the same unnatural amino acid. In certain embodiments, X_1 and X_2 are different unnatural amino acids. In certain embodiments, X_1 is N-acetylglycine and X_2 is glycine amide.

[0234] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVLQPYWGCGPDIDCXSNCICHWYNSCG (SEQ ID NO: 447), wherein X is 3-cyclobutyl-L-alanine or t-butyl-L-alanine (L-Nepentylglycine).

[0235] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVLQPYWGCGPDIDCLSNCXCHWYNSCG (SEQ ID NO: 448), wherein X is cyclobutyl-L-glycine.

[0236] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVX_1QPYWGCGPDIDCX_2SNC2X_3CHWYNSCG (SEQ ID NO: 449). In certain embodiments, X_1, X_2, and/or X_3 are the same unnatural amino acid. In certain embodiments, X_1, X_2, and/or X_3 are not the same unnatural amino acid. In certain embodiments, X_1 is norleucine, X_2 is 3-cyclobutyl-L-alanine, and X_3 is cyclobutyl-L-glycine. In certain embodiments, X_1 is norleucine, X_2 is 3-cyclobutyl-L-alanine, and X_3 is 3-cyclobutyl-L-alanine.

[0237] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCDVLQPYWGCGPDIDCX$_1$SNC2X$_2$CHWYNSCG (SEQ ID NO: 450). In certain embodiments, X$_1$ and X$_2$ are the same unnatural amino acid. In certain embodiments, X$_1$ and X$_2$ are different unnatural amino acids. In certain embodiments, X$_1$ is 3-cyclobutyl-L-alanine, and X$_2$ is cyclobutyl-L-glycine. In certain embodiments, X$_1$ is 3-cyclobutyl-L-alanine, and X$_2$ is 3-cyclobutyl-L-alanine.

[0238] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X$_1$CDVLQPYWGCGPDIDCX$_2$SNC2X$_3$CHWYNSCX$_4$ (SEQ ID NO: 451). In certain embodiments, X$_1$, X$_2$, X$_3$, and/or X$_4$ are the same unnatural amino acid. In certain embodiments, X$_1$, X$_2$, X$_3$, and/or X$_4$ are not the same unnatural amino acid. In certain embodiments, X$_1$ is N-acetylglycine, X$_2$ is 3-cyclobutyl-L-alanine, X$_3$ is cyclobutyl-L-glycine, and X$_4$ is glycine amide In certain embodiments, X$_1$ is acetylglycine, X$_2$ is cyclobutyl-L-alanine, X$_3$ is cyclobutyl-L-alanine, and X$_4$ is glycine amide

[0239] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X$_1$CDVX$_2$QPYWGCGPDIDCLSNCICHWYNSCX$_3$ (SEQ ID NO: 452). In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are the same unnatural amino acid. In certain embodiments, X$_1$, X$_2$, and/or X$_3$ are not the same unnatural amino acid. In certain embodiments, X$_1$ is N-acetylglycine, X$_2$ is norleucine, and X$_3$ is glycine amide

[0240] In certain embodiments, the non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence X$_1$CDVLQPYWGCGPDIDCLSNCICHWYNSCX$_2$ (SEQ ID NO: 453). In certain embodiments, X$_1$ and X$_2$ are the same unnatural amino acid. In certain embodiments, X$_1$ and X$_2$ are different unnatural amino acids. In certain embodiments, X$_1$ is N-acetylglycine and X$_2$ is glycine amide

[0241] In certain embodiments, the C-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, the N-terminus of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is modified (such as capped). In certain embodiments, both the C- and N-termini of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A are modified (such as capped). In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is amidated. In certain embodiments, the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated. In certain embodiments, the C-terminal carboxyl group of the non-naturally occurring cystine knot peptide (CKP) that binds to

VEGF-A is amidated and the N-terminal amine of the non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A is acetylated.

Structural Characteristics

[0242] In certain embodiments, the structure of a non-naturally occurring VEGF-Abinding CKP provided herein has a disulfide bond connectivity that is different from the WT EETI-II protein, *i.e.*, different from the C1-C4, C2-C5, and C3-C6 disulfide bond pattern characteristic of WT EETI-II. In certain embodiments, a non-naturally occurring VEGF-A binding CKP provided herein has a disulfide bond connectivity of C1-C4, C2-C3, and C5-C6. Methods of determining the disulfide bond connectivity of, e.g., a non-naturally occurring VEGF-A binding CKP, include, e.g., by solving and analyzing the co-crystal structure of a non-naturally occurring VEGF-A binding CKP in complex with VEGF-A, via mass spectrometry following partial reduction alkylation, or via mass spectrometry following proteolytic digestion, performing structure calculations as described in Sampoli et al. (2000) *Proteins Struct Funct Gen*. 40, 168-174, etc.

[0243] In certain embodiments, the non-naturally occurring VEGF-A binding CKP and has an alpha helix content of at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%, including any range in between these values. In certain embodiments, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, or at least 18 amino acids of the non-naturally occurring VEGF-A binding CKP form the alpha helix. In certain embodiments, the non-naturally occurring VEGF-A binding CKP has a disulfide bond connectivity of C1-C4, C2-C3, and C5-C6 and an alpha helix content of at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%, including any range in between these values. In certain embodiments, the alpha helix content of a non-naturally occurring VEGF-A binding CKP is determined by, e.g., circular dichroism (CD), optical rotary dispersion (ORD), nuclear magnetic resonance (NMR), by solving and analyzing the co-crystal structure of a non-naturally occurring VEGF-A binding CKP in complex with VEGF-A, via mass spectrometry following partial reduction alkylation, or via mass spectrometry following proteolytic digestion.

[0244] In certain embodiments, a non-naturally occurring VEGF-A binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG (SEQ ID NO: 52).

[0245] In certain embodiments, a non-naturally occurring VEGF-A binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG (SEQ ID NO: 52).

[0246] In certain embodiments, the non-naturally VEGF-A binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A binding CKP, wherein the second VEGF-A binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence GRDFE (SEQ ID NO: 97); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A binding CKP comprises the amino acid sequence GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102).

[0247] In certain embodiments, the non-naturally occurring VEGF-A binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence GRDFE (SEQ ID NO: 97); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises the amino acid sequence GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102).

[0248] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises the amino acid sequence GCDVMQPYWGCKQDSDCLAGCVCHWYNSCG (SEQ ID NO: 55).

[0249] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-

binding CKP comprises the amino acid sequence GCDVMQPYWGCKQDSDCLAGCVCHWYNSCG (SEQ ID NO: 55).

[0250] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP protein provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A-binding CKP, wherein the second VEGF-A-binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence GPDID (SEQ ID NO: 118); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein competes for binding to VEGF-A with a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises the amino acid sequence GCDVMQPYWGCGPDIDCLAGCVCHWYNSCG (SEQ ID NO: 123).

[0251] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence GPDID (SEQ ID NO: 118); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP provided herein binds the same epitope on VEGF-A bound by a second non-naturally occurring VEGF-A-binding CKP, wherein the second non-naturally occurring VEGF-A-binding CKP comprises the amino acid sequence GCDVMQPYWGCGPDIDCLAGCVCHWYNSCG (SEQ ID NO: 123).

[0252] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, or more than ten amino acids selected from the group consisting of V14, V15, F17, D19,Y21, Q22, Y25, I46, K48, N62, D63, L66, M81, I83, K84, P85, H86, Q87, G88, Q89, I91, C104, R105, and P106. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising K48, N62, and D63. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising H86. In certain embodiments, non-naturally

occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising Y21, Y25, and P106. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising M81, D19, and Q22. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising F17, M81, and I91. In certain embodiments, non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising V14, F17, D19, Q22, M81, and I91. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope of VEGF-A comprising Q22 and Y25.

[0253] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope on VEGF-A that overlaps the epitope of VEGF-A bound by the anti-VEGF-A antibody G6.31 (Fuh et al. (2006) *J. Biol. Chem.* 281, 6625-6631). In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope on VEGF-A that overlaps with the epitope of VEGF-A bound by Flt-1. In certain embodiments, a non-naturally occurring VEGF-A-binding CKP provided herein binds an epitope on VEGF-A that overlaps with the epitope of VEGF-A bound by bevacizumab.

Functional Characteristics

[0254] In certain embodiments, a non-naturally occurring CKP that "specifically binds" VEGF-A (such as a human VEGF-A, a mouse VEGF-A, and/or a rat VEGF-A) has a binding affinity (Kd) value of no more than about 1 x 10^{-7} M, preferably no more than about 1 x 10^{-8} and most preferably no more than about 1 x 10^{-9} M) but has a binding affinity for a homologue of VEGF-A or other growth factor which is at least about 50-fold, or at least about 500-fold, or at least about 1000-fold, weaker than its binding affinity for VEGF-A.

[0255] In certain embodiments, the extent of binding of a non-naturally occurring VEGF-A-binding CKP provided herein to, e.g., a non-target protein (e.g., a homolog of VEGFA such as VEGF-B, VEGF-C and VEGF-D) or other growth factors (such as P1GF, EGF, NGF, IGF and PDGF) is less than about 10% of the binding of the non-naturally occurring VEGF-A-binding CKP to VEGF-A as determined by methods known in the art, such as ELISA, fluorescence activated cell sorting (FACS) analysis, or radioimmunoprecipitation (RIA). Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by

competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. Other methods of assessing the binding of a non-naturally occurring CKP that "specifically binds" VEGF-A are described in the Examples.

[0256] The term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a Kd for the target of at least about 10^{-4} M, alternatively at least about 10^{-5} M, alternatively at least about 10^{-6} M, alternatively at least about 10^{-7} M, alternatively at least about 10^{-8} M, alternatively at least about 10^{-9} M, alternatively at least about 10^{-10} M, alternatively at least about 10^{-11} M, alternatively at least about 10^{-12} M, or greater. In one embodiment, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

[0257] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP binds VEGF-A with a Kd between about 1 pM to about 500 nM. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP binds VEGF-A with a Kd between about 1 pM to about 50 pM, between about 50 pM to about 250 pM, between about 250 pM to about 500 pM, between about 500 pM to 750 pM, between about 750 pM to about 1 nM, between about 1 nM to about 25 nM, between about 25 nM to about 50 nM, between 50 nM to about 100 nM, between about 100 nM to about 250 nM, or between about 250 nM to about 500 nM.

[0258] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP binds human VEGF-A, a mouse VEGF-A, and/or a rat VEGF-A. In certain embodiments, non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises the amino acid sequence GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG (SEQ ID NO: 52).

[0259] In certain embodiments, non-naturally occurring VEGF-A-binding CKP binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises an L1 comprising the amino acid sequence NIMLPFWG (SEQ ID NO: 33); an L2 comprising the amino acid sequence GRDFE (SEQ ID NO: 97); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence QYYQS (SEQ ID NO: 45), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises the amino acid sequence GCNIMLPFWGCGRDFECLAGCVCQYYQSCG (SEQ ID NO: 102).

[0260] In certain embodiments, non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises the amino acid sequence GCDVMQPYWGCKQDSDCLAGCVCHWYNSCG (SEQ ID NO: 55).

[0261] In certain embodiments, non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises an L1 comprising the amino acid sequence DVMQPYWG (SEQ ID NO: 35); an L2 comprising the amino acid sequence GPDID (SEQ ID NO: 118); an L3 comprising the amino acid sequence LAG; an L4 comprising the amino acid V, and an L5 comprising the amino acid sequence HWYNS (SEQ ID NO: 46), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP that binds human VEGF-A, a mouse VEGF-A, and a rat VEGF-A comprises the amino acid sequence GCDVMQPYWGCGPDIDCLAGCVCHWYNSCG (SEQ ID NO: 123).

[0262] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP described herein has an IC_{50} value of less than about 0.5 nM, less than about 0.6 nM, less than about 0.7 nM, less than about 0.8 nM, less than about 0.9 nM, or less than about 1.0nM, including any range in between these values.

[0263] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP does not inhibit trypsin protease activity as measured in a peptide substrate cleavage assay (e.g., the peptide substrate cleavage assay described in the Examples).

[0264] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP is resistant to trypsin digestion. In certain embodiments, about 30% or less, about 25% or less, or about 20% or less of the non-naturally occurring VEGF-A-binding CKP is cleaved at Arg13 within loop 2 after 24 h incubation with trypsin at 37°C.

[0265] Nucleic acid molecules encoding non-naturally occurring VEGF-A-binding CKPs described herein, expression vectors comprising nucleic acid molecules encoding the non-naturally occurring VEGF-A-binding CKP, and cells comprising the nucleic acid molecules are also contemplated. Also provided herein are methods of producing a non-naturally occurring VEGF-A-binding CKP described herein by culturing such cells, expressing the non-naturally occurring VEGF-A-binding CKP, and recovering the non-naturally occurring VEGF-A-binding CKP from the cell culture.

[0266] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP is produced via *in vitro* translation, as described elsewhere herein.

[0267] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP is generated via chemical peptide synthesis, e.g., by grafting chemically synthesized L1, L2, L3, L4, and/or L5 peptides onto an scaffold framework (such as scaffold structure I), or by chemically synthesizing the entire non-naturally occurring VEGF-A-binding CKP.

Non-Naturally Occurring Cystine Knot Peptides (CKPs) That Bind Human Low Density Lipoprotein Receptor (LDL)-Related Protein 6 (LRP6)

[0268] LDL receptors are transmembrane cell surface proteins involved in receptor-mediated endocytosis of lipoprotein and protein ligands. Human LDL receptor-related protein 6 (LRP6) (Accession Nos: NM_002336 (mRNA) and NP_002327 (protein); UniProtKB: O75581) functions as a receptor or, with Frizzled, a co-receptor for Wnt and thereby transmits the canonical Wnt/beta-catenin signaling cascade (Katoh *et al.* (2007) *Clin Cancer Res* 13:4042-4045). Through its interaction with the Wnt/beta-catenin signaling cascade, LRP6 plays a role in the regulation of cell differentiation, proliferation, and migration, and in the development of many cancer types (Li *et al.* (2004) *Oncogene* 23:9129-9135; Tung *et al.* (2012) PLoS ONE 7(5): e36565. doi:10.1371/journal.pone.0036565; Liu *et al.* (2010) *Proc Natl Acad Sci USA* 107:5136-5141).

[0269] Wnt signaling is involved in many biological pathways. With respect to diseases it is involved with cancer and metastatic disease, osteoporosis and other bone metabolism and disease, neuronal and neurodegenerative disease, rheumatoid arthritis and other inflammatory

disease. This inhibition of Wnt signaling by blockade of LRP6 may have a wide range of therapeutic utility. Bone loss is a serious medical problem, not only during postmenopausal osteoporosis, but also in rheumatoid arthritis. Bone is degraded in multiple myeloma and in bone metastases. Therapeutic strategies aimed at strengthening bone, fracture prevention, or restoration of damaged bone are therefore of very high interest (Kawai *et al.* (2011) *Nat. Rev. Drug Discov.* 10, 141–156; Mason and Williams (2010) *J. Osteoporosis*, vol. 2010, Article ID 460120, 9 pages; doi:10.4061/2010/460120). The Wnt pathway inhibitors DKK1 and SOST, because of their roles in suppressing new bone formation, are considered highly promising therapeutic targets; antibodies with neutralizing the function of SOST show significant preclinical activity (Ominsky *et al.* (2010) *J. Bone Miner. Res.* 25, 948–959) and are now in human clinical trials (Padhi *et al.* (2011) *J. Bone Miner. Res.* 26, 19–26.

[0270] Misregulated Wnt signaling is implicated in diseases ranging from osteoporosis to cancer (Clevers (2006) *Cell* 127: 469-80; MacDonald *et al.* 2009. *Dev Cell* 17: 9-26; Nusse (2008) *Cell Res* 18: 523-7; Polakis (2007) *Curr Opin Genet Dev* 17: 45-51). This list has expanded to include metabolic disorders (Mani *et al.* (2007) *Science* 315: 1278-82 and neurodegeneration (Caricasole *et al.* (2004) *J* Neurosci 24: 6021-7; De Ferrari *et al.* (2007) *Proc Natl Acad Sci USA* 104: 9434-9). An especially clear link exists between mutations of the protein adenomatous polyposis coli (APC), which prevent effective regulation of β-catenin levels, and colorectal cancers (Polakis (2007) *Curr Opin Genet Dev* 17: 45-51). Also of particular note is the strong genetic relationship between LRP5 and bone homeostasis. Loss-of-function mutations in LRP5 cause the autosomal recessive disorder osteoporosispseudoglioma syndrome (OPPG), characterized by low bone mass, ocular defects and a predisposition to fractures (Gong *et al.* (2001) *Cell* 107: 513-23.

[0271] Provided herein is a non-naturally occurring CKP that binds to human low density lipoprotein receptor-related protein 6 (LRP6), wherein the non-naturally CKP comprises the following cystine scaffold structure (i.e., scaffold structure I):

$$Z_1C_1\mathbf{L1}C_2\mathbf{L2}C_3\mathbf{L3}C_4\mathbf{L4}C_5\mathbf{L5}C_6Z_2 \qquad (I)$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6$, $X_1X_2X_3X_4X_5X_6X_7$, $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of $X_1 - X_3$ is any amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid; and

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid.

[0272] In certain embodiments, Z_1 and/or Z_2 of the non-naturally occurring cystine knot peptide (CKP) that binds to LRP6 is G. In certain embodiments, Z_1 and/or Z_2 comprise more than one amino acid. In certain embodiments, Z_1 and/or Z_2 comprise 4 amino acids. In certain embodiments, Z_1 and/or Z_2 comprise 5 amino acids.

[0273] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 wherein X_1 is an amino acid selected from R, V, M, A, G, N, S, and E; wherein X_2 is an amino acid selected from T, N, S, G, R, and A; wherein X_3 is an amino acid selected from N, R, H, V, K, S, G, I, and Y; wherein X_4 is an amino acid selected from R, V, N, I, K, S, and T; wherein X_5 is an amino acid selected from V, R, K, I, T, S, L, and N; and wherein X_6 is an amino acid selected from K, G, A, I, R, N, S, and V. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 wherein X_7 is an amino acid selected from G, R, K, E, P, and T. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 wherein X_8 is an amino acid selected from G, R, K, Q, A, and S. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 wherein X_9 is an amino acid selected from R or G. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 wherein X_{10} is an amino acid selected from E, W, and G. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L5 wherein X_1 is an amino acid selected from G, S, N, Y, A, and R; wherein X_2 is an amino acid selected from P, G, S, V, E, R, F, and D; wherein X_3 is an amino acid selected from N, G, S, E, P, K, H, and R; wherein X_4 is an amino acid selected from G, R, H, S, Q, V, and D; and wherein X_5 is an amino acid selected from F, D, N, R, G, Y, S, and T. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L2 wherein X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L3 wherein X_1 is L, X_2 is A, and X_3 is G. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L4 wherein X_1 is V.

[0274] In certain embodiments, the non-naturally occurring LRP6-binding CKP competitively inhibits the binding of a competing molecule to human LRP6. In certain embodiments, the competing molecule is an anti-LRP6 antibody. In certain embodiments, the competing molecule is a second non-naturally occurring LRP6-binding CKP.

[0275] Non-naturally occurring LRP6-binding CKPs that bind to overlapping or similar areas on a target can be identified by competitive inhibition/binding assays. Such assays are well known in the art and are described in, e.g., S. J. Mather (ed.) *1996. Current Directions in Radiopharmaceutical Research and Development,* 169- 179, Kluwer Academic Publishers; Zettner (1973) *Clin. Chem.* 19, 699-705; Gao (2012) *Analytical Methods* 4, 3718-3723.

[0276] In certain embodiments, the non-naturally occurring LRP6-binding CKP binds the same epitope of human LRP6 bound by a second non-naturally occurring LRP6-binding CKP comprising an L1 that comprises the amino acid sequence V/R/N/S/E/G-N/S/G/R-R/V/K/S/N/I/Y-V/N/I/R/S/T-R/K/I/N-G/I/R/K/S/A (SEQ ID NO: 185) or A/R/M/V/G/S-N/T/S/A-R/N/H-V/R/K-K/V/I-R/K/A/N/S/V-T/G/R/K/P-S/G/R/A (SEQ ID NO: 186) or R/A/Q-S/A-G/S/N/I-N/K-T/S/L/R-I/R/V-R/E/K-K/Q/A/R-R/G/Q-E/W/G/R (SEQ ID NO: 187); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence G/S/N/Y/A/R-P/G/S/V/E/R/F/D-N/G/S/E/P/K/H/R-G/R/H/S/Q/V/D-F/D/N/R/G/Y/S/T (SEQ ID NO: 188), with reference to scaffold structure I.

[0277] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence V/R/N/S/E/G-N/S/G/R-R/V/K/S/N/I/Y-V/N/I/R/S/T-R/K/I/N-G/I/R/K/S/A (SEQ ID NO: 185) or A/R/M/V/G/S-N/T/S/A-R/N/H-V/R/K-K/V/I-R/K/A/N/S/V-T/G/R/K/P-S/G/R/A (SEQ ID NO: 186) or R/A-S-G/S/N-N/K-T/S/L-I/R-R/E-K/Q/A-R/G-E/W/G (SEQ ID NO: 187)), with reference to scaffold structure I. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93). In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L3 that comprises the amino acid sequence LAG. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L4 that comprises the amino acid V. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L5 that comprises the amino acid sequence G/S/N/Y/A/R-P/G/S/V/E/R/F/D-N/G/S/E/P/K/H/R-G/R/H/S/Q/V/D-F/D/N/R/G/Y/S/T (SEQ ID NO: 188

[0278] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 and/or L5 of any one of the non-naturally occurring LRP6-binding CKPs

disclosed herein. In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises an amino acid sequence set forth in any one of SEQ ID NOs: 147-168 and 367, with respect to scaffold structure I. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L2 that comprises the amino acid sequence set forth in SEQ ID NO: 93. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L3 that comprises the amino acid sequence LAG. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L4 comprising the amino acid V. In certain embodiments, the non-naturally occurring LRP6-binding CKP further comprises an L5 that comprises an amino acid sequence set forth in any one of SEQ ID NOs: 19 and 169-184.

[0279] The L1 and L5 amino acid sequences described above are provided in **Table 18** below:

Table 18

RTNRVKGG (SEQ ID NO: 147)	GPNGF (SEQ ID NO: 19)
VNRVRG (SEQ ID NO: 148)	SGGRD (SEQ ID NO: 169)
MNHVKARR (SEQ ID NO: 149)	GPNGF (SEQ ID NO: 19)
RSVNKI (SEQ ID NO: 150)	GSSRN (SEQ ID NO: 170)
VNKIKG (SEQ ID NO: 151)	GVEGR (SEQ ID NO: 171)
RNSIKR (SEQ ID NO: 152)	SVGHG (SEQ ID NO: 172)
VSNRVNKG (SEQ ID NO: 153)	GPNGF (SEQ ID NO: 19)
RGNIIK (SEQ ID NO: 154)	NESRG (SEQ ID NO: 173)
RSGNTIRKRE (SEQ ID NO: 155)	GGPGG (SEQ ID NO: 174)
ASSNSIRQGW (SEQ ID NO: 156)	GPKSN (SEQ ID NO: 175)
RSNRIR (SEQ ID NO: 157)	YGHGD (SEQ ID NO: 176)
RSNKLREARG (SEQ ID NO: 158)	GSRQD (SEQ ID NO: 177)
VNSVKR (SEQ ID NO: 159)	SRGVN (SEQ ID NO: 178)
GSNKIRPR (SEQ ID NO: 160)	GPNDF (SEQ ID NO: 179)
NRIRNS (SEQ ID NO: 161)	GRGDY (SEQ ID NO: 180)
SRNSIK (SEQ ID NO: 162)	ASGSS (SEQ ID NO: 181)
SNYVKR (SEQ ID NO: 163)	SPGGR (SEQ ID NO: 182)
RANRVSGR (SEQ ID NO: 164)	GPNGF (SEQ ID NO: 19)
SNRVKVRA (SEQ ID NO: 165)	GPNGF (SEQ ID NO: 19)
ENRTKG (SEQ ID NO: 166)	GFRGT (SEQ ID NO: 183)
GNKIRA (SEQ ID NO: 167)	RDRVG (SEQ ID NO: 184)
ANRVKRTS (SEQ ID NO: 168)	GPNGF (SEQ ID NO: 19)

QAINRVKRQR (SEQ ID NO: 367)	
V/R/N/S/E/G-N/S/G/R-R/V/K/S/N/I/Y-V/N/I/R/S/T-R/K/I/N-G/I/R/K/S/A (SEQ ID NO: 185)	A/R/M/V/G/S-N/T/S/A-R/N/H-V/R/K-K/V/I-R/K/A/N/S/V-T/G/R/K/P-S/G/R/A (SEQ ID NO: 186)
R/A-S-G/S/N-N/K-T/S/L-I/R-R/E-K/Q/A-R/G-E/W/G (SEQ ID NO: 187)	G/S/N/Y/A/R-P/G/S/V/E/R/F/D-N/G/S/E/P/K/H/R-G/R/H/S/Q/V/D-F/D/N/R/G/Y/S/T (SEQ ID NO: 188)

[0280] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RTNRVKGG (SEQ ID NO: 147); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to with reference to scaffold structure I.

[0281] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence VNRVRG (SEQ ID NO: 148); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence SGGRD (SEQ ID NO: 169), with reference to with reference to scaffold structure I.

[0282] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence MNHVKARR (SEQ ID NO: 149); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0283] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RSVNKI (SEQ ID NO: 150); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GSSRN (SEQ ID NO: 170), with reference to scaffold structure I.

[0284] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence VNKIKG (SEQ ID NO: 151); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that

comprises the amino acid sequence GVEGR (SEQ ID NO: 29), with reference to scaffold structure I.

[0285] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RNSIKR (SEQ ID NO: 152); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence SVGHG (SEQ ID NO: 172), with reference to scaffold structure I.

[0286] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence VSNRVNKG (SEQ ID NO: 153); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0287] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RGNIIK (SEQ ID NO: 154); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence NESRG (SEQ ID NO: 173), with reference to scaffold structure I.

[0288] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RSGNTIRKRE (SEQ ID NO: 155); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GGPGG (SEQ ID NO: 174), with reference to scaffold structure I.

[0289] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence ASSNSIRQGW (SEQ ID NO: 156); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPKSN (SEQ ID NO: 175), with reference to scaffold structure I.

[0290] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RSNRIR (SEQ ID NO: 157); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the

amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence YGHGD (SEQ ID NO: 176), with reference to scaffold structure I.

[0291] In certain embodiments, non-naturally occurring LRP6-binding CKP an L1 that comprises the amino acid sequence RSNKLREARG (SEQ ID NO: 158); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GSRQD (SEQ ID NO: 177), with reference to scaffold structure I.

[0292] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence VNSVKR (SEQ ID NO: 159); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence SRGVN (SEQ ID NO: 178), with reference to scaffold structure I.

[0293] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence GSNKIRPR (SEQ ID NO: 160); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNDF (SEQ ID NO: 179), with reference to scaffold structure I.

[0294] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence NRIRNS (SEQ ID NO: 161); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GRGDY (SEQ ID NO: 180), with reference to scaffold structure I.

[0295] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence SRNSIK (SEQ ID NO: 162); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence ASGSS (SEQ ID NO: 181), with reference to scaffold structure I.

[0296] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence SNYVKR (SEQ ID NO: 163); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that

comprises the amino acid sequence SPGGR (SEQ ID NO: 182), with reference to scaffold structure I.

[0297] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence RANRVSGR (SEQ ID NO: 164); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0298] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence SNRVKVRA (SEQ ID NO: 165); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0299] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence ENRTKG (SEQ ID NO: 166); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GFRGT (SEQ ID NO: 183), with reference to with reference to scaffold structure I.

[0300] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence GNKIRA (SEQ ID NO: 167); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence RDRVG (SEQ ID NO: 184), with reference to scaffold structure I.

[0301] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence ANRVKRTS (SEQ ID NO: 168); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0302] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an L1 that comprises the amino acid sequence QAINRVKRQR (SEQ ID NO: 367); an L2 that comprises the amino acid sequence KQDSD (SEQ ID NO: 93); an L3 that

comprises the amino acid sequence LAG; an L4 that comprises the amino acid V; and an L5 that comprises the amino acid sequence GPNGF (SEQ ID NO: 19), with reference to scaffold structure I.

[0303] In certain embodiments, the non-naturally occurring LRP6-binding CKP comprises an amino acid sequence set forth in any one of SEQ ID NOs: 189-210 and 366. SEQ ID NOs: 189-210 and 366 are provided below.

GCRTNRVKGGCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 189)
GCVNRVRGCKQDSDCLAGCVCSGGRDCG (SEQ ID NO: 190)
GCMNHVKARRCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 191)
GCRSVNKICKQDSDCLAGCVCGSSRNCG (SEQ ID NO: 192)
GCVNKIKGCKQDSDCLAGCVCGVEGRCG (SEQ ID NO: 193)
GCRNSIKRCKQNSDCLAGCVCSVGHGCG (SEQ ID NO: 194)
GCVSNRVNKGCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 195)
GCRGNIIKCKQDSDCLAGCVCNESRGCG (SEQ ID NO: 196)
GCRSGNTIRKRECKQDSDCLAGCVCGGPGGCG (SEQ ID NO: 197)
GCASSNSIRQGWCKQDSDCLAGCVCGPKSNCG (SEQ ID NO: 198)
GCRSNRIRCKQDSDCLAGCVCYGHGDCG (SEQ ID NO: 199)
GCRSNKLREARGCKQDSDCLAGCVCGSRQDCG (SEQ ID NO: 200)
GCVNSVKRCKQDSDCLAGCVCSRGVNCG (SEQ ID NO: 201)
GCGSNKIRPRCKQDSDCLAGCVCGPNDFCG (SEQ ID NO: 202)
GCNRIRNSCKQDSDCLAGCVCGRGDYCG (SEQ ID NO: 203)
GCSRNSIKCKQDSDCLAGCVCASGSSCG (SEQ ID NO: 204)
GCSNYVKRCKQDSDCLAGCVCSPGGRCG (SEQ ID NO: 205)
GCRANRVSGRCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 206)
GCSNRVKVRACKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 207)
GCENRTKGCKQDSDCLAGCVCGFRGTCG (SEQ ID NO: 208)
GCGNKIRACKQDSDCLAGCVCRDRVGCG (SEQ ID NO: 209)
GCANRVKRTSCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 210)
GCQAINRVKRQRCKQDSDCLAGCVCGPNGFCG (SEQ ID NO: 366)

[0304] In certain embodiments, the non-naturally occurring LRP6-binding CKP is a variant of a non-naturally occurring LRP6-binding CKP described herein. In certain embodiments, such a variant comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 amino acid substitutions in one or more of the sequences set forth in SEQ ID NOs: 19, 93, 147-168, 169-184, and 189-210 and/or in the amino acid sequence LAG. In certain embodiments, the amino acid substitution(s) are conservative amino acid substitution(s). In certain embodiments, the amino acid substitutions do not substantially reduce the ability of the non-naturally occurring LRP6-binding CKP to bind human LRP6. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce LRP6 binding affinity may be made. The

binding affinity of a variant of a non-naturally occurring LRP6-binding CKP can be assessed using a method described in the Examples below.

[0305] Conservative substitutions are shown in **Table 17** above under the heading of "conservative substitutions." More substantial changes are provided in **Table 17** under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into a variant of a non-naturally occurring LRP6-binding CKP and the products screened for a desired activity, e.g., retained/improved LRP6 binding.

[0306] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. An exemplary substitutional variant is an affinity matured non-naturally occurring LRP6-binding CKP, which may be conveniently generated, e.g., using phage display based affinity maturation techniques such as those described herein. Briefly, one or more residues in L1, L2, L3, L4, and/or L5 is altered (i.e., added, deleted, or substituted) and the variant LRP6-binding CKP is displayed on phage and screened for LRP6 binding affinity. In certain embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, loop shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any non-naturally occurring CKP variants with the desired affinity for LRP6. In certain embodiments, introducing diversity involves loop-directed approaches, in which several residues in L1, L2, L3, L4, and/or L5 (e.g., about 5, about 4-6, or about 6-10 residues at a time) are randomized. L1, L2, L3, L4, and/or L5 residues involved in binding a target ligand may be identified, e.g., using alanine scanning mutagenesis or modeling.

[0307] In certain embodiments, a non-naturally occurring CKP that "specifically binds" human LRP6 (i.e., has a binding affinity (Kd) value of no more than about 1×10^{-7} M, preferably no more than about 1×10^{-8} and most preferably no more than about 1×10^{-9} M) but has a binding affinity for another LRP protein which is at least about 50-fold, or at least about 500-fold, or at least about 1000-fold, weaker than its binding affinity for LRP6.

[0308] In certain embodiments, the extent of binding of the non-naturally occurring LRP6-binding CKP to a non-target protein (e.g., a LRP6 homolog such as LRP1, LRP1B, LRP2, LRP3, LRP4, LRP5, LRP8, LRP10, LRP11, and LRP12) is less than about 10% of the binding of the non-naturally occurring LRP6-binding CKP to human LRP6 as determined by methods known in the art, such as ELISA, fluorescence activated cell sorting (FACS) analysis, or radioimmunoprecipitation (RIA). Specific binding can be measured, for

example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. The term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a Kd for the target of at least about 10^{-4} M, alternatively at least about 10^{-5} M, alternatively at least about 10^{-6} M, alternatively at least about 10^{-7} M, alternatively at least about 10^{-8} M, alternatively at least about 10^{-9} M, alternatively at least about 10^{-10} M, alternatively at least about 10^{-11} M, alternatively at least about 10^{-12} M, or greater. In one embodiment, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

[0309] In certain embodiments, the non-naturally occurring LRP6-binding CKP binds a human LRP6 with a Kd between about 1 pM to about 500 nM. In certain embodiments, the non-naturally occurring LRP6-binding CKP protein that specifically binds LRP6 binds a human LRP6 with a Kd between about 1 pM to about 50 pM, between about 50 pM to about 250 pM, between about 250 pM to about 500 pM, between about 500 pM to 750 pM, between about 750 pM to about 1 nM, between about 1 nM to about 25 nM, between about 25 nM to about 50 nM, between 50 nM to about 100 nM, between about 100 nM to about 250 nM, or between about 250 nM to about 500 nM, including any range in between these values.

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[0310] In certain embodiments, the non-naturally occurring LRP6-binding CKP inhibits Wnt1 signaling, e.g., as determined using methods described in the Examples below.

[0311] Nucleic acid molecules encoding the non-naturally occurring LRP6-binding CKPs described, expression vectors comprising nucleic acid molecules encoding the non-naturally occurring LRP6-binding CKPs, and cells comprising the nucleic acid molecules are also contemplated. Also provided herein are methods of producing a non-naturally occurring LRP6-binding CKP by culturing such cells, expressing the non-naturally occurring LRP6-binding CKP, and recovering the non-naturally occurring LRP6-binding CKP from the cell culture.

[0312] In certain embodiments, a non-naturally occurring LRP6-binding CKP is produced via *in vitro* translation, as described elsewhere herein.

[0313] As described elsewhere herein, a non-naturally occurring LRP6-binding CKP is generated via chemical peptide synthesis, e.g., by grafting chemically synthesized L1, L2, L3, L4, and/or L5 peptides onto an EETI-II framework, or by chemically synthesizing the entire non-naturally occurring LRP6-binding CKP.

[0314] In certain embodiments, the non-naturally occurring LRP6-binding CKP is as a therapeutic agent in the treatment of diseases or conditions wherein excessive LRP6 activity is involved.

Methods of Production

[0315] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is generated via genetic engineering. A variety of methods for mutagenesis have been previously described (along with appropriate methods for screening or selection). Such mutagenesis methods include, but are not limited to, e.g., error-prone PCR, loop shuffling, or oligonucleotide-directed mutagenesis, random nucleotide insertion or other methods prior to recombination. Further details regarding these methods are described in, e.g., Abou-Nadler *et al.* (2010) *Bioengineered Bug*s 1, 337-340; Firth *et al.* (2005) *Bioinformatics* 21, 3314-3315; Cirino *et al.* (2003) *Methods Mol Biol* 231, 3-9; Pirakitikulr (2010) *Protein Sci* 19, 2336-2346; Steffens *et al.* (2007) *J. Biomol Tech* 18, 147-149; and others. Accordingly, in certain embodiments, provided is a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP generated via genetic engineering techniques.

[0316] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is generated via *in vitro* translation. Briefly, *in vitro* translation entails cloning the protein-coding sequence(s) into a vector containing a promoter, producing mRNA by transcribing the cloned sequence(s) with an RNA polymerase, and synthesizing the protein by translation of this mRNA *in vitro*, e.g., using a cell-free extract. A desired variant protein can be generated simply by altering the cloned protein-coding sequence. Many mRNAs can be translated efficiently in wheat germ extracts or in rabbit reticulocyte lysates. Further details regarding *in vitro* translation are described in, e.g., Hope et al. (1985) *Cell* 43, 177-188; Hope et al. (1986) *Cell* 46, 885-894; Hope et al.

(1987) *EMBO J.* 6, 2781-2784; Hope et al. (1988) *Nature* 333, 635-640; and Melton et al. (1984) *Nucl. Acids Res.*12, 7057-7070.

[0317] Accordingly, provided are nucleic acid molecules encoding a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP described herein. An expression vector operably linked to a nucleic acid molecule encoding a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is also provided. Host cells (including, e.g., prokaryotic host cells such as E. coli, eukaryotic host cells such as yeast cells, mammalian cells, CHO cells, etc.) comprising a nucleic acid encoding a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP are also provided.

[0318] In certain embodiments, non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is generated via *in vitro* translation. Briefly, *in vitro* translation entails cloning the protein-coding sequence(s) into a vector containing a promoter, producing mRNA by transcribing the cloned sequence(s) with an RNA polymerase, and synthesizing the protein by translation of this mRNA *in vitro*, e.g., using a cell-free extract. A desired mutant protein can be generated simply by altering the cloned protein-coding sequence. Many mRNAs can be translated efficiently in wheat germ extracts or in rabbit reticulocyte lysates. Further details regarding *in vitro* translation are described in, e.g., Hope et al. (1985) *Cell* 43, 177-188; Hope et al. (1986) *Cell* 46, 885-894; Hope et al. (1987) *EMBO J.* 6, 2781-2784; Hope et al. (1988) *Nature* 333, 635-640; and Melton et al. (1984) *Nucl. Acids Res.*12, 7057-7070.

[0319] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is generated via chemical synthesis. In certain embodiments, chemically synthesized L1, L2, L3, L4, and/or L5 peptides are grafted onto an EETI-II-based framework (such as scaffold structure I) to generate non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP. In certain embodiments the entire non-naturally occurring VEGF-A-binding CKP or the entire non-naturally occurring LRP6-binding CKP is chemically synthesized. Methods of solid phase and liquid phase peptide synthesis are well known in the art and described in detail in, e.g., Methods of Molecular Biology, *35*, Peptide Synthesis Protocols, (M. W. Pennington and B. M. Dunn Eds), Springer, 1994; Welsch *et al.* (2010) *Curr Opin Chem Biol* 14, 1-15; Methods of Enzymology, *289*, Solid Phase Peptide Synthesis, (G. B. Fields Ed.), Academic Press, 1997; Chemical Approaches to the Synthesis of Peptides and Proteins, (P. Lloyd-Williams, F. Albericio, and E. Giralt Eds), CRC Press, 1997; Fmoc Solid Phase Peptide Synthesis, A

Practical Approach, (W. C. Chan, P. D. White Eds), Oxford University Press, 2000; Solid Phase Synthesis, A Practical Guide, (S. F. Kates, F Albericio Eds), Marcel Dekker, 2000; P. Seneci, Solid-Phase Synthesis and Combinatorial Technologies, John Wiley & Sons, 2000; Synthesis of Peptides and Peptidomimetics (M. Goodman, Editor-in-chief, A. Felix, L. Moroder, C. Tmiolo Eds), Thieme, 2002; N. L. Benoiton, Chemistry of Peptide Synthesis, CRC Press, 2005; Methods in Molecular Biology, 298, Peptide Synthesis and Applications, (J. Howl Ed) Humana Press, 2005; and Amino Acids, Peptides and Proteins in Organic Chemistry, Volume 3, Building Blocks, Catalysts and Coupling Chemistry, (A. B. Hughs, Ed.) Wiley-VCH, 2011.

Chimeric Molecules Comprising a Non-Naturally Occurring EETI-II Protein

[0320] A non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) can also be modified if advantageous in a way to form a chimeric molecule comprising the non-naturally occurring CKP fused (e.g., recombinantly fused) to another, heterologous polypeptide or amino acid sequence. In certain embodiments, such a chimeric molecule comprises a fusion of a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) with an antibody to form, e.g., a divalent molecule or a bispecific molecule.

[0321] In certain embodiments, a chimeric molecule comprises a fusion of a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) with a second moiety (such as a protein transduction domain) which targets the chimeric molecule for delivery to various tissues, or , e.g., across brain blood barrier, using, for example, the protein transduction domain of human immunodeficiency virus TAT protein (Schwarze *et al.*, 1999, *Science* 285: 1569-72).

[0322] In certain embodiments, the non-naturally occurring CKP provided herein can be used as bi- or multi-specific (for different target ligands or different epitopes on the same target ligand) in multimer form. For example, a dimeric bispecific non-naturally occurring CKP has one subunit with specificity for a first target protein or epitope and a second subunit with specificity for a second target protein or epitope. Non-naturally occurring CKP protein subunits can be joined in a variety of conformations that can increase the valency and thus the avidity of binding to a target ligand.

[0323] In certain embodiments a chimeric molecule provided herein comprises two or more (such as three, four, five, six, seven, eight, nine, ten, or more than ten) non-naturally occurring CKP proteins. In certain embodiments, a nucleic acid can be engineered to encode two or more copies of a single non-naturally occurring CKP, which copies are transcribed and translated in tandem to produce a covalently linked multimer of identical subunits. In certain embodiments, the nucleic acid can be engineered to encode two or more different non-naturally occurring CKPs, which copies are transcribed and translated in tandem to produce a covalently linked multimer of different subunits that bind, e.g., different epitopes of a single target ligand, or, e.g., different target ligands.

[0324] In another embodiment, such a chimeric molecule comprises a fusion of a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the non-naturally occurring CKP. The presence of such epitope-tagged forms of the non-naturally occurring CKP protein can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the non-naturally occurring CKP to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. Various tag polypeptides and their respective antibodies are known in the art. Examples include poly-histidine (poly-His) or poly-histidine-glycine (poly-His-Gly) tags; the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.* (1988) *Mol. Cell. Biol.* 8, 2159-2165); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.* (1985) *Mol. Cell. Biol.* 5, 3610-3616]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.* (1990) *Protein Eng.*, 3, 547-553). Other tag polypeptides include the Flag-peptide (Hopp *et al.* (1988) *BioTechnology*, 6,1204-1210); the KT3 epitope peptide (Martin *et al.* (1992) *Science, 255*, 192-194]; an α-tubulin epitope peptide (Skinner *et al.* (1991) *J. Biol. Chem.* 266, 15163-15166); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 6393-6397].

[0325] In certain embodiments, the chimeric molecule can comprise a fusion of a non-naturally occurring CKP protein described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule (e.g., an "immunoadhesin"), such a fusion could be to the Fc region of an IgG molecule. Ig fusions provided herein include polypeptides that comprise approximately or only residues 94-243,

residues 33-53 or residues 33-52 of human in place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH2 and CH3, or the hinge, CH1, CH2 and CH3 regions of an IgG1 molecule. For the production of immunoglobulin fusions *see* also, U.S. Patent No. 5,428,130 issued June 27, 1995. In certain embodiments, a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) is fused, e.g., at the N or C terminus, to the constant region of an IgG (Fc). In certain embodiments, the non-naturally occurring CKP/Fc fusion molecule activates the complement component of the immune response. In certain embodiments, the non-naturally occurring CKP/Fc fusion protein increases the therapeutic value of the non-naturally occurring CKP. In certain embodiments, a non-naturally occurring CKP protein described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) is fused (such as recombinantly fused), e.g., at the N or C terminus, to a complement protein, such as C1q. Various publications describe methods for obtaining non-naturally occurring proteins whose half-lives are modified either by introducing an FcRn-binding polypeptide into the molecules (WO 1997/43316, US 5869046, US 5747035, WO 1996/32478, WO 1991/14438) or by fusing the proteins with antibodies whose FcRn-binding affinities are preserved but affinities for other Fc receptors have been greatly reduced (WO 1999/43713) or fusing with FcRn binding domains of antibodies (WO 2000/09560, US 4703039). Specific techniques and methods of increasing half-life of physiologically active molecules (e.g., non-naturally occurring CKP) can also be found in US 7083784. In certain embodiments, a non-naturally occurring CKP protein described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) is fused to an Fc region from an IgG that comprises amino acid residue mutations (as numbered by the EU index in Kabat): M252Y/S254T/T256E or H433K/N434F/Y436H.

[0326] In certain embodiments, non-naturally occurring CKP proteins described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) are fused with molecules that increase or extend *in vivo* or serum half-life. In certain embodiments, a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) is fused with albumin, such as human serum albumin (HSA), polyethylene glycol (PEG), polysaccharides, immunoglobulin molecules (IgG), complement, hemoglobin, a binding peptide, lipoproteins or other factors to increase its half-life in the bloodstream and/or its tissue penetration.

[0327] Additional chimeric molecules comprising non-naturally occurring VEGF-A-binding CKPs or non-naturally occurring LRP6-binding CKPs may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to alter the activities of the non-naturally occurring CKPs (e.g., non-naturally occurring CKPs with higher affinities and lower dissociation rates). See, generally, US 5605793, US5811238, US 5830721, US 5834252, US 5837458, Patten *et al.* (1997) *Curr. Opinion Biotechnol.* 8, 724-33; Harayama (1998) *Trends Biotechnol.* 16, 76-82; Hansson, *et al.*, (1999) *J. Mol. Biol.* 287, 265-76; and Lorenzo and Blasco, (1998) *Biotechniques* 24, 308-313

[0328] In certain embodiments, a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP provided herein is altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding a scaffold that binds to a specific target may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

[0329] Any of these fusions can generated by standard techniques, for example, by expression of the fusion protein from a recombinant fusion gene constructed using publicly available gene sequences, or by chemical peptide synthesis.

Conjugates Comprising a Non-Naturally Occurring VEGF-A-Binding CKP or a Non-Naturally Occurring LRP6-binding CKP)

[0330] Provided herein are immunoconjugates comprising a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

[0331] Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa),* ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *Momordica charantia* inhibitor, curcin, crotin, *Saponaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. Other toxins include maytansine and maytansinoids, calicheamicin and other cytotoxic agents. A variety

of radionuclides are available for the production of radioconjugated non-naturally occurring CKPs. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re.

[0332] Conjugates of a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) and, e.g., cytotoxic agent, are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (*p*-azidobenzoyl) hexanediamine), bisdiazonium derivatives (such as bis-(*p*-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al., Science,* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionuclide to a non-naturally occurring CKP provided herein. *See,* WO94/11026.

[0333] In another embodiment, the non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) can be conjugated to a "receptor" (such as streptavidin) for utilization in ocular "pre-targeting" wherein the non-naturally occurring EETI-II scaffold protein-receptor conjugate is administered to the eye patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) that is conjugated to a cytotoxic agent (e.g., a radionuclide) or a therapeutic agent.

[0334] In certain embodiments, the non-naturally occurring CKPs provided herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) can be used as bi- or multi-specific (for different target ligands or different epitopes on the same target ligand) in multimer form. The attachments may be covalent or non-covalent. For example, a dimeric bispecific non-naturally occurring CKP has one subunit with specificity for a first target protein or epitope and a second subunit with specificity for a second target protein or epitope. Non-naturally occurring CKP subunits can be joined, e.g., via conjugation, in a variety of conformations that can increase the valency and thus the avidity of binding to a target ligand or to bind multiple target ligands.

[0335] In certain embodiments, non-naturally occurring CKPs provided herein are engineered to provide reactive groups for conjugation. In certain embodiments, the N-terminus and/or C- terminus may also serve to provide reactive groups for conjugation. In

certain embodiments, the N- terminus is conjugated to one moiety (such as, but not limited to PEG) while the C-terminus is conjugated to another moiety (such as, but not limited to biotin), or vice versa.

[0336] Provided is a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) conjugated to one or more moieties, including but not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. Also provided is the use of a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide (or fragment thereof, to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids). The fusion does not necessarily need to be direct, but may occur through linker sequences described herein.

[0337] In certain embodiments, a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP), or analogs or derivatives thereof may be conjugated to a diagnostic or detectable agent. Such non-naturally occurring CKP conjugates can be useful for monitoring or prognosing the development or progression of a disease as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can be accomplished by coupling the non-naturally occurring CKP to detectable substances including, but not limited to various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidinlbiotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as, but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not limited to iodine (^{131}I, ^{125}I, ^{123}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium(^{3}H), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In,), and technetium (^{99}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc , ^{186}Re, ^{188}Re, ^{142}Pr , ^{105}Rh , ^{97}Ru, ^{68}Ge , ^{57}Co , ^{65}Zn, ^{85}Sr, ^{32}P , ^{153}Gd , ^{169}Yb , ^{51}Cr , ^{54}Mn , ^{75}Se, ^{113}Sn, and ^{117}Tn; positron emitting metals using various positron emission

tomographies, nonradioactive paramagnetic metal ions, and molecules that are radiolabeled or conjugated to specific radioisotopes.

[0338] Also provided is a non-naturally occurring CKPs (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) conjugated to a therapeutic moiety. In certain embodiments, a non-naturally occurring CKP may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells.

[0339] In certain embodiments, a non-naturally occurring CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP is conjugated to therapeutic moieties such as a radioactive metal ion, such as alpha-emitters such as ^{213}Bi or macrocyclic chelators useful for conjugating radiometal ions, including but not limited to, ^{131}In, ^{131}Lu, ^{131}Y, ^{131}Ho, ^{131}Sm, to polypeptides. In certain embodiments, the macrocyclic chelator is 1, 4, 7, 10- tetraazacyclododecane-N,N',N",N"'-tetra-acetic acid (DOTA) which can be attached to the non-naturally occurring CKP via a linker molecule. Such linker molecules are commonly known in the art and described in, e.g., Denardo *et al.* (1998) *Clin Cancer Res.* 4, 2483-90; Peterson *et al.* (1999) *Bioconjug. Chem.* 10, 553-557; and Zimmerman *et al.* (1999) *Nucl. Med. Biol.* 26, 943-50.

[0340] Techniques for conjugating therapeutic moieties to antibodies are well known and can be applied to the non-naturally CKPs disclosed herein, see, e.g., Amon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56. (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies 84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radio labeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, 1982, Immunol. Rev. 62:119-58. Similar approaches may be adapted for use with the non-naturally occurring CKPs provided herein.

[0341] The therapeutic moiety or drug conjugated to a non-naturally CKP described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) should be chosen to achieve the desired prophylactic or therapeutic effect(s) for a particular disorder in a subject. A clinician or other medical personnel should

consider the following when deciding on which therapeutic moiety or drug to conjugate to a scaffold: the nature of the disease, the severity of the disease, and the condition of the subject.

[0342] In certain embodiments, non-naturally occurring CKPs described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) can also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Covalent Modifications

[0343] Covalent modifications of non-naturally occurring CKPs described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) are also contemplated. One type of covalent modification includes reacting targeted amino acid residues of a non-naturally occurring CKP with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the non-naturally occurring CKP. Derivatization with bifunctional agents is useful, for instance, for crosslinking the non-naturally occurring CKP to a water-insoluble support matrix or surface for use in the method for purifying a target ligand, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(*p*-azidophenyl)-dithio]propioimidate.

[0344] Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[0345] Another type of covalent modification of a non-naturally occurring CKP comprises linking the non-naturally occurring CKP to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in US 4640835, US 4496689, US 4301144, US 4670417, US 4791192 or US 4179337

[0346] The term “polyethylene glycol” or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (e.g., with thiol, triflate, tresylate, azirdine, oxirane, N-hydroxysuccinimide or a maleimide moiety). The term "PEG" is intended to indicate polyethylene glycol of a molecular weight between 500 and 150,000 Da, including analogues thereof, wherein for instance the terminal OR-group has been replaced by a methoxy group (referred to as mPEG).

[0347] In certain embodiments, non-naturally occurring CKPs described herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) are derivatized with polyethylene glycol (PEG). PEG is a linear, water-soluble polymer of ethylene oxide repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights which typically range from about 500 daltons to about 40,000 daltons. In a presently preferred embodiment, the PEGs employed have molecular weights ranging from 5,000 daltons to about 20,000 daltons. PEGs coupled to the non-naturally occurring CKPs described herein can be either branched or unbranched (for example, Monfardini, C. *et al.* 1995 *Bioconjugate Chem* 6:62-69). PEGs are commercially available from Nektar Inc., Sigma Chemical Co. and other companies. Such PEGs include, but are not limited to, monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM).

[0348] In certain embodiments, the hydrophilic polymer which is employed, for example, PEG, is capped at one end by an unreactive group such as a methoxy or ethoxy group. Thereafter, the polymer is activated at the other end by reaction with a suitable activating agent, such as cyanuric halides (for example, cyanuric chloride, bromide or fluoride), diimadozle, an anhydride reagent (for example, a dihalosuccinic anhydride, such as dibromosuccinic anhydride), acyl azide, *p*-diazoiumbenzyl ether, 3-(*p*-diazoniumphenoxy)-2-hydroxypropylether) and the like. The activated polymer is then reacted with a non-naturally occurring CKP herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) to produce a non-naturally occurring CKP derivatized with a polymer. Alternatively, a functional group in the non-naturally occurring CKP provided herein can be activated for reaction with the polymer, or the two groups can be joined in a concerted coupling reaction using known coupling methods. It will be readily

appreciated that the non-naturally occurring CKPs provided herein can be derivatized with PEG using a myriad of other reaction schemes known to and used by those of skill in the art.

Liposomes

[0349] Non-naturally occurring CKPs disclosed herein (such as a non-naturally occurring VEGF-A-binding CKP or a non-naturally occurring LRP6-binding CKP) can also be formulated as liposomes. Liposomes containing a non-naturally occurring EETI-II scaffold protein described herein can be prepared by methods known in the art, such as described in Epstein *et al., Proc Natl Acad Sci USA,* 82: 3688 (1985); Hwang *et al., Proc Natl Acad Sci USA,* 77: 4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

[0350] Particularly useful liposomes can be generated by the reverse-phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. A second therapeutic agent is optionally also contained within the liposome. *See,* Gabizon *et al., J. National Cancer Inst.*, 81(19): 1484 (1989). Pharmaceutical Compositions and Formulations Comprising Non-Naturally Cystine Knot Peptides (CKPs) That Bind Human Vascular Endothelial Growth Factor A(VEGF-A)

[0351] In certain embodiments, provided herein is a pharmaceutical composition comprising a non-naturally occurring VEGF-A-binding CKP and a pharmaceutically acceptable excipient. In certain embodiments the composition may also contain, buffers, carriers, stabilizers, preservatives and/or bulking agents, to render the composition suitable for ocular administration to a patient to achieve a desired effect or result. In certain embodiments, the pharmaceutical composition comprises one or more permeability enhancers that permit a non-naturally occurring VEGF-A-binding CKP to penetrate the cornea. Examples of such permeability enhancers include, e.g., surfactants, bile acids, chelating agents, preservatives, cyclodextrins (i.e., cylindrical oligonucleotides with a hydrophilic outer surface and a lipophilic inner surface that form complexes with lipophilic drugs), etc. Such permeability enhancers increase chemical stability and bioavailability and decrease local irritation. In certain embodiments, a pharmaceutical composition provided herein additionally comprises agents that increase the absorption and distribution of non-naturally occurring VEGF-A-binding CKP in various ocular compartments. In certain embodiments, a

pharmaceutical composition provided herein comprises a cross-linked polyacrylic acid, which can enhance ocular bioavailability by virtue of its mucoadhesive properties. In certain embodiments, a pharmaceutical composition provided herein comprises a bioadhesive polymer.

[0352] In certain embodiments, a pharmaceutical composition provided herein is formulated as an in-situ gelling system, e.g., a viscous polymer-based liquid that exhibits sol-to-gel phase transition on the ocular surface due to change in a specific physicochemical parameter (ionic strength, temperature, pH, or solvent exchange) when the composition comes into contact with tear fluid. In certain embodiments, a pharmaceutical composition provided herein is formulated as an eye spray. In certain embodiments, a pharmaceutical composition provided is formulated as liposomes. In certain embodiments, a pharmaceutical composition provided herein is formulated as niosomes (i.e., non-ionic surfactant-based vesicles containing, e.g., cholesterol as an excipient). In certain embodiments, a pharmaceutical composition provided herein is formulated as pharmacosomes (i.e., vesicles formed by amphiphilic drugs). In certain embodiments, a pharmaceutical composition provided herein is formulated as a microemulsion. Further details regarding various ophthalmic pharmaceutical formulations are provided in, e.g., Gaikwad et al. (2013) *Indo Amer J Pharm Res.* 3, 3216-3232; Achouri et al. (2012) *Drug Dev Indust Pharm.* 39, 1599-1617; Lu (2010) *Recent Pat Drug Deliv Formul.* 4, 49-57; Baranowski et al. (2014) *Sci World J.* doi.org/10.1155/2014/861904; Lang (1995) *Adv Drug Deliv Rev.* 16, 39-43; Short (2008) *Toxicologic Path.* 36, 49-62; and others.

[0353] In certain embodiments, a pharmaceutical composition comprising non-naturally occurring VEGF-A-binding CKP described herein is stable at room temperature (such as at about 20-25°C) for about 0.5 weeks, about 1.0 weeks, about 1.5 weeks, about 2.0 weeks, about 2.5 weeks, 3.5 weeks, about 4.0 weeks, about 1 month, about 2 months about 3 months, about 4 months about 5 months, about 6 months, or greater than 6 months, including any range in between these values. In certain embodiments, a pharmaceutical composition comprising non-naturally occurring VEGF-A-binding CKP described herein is stable under accelerated conditions (such as storage at about 37°C) for about 0.5 weeks, about 1.0 weeks, about 1.5 weeks, about 2.0 weeks, about 2.5 weeks, 3.5 weeks, about 4.0 weeks, about 1 month, about 2 months about 3 months, about 4 months about 5 months, about 6 months, or greater than 6 months, including any range in between these values.

Methods of Treatment Using Non-Naturally Occurring Cystine Knot Peptides (CKPs) That Bind Vascular Endothelial Growth Factor A (VEGF-A)

[0354] Vascular endothelial growth factor (VEGF-A), a dimeric glycoprotein of approximately 40 kDa, is a potent, endothelial cell mitogen that stimulates proliferation, migration and tube formation leading to angiogenic growth of new blood vessels and increased vascular permeability. Low oxygen conditions in the retina or cornea induce the expression of vascular endothelial growth factor (VEGF-A), and the abnormal (such as excessive or otherwise inappropriate) growth of leaky blood vessels contributes to the pathology of several debilitating ocular diseases including, e.g., diabetic blindness, retinopathies, primarily diabetic retinopathy, age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP), choroidal neovascularization (CNV), diabetic macular edema, pathological myopia, von Rippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (both branched retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO), corneal neovascularization, retinal neovascularization and rubeosis. The VEGF-A-induced formation of new blood vessels is detrimental, and retinal, intertrabecular or corneal neovascularization can ultimately lead to vision loss.

[0355] In certain embodiments, provided herein is a method of treating an ocular disease or disorder in a subject comprising administering to the subject an effective amount of a non-naturally occurring VEGF-A-binding CKP described herein or a composition (such as a pharmaceutical composition) comprising a non-naturally occurring VEGF-A-binding CKP described herein. In certain embodiment, provided are compositions (such as pharmaceutical compositions) comprising a non-naturally occurring VEGF-A-binding CKP described herein for use in treating an ocular disease or disorder in a subject. In certain embodiments, provided is the use of a non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) in the manufacture of a medicament for the treatment of an ocular disease or disorder in a subject.

[0356] In certain embodiments, the subject to be treated is a mammal (e.g., human, non-human primate, rat, mouse, cow, horse, pig, sheep, goat, dog, cat, etc.). In certain embodiments, the subject is a human. In certain embodiments, the subject is a clinical patient, a clinical trial volunteer, an experimental animal, etc. In certain embodiments, the subject is suspected of having or at risk for having an ocular disease or disorder characterized by abnormal angiogenesis and/or abnormal vascular permeability (such as those described

herein). In certain embodiments, the subject has been diagnosed with an ocular disease or disorder characterized by abnormal angiogenesis and/or abnormal vascular permeability (such as those described herein).

[0357] In certain embodiments, the ocular disease or disorder is an ocular vascular proliferative disease, such as an ocular vascular proliferative disease selected from the group consisting of diabetic blindness, retinopathies, primarily diabetic retinopathy, age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP), choroidal neovascularization (CNV), diabetic macular edema, pathological myopia, von Rippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (both branched retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO), corneal neovascularization, retinal neovascularization, and rubeosis. In certain embodiments, the corneal neovascularization results infection of the eye, inflammation in the eye, trauma to the eye (including chemical burns), or loss of the limbal stern cell barrier. In certain embodiments, the corneal neovascularization results from herpetic keratitis, trachoma, or onchocerciasis.

[0358] In certain embodiments, the effective amount of the non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring VEGF-A-binding CKP described herein) is administered directly to the eye of the subject (such as intravitreally or topically), as described in further detail elsewhere herein.

[0359] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) is administered in combination with a second agent. For patients in whom the ocular disease or disorder is triggered by an inflammatory response, combination therapy with an anti-inflammatory agent can be considered. For example, the combined use of steroids and a non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally CKP) to reduce inflammation and prevent formation of new blood vessels, respectively, may be particularly advantageous in patients with, e.g., corneal neovascularization. Patients who suffer from an ocular disease or disorder secondary to bacterial, viral, fungal or acanthamoebal infection may benefit from administration of a non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) in combination with an antimicrobial agent and optionally an anti-inflammatory agent. Patients with corneal stromal blood vessels as a result of an ocular disease or disorder are at a significant risk for immune rejection after corneal transplantation. Administration of a non-naturally occurring VEGF-A-binding CKP described herein (or

composition comprising such non-naturally occurring CKP) prior to (and optionally also subsequent to) corneal transplantation therefore may be particularly beneficial to patients with corneal stromal blood vessels as successful reduction of corneal vascularization will reduce the risk of graft rejection. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) is administered in combination with a second anti-angiogenic agent. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) is administered in combination with a matrix metalloprotease (MMP) inhibitor.

[0360] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP described herein (or composition comprising such non-naturally occurring CKP) is administered in combination with a second therapy. In certain embodiments, the second therapy is laser photocoagulation therapy (LPT). LPT uses laser light to cause controlled damage of the retina to produce a beneficial therapeutic effect. Small bursts of laser light can seal leaky blood vessels, destroy abnormal blood vessels, seal retinal tears, or destroy abnormal tissue in the back of the eye. It is quick, non-invasive, and usually requires no anesthesia other than an anesthetic eye drop. LPT techniques and apparatuses are readily available to ophthalmologists (see Lock *et al.* (2010) *Med J Malaysia* 65:88-94). Additional details regarding LPT can be found in, e.g., WO 2014/033184.

[0361] In certain embodiments, the second therapy is photodynamic therapy (PDT). PDT uses a light-activated molecule to cause localized damage to neovascular endothelium, resulting in vessel occlusion. Light is delivered to the retina as a single circular spot via a fiber optic cable and a slit lamp, using a suitable ophthalmic magnification lens (laser treatment). The light-activated compound is injected into the circulation prior to the laser treatment, and damage is inflicted by photoactivation of the compound in the area afflicted by neovascularization. One commonly used light-activated compound is verteporfin (Visudyne®). Verteporfin is transported in the plasma primarily by lipoproteins. Once verteporfin is activated by light in the presence of oxygen, highly reactive, short-lived singlet oxygen and reactive oxygen radicals are generated which damages the endothelium surrounding blood vessels. Damaged endothelium is known to release procoagulant and vasoactive factors through the lipo-oxygenase (leukotriene) and cyclooxygenase (eicosanoids such as thromboxane) pathways, resulting in platelet aggregation, fibrin clot formation and vasoconstriction. Verteporfin appears to somewhat preferentially accumulate in neovasculature. The wavelength of the laser used for photoactivation of the light-activated

compound may vary depending on the specific light-activated compound used. Additional details regarding PDT can be found in, e.g., WO 2014/033184.

[0362] In certain embodiments, the second therapy is diathermy and cautery, wherein vessels are occluded either by application of a coagulating current through a unipolar diathermy unit or by thermal cautery using an electrolysis needle inserted into feeder vessels at the limbus.

Administration

[0363] In certain embodiments the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is administered, *e.g.,* via injection, e.g., subconjunctival injection, intracorneal injection, or intravitreal injection. Administration in aqueous form is usual, with a typical volume of 20-150µl e.g. 40-60µl, or 50µl. Injection can be via a 30-gauge x 1/2-inch (12.7 mm) needle. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is provided in a pre-filled sterile syringe ready for administration. In certain embodiments, the syringe has low silicone content or is silicone free. The syringe may be made of glass. Using a pre-filled syringe for delivery has the advantage that any contamination of the sterile antagonist solution prior to administration can be avoided. Pre-filled syringes also provide easier handling for the administering ophthalmologist. See, e.g., WO 2014/033184, Fagan et al. (2013) *Clin Exp Ophthalmol.* 41, 500-507; Avery et al. (2014) *Retina.* 34 Suppl 12, S1-S18; and Doshi et al. (2015) *Seminar Ophthalmol.* 26, 104-113 for further details regarding intravitreal administration.

[0364] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is administered topically, *e.g.* in form of eye drops. Additional details regarding topical drug delivery to the eye are found in, e.g., Loftsson et al. (2012) Acta Ophthalmologica. 90, 603-608; Patel et al. (2013) World J. Pharmacol. 2, 47-64; Freeman et al. (2009) Exp Rev Ophthalmol. 4, 59-64; and Boddu et al. (2014) Recent Patents on Drug Delivery and Formulation. 8, 27-36.

[0365] In certain embodiments, an intravitreal device is used to continuously deliver the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) into the eye. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is administered via ocular insert (including, but not limited to, e.g., Ocuserts, Lactisers, Soluble

Ocular Drug Inserts (SODIs), Minidiscs, contact lenses, films, filter paper strips, artificial tear inserts, and collagen shields). See, e.g., Gaikwad et al. (2013) *Indo Amer J Pharm Res.* 3, 3216-3232). In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is administered as a slow-release depot, an ocular plug/reservoir, an ocular implant (such as a scleral or vitreal implant). Various scleral and intravitreal delivery systems are known in the art. These delivery systems are typically non-biodegradable, and may be active or passive. For example, WO 2010/088548 describes a delivery system having a rigid body using passive diffusion to deliver a therapeutic agent. WO 2002/100318 discloses a delivery system having a flexible body that allows active administration via a pressure differential. Alternatively, active delivery can be achieved by implantable miniature pumps. An example for an intravitreal delivery system using a miniature pump to deliver a therapeutic agent is the Ophthalmic MicroPump System™ marketed by Replenish, Inc. which can be programmed to deliver a set amount of a therapeutic agent for a pre-determined number of times. In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is encased in a small capsule-like container (e.g., a silicone elastomer cup). The container is usually implanted in the eye above the iris. The container comprises a release opening. Release of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) may be controlled by a membrane positioned between the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) and the opening, or by means of a miniature pump connected to the container. Alternatively, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) may be deposited in a slow-release matrix that prevents rapid diffusion of the antagonist out of the container. Preferably, the intravitreal device is designed to release the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) at an initial rate that is higher in the first month. The release rate slowly decreases, e.g., over the course of the first month after implantation, to a rate that is about 50% less than the initial rate. The container may have a size that is sufficient to hold a supply of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) that lasts for about four to six months. Since a reduced dose of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) may be sufficient for effective treatment when administration is continuous, the supply in the container may last for one year or longer, preferably about two years, more preferably

about three years. Because only a small surgery is required to implant a delivery system and intravitreal injections are avoided, patient compliance issues with repeated intravitreal injections can be avoided. Intravitreal concentrations of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) are reduced, and therefore the potential risk of side-effects from the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) entering the circulation is decreased.

[0366] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is administered via iontophoresis. Iontophoresis is a noninvasive technique in which a small electric current is applied to enhance ionized drug penetration into tissue (*see*, e.g., Myles et al. (2005) *Adv Drug Deliv Rev* 57, 2063-79 and Eljarrat-Binstock et al. (2006) *J Controlled Release* 110, 479-89). The drug is applied with an electrode carrying the same charge as the drug, and the ground electrode, which is of the opposite charge, is placed elsewhere on the body to complete the circuit. The drug serves as the conductor of the current through the tissue.

[0367] Additional details regarding administration of drug to the eye are provided in, e.g., Kuno et al. (2011) *Polymers* 3, 193-221; Short (2008) *Toxicologic Path.* 36, 49-62; Ghateet al. (2006) *Expert Opin Drug Deliv* 3, 275-87; Davis et al. (2004) *Curr Opin Mol Therap* 6, 195-205; Gaudana et al. (2010) *AAPS J.* 12, 348-360; and others.

Slow Release / Long Acting Delivery Formulations

[0368] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is provided as slow-release formulations. Slow-release formulations are typically obtained by mixing a therapeutic agent with a biodegradable polymer or encapsulating it into microparticles.

[0369] A slow-release formulation in accordance with the invention typically comprises the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP), a polymeric carrier, and a release modifier for modifying a release rate of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) from the polymeric carrier. By varying the manufacture conditions of polymer-based delivery compositions, the release kinetic properties of the resulting compositions can be modulated. The polymeric carrier usually comprises one or more biodegradable polymers or co-polymers or combinations thereof. For example, the

polymeric carrier may be selected from poly-lactic acid (PLA), poly-glycolic acid (PGA), polylactide-co-glycolide (PLGA), polyesters, poly (orthoester), poly(phosphazine), poly (phosphate ester), polycaprolactones, or a combination thereof.

[0370] In certain embodiments the polymeric carrier is PLGA. The release modifier is typically a long chain fatty alcohol, preferably comprising from 10 to 40 carbon atoms. Commonly used release modifiers include capryl alcohol, pelargonic alcohol, capric alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol, palmitoleyl alcohol, stearyl alcohol, isostearyl alcohol, elaidyl alcohol, oleyl alcohol, linoleyl alcohol, polyunsaturated elaidolinoleyl alcohol, polyunsaturated linolenyl alcohol, elaidolinolenyl alcohol, polyunsaturated ricinoleyl alcohol, arachidyl alcohol, behenyl alcohol, erucyl alcohol, lignoceryl alcohol, ceryl alcohol, montanyl alcohol, cluytyl alcohol, myricyl alcohol, melissyl alcohol, and geddyl alcohol.

[0371] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is incorporated into a microsphere-based sustained release composition. In certain embodiments, the microspheres are prepared from PLGA. The amount of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) incorporated in the microspheres and the release rate of the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) can be controlled by varying the conditions used for preparing the microspheres. Processes for producing such slow-release formulations are described in US 2005/0281861 and US 2008/0107694.

[0372] In certain embodiments, the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) is incorporated into a biodegradable implant (such as a microneedle). Matrix implants (such as microneedles) are typically used to treat ocular diseases that require a loading dose followed by tapering doses of the drug during a 1-day to 6-month time period (Davis et al. (2004) *Curr Opin Mol Therap* 6, 195–205). They are most commonly made from the copolymers poly-lactic-acid (PLA) and/or poly-lactic-glycolic acid (PLGA), which degrade to water and carbon dioxide. The rate and extent of drug release from the implant can be decreased by altering the relative concentrations of lactide (slow) and glycolide (fast), altering the polymer weight ratios, adding additional coats of polymer, or using hydrophobic, insoluble drugs. The release of drug generally follows first-order kinetics with an initial burst of drug release followed by a rapid decline in drug levels. Biodegradable implants do not require removal, as they dissolve over time (Hsu (2007) *Curr Opin Ophthalmol* 18, 235–9). Biodegradable implants also allow flexibility in dose and treatment from short duration (weeks) to longer duration (months to a

year), depending on the polymer PLA/PLGA ratio, which is another benefit in tailoring drug delivery to disease progression, because dose and treatment requirements may change over time. Additional details regarding the manufacture and implantation of biodegradable implants (such as PLGA or PLA implants) for the ocular administration are provided in, e.g., WO 2006/093758, US 2006/0182783, WO 2009/026461, US 2008/0181929, US 2009/0263460, US 2010/0015158, US 2011/0207653, and US 2014/0154321. Additional details regarding microneedles for ocular drug delivery are provided in, e.g., Donnelly et al. (2010) *Drug Deliv* 14, 187-207; USP7918814, Yavux et al. (2013) *Sci World J.* doi.org/10.1155/2013/732340, and elsewhere.

Articles of Manufacture and Kits

[0373] In certain embodiments, provided is an article of manufacture containing a non-naturally occurring VEGF-A-binding CKP described herein and materials useful for the treatment of an ocular disease or disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis). The article of manufacture can comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. In certain embodiments, the container holds a composition which is effective for treating the ocular disease or disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis) and may have a complete set of items needed to implant a slow release ocular or intraocular drug delivery system, including, but not limited to, injection devices, topical and injectable medications, surgical instruments, sutures and suturing needles, and eye covers. In certain embodiments, the container fold sterile unit-dose packages. At least one active agent in the composition is non-naturally occurring VEGF-A-binding CKP described herein. The label or package insert indicates that the composition is used for treating an ocular disease or disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis). The label or package insert will further comprise instructions for administering the non-naturally occurring VEGF-A-binding CKP (or composition comprising such non-naturally occurring CKP) to the patient. Articles of manufacture and kits comprising combinatorial therapies described herein are also contemplated.

[0374] Package insert refers to instructions customarily included in commercial packages of therapeutic products that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products. In certain embodiments, the package insert indicates that the composition comprising the non-naturally occurring VEGF-A-binding CKP is used for treating an ocular disease or disorder (such as an ocular vascular proliferative disease or ocular disorder characterized by excessive angiogenesis described herein).

[0375] Kits are also provided that are useful for various purposes, e.g., for isolation or detection VEGF-A, optionally in combination with the articles of manufacture. For isolation and purification of VEGF-A, the kit can contain non-naturally occurring VEGF-A-binding CKP described herein coupled to beads (e.g., sepharose beads). Kits can be provided which contain the non-naturally occurring VEGF-A-binding CKP described herein for detection and quantitation of VEGF-A *in vitro*, e.g. in an ELISA or blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. For example, the container holds a composition comprising at least one non-naturally occurring VEGF-A-binding CKP described herein. Additional containers may be included that contain, e.g., diluents and buffers, control antibodies, etc. The label or package insert may provide a description of the composition as well as instructions for the intended *in vitro* or diagnostic use.

EXAMPLES

Example 1: Materials and Methods for Examples 2-3

Display of EETI-II on M13 phage.

[0376] EETI-II was displayed on the surface of M13 bacteriophage by modifying a previously described phagemid pS2202b (Skelton, N. J., Koehler, M. F., Zobel, K., Wong, W. L., Yeh, S., Pisabarro, M. T., Yin, J. P., Lasky, L. A., and Sidhu, S. S. (2003) Origins of PDZ domain ligand specificity. Structure determination and mutagenesis of the Erbin PDZ domain. *J Biol Chem* **278**, 7645-7654). Standard molecular biology techniques were used to replace the fragment of pS2202d encoding Erbin PDZ domain with a DNA fragment encoding for EETI-II. The resulting phagemid (p8EETI-II) contained an open reading frame that encoded for the maltose binding protein secretion signal, followed by a gD tag and EETI-II and ending with M13 major coat protein p8. *E. Coli* harboring p8EETI-II were co-infected with M13-KO7 helper phage and cultures were grown in 30 ml 2YT medium supplemented with 50 μg/ml Carbenecillin and 25 μg/ml Kanamycin at 30 °C overnight. The propagated

phage was purified according to the standard protocol (Tonikian, R., Zhang, Y., Boone, C., and Sidhu, S. S. (2007) Identifying specificity profiles for peptide recognition modules from phage-displayed peptide libraries. *Nat Protoc* **2**, 1368-1386) and re-suspended in 1 ml PBT buffer (PBS, 0.5% BSA and 0.1% TWEEN®20), resulting in the production of phage particles that encapsulated p8EETI-II DNA and displayed EETI-II. The display level was analyzed using a phage ELISA.

Library Construction and Sorting.

[0377] The EETI-II libraries were constructed following Kunkel mutagenesis method (Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 154, 367-382). Three libraries were constructed: Library 1, in which loop 1 (3-8) was randomized with the degenerated codon encoding all natural amino acids except Cys at 6, 8 or 10 amino acids in length; or Library 2, in which loop 5 (22-26) was randomized with the same set of degenerated codon with fixed length of 5 amino acids; or Library 3, in which both loop 1 were randomized with 6, 8, and 10 amino acids and loop 5 with 5 amino acids simultaneously with degenerated codon encoding for 19 amino acids. Oligonucleotides for mutagenesis were synthesized using custom mixes of trimer phosphoramidites encoding for 19 amino acids at equimolar concentration. (Glen Research, Sterling, VA). The stop template is the single strand DNA of p8EETI-II containing three stop codons in region of 3-26 and was used to construct all three libraries. The pool of three libraries contained ~3 x 10^{10} unique members and was cycled through rounds of binding selection against hVEGF (8-109) captured on plate for four rounds following the standard protocol (Tonikian, R., Zhang, Y., Boone, C., and Sidhu, S. S. (2007) Identifying specificity profiles for peptide recognition modules from phage-displayed peptide libraries. *Nat Protoc* **2**, 1368-1386) with the variation that, 25ug/ml of hVEGF(8-109) was used to coat the plate and eluted phage were propagated by growing the overnight culture at 30°C.

Spot Phage ELISA.

[0378] After four rounds of binding selection, individual phage clones were picked and inoculated into 450µl 2YT media containing 50µg/ml Carbenecillin and M13-KO7 helper phage in 96-well blocks, which were grown at 37°C overnight. The supernatant was analyzed with spot phage ELISA as follows: hVEGF(8-109) or BSA were coated on 384-well MAXISORP™ immunoplates and phage supernatant diluted (1:3) with PBT buffer was

added to the wells. The plates were washed and bound phage was detected with anti-M13-HRP followed by TMB substrate. In these assays, phage binding to BSA alone was tested in parallel to assess background binding. Clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were considered positive. Positive clones were subjected to DNA sequence analysis.

Crystallography.

[0379] To form a stable complex, VEGF-A was concentrated to 7 mg/ml and incubated with a 6-fold molar excess of VEGF_CKP9.54.90 variant. VEGFA/ VEGF_CKP9.54.90 crystals of the primitive monoclinic space group $P12_11$ were grown at 19°C by the hanging-drop vapor diffusion method using a drop ratio of 2:1 protein: reservoir solution. Reservoir solution contained 100 mM HEPES pH 7.4 and 26% PEG 3350. Crystals were cryoprotected in reservoir solution supplemented with 25% PEG 200 and flash-frozen in liquid nitrogen prior to data collection.

Data Collection and Structure Determination.

[0380] X-ray diffraction data were collected to 1.64 Å at beamline 21IDF at the Advanced Photon Source. Data were processed using iMosflm. The structure was solved by molecular replacement using Phaser in Phenix with the previously published apo VEGF-A structure (PDB: 1VPF) as a search model and one VEGF-A dimer in the asymmetric unit. Clear $F_o - F_c$ density was present for the VEGF_CKP9.54.90 variant, so the structure of this variant was built into the density manually using Coot and then subjected to iterative rounds of refinement and rebuilding using Phenix and Coot.

KDR-CHO VEGF Assay to Determine Cellular IC_{50}

[0381] KDR-CHO cells (CHO cells stably transfected with gD tagged-KDR) were grown in cell growth medium (DMEM/Ham's F-12, 10% diafiltered FBS (GIBCO catalog no. 26400), 25 mM HEPES, 2 mM L-GLUTAMAX™). For VEGF stimulation assay, $5x10^4$ cells/well were plated in 100 µl of cell plating medium (DMEM/Ham's F-12, 0.2% BSA, 0.25% diafiltered FBS, 25 mM HEPES, 2 mM L-GLUTAMAX™) in 96-well tissue culture plate and incubated at 37°C overnight. The medium was replaced with 100 µl of serum-free cell stimulation medium (DMEM/Ham's F-12, 0.5% BSA, 25 mM HEPES) and cells were incubated at 37°C for 2 hr. One hour before stimulation, the medium was replaced with 50 µl of serum-free cell stimulation medium. Concurrently, VEGF (50 ng/ml for hVEGF, 100

ng/ml for mVEGF and rVEGF) was pre-incubated with titrated amount of CKP or anti-VEGF in 50 µl of serum-free cell stimulation medium at 37°C for 1 hour and added to the cells. The cells were stimulated for 15 min at 37C and the medium is removed. The cells were lysed with 130 µl of ice-cold cell lysis buffer (150 mM NaCl, 50 mM HEPES, 0.5% Triton-X 100, HALT protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Inc.catalog no. 78444), 5 mM EDTA). VEGF mediated Tyr phosphorylation of KDR was determined by ELISA-based assay. Briefly, MAXISORP™ 96 well plates (ThermoFisher Scientific, Inc. catalog no. 439454) were coated with 100 µl of anti-gD antibody diluted in PBS (1 µg/ml) at 4°C overnight and washed three times with washing buffer (PBS, 0.05% TWEEN®20, pH 7.4). The plates were blocked with 300 µl of blocking buffer (PBS, 0.5% BSA) at room temperature for 1 hour followed by washing three times with washing buffer. The above KDR-CHO cell lysate (100 µl) was added to each well and incubated at room temperature for 2 hours. The plates were washed four times with washing buffer followed by incubation with 100 µl of 0.5 µg/ml biotin-conjugated anti-phosphotyrosine (clone 4G10, Millipore catalog no. 16-103) in blocking buffer at room temperature for 2 hours. After washing four times, the plates were incubated with 100 µl of HRP-conjugated streptavidin in blocking buffer at room temperature for 30 min. After washing four times, the plates were developed with 100 µl of TMB substrate (BD Biosciences) at room temperature for 20~30 min and stopped by addition of 50 µl of H_2SO_4 solution. The optical density of each well was determined using a microplate reader set to 450 nm.

Competition ELISAs

[0382] Binding specificity of each peptide was established by competition ELISA. First, binding of each growth factor to their corresponding receptor in a plate-ELISA format was confirmed by coating VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGf-2, NGF, EGF, PDGF-ββ, or IGF-1 at 2 or 5 µg/mL in MAXISORP™ plates overnight at 4° C in PBS. After blocking with block buffer (PBS with 0.5% BSA and 0.05% TWEEN®20) for 2 hours at room temperature, the receptor-Fc fusions or biotinylated receptors were serially diluted using assay buffer (PBS with 0.5% BSA and 0.05% TWEEN®20) and incubated for 1 hour at room temperature. Amount of bound receptor-Fc or biotinylated receptor was detected by incubating with anti-human-Fc-HRP (Life Technologies) or high affinity streptavidin-HRP (ThermoFisher Scientific, Inc.) respectively for 30 min. Competition ELISA was conducted in an identical fashion as described above except after blocking, a mixture of serially diluted

peptide containing a constant concentration of receptor-Fc fusion or biotinylated receptor (concentration of receptor was set to EC_{60}) was added and incubated for 1 hour. All recombinant human proteins and antibodies were purchased from R &D Systems (Minneapolis, MN).

SPR Binding Assays.

[0383] Binding kinetics and affinities of inhibitors of VEGF-A were assessed using surface plasmon resonance technology on a BIACORE™ 3000 instrument (GE Healthcare) at 37°C using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% v/v surfactant P20) containing 0.1% DMSO (v/v). Depending on the format of the assay either a streptavidin sensor (SA) or a dextran-coated (CM5) sensor was utilized as described below.

[0384] For use with the SA sensor, VEGF-A was first biotinylated (no more than 2 biotin/ VEGF-A) by incubating the protein with EZ-link NHS-PEG4-Biotin (Pierce) in a 1:1.5 molar ratio respectively, in PBS for 2 hours on ice. Reaction was then quenched by addition of 10 molar excess of Glycine pH 8.0 and the sample was buffer exchanged into PBS using an Amicon 0.5 mL 3000 MWCO ultra-centrifugal filters (EMD Millipore). The biotinylation state of the protein was verified by LC-MS analysis. Biotinylated VEGF-A was then captured on the surface until a resonance unit (RU) signal of about ~400. For immobilization of VEGF-A on CM5 sensor, the surface was first activated with a mixture of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS) according to the supplier instructions. VEGF-A was then diluted into coupling buffer (0.1 M Acetate Buffer, pH 5.0) and injected until the signal reached about ~400 RU followed by a wash with 1 M ethanolamine pH 8.0 to quench remaining activated sites.

[0385] Following the capture step, a series of the peptide concentrations were prepared in HBS-EP buffer with matching DMSO concentrations to 0.1% and injected at a flow rate of 80 μL/min. The resulting sensorgrams were then analyzed using a 1:1 binding model to obtain kinetic data and affinities using Scrubber 2.0 (BioLogic Software).

CKP synthesis and folding.

[0386] Linear precursor LRP6 peptides were dissolved into DMSO (0.5mg/mL) into 0.1M ammonium bicarbonate (pH 9), 1mM reduced glutathione in 50% DMSO and incubated while shaking at room temperature for 24h. Folded CKPs were purified by RP-

HPLC on a C18 column and then collected fractions were analyzed by mass spectrometry, pooled and lyophilized prior to use.

Cell culture and transfection.

[0387] HEK293 cells stably transfected with a firefly luciferase Wnt reporter (Gong et al. (2010) *PLoS ONE* 5,9: e12682). and pRL-SV40 Renilla luciferase (Promega) were grown to 90% confluence in DMEM:F12 (50:50) supplemented with 10% FBS, 2 mM GLUTAMAX™ and 40 μg/ml hygromycin. Cells were incubated in a 5% CO_2 humidified incubator at 37 °C for 24 h. Following the incubation, the cells were trypsinized (0.05% Gibco 15400-54 in PBS) then diluted to $4x10^5$ cells/ml in DMEM:F12 (50:50) supplemented with 10% FBS, 2 mM GLUTAMAX™. 20,000 cells were loaded into individual wells of white microtest 96-well optilux plates (catalog no. 353947) and incubated for ~24h. Each well was transfected using FUGENE® HD with Wnt1-pCDNA3.2 (5ng/well) or Wnt3a-pCDNA3.2 (25ng/well) then grown for 24h. All LRP6-binding variants were diluted in DMSO and added to cells at a final DMSO concentration of 1% at peptide concentrations of 0, 0.1, 0.1, 1.0, 10, and 100 μM for 6 hours. For stimulation with recombinant Wnt3a (5036-WN-010/CF, R&D Systems) was diluted in PBS to 50ng/mL and added to the incubation media with the indicated CKP.

[0388] Luciferase response in all assays was then measured with Promega's DUAL-GLO® kit according to the manufacturer's instructions, except using half the volume of each reagent. Firefly luminescence and Renilla luminescence were measured on a Perkin Elmer ENVISION™ Multilabel Reader. The ratios of firefly luminescence: Renilla luminescence were calculated and normalized to the ratio in control cells expressing or treated with the indicated Wnt protein. Inhibitory constants were calculated using normalized data in Prism Graphpad using the using the log(inhibitor) vs. normalized response – variable slope Y=100/(1+10^((LogIC_{50}-X)*HillSlope)). Statistical significance was determined using the Holm-Sidak method, with alpha=5.000%. Computations assume that all rows are sample from populations with the same scatter (SD) and IC_{50} were identified as significantly different using the Extra sum-of-squares F test where $P<0.05$ when significant.

Example 2A: Generation of non-naturally occurring EETI-II variants that bind VEGF-A

[0389] EETI-II (**FIG. 1**) was chosen as a scaffold for display on the surface of M13 bacteriophage. EETI-II was fused to the N terminus of M13 major coat protein p8. Furthermore, a gD-tag was engineered N terminal of EETI-II sequence in order to verify

display levels. Three peptide phage libraries were generated based on the EETI-II framework as follows: library I in which loop 1 amino acid residues were randomized and the loop length was varied (6, 8, 10 residues); library II in which loop 5 amino acid residues were randomized and the native loop length was fixed; and library III in which both loops 1 and 5 were randomized in amino acid content simultaneously and loop 1 length was varied from 6 to 10 amino acid residues while loop 5 length was fixed. Altogether, the three libraries contained 3 x 10^{10} unique members and were cycled through rounds of selection against VEGF-A.

[0390] Panning against VEGF-A generated thirteen unique variants that bound to $hVEGF_{8-109}$ (*see* **Table 19**). These variants contained variations in amino acid composition in loop 1 or both loops 1 and 5 simultaneously. Also, a number of variants had a longer loop compared to the native loop present in EETI-II. A conserved **YXS** motif was also apparent in loop 5. We generated soluble folded cystine-knot peptides that correspond to seven of these unique variants, and they all demonstrated binding to hVEGF-A in a phage competition ELISA (**Table 19**). Moreover, we assessed some of the variants in a cellular assay and they demonstrated cross-species inhibition of human, mouse and rat VEGF-A activity with IC_{50} in low μM.

Table 19: EETI-II-based binders against hVEGF-A

VARIANT	LOOP 1	LOOP 5	n	ELISA	S/N*
EETI-II	PRILMR (SEQ ID NO: 92)	GPNGF (SEQ ID NO: 15)		0.01	1.11
VEGF_CKP1	ETDWYPHQID (SEQ ID NO: 225)	GPNGF (SEQ ID NO: 15)	2	0.9	16.9
VEGF_CKP2	GETVFEQFLW (SEQ ID NO: 226)	GPNGF (SEQ ID NO: 15)	2	3.2	48.1
VEGF_CKP3	HMMYDY (SEQ ID NO: 227)	EMYDA (SEQ ID NO: 235)	2	3.1	42.9
VEGF_CKP4	KKWQWWYM (SEQ ID NO: 228)	YPWTE (SEQ ID NO: 236)	5	2.6	35.3
VEGF_CKP5	PAIQNWKEHP (SEQ ID NO: 229)	SWWPSL (SEQ ID NO: 237)	2	1.9	28.4
VEGF_CKP6	PTTRFKQY	GPNGF	28	3.5	51.9

	(SEQ ID NO: 8)	(SEQ ID NO: 15)			
VEGF_CKP7	QDPTFNWALY (SEQ ID NO: 9)	QMYQS (SEQ ID NO: 16)	2	3.4	54.5
VEGF_CKP8	QLMHPFWG (SEQ ID NO: 230)	HWYRS (SEQ ID NO: 238)	2	3.9	59.4
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)	HWYQS (SEQ ID NO: 17)	11	3.3	36.0
VEGF_CKP10	RDLDVKWD (SEQ ID NO: 11)	QYYSS (SEQ ID NO: 18)	3	3.2	43.7
VEGF_CKP11	RTPWEPHDIT (SEQ ID NO: 12)	GPNGF (SEQ ID NO: 15)	16	4.1	57.5
VEGF_CKP12	TTPWPPHEIM (SEQ ID NO: 13)	GPNGF (SEQ ID NO: 15)	75	3.5	55.2
VEGF_CKP13	VTPWKPHWIN (SEQ ID NO: 14)	GPNGF (SEQ ID NO: 15)	2	3.5	56.3

*S/N = signal to noise ratio as compared to BSA control

Table 20: Phage Competition ELISA and cellular inhibitory activities of soluble EETI-II-based binders against hVEGF-A

VARIANT	In vitro IC_{50} (μM)	Cellular Assay		
		hVEGF-A (25 ng/ml)	mVEGF (50 ng/ml)	rVEGF (50 ng/ml)
VEGF_CKP6	3	47	169	711.2 (partial)
VEGF_CKP7	12	18	39	7
VEGF_CKP9	0.6	12	10	1
VEGF_CKP10	1	45	ND*	18
VEGF_CKP11	80	ND*	ND*	ND*
VEGF_CKP12	60	102	243	1220 (partial)
VEGF_CKP13	80	ND*	ND*	ND*

*ND= not determined

[0391] To further improve the potency of these variants, we followed up on VEGF_CKP7 and VEGF_CKP9. Soft randomization was done on loops 1 and 5 within the VEGF-CKP7 framework, resulting in 16 unique variants that bound to human VEGF-A (*see* **Table 21** below).

Table 21: CKP_7 Affinity-matured binders against hVEGF-A

VARIANT	LOOP 1	LOOP 5	n	ELISA	S/N*
VEGF_CKP7	QDPTFNWALY (SEQ ID NO: 9)	QMYQS (SEQ ID NO: 16)	1	0.1	1.3
VEGF_CKP7.2	DDPSFDWSVY (SEQ ID NO: 287)	RMYDS (SEQ ID NO: 292)	1	1.2	21.5
VEGF_CKP7.8	KNPLFNWALY (SEQ ID NO: 60)	QLFDS SEQ ID NO: 71)	2	0.5	7.5
VEGF_CKP7.17	QDPTVNWAVY (SEQ ID NO: 61)	QFYQS (SEQ ID NO: 72)	1	0.8	13.4
VEGF_CKP7.19	QDPTFNWAEY (SEQ ID NO: 62)	QLYQS (SEQ ID NO: 73)	2	0.6	11.1
VEGF_CKP7.24	WDPTFNWALY (SEQ ID NO: 288)	QMYDS (SEQ ID NO: 76)	2	0.8	13.4
VEGF_CKP7.35	QDPTFNWAEY (SEQ ID NO: 62)	QMYQS (SEQ ID NO: 16)	3	0.6	10.6
VEGF_CKP7.43	QDPTLNWATY (SEQ ID NO: 289)	QMYQS (SEQ ID NO: 16)	1	0.5	6.3
VEGF_CKP7.46	EDPTVDWAQY (SEQ ID NO: 290)	QMYQS (SEQ ID NO: 16)	1	0.3	4.9
VEGF_CKP7.50	QDPSLNWADY (SEQ ID NO: 63)	QMHQS (SEQ ID NO: 74)	1	0.8	14.3
VEGF_CKP7.54	LDRTLNWALY (SEQ ID NO: 64)	QMYNS (SEQ ID NO: 75)	1	0.5	9.3
VEGF_CKP7.57	LDPSFNWSLY (SEQ ID NO: 65)	QMYDS (SEQ ID NO: 76)	2	1.0	17.4
VEGF_CKP7.73	RDLTINWALF (SEQ ID NO: 66)	QMFNS (SEQ ID NO: 274)	1	1.2	19.2
VEGF_CKP7.78	KDTTFNWGLF (SEQ ID NO: 291)	QLYQS (SEQ ID NO: 73)	1	0.7	11.8
VEGF_CKP7.81	LDPTVNWALF (SEQ ID NO: 67)	QHYKT (SEQ ID NO: 77)	1	1.1	18.6

VEGF_CKP7.88	QDPKLNWAVY (SEQ ID NO: 68)	QLFNS (SEQ ID NO: 78)	2	0.5	7.7
LRP6_CKP7.89	LDPSFDWALY (SEQ ID NO: 69)	QLYNS (SEQ ID NO: 79)	1	0.5	8.1

*S/N = signal to noise ratio as compared to BSA control

[0392] Soft randomization was done on loops 1 and 5 within the VEGF-CKP9 framework, resulting in 16 unique variants that bound to human VEGF-A (*see* **Tables 22-24** below).

Table 22: VEGF_CKP9 Loop1 Affinity-Matured Variants Against VEGF-A

VARIANT	L1 SEQUENCE	n	ELISA	S/N*
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)		0.4	7.4
VEGF_CKP9.L1.2	HLFEPLWG (SEQ ID NO: 245)	17	1.6	12.5
VEGF_CKP9.L1.7	QVMRPFWG (SEQ ID NO: 246)	3	1.5	8.9
VEGF_CKP9.L1.8	QVMQPAWG (SEQ ID NO: 247)	1	1.2	12.8
VEGF_CKP9.L1.19	HRLQPLWG (SEQ ID NO: 248)	3	1.4	11.6
VEGF_CKP9.L1.26	ELLQPSWG (SEQ ID NO: 249)	4	1.7	11.9
VEGF_CKP9.L1.57	NPMLPFWG (SEQ ID NO: 368)	3	3.7	31.8
VEGF_CKP9.L1.64	NVLLPLWG (SEQ ID NO: 250)	1	2.3	19.9
VEGF_CKP9.L1.68	DIMQPLWG (SEQ ID NO: 36)	1	2.0	23.2
VEGF_CKP9.L1.76	DLMQPLWG (SEQ ID NO: 251)	2	2.5	17.4
VEGF_CKP9.L1.78	NPMLPLWG (SEQ ID NO: 252)	1	3.0	25.1
VEGF_CKP9.L1.79	QVLQPSWG (SEQ ID NO: 253)	1	1.2	10.0

*S/N = signal to noise ratio as compared to BSA control

Table 23: VEGF_CKP9 Loop5 Affinity-Matured Variants Against VEGF-A

VARIANT	L5 SEQUENCE	n	ELISA	S/N*
VEGF_CKP9	HWYQS (SEQ ID NO:17)		0.9	11.6
VEGF_CKP9.L5.7	RWYNS (SEQ ID NO: 133)	11	1.4	18.9
VEGF_CKP9.L5.18	HWYQS (SEQ ID NO: 17)	1	1.6	20.4
VEGF_CKP9.L5.43	RWYHS (SEQ ID NO: 43)	2	0.9	13.6

*S/N = signal to noise ratio as compared to BSA control

Table 24: VEGF_CKP9 Loop1/Loop5 Affinity-Matured Variants Against VEGF-A

VARIANT	LOOP 1	LOOP 5	n	ELISA	S/N*
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)	HWYQS (SEQ ID NO:17)		0.4	7.4
VEGF_CKP9.2	DVLQPFWG (SEQ ID NO: 28)	HWYQS (SEQ ID NO: 17)	20	2.4	31.1
VEGF_CKP9.3	QISQPFWG (SEQ ID NO: 29)	HFYNS (SEQ ID NO: 41)	1	1.6	24.5
VEGF_CKP9.4	DRMQPLWG (SEQ ID NO: 30)	LWYKS (SEQ ID NO: 42)		N/D	N/D
VEGF_CKP9.9	QLLEPMWG (SEQ ID NO: 254)	HWYNS (SEQ ID NO: 46)	1	1.1	19.7
VEGF_CKP9.14	KLLQPMWG (SEQ ID NO: 255)	RWYQS (SEQ ID NO: 44)	1	2.0	30.1
VEGF_CKP9.11	DRMQPYWG (SEQ ID NO: 256)	QWYKS (SEQ ID NO: 262)	1	1.1	14.1
VEGF_CKP9.20	NLMLPFWG (SEQ ID NO: 31)	RWYHS (SEQ ID NO: 43)	1	1.7	13.3
VEGF_CKP9.22	QRTQPFWG (SEQ ID NO: 32)	RWYQS (SEQ ID NO: 44)	1	1.2	17.2
VEGF_CKP9.47	KIMQPLWG (SEQ ID NO: 257)	LWYDS (SEQ ID NO: 263)	1	1.0	14.6
VEGF_CKP9.51	NLMHPFWG (SEQ ID NO: 258)	HWYQS (SEQ ID NO: 17)	1	1.0	11.0
VEGF_CKP9.54	NIMLPFWG (SEQ ID NO: 33)	QYYQS (SEQ ID NO: 45)	1	2.1	28.4
VEGF_CKP9.59	DPMQPFWG	RWYQS		N/D	N/D

	(SEQ ID NO: 34)	(SEQ ID NO: 44)			
VEGF_CKP9.63	DVMQPYWG (SEQ ID NO: 35)	HWYNS (SEQ ID NO: 46)	1	2.0	29.7
VEGF_CKP9.69	ALLQPLWG (SEQ ID NO: 259)	RWYNS (SEQ ID NO: 133)	1	1.0	14.3
VEGF_CKP9.71	QLLQPLWG (SEQ ID NO: 37)	RWYQS (SEQ ID NO: 44)	1	1.0	16.5
VEGF_CKP9.72	RLLEPSWG (SEQ ID NO: 260)	QWYQS (SEQ ID NO: 264)	1	0.6	10.0
VEGF_CKP9.76	HLLLPLWG (SEQ ID NO: 261)	RWYHS (SEQ ID NO: 43)	1	1.3	15.5
VEGF_CKP9.96	KLFEPLWG (SEQ ID NO: 39)	RWYES (SEQ ID NO: 567)	1	1.2	18.4

*S/N = signal to noise ratio as compared to BSA control

[0393] These clones were selected for further validation in a phage titration assay, and soluble folded forms corresponding to ten of these sequences were generated for further in vitro assessment (see **Table 25** below). From this set of variants, VEGF_CKP9.2, VEGF_CKP9.54 and VEGF_CKP9.63 exhibited improved potency against VEGF-A compared to parent VEGF-CKP9 in in vitro and cellular assays, with IC_{50} in 100 – 200 nM range (see **Table 25**).

Table 25: Inhibitory activity in phage competition ELISA and VEGF-A-KDR interaction ELISA

VARIANT	phage ELISA $IC_{50}(\mu M)$	Cellular $IC_{50}(nM)$
VEGF_CKP9	1.36	11700
VEGF_CKP9.2	0.168	270
VEGF_CKP9.3	1.50	N/D
VEGF_CKP9.4	1.95	N/D
VEGF_CKP9.20	1.34	N/D
VEGF_CKP9.22	>100	N/D
VEGF_CKP9.54	0.45	188
VEGF_CKP9.59	5.82	N/D
VEGF_CKP9.63	0.146	140
VEGF_CKP9.96	49.00	N/D

[0394] To enhance potency, we selected the lead 9.54 and 9.63 molecules and generated new phage libraries based on these frameworks in which loop 2 was randomized. The new libraries were panned against hVEGF-A and yielded a number of loop 2 variants which demonstrated significantly improved potency against VEGF-A compared to parent 9.54 and 9.63 molecules (*see* **Tables 26** and **27** below, respectively), with the most potent molecules exhibiting IC_{50} in 0.5 – 2 nM range (*see* **Table 28** below).

Table 26: Affinity-matured VEGF-A binding loop 2 variants based on 9.54 framework

VARIANT	LOOP 1	LOOP 2	LOOP 5	n	ELISA	S/N*
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)	KQDSD (SEQ ID NO: 93)	HWYQS (SEQ ID NO: 17)			
VEGF_CKP9.54	NIMLPFWG (SEQ ID NO: 33)	KQDSD (SEQ ID NO: 93)	QYYQS (SEQ ID NO: 45)			
VEGF_CKP9.54.1	NIMLPFWG (SEQ ID NO: 33)	GQSFE (SEQ ID NO: 94)	QYYQS (SEQ ID NO: 45)	80	2.4	29.9
VEGF_CKP9.54.2	NIMLPFWG (SEQ ID NO: 33)	GLDYD (SEQ ID NO: 95)	QYYQS (SEQ ID NO: 45)	1	0.1	26
VEGF_CKP9.54.12	NIMLPFWG (SEQ ID NO: 33)	GPELN (SEQ ID NO: 298)	QYYQS (SEQ ID NO: 45)	1	2.4	38.8
VEGF_CKP9.54.14	NIMLPFWG (SEQ ID NO: 33)	QADYA (SEQ ID NO: 299)	QYYQS (SEQ ID NO: 45)	1	2.5	23.5
VEGF_CKP9.54.16	NIMLPFWG (SEQ ID NO: 33)	GVDYL (SEQ ID NO: 300)	QYYQS (SEQ ID NO: 45)	1	2.4	30.8
VEGF_CKP9.54.31	NIMLPFWG (SEQ ID NO: 33)	GTNFL (SEQ ID NO: 301)	QYYQS (SEQ ID NO: 45)	1	2.3	32.5
VEGF_CKP9.54.44	NIMLPFWG (SEQ ID NO: 33)	SRDFD (SEQ ID NO: 302)	QYYQS (SEQ ID NO: 45)	1	2.4	34.4
VEGF_CKP9.54.48	NIMLPFWG (SEQ ID NO: 33)	NRDFL (SEQ ID NO: 303)	QYYQS (SEQ ID NO: 45)	1	2.5	34.9
VEGF_CKP9.54.51	NIMLPFWG (SEQ ID NO: 33)	GWDQF (SEQ ID NO: 304)	QYYQS (SEQ ID NO: 45)	1	2.5	44.5

VEGF_CKP9.54.56	NIMLPFWG (SEQ ID NO: 33)	GKDFH (SEQ ID NO: 305)	QYYQS (SEQ ID NO: 45)	1	2.3	35.8
VEGF_CKP9.54.59	NIMLPFWG (SEQ ID NO: 33)	GPDLQ (SEQ ID NO: 96)	QYYQS (SEQ ID NO: 45)	1	2.3	35.4
VEGF_CKP9.54.64	NIMLPFWG (SEQ ID NO: 33)	SGDFA (SEQ ID NO: 306)	QYYQS (SEQ ID NO: 45)	1	2.2	22.4
VEGF_CKP9.54.69	NIMLPFWG (SEQ ID NO: 33)	GKELN (SEQ ID NO: 307)	QYYQS (SEQ ID NO: 45)	1	2.5	21.7
VEGF_CKP9.54.76	NIMLPFWG (SEQ ID NO: 33)	GWSMD (SEQ ID NO: 308)	QYYQS (SEQ ID NO: 45)	1	2.7	42.2
VEGF_CKP9.54.87	NIMLPFWG (SEQ ID NO: 33)	GYDLQ (SEQ ID NO: 309)	QYYQS (SEQ ID NO: 45)	1	2.4	26.1
VEGF_CKP9.54.90	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	QYYQS (SEQ ID NO: 45)	1	2.3	29.5

*S/N = signal to noise ratio as compared to BSA control

Table 27: Affinity-matured VEGF-A binding loop 2 variants based on VEGF_CKP9.63 framework

***S/N = signal to noise ratio as compared to BSA control**

VARIANT	LOOP 1	LOOP 2	LOOP 5	ELISA	S/N*
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)	GRDLQ (SEQ ID NO: 322)	HWYQS (SEQ ID NO: 17)		
VEGF_CKP9.63	DVMQPYWG (SEQ ID NO: 35)	GVDLS (SEQ ID NO: 323)	HWYNS (SEQ ID NO: 46)		
VEGF_CKP9.63.1	DVMQPYWG (SEQ ID NO: 35)	GPDID (SEQ ID NO: 118)	HWYNS (SEQ ID NO: 46)	2.0	25.2
VEGF_CKP9.63.2	DVMQPYWG (SEQ ID NO: 35)	GDDLE (SEQ ID NO: 324)	HWYNS (SEQ ID NO: 46)	2.0	15.0
VEGF_CKP9.63.3	DVMQPYWG (SEQ ID NO: 35)	GVDMT (SEQ ID NO: 325)	HWYNS (SEQ ID NO: 46)	1.7	20.7
VEGF_CKP9.63.12	DVMQPYWG (SEQ ID NO:	GMDIE (SEQ ID NO:	HWYNS (SEQ ID NO:	2.6	39.9

	35)	326)	46)		
VEGF_CKP9.63.14	DVMQPYWG (SEQ ID NO: 35)	DGDYQ (SEQ ID NO: 327)	HWYNS (SEQ ID NO: 46)	1.5	21.3
VEGF_CKP9.63.15	DVMQPYWG (SEQ ID NO: 35)	GNDVS (SEQ ID NO: 328)	HWYNS (SEQ ID NO: 46)	1.5	21.4
VEGF_CKP9.63.16	DVMQPYWG (SEQ ID NO: 35)	GRDMD (SEQ ID NO: 119)	HWYNS (SEQ ID NO: 46)	2.2	10.3
VEGF_CKP9.63.18	DVMQPYWG (SEQ ID NO: 35)	AGDEL (SEQ ID NO: 329)	HWYNS (SEQ ID NO: 46)	2.3	17.3
VEGF_CKP9.63.24	DVMQPYWG (SEQ ID NO: 35)	GLDEE (SEQ ID NO: 330)	HWYNS (SEQ ID NO: 46)	1.6	20.4
VEGF_CKP9.63.27	DVMQPYWG (SEQ ID NO: 35)	DGDFD (SEQ ID NO: 331)	HWYNS (SEQ ID NO: 46)	2.1	26.0
VEGF_CKP9.63.30	DVMQPYWG (SEQ ID NO: 35)	AGDFE (SEQ ID NO: 332)	HWYNS (SEQ ID NO: 46)	2.0	26.0
VEGF_CKP9.63.37	DVMQPYWG (SEQ ID NO: 35)	EMDFD (SEQ ID NO: 120)	HWYNS (SEQ ID NO: 46)	0.6	8.6
VEGF_CKP9.63.39	DVMQPYWG (SEQ ID NO: 35)	GNSFE (SEQ ID NO: 333)	HWYNS (SEQ ID NO: 46)	1.6	18.9
VEGF_CKP9.63.42	DVMQPYWG (SEQ ID NO: 35)	GQDLT (SEQ ID NO: 334)	HWYNS (SEQ ID NO: 46)	1.7	23.1
VEGF_CKP9.63.44	DVMQPYWG (SEQ ID NO: 35)	GENLA (SEQ IDNO: 335	HWYNS (SEQ ID NO: 46)	1.7	19.5
VEGF_CKP9.63.47	DVMQPYWG (SEQ ID NO: 35)	GQDYN (SEQ ID NO: 336)	HWYNS (SEQ ID NO: 46)	1.7	20.7
VEGF_CKP9.63.50	DVMQPYWG (SEQ ID NO: 35)	GADLS (SEQ ID NO: 337)	HWYNS (SEQ ID NO: 46)	0.9	12.7
VEGF_CKP9.63.54	DVMQPYWG (SEQ ID NO: 35)	GFDMD (SEQ ID NO: 338)	HWYNS (SEQ ID NO: 46)	1.4	19.9
VEGF_CKP9.63.56	DVMQPYWG (SEQ ID NO: 35)	GESLS (SEQ ID NO: 211)	HWYNS (SEQ ID NO: 46)	1.8	8.4

VEGF_CKP9.63.62	DVMQPYWG (SEQ ID NO: 35)	DLNYE (SEQ ID NO: 339)	HWYNS (SEQ ID NO: 46)	1.8	25.4
VEGF_CKP9.63.65	DVMQPYWG (SEQ ID NO: 35)	GRDLQ (SEQ ID NO: 322)	HWYNS (SEQ ID NO: 46)	2.0	27.1
VEGF_CKP9.63.69	DVMQPYWG (SEQ ID NO: 35)	GVDLS (SEQ ID NO: 323)	HWYNS (SEQ ID NO: 46)	2.9	23.7
VEGF_CKP9.63.87	DVMQPYWG (SEQ ID NO: 35)	GPDID (SEQ ID NO: 118)	HWYNS (SEQ ID NO: 46)	0.9	8.6

Table 28. Inhibitory activity of VEGF_CKP9.54- and VEGF_CKP9.63-derived loop 2 variants against VEGF-A

VARIANT	LOOP 1	LOOP 2	LOOP 5	KDR-VEGF IC_{50} (nM)	Cellular IC_{50} (nM)
VEGF_CKP9	QLMQPFWG (SEQ ID NO: 10)	KQDSD (SEQ ID NO: 93)	HWYQS (SEQ ID NO: 17)	569	11700
VEGF_CKP9.54	NIMLPFWG (SEQ ID NO: 33)	KQDSD (SEQ ID NO: 93)	QYYQS (SEQ ID NO: 45)	5.8	188
VEGF_CKP9.54.1	NIMLPFWG (SEQ ID NO: 33)	GQSFE (SEQ ID NO: 94)	QYYQS (SEQ ID NO: 45)	0.2	1.47
VEGF_CKP9.54.2	NIMLPFWG (SEQ ID NO: 33)	GLDYD (SEQ ID NO: 95)	QYYQS (SEQ ID NO: 45)	0.2	4.3
VEGF_CKP9.54.59	NIMLPFWG (SEQ ID NO: 33)	GPDLQ (SEQ ID NO: 96)	QYYQS (SEQ ID NO: 45)	0.5	3.06
VEGF_CKP9.54.90	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	QYYQS (SEQ ID NO: 45)	0.2	1.35
VEGF_CKP9.63	DVMQPYWG (SEQ ID NO: 35)	KQDSD (SEQ ID NO: 93)	HWYNS (SEQ ID NO: 46)	10.8	140
VEGF_CKP9.63.1	DVMQPYWG (SEQ ID NO: 35)	GENFL (SEQ ID NO: 117)	HWYNS (SEQ ID NO: 46)	0.4	0.49
VEGF_CKP9.63.27	DVMQPYWG (SEQ ID NO:	GRDMD (SEQ ID NO:	HWYNS (SEQ ID NO:	0.3	5.28

	35)	119)	46)		
VEGF_CKP9.63.44	DVMQPYWG (SEQ ID NO: 35)	EMDFD (SEQ ID NO: 120)	HWYNS (SEQ ID NO: 46)	0.2	2.05
VEGF_CKP9.63.69	DVMQPYWG (SEQ ID NO: 35)	GESLS (SEQ ID NO: 211)	HWYNS (SEQ ID NO: 46)	2.1	26.4
VEGF_CKP9.63.12	DVMQPYWG (SEQ ID NO: 35)	GPDID (SEQ ID NO: 118)	HWYNS (SEQ ID NO: 46)	0.7	1.83

[0395] The affinities / potencies of VEGF_CKP9.63.1, VEGF_CKP9.63.27, VEGF_CKP9.63.44, VEGF_CKP9.63.69, and VEGF_CKP9.63.12 for hVEGF-A (8-109) are shown below in **Table 29**.

Table 29

VARIANT	k_a	k_d	K_D
EM63	0.16 ± 0.03	1.6 ± 0.5	100 ± 9 nM
L2.9.63.1	6 ± 1	0.37 ± 0.13	5.8 ± 1.2
L2.9.63.12	8 ± 1	0.10 ± 0.04	1.1 ± 0.2
L2.9.63.27	11 ± 4	0.15 ± 0.04	1.4 ± 0.2
L2.9.63.44	10 ± 2	0.11± 0.02	1.2 ± 0.2
L2.9.63.69	3 ± 1	0.20 ± 0.04	6.9 ± 0.7

[0396] Variants VEGF_CKP9.54.90 (*see* row 2 of **Table 26**) and VEGF_CKP9.63.12 (*see* row 6 of **Table 27**), as well as parental variants VEGF_CKP9.54 (*see* row 12 of **Table 24**) and VEGF_CKP9.63 (*see* row 15 of **Table 24**), bind with similar affinity to human, mouse, rat and rabbit VEGF-A, as determined by surface plasmon resonance. *See* **Table 30** below.

Table 30: Binding kinetics and affinities of VEGF_CKP9.54.90, VEGF_CKP9.63.12, VEGF_CKP9.54, and VEGF_CKP9.63 for various VEGF isoforms.

VARIANT	VEGF Isoform	ka	ka (error)	kd	kd (error)	KD (nM)	KD (error)
9.54	human 8-109	$1.26x10^{6}$	$1.10x10^{5}$	$2.18x10^{-1}$	$1.19x10^{-2}$	175.88	18.21

	human 165	$8.23x10^{5}$	$1.11x10^{5}$	$1.49x10^{-1}$	$1.29x10^{-2}$	189.67	34.57
	mouse 164	$8.26x10^{5}$	$2.93x10^{4}$	$2.07x10^{-1}$	$2.44x10^{-2}$	249.87	21.19
	rat	$1.93x10^{6}$	$8.91x10^{5}$	$2.96x10^{-1}$	$9.36x10^{-2}$	175.64	26.27
	rabbit	$2.10x10^{6}$	$7.12x10^{5}$	$2.74x10^{-1}$	$9.14x10^{-2}$	133.22	9.47
9.54.90	human 8-109	$5.15x10^{7}$	$1.62x10^{7}$	$4.05x10^{-2}$	$8.77x10^{-3}$	0.87	0.13
	human 165	$1.58x10^{7}$	$3.87x10^{6}$	$1.33x10^{-2}$	$1.70x10^{-3}$	0.89	0.10
	mouse 164	$8.71x10^{6}$	$2.79x10^{6}$	$1.01x10^{-2}$	$2.12x10^{-3}$	1.31	0.24
	rat	$1.72x10^{7}$	$7.43x10^{6}$	$1.35x10^{-2}$	$3.33x10^{-3}$	0.90	0.14
	rabbit	$5.15x10^{7}$	$1.36x10^{7}$	$6.75x10^{-2}$	$8.99x10^{-3}$	1.14	0.18
9.63	human 8-109	$6.62x10^{5}$	$9.83x10^{4}$	$1.81x10^{-1}$	$2.56x10^{-2}$	281.44	43.53
	human 165	$3.40x10^{5}$	$2.79x10^{4}$	$1.30x10^{-1}$	$1.33x10^{-2}$	381.89	19.15
	mouse 164	$5.57x10^{5}$	$4.60x10^{4}$	$1.60x10^{-1}$	$1.35x10^{-2}$	288.75	11.08
	rat	$4.56x10^{5}$	$1.49\ x10^{5}$	$2.46x10^{-1}$	$9.23x10^{-2}$	523.93	25.06
	rabbit	$4.24x10^{5}$	$3.22x10^{4}$	$1.30x10^{-1}$	$1.95x10^{-2}$	311.43	52.25
9.63.12	human 8-109	$6.54x10^{6}$	$7.25x10^{5}$	$2.50x10^{-2}$	$3.45x10^{-3}$	3.20	0.22
	human 165	$4.65x10^{6}$	$8.39x10^{5}$	$2.01x10^{-2}$	$3.95x10^{-3}$	4.32	0.15
	mouse 164	$1.04x10^{6}$	$2.81x10^{5}$	$1.32x10^{-2}$	$3.04x10^{-3}$	13.07	1.74
	rat	$6.44x10^{6}$	$3.87x10^{6}$	$2.59x10^{-2}$	$1.01x10^{-2}$	5.74	1.54
	rabbit	$6.91x10^{6}$	$1.35x10^{6}$	$1.78x10^{-2}$	$4.46x10^{-3}$	2.54	0.40

[0397] VEGF_CKP9.54.90 is also highly selective to VEGF-A and does not bind to or inhibit the activity of other VEGF isoforms such as VEGF-B, VEGF-C and VEGF-D or other growth factors such as P1GF, EGF, NGF, IGF and PDGF. As shown in **FIGS. 2A** and **2B**, the variant VEGF_CKP9.54.90 disrupts the interaction between VEGF-A and KDR as well as the interaction between VEGF-A and Flt-1, but not disrupt the interaction between VEGF-B and Flt-1, between VEGF-C and Flt-4, between VEGF-D and Flt-4, between PIGF-2 and Flt-1, between EGF and EGFR, between PDGF and PDGFR, between NGF and NGFR, or between IGF and IGFR.

[0398] Unlike EETI-II, VEGF_CKP9.54.90, VEGF_CKP9.54, and VEGF_CKP9.63.12 do not inhibit trypsin protease activity as measured in a peptide substrate cleavage assay (Stanger et al. (2014) *FEBS Lett. 588* (23), 4487-96). *See* **FIG. 3**. However, VEGF_CKP9.54.90 and VEGF_CKP9.63.12 maintain a degree of resistance to trypsin

digestion (*see* **FIG. 4**). Approximately 20% of VEGF_CKP9.54.90 was cleaved at Arg13 within loop 2 after 24 h incubation with trypsin at 37°C.

[0399] VEGF_CKP9.54, VEGF_CKP9.63, and VEGF_CKP9.54.90 each contains roughly a 3-turn alpha-helix and each adopts a disulfide signature that is distinct from that of wild-type EETI-II (C1-C4, C2-C3, C5-C6 for VEGF_CKP9.54.90 vs. C1-C4, C2-C5, C3-C6 for wild-type EETI-II). *See* **FIG. 5**. On one side of the helix, VEGF_CKP9.54.90 forms a fused bicyclic structure that is bridged by two disulfide bonds (C1-C4 and C2-C3), encompassing loops 1, 2 and 3, and ~ 1.5 turn of the alpha-helix. Loop 5 forms on the opposite side of the helix and is constrained by C5-C6 disulfide bond.

[0400] The co-crystal structures of VEGF_CKP9.54, VEGF_CKP9.63, and VEGF_CKP9.54.90 in complex with VEGF-A were obtained. co-crystal structures of VEGF_CKP9.54, VEGF_CKP9.63, and VEGF_CKP9.54.90 in complex with VEGF-A are highly similar. *See* **FIGS. 5** and **6** for the co-crystal structure of VEGF_CKP9.54 in complex with VEGF-A. Given that the structures of VEGF_CKP9.54, VEGF_CKP9.63, and VEGF_CKP9.54.90 are highly similar, (*see* **FIGS. 5** and **6**) further studies were performed with VEGF_CKP9.54.90. The helix defined by residues Phe15 – Tyr26 of VEGF_CKP9.54.90 forms extensive hydrophobic and polar interactions with the VEGF-A surface (*see* **Table 31** below). Additionally, there is a network of backbone H-bonds which forms within and stabilizes the ~3-turn alpha-helix. In general, VEGF_CKP9.54.90 exhibits a compact and rigid structure, stabilized by intramolecular polar and hydrophobic contacts, including backbone-backbone, side chain-backbone and side chain-side chain interactions (**Table 32**). The surface of VEGF_CKP9.54.90 that contacts VEGF-A is mainly hydrophobic in nature with few polar side chains (**Table 31**), whereas the opposite surface of the peptide that is not interacting with VEGF-A is solvent-exposed and primarily polar in nature.

Table 31: VEGF_CKP9.54.90 residues that are within 4 Å of the VEGF-A dimer

VEGF_CKP9.54.90 residues within 4 Å of VEGFA	
VEGF_CKP9.54.90 (Chain 1)	VEGF_CKP9.54.90 (Chain 2)
I4	I4
M5	M5
L6	L6
P7	P7
F8	F8

W9	W9
R13	R13
D14	D14
F15	F15
L18	L18
A19	A19
V22	V22
C23	C23
Y25	Y25
Y26	Y26
Q27	Q27
S28	S28
G30	G30

Table 32: Summary of VEGF_CKP9.54.90 intra-molecular interactions

Residue 1	Residue 2	Comments
Cys2	Cys21	Disulfide
Asn3	Trp9	Main chain H-bond
Asn3	Met5	Asp3 makes H-bond with M5 main chain nitrogen
Asn3	Leu6	Main chain H-bond
Leu6	Trp9	Main chain H-bond
Pro7	Gly10	Main chain H-bond
Phe8	Cys11	Main chain H-bond
Phe8	Leu18	Van der Waals interaction
Trp9	Ile4, Leu18, Val22, Tyr25, Tyr26	Core Trp makes a network of Van der Waals interactions
Cys11	Cys17	Disulfide
Gly12	Asp14	Main chain H-bond
Asp14	Glu16, C17	Asp14 makes stabilizing H-bond with N-terminus of helix
Phe15	Leu18, Ala19	Van der Waals interactions stabilizing helix
Leu18	Val22	Van der Waals interactions stabilizing helix
Val22	Tyr26	Van der Waals interactions stabilizing helix
Phe15 – Tyr26		Network of backbone H-bonds form stabilizing a ~3-turn helix
Cys23	Cys29	Disulfide
Tyr25	Tyr26	Van der Waals interactions stabilizing helix

Gln27	Cys23	Main chain H-bond
Ser28	Cys23	Main chain H-bond

[0401] The binding interface of VEGF_CKP9.54.90 on VEGF-A overlaps with that of the natural receptors and G6.31 antibody (**FIGS. 7** and **8**). Contact residues on VEGF-A that are in the peptide interface are summarized in **Table 33** and shown in **FIG. 9**. The binding epitope of VEGF_CKP9.54.90 on VEGF-A is distinct from that of ranibizumab and bevacizumab (**FIG. 10**), which do not bind to mouse or rat VEGF-A because their interaction with human VEGF-A is dependent on a key Gly88 residue that is substituted with Ser in rodents. The binding mode of VEGF_CKP9.54.90 suggests that it is not substantially dependent on Gly88, and this notion is validated by the observation that the peptide bound efficiently to both human and rodent VEGF-A. Site-directed mutagenesis was utilized to validate a number of contacts in the protein – peptide interface observed from the crystal structure. As expected, Y21A, Q89A and F17A/M81A mutations on VEGF-A led to reduced binding of VEGF_CKP9.54.90 on VEGF-A. *See* **FIG. 11**. However, K48A mutation enhanced the binding of VEGF_CKP9.54.90 by ~ 2-3 fold, a behavior that is similar to that observed with the G6.31 antibody (Fuh et al. (2006) *J. Biol. Chem.* 281, 6625-6631). *See* **Table 34** below and **FIG. 11**.

Table 33. VEGF-A dimer residues that are within 4 Å of VEGF_CKP9.54.90

VEGF-A residues within 4 Å of VEGF_CKP9.54.90	
VEGF-A (Dimer Chain A)	**VEGF-A (Dimer Chain B)**
	V14
	V15
F17	F17
M18	M18
	D19
Y21	Y21
Q22	Q22
Y25	Y25
I46	I46
K48	K48
N62	N62
D63	D63
L66	L66

M81	M81
I83	I83
K84	K84
P85	P85
H86	H86
Q87	Q87
G88	G88
Q89	Q89
I91	
	C104
	R105
P106	P106

Table 34: Binding kinetics and affinities of VEGF_CKP9.54.90, VEGF_CKP9.63.12, VEGF_CKP9.54, and VEGF_CKP9.63 for various hVEGF-A mutants.

VARIANT	VEGF MUTANT	ka	ka (error)	kd	kd (error)	KD (nM)	KD (error)
9.54.90	WT	$7.81x10^{7}$	$1.86x10^{7}$	0.0300	$9.79x10^{-3}$	0.37	0.09
	Y21A	$3.72x10^{7}$	$1.30x10^{7}$	0.1202	$2.44x10^{-2}$	3.50	0.30
	K48A	$6.04x10^{7}$	$2.12x10^{7}$	0.0116	$3.77x10^{-3}$	0.19	0.02
	Q89A	$1.71x10^{7}$	$8.38x10^{6}$	0.1458	$5.99x10^{-2}$	8.80	0.45
	F17A/M81A						
9.63.12	WT	$8.43x10^{6}$	$1.47x10^{6}$	0.0096	0.0035	1.07	0.20
	Y21A	$1.99x10^{7}$	$6.84x10^{6}$	$1.48x10^{-1}$	$3.13x10^{-2}$	9.84	4.41
	K48A	$5.30x10^{7}$	$3.77x10^{7}$	0.017	0.013	0.33	0.01
	Q89A	$7.43x10^{6}$	$2.09x10^{6}$	0.47	$2.71x10^{-1}$	36.49	1.62
	F17A/M81A						
9.54	WT	$4.10x10^{6}$	$8.59x10^{5}$	0.2349	0.0778	55	7
	Y21A	$6.63x10^{5}$	$6.39x10^{3}$	0.2101	0.0389	317	56
	K48A	$2.64x10^{6}$	$2.24x10^{5}$	0.0587	0.0010	22	3
	Q89A	$3.37x10^{5}$	$1.41x10^{5}$	0.7932	0.5101	1882	460
	F17A/M81A						
9.63	WT	$1.57x10^{6}$	$3.14x10^{5}$	0.1624	0.05	100.34	9.40
	Y21A	$7.52x10^{5}$	$2.45x10^{5}$	0.4814	$2.13x10^{-1}$	584.85	112.171
	K48A	$5.72x10^{5}$	$1.75x10^{5}$	0.02	$5.24x10^{-3}$	28.4	4.8
	Q89A	$2.66x10^{5}$	$8.01x10^{4}$	0.51	$1.22x10^{-1}$	1999.8	127.5
	F17A/M81A						

[0402] Next, VEGF-A binding variants VEGF_CKP9.54.90, VEGF_CKP9.63.12, and VEGF_CKP9.63.44 were assessed for their *in vivo* efficacy in a VEGF-A driven model of choroidal neovascularization. Laser-burnt spots were created in rat eyes and the formation of new vessels was monitored after a 14-day period in the presence and absence of peptide that was administered intravitreally at different intervals. Peptide VEGF_CKP9.54.90 demonstrated effective inhibition of laser-induced choroidal neovascularization in rat eyes, as measured by the significant reduction observed in neovascular area in peptide-treated eyes compared to control eyes. *See* **FIG. 12**.

[0403] The co-crystal structure of VEGF_CKP9.54.90 in complex with VEGF-A revealed that the native amino acid residues in loops 3 and 4 are not necessarily in optimal orientations for binding to VEGF-A (*see* **FIG. 9**) and could be modified to enhance their interaction with the VEGF-A surface or to elicit intramolecular interactions within the peptide that could improve peptide folding and stability. Therefore, with the goal of further improving the potency and behavior of the lead molecules, new phage libraries were constructed based on the sequences of 9.54, 54.1 and 9.63, 63.12 in which only loops 3 and 4 were randomized. These specific frameworks, though slightly weaker than the lead molecules, were selected in order to allow for a sufficient dynamic range in the assay to detect improvement in affinity. Many new clones containing variations in loops 3 and 4 only were identified that showed improved binding to VEGF-A. Fourteen of the obtained sequences were selected and grafted onto loops 3 and 4 within the lead VEGF_CKP9.54.90 or VEGF_CKP9.63.12 molecules, and the corresponding soluble molecules were then generated in folded form. The amino acid sequences of the fourteen affinity-matured variants are provided in **Table 35** below.

Table 35: Affinity-matured VEGF-A binding loop 3/loop 4 variants based on VEGF_CKP9.54.90 or VEGF_CKP9.63.12 frameworks

VARIANT	LOOP 1	LOOP 2	LOOP 3	LOOP 4	LOOP 5
9.54.90.7	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	LQQ	I	QYYQS (SEQ ID NO: 45)
9.54.90.10	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	VER	I	QYYQS (SEQ ID NO: 45)
9.54.90.12	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	MSD	I	QYYQS (SEQ ID NO: 45)
9.54.90.13	NIMLPFWG	GRDFE	MNQ	I	QYYQS

	(SEQ ID NO: 33)	(SEQ ID NO: 97)			(SEQ ID NO: 45)
9.54.90.25	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	MQT	I	QYYQS (SEQ ID NO: 45)
9.54.90.31	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	VYQ	I	QYYQS (SEQ ID NO: 45)
9.54.90.44	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	FIN	I	QYYQS (SEQ ID NO: 45)
9.54.90.53	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	VSQ	I	QYYQS (SEQ ID NO: 45)
9.54.90.55	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	VTE	I	QYYQS (SEQ ID NO: 45)
9.54.90.62	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	FYE	I	QYYQS (SEQ ID NO: 45)
9.54.90.67	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	MEQ	I	QYYQS (SEQ ID NO: 45)
9.54.90.71	NIMLPFWG (SEQ ID NO: 33)	GRDFE (SEQ ID NO: 97)	VYR	I	QYYQS (SEQ ID NO: 45)
9.63.12.8	DVMQPYWG (SEQ ID NO: 35)	GPDID (SEQ ID NO: 118)	FVR	L	HWYNS (SEQ ID NO: 46)
9.63.12.12	DVMQPYWG (SEQ ID NO: 35)	GPDID (SEQ ID NO: 118)	LSN	I	HWYNS (SEQ ID NO: 46)

[0404] All soluble molecules containing L3/L4 variations showed improved potency in the cellular assay relative to 54.90 or 63.12.12. Three lead molecules, VEGF_CKP9.54.90.67, VEGF_CKP9.54.90.53 and VEGF_CKP9.63.12.12 had cellular IC_{50} values in the range of about 0.5 to about 1 nM . *See* **FIG. 13** and **Table 36** below.

Table 36: IC_{50} values for variants in Table 35

VARIANT	Cellular IC_{50} (nM)	FOLD IMPROVEMENT RELATIVE TO VARIANT 9.54.90
9.54.90	1.35	1
9.54.90.12	1.20	1.125
9.54.90.13	0.96	1.41
9.54.90.25	1.26	1.07
9.54.90.44	1.16	1.16
9.54.90.62	0.92	1.47
9.54.90.67	1.10	1.23

9.63.12	1.83	0.74
9.63.12.8	N/D	
9.63.12.12	0.56	2.41

[0405] The co-crystal structure of VEGF_CKP9.63 in complex with VEGF-A revealed that Tyr residue at position 8 within loop1 could form a hydrogen bond with the side chain of Gln22 on VEGF-A. *See* **FIG. 14**. In variants derived from VEGF_CKP9.54 (such as the variants in **Table 35**) the amino acid at position 8 is Phe. Therefore, we sought to mutate Phe8 to Tyr I in some of the variants in **Table 35**, with the goal of improving affinity and/or solubility of the resulting F8Y variant. The F8Y mutation showed a modest improvement on affinity / potency of some of the molecules (e.g., VEGF_CKP9.54.1.F8Y, VEGF_CKP9.54.90.F8Y, and VEGF_CKP9.54.90.67.F8Y), whereas in few other cases it demonstrated minimal or a slightly negative effect (e.g., VEGF_CKP9.54.90.13.F8Y and VEGF_CKP9.54.90.62.F8Y). See **Table 37**, in which the binding affinities of certain variants (as determined by surface plasmon resonance) are compared, and **Table 38** in which the potencies of certain variants (as determined by cellular IC_{50}) are compared. The F8Y substitution helped to improve the solubility of VEGF_CKP9.54.90.67.F8Y by about 2 mg/ml. VEGF_CKP9.54.90.67.F8Y was selected for further follow-up studies.

Table 37: Binding kinetics and affinities of VEGF_CKP9.54.1.F8Y, VEGF_CKP9.54.90.F8Y, VEGF_CKP9.54.90.67.F8Y, VEGF_CKP9.54.90.13.F8Y and VEGF_CKP9.54.90.62.F8Y for VEGF-A

VARIANT	k_a	k_d	K_D
VEGF_CKP9	1.2 ± 0.3	40 ± 20	5 ± 1 μM
VEGF_CKP9.54	3.4 ± 0.2	2.3 ± 0.8	44 ± 6 nM
VEGF_CKP9.54.1	15 ± 2	0.36 ± 0.17	2.2 ± 0.7 nM
VEGF_CKP9.54.1.F8Y	53 ± 12	0.38 ± 0.06	0.78 ± 0.11 nM
VEGF_CKP9.54.90	63 ± 16	0.17 ± 0.05	0.40 ± 0.08 nM
VEGF_CKP9.54.90.F8Y	70 ± 10	0.27 ± 0.01	0.40 ± 0.05 nM
VEGF_CKP9.54.90-Alkyn	50 ± 0.3	0.27 ± 0.004	0.49 ± 0.05 nM

[0406] The oxidative stability of various CKP variants was assayed as follows: 5μL of 11mM AAPH (Calbiochem catalog no. 100110) in water was added to 50uL of variant peptide sample (prepared as 1mg/mL peptide in 20mM histidine acetate pH 5.5) and the mix was incubated for 16 hours at 40°C. At the end of the incubation, the sample was quenched

by addition of 27.5uL of 40mM methionine, followed by addition of 160ul of 20mM Histidine acetate, 100mM sucrose at pH 5.5 to dilute the samples. The reactions were analyzed by LC-MS.

[0407] It was observed that VEGF_CKP9.54.90 underwent ~ 30% oxidation at Met5 within loop 1. Replacement of Met 5 with the unnatural amino acid norleucine rendered VEGF_CKP9.54.90 completely resistant to oxidation. The replacement of Met5 with norleucine also had a favorable effect on binding efficiency (~ 2-fold improvement). Variants VEGF_CKP9.54.90.67 F8Y M5Nle, VEGF_CKP9.54.90.53 M5Nle and VEGF_CKP9.63.12.12 M5Nle were produced. All three Met5Nle of the Met5Nle variants showed modest improvement in cellular potency by ~ 1.5 – 2 x compared to their parent molecules.

[0408] Next, the effect of naphthalene-based amino acid substitutions at Trp9 in loop 1 of VEGF_CKP9.54.90 on VEGF-A binding affinity was assessed. The crystal structures of VEGF_CKP9.54.90 complexed with VEGF indicate that the Trp9 residue of VEGF_CKP9.54.90 (and variants derived therefrom) interacts with the VEGF-A surface, with residual space that might allow larger ring systems to fit in. To test this hypothesis, we generated soluble variants of VEGF_CKP9.54.90 in which the indole ring of Trp9 was replaced with 1- or 2-naphthyl isomers. These molecules showed reduced cellular potency relative to parent VEGF_CKP9.54.90. Further data regarding the potency of VEGF_CKP9.54.90-derived variants comprising the F8Y substitution and/or an unnatural amino acid substitution are provided in **Table 38**:

Table 38: IC_{50} values for VEGF_CKP9.54.90-derived variants comprising the F8Y substitution and/or an unnatural amino acid substitution.

VARIANT	Cellular IC_{50} (nM)	FOLD IMPROVEMENT RELATIVE TO VARIANT 9.54.90
9.54.90	1.35	1
9.54.90.F8Y	1.58	0.85
9.54.90.F7Y.Δ2G	3.01	0.44
9.54.90.M5.Nle	1.95	0.69
9.54.90.Naph1	5.42	0.24
9.54.90.Naph2	17.1	0.08

9.54.90.13	0.96	1.41
9.54.90.13.F8Y	2.02	0.67
9.54.90.62	0.92	1.47
9.54.90.62.F8Y	1.14	1.18
9.54.90.67	1.1	1.23
9.54.90.67.F8Y	0.66	2.05

[0409] Shortening the lead VEGF_CKP9.54.90. F8Y by trimming the two glycine residues at the N- and C-termini to generate variant VEGF_CKP9.54.90 F7Y Δ2G) resulted in a slight reduction of cellular potency relative to VEGF_CKP9.54.90. *See* **Table 38** above.

Example 2B: Generation of VEGF-A-binding non-naturally occurring EETI-II variants comprising C-terminal amino acid extensions

[0410] To identify additional peptide variants with enhanced affinity for VEGF-A, we selected the 9.54 (SEQ ID NO: 52), 9.54.1 (SEQ ID NO: 99) molecules and generated new phage libraries based on these frameworks in which two additional amino acids were added to their C-termini.

[0411] From the 9.54 library, twenty-two clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 39**). These hits contained variations in amino acid composition within loop 2, within loops 2 and 4, or within loops 2, 4, and 5.

Table 39: C-terminal Two-residue Extension Variants Based on 9.54

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.54	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG	52
9.54-28	GCNIMLPFWGCKQDFDCLAGCICQYYQSCGFH	455
9.54-39	GCNIMLPFWGCKQDFDCLAGCICQYYQSCGGE	457
9.54-10	GCNIMLPFWGCKQDSDCLVGCICQYYQSCGSI	458
9.54-32	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGGR	459
9.54-13	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGRP	460
9.54-6	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGQY	461
9.54-24	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGEN	462
9.54-34	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGDT	463
9.54-9	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGQH	464

9.54-12	GCNIMLPFWGCKQDSDCLAGCICQYYQSCGQN	465
9.54-17	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGEE	466
9.54-19	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGDD	467
9.54-43	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGDG	468
9.54-5	GCNIMLPFWGCKQDFDCLAGCVCQYYQSCGLE	469
9.54-1	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGTD	470
9.54-4	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGSE	471
9.54-15	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGPE	472
9.54-42	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGTN	473
9.54-27	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGPH	474
9.54-2	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGMD	475
9.54-21	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCGSD	476

[0412] From the 9.54.1 library, clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 40**).

[0413] Table 40: C-terminal Two-residue Extension Variants Based on 9.54.1

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.54.1	GCNIMLPFWGCGQSFECLAGCVCQYYQSCG	99
9.54.1-2	GCNIMLPFWGCGQSFECLAGCICQYYQSCGIA	477
9.54.1-63	GCNIMLPFWGCGQSFECLAGCICQYYQSCGGS	478
9.54.1-36	GCNIMLPFWGCGQSFECLAGCICQYYQSCGTR	479
9.54.1-42	GCNIMLPFWGCGQSFECLAGCICQYYQSCGLS	533
9.54.1-90	GCNIMLPFWGCGQSFECLAGCICQYYQSCGVH	480

[0414] Clone 9.54-28 (in **Table 39**) showed approximately 10-fold improved binding affinity for hVEGF-A (8-109) compared to 9.54, as determined by phage competition ELISA (described above). (*See* **FIG. 15**). Clones 9.54.1-2, 9.54.1-36, 9.54.1-42, 9.54.1-63, and 9.54.1-90 (in **Table 40**) also showed approximately 10-fold improved binding affinity for hVEGF-A (8-109) compared to 9.54.1, as determined by phage competition ELISA. (*See* **FIG. 15**).

[0415] Peptides 9.63 (SEQ ID NO: 55), and 9.63.44 (SEQ ID NO: 125) were selected for further modification as described above. New phage libraries based on these frameworks were generated in which two additional amino acids were added to their C-termini.

[0416] From the 9.63 library, 28 clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 41**). These hits contained variations in amino acid composition within loops 2 and 4.

Table 41: C-terminal Two-residue Extension Variants Based on 9.63

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.63	G**C**DVMQPYWG**C**KQDSD**C**LAG**C**V**C**HWYNS**C**G	55
9.63-1	G**C**DVMQPYWG**C**KQDFD**C**LAG**C**V**C**HWYNS**C**GPS	481
9.63-4	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GFS	482
9.63-7	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GGK	483
9.63-10	G**C**DVMQPYWG**C**KQDFD**C**LAG**C**I**C**HWYNS**C**GYL	484
9.63-16	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GDL	485
9.63-17	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GEK	486
9.63-19	G**C**DVMQPYWG**C**KQDSD**C**LAG**C**I**C**HWYNS**C**GTD	487
9.63-20	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GQV	488
9.63-21	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GRL	489
9.63-22	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GYA	490
9.63-23	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GA**S**	491
9.63-25	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GSR	492
9.63-30	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GPT	493
9.63-36	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GSL	456
9.63-40	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GWD	494
9.63-45	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GSM	495
9.63-61	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GTR	496
9.63-62	G**C**DVMQPYWG**C**KQDSD**C**LAG**C**V**C**HWYNS**C**GEN	497
9.63-65	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GNN	498
9.63-66	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GPE	499
9.63-67	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GGI	500
9.63-68	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GVE	501
9.63-70	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GPL	503
9.63-72	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GTS	527
9.63-74	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GRP	504
9.63-77	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**V**C**HWYNS**C**GND	505
9.63-79	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GLQ	506
9.63-93	G**C**DVMQPYWG**C**EMDFD**C**LAG**C**I**C**HWYNS**C**GDE	507

[0417] From the 9.63.44 library, 17 clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 42**). These hits contained a variation in amino acid composition within loop 4. Clone 9.63.44-55 contained a variation in amino acid composition within loop 2, and clone 9.63.44-10 contained a variation in amino acid composition within loop 3. Interestingly clone 9.63.44-12 in **Table 42** and clone 9.63-70 in Table 41 have the same amino acid sequence.

Table 42: C-terminal Two-residue Extension Variants Based on 9.63.44

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.63.44	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCG	125
9.63.44-2-A	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGRT	508
9.63.44-55	GCDVMQPYWGCEIDFDCLAGCVCHWYNSCGQV	509
9.63.44-10-A	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGGI	510
9.63.44-54	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGYM	511
9.63.44-19	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGGQ	512
9.63.44-44	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGTP	513
9.63.44-14	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGVN	514
9.63.44-73	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGFN	515
9.63.44-16	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGEP	516
9.63.44-80	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGNS	517
9.63.44-41	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGST	518
9.63.44-82	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGRY	519
9.63.44-1	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGFS	520
9.63.44-2	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGQV	521
9.63.44-3	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGYA	522
9.63.44-4	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGSR	523
9.63.44-5	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGPT	524
9.63.44-6	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGSM	525
9.63.44-7	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGGI	526
9.63.44-8	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGTS	527
9.63.44-9	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGLQ	528
9.63.44-10	GCDVMQPYWGCEMDFDCLVGCVCHWYNSCGDE	529
9.63.44-11	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGDL	530
9.63.44-12	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGPL	503
9.63.44-13	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCGQF	531

9.63.44-14	GCDVMQPYWGCEMDFDCLAGCICHWYNSCGWK	532

[0418] Clones 9.63.44-1 through 9.63.44-14 (in **Table 42**) showed improved binding affinity for hVEGF-A (8-109) compared to 9.63.44, as determined by phage competition ELISA. (*See* **FIGS. 16A** and **16B**).

[0419] Taken together, the results above indicate that extending lead peptides 9.54 (SEQ ID NO: 52), 9.54.1 (SEQ ID NO: 99), 9.63 (SEQ ID NO: 55), and 9.63.44 by adding two amino acids to their C-termini produced variants having ~10-fold greater binding affinity for hVEGF-A (8-109).

[0420] Next, peptides 9.54.90 (SEQ ID NO: 102) and 63.12.12.M5L (SEQ ID NO: 386) were selected for further modification as described above. Briefly new phage libraries were generated based on 9.54.90 in which two additional amino acids, three additional amino acids, or four additional amino acids were added at the C-terminus. A second set of libraries was generated based on 63.12.12.M5L in which two additional amino acids were added at the C-terminus.

[0421] From the 9.54.90 libraries comprising 2-amino acid C-terminal extensions, 6 clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 43**).

Table 43: C-terminal Two-residue Extension Variants Based on 9.54.90

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.54.90	GCNIMLPFWGCGRDFECLAGCVCQYYQSCG	102
9.54.90-2x28	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGFH	379
9.54.90-2x2	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGIA	380
9.54.90-2x63	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGGS	381
9.54.90-2x36	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGTR	382
9.54.90-2x90	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGVH	383
9.54.90-2x42	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGLS	384

[0422] From the 9.54.90 libraries comprising 3-amino acid C-terminal extensions, 10 clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 44**).

Table 44: C-terminal Three-residue Extension Variants Based on 9.54.90

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
9.54.90	GCNIMLPFWGCGRDFECLAGCVCQYYQSCG	102
9.54.90-3X83	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPLI	369
9.54.90-3X50	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGNYQ	370
9.54.90-3x49	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPLQ	371
9.54.90-3x10	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGTFQ	372
9.54.90-3x91	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGDLV	373
9.54.90-3x42	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGEHK	374
9.54.90-3x88	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGYLS	375
9.54.90-3x9	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGWDY	376
9.54.90-3x13	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGWPH	377
9.54.90-3x33	GCNIMLPFWGCGRDFECLAGCVCQYYQSCGPHQ	378

[0423] All peptides from the 9.54.90 libraries comprising 4-amino acid C-terminal extensions, contained 3amino acid C-terminal extensions.

[0424] From the 63.12.12.M5L libraries comprising 2-amino acid C-terminal extensions, 9 clones whose binding signals for hVEGF-A (8-109) were more than 3 times higher than to BSA (background) were identified (**Table 45**).

Table 45: C-terminal Two-residue Extension Variants Based on 63.12.12.M5L

CLONE ID	AMINO ACID SEQUENCE	SEQ ID NO
63.12.12.M5L	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**G	386
63.12.12.M5L.2x2	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GRT	387
63.12.12.M5L.2x77	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GWK	388
63.12.12.M5L.2x48	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GPL	389
63.12.12.M5L.2x25	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GDE	390
63.12.12.M5L.2x69	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GQF	391
63.12.12.M5L.2x12	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GEQ	392
63.12.12.M5L.2x30	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GPT	393
63.12.12.M5L.2x21	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GRL	394
63.12.12.M5L.2x29	G**C**DVLQPYWG**C**GPDID**C**LSN**C**I**C**HWYNS**C**GSL	395

Example 2C: Characterization of VEGF-A-binding non-naturally occurring EETI-II variants comprising C-terminal amino acid extensions

[0425] The variants provided in **Tables 43-45** above are assayed via phage competition ELISA as described above to identify variants with greater binding affinity for hVEGF-A (8-109).

[0426] Clones (e.g., such as those provided in **Tables 39-45**) demonstrating greater affinity for hVEGF (8-109), including, *e.g.*, 9.54.1-2, 9.54.1-36, 9.54.1-42, 9.54.1-63, and 9.54.1-90, and 9.63.44-1 – 9.63.44-14, are then selected for further in vitro assessments, such as inhibitory activity in phage competition ELISAs and VEGF-KDR interaction ELISAs, as described above.

[0427] Clones are then analyzed via surface plasmon resonance to determine their affinities for various VEGF isoforms, including hVEGF-A (8-109), hVEGF-A 165, mouse VEGF-A 164, rat VEGF-A, and rabbit VEGF-A.

[0428] Further analyses are performed to assess the clones specificity for VEGF-A. For example, competition ELISAs are performed as described above with VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGf-2, NGF, EGF, PDGF-β, or IGF-1.

[0429] The clones are also assayed for their abilities to inhibit trypsin protease activity as measured in a peptide substrate cleavage assay (Stanger et al. (2014) *FEBS Lett. 588* (23), 4487-96).

[0430] Binding kinetics and affinities of the clones for various hVEGF mutants, including, e.g., Y21A, K48A, Q89A, and F17A/M81A, are determined as described above.

[0431] Next, the clones are assessed for their *in vivo* efficacy in a VEGF-A driven model of choroidal neovascularization, as described above.

[0432] The oxidative stability of the variants is assayed as described above.

Example 3: Generation of Non-naturally Occurring EETI-II variants that bind LRP6

[0433] The naïve EETI-II libraries described in Example 2A were cycled through rounds of selection against LRP6 E1E2 protein. Twenty-two unique clones were identified which bound LRP6 E1E2 (**Table 46**). These initial hits contained variations in amino acid content within loops 1 and 5. In several variants, loop 1 exhibited a longer length compared to that of the native EETI-II framework. Notably, the newly evolved sequences that bound to LRP6 contained a consensus motif in loop 1 (**NXI**) that is similar to a motif (NAI) present within

the native Dkk1 molecules which are endogenous LRP6 ligands. The newly evolved variants recapitulated a motif which occurs in natural ligands.

Table 46: EETI-II-based binders against LRP6 E1E2

VARIANT	LOOP 1	LOOP 5	ELISA	S/N*
LRP6_CKP1	RTNRVKGG (SEQ ID NO: 147)	GPNGF (SEQ ID NO: 19)	3.23	45.49
LRP6_CKP2	VNRVRG (SEQ ID NO: 148)	SGGRD (SEQ ID NO: 169)	3.41	41.62
LRP6_CKP3	MNHVKARR (SEQ ID NO: 149)	GPNGF (SEQ ID NO: 19)	2.93	40.18
LRP6_CKP4	RSVNKI (SEQ ID NO: 150)	GSSRN (SEQ ID NO: 170)	2.82	25.39
LRP6_CKP5	VNKIKG (SEQ ID NO: 151)	GVEGR (SEQ ID NO: 171)	3.04	35.71
LRP6_CKP6	RNSIKR (SEQ ID NO: 152)	SVGHG (SEQ ID NO: 172)	3.10	37.36
LRP6_CKP7	VSNRVNKG (SEQ ID NO: 153)	GPNGF (SEQ ID NO: 19)	3.30	28.96
LRP6_CKP8	RGNIIK (SEQ ID NO: 154)	NESRG (SEQ ID NO: 173)	3.23	37.56
LRP6_CKP9	RSGNTIRKRE (SEQ ID NO: 155)	GGPGG (SEQ ID NO: 174)	2.97	37.62
LRP6_CKP10	ASSNSIRQGW (SEQ ID NO: 156)	GPKSN (SEQ ID NO: 175)	3.29	37.38
LRP6_CKP11	RSNRIR (SEQ ID NO: 157)	YGHGD (SEQ ID NO: 176)	2.65	36.76
LRP6_CKP12	RSNKLREARG (SEQ ID NO: 158)	GSRQD (SEQ ID NO: 177)	0.60	6.78
LRP6_CKP13	VNSVKR (SEQ ID NO: 159)	SRGVN (SEQ ID NO: 178)	3.28	37.75
LRP6_CKP14	GSNKIRPR (SEQ ID NO: 160)	GPNDF (SEQ ID NO: 179)	3.18	43.53
LRP6_CKP15	NRIRNS (SEQ ID NO: 161)	GRGDY (SEQ ID NO: 180)	2.03	26.31
LRP6_CKP16	SRNSIK (SEQ ID NO: 162)	ASGSS (SEQ ID NO: 181)	3.36	31.11
LRP6_CKP17	SNYVKR (SEQ ID NO: 163)	SPGGR (SEQ ID NO: 182)	3.09	35.88
LRP6_CKP18	RANRVSGR (SEQ ID NO: 164)	GPNGF (SEQ ID NO: 19)	1.67	18.32
LRP6_CKP19	SNRVKVRA (SEQ ID NO: 165)	GPNGF (SEQ ID NO: 19)	3.27	41.96
LRP6_CKP20	ENRTKG	GFRGT	3.10	38.69

	(SEQ ID NO: 166)	(SEQ ID NO: 183)		
LRP6_CKP21	GNKIRA (SEQ ID NO: 167)	RDRVG (SEQ ID NO: 184)	2.80	33.69
LRP6_CKP22	ANRVKRTS (SEQ ID NO: 168)	GPNGF (SEQ ID NO: 19)	3.43	42.86

*S/N = signal to noise ratio as compared to BSA control

[0434] The extracellular domain of the LRP6 consists of four propeller domains (E1-E4) that interact with Frizzled receptors and Wnt proteins to propagate Wnt signaling. Utilizing a modular approach, LRP6 distinguishes between Wnt1 or Wnt3a signaling through selective binding of either its E1-E2 or E3-E4 domains to specific Wnt isoforms, respectively (Hannoush et al. (2010) *J. Biol. Chem.* 285, 9172-9179). To pharmacologically delineate Wnt1 and Wnt3a signaling arms, we sought to identify ligands that bind selectively to LRP6 E1-E2.

[0435] Of the identified sequences, R1, LRP6_CKP6 and LRP6_CKP19 were generated in soluble folded form in order to test their pharmacological activity against either Wnt1 or Wnt3a signaling. As shown in **Tables 47** and **48** below, no significant selectivity was observed by R77 towards Wnt1 or Wnt3a in a cell-based signaling reporter assay. On the other hand, R1 and R19 showed selective inhibition towards Wnt1 signaling relative to Wnt3a (160-fold and 11-fold for Wnt1 over Wnt3a, respectively) as measured in a luciferase reporter assay, supporting the notion that these variants do not target the LRP6 E3-E4 domains (IC_{50} > 44 μM). Altogether, the data highlight the specificity of the newly evolved variants and their effects in mimicking a motif which occurs in natural ligands. More importantly, the identified variants provide a pharmacological means to interrogate Wnt1 and Wnt3 signaling.

Table 47: Inhibitory activity of LRP6-binding CKP variants against Wnt1 signaling

Lrp6-CKP (n=4)	Best Fit IC_{50} (nM) for Wnt1 (5ng/well)	95% confidence interval (nM)
R1 F1	241.7	185.8 to 314.5
R1 F2	193.8	140.1 to 268.2
LRP6_CKP6 F1	22,866	13,593 to 38,463
LRP6_CKP6 F2	23,760	14,458 to 39,046
LRP6_CKP6 F3	4,625	3,037 to 7,044
LRP6_CKP19 F1	23,132	15,397 to 34,754

LRP6_CKP19 F2	49,330	31,391 to 77,520

Table 48: Inhibitory activity of LRP6-binding CKP variants against Wnt3a signaling

Lrp6-CKP (n=4)	Best Fit IC_{50} (nM) for Wnt3a (25ng/well)	95% confidence interval (nM)
R1 F1	38,594	16,093 to 92,554
R1 F2	16,596	9,037 to 30,478
LRP6_CKP6 F1	350,240	11840 to $1.036x10^7$
LRP6_CKP6 F2	275,584	24,695 to $3.075x10^6$
LRP6_CKP6 F3	Not converge	Not converge
LRP6_CKP19 F1	59,287	32,600 to 107,823
LRP6_CKP19 F2	69,827	26,179 to 186,252

[0436] F1, F2, and F3 in **Tables 47** and **48** refer to peak fractions 1, 2, and 3, respectively that were obtained during the purification of R1, LRP6_CKP6, and LRP6_CKP19.

The preceding Examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

CLAIMS

WHAT IS CLAIMED IS:

1. A non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A), wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6$ (SEQ ID NO: 2), $X_1X_2X_3X_4X_5X_6X_7$ (SEQ ID NO: 3), $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), $X_1X_2X_3X_4X_5X_6X_7X_8X_9$ (SEQ ID NO: 5), and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 6), wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$ (SEQ ID NO: 7), wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of X_1 – X_3 is any amino acid or an unnatural amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid or an unnatural amino acid;

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$ (SEQ ID NO: 7), wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

wherein the unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine,

benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide;

wherein the CKP binds to VEGF-A with an affinity of 500 pM or better.

2. The CKP of claim 1, wherein the CKP has an altered disulfide bond connectivity-with reference to a wild-type *Ecballium elaterium* trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6.

3. The CKP of claim 1 or claim 2, wherein Z_1 and/or Z_2 is G.

4. The CKP of any one of claims 1-3, wherein in L1:

X_3 is not I;

X_5 is not M; and/or

X_6 is not R.

5. The CKP of any one of claims 1-4, wherein in L1:

X_1 is an amino acid selected from P, Q, R, T, V, D, N, K, L, and X;

X_2 is an amino acid selected from T, D, L, V, I, R, P, N and X;

X_3 is an amino acid selected from T, P, M, L, S, F, R, and X;

X_4 is an amino acid selected from R, T, Q, D, W, L, E, S, K, and X;

X_5 is an amino acid selected from F, P, V, E, K, L, I, and X;

X_6 is an amino acid selected from K, N, F, P, L, Y, T, D, M, and X;

X_7 is an amino acid selected from Q, W, H and/X; and/or

X_8 is an amino acid selected from Y, A, G, D, E, W, S, and X,

wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine,

gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

6. The CKP of claim 5, wherein in L1:

X_9 is an amino acid selected from L, I, V, D, E and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-

L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

7. The CKP of claim 5 or claim 6, wherein in L1:

X_{10} is an amino acid selected from Y, T, M, N, F, and X, wherein X is and unnatural amino acid is selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

8. The CKP of any one of claims 1-7 wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P).

9. The CKP of any one of claims 1-7 wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G).

10. The CKP of any one of claims 1-7, wherein in L5:

X_1 is an amino acid selected from G, Q, H, R, L, and Q;

X_2 is an amino acid selected from P, M, W, Y, F, L, and H;

X_3 is an amino acid selected from N, F, H, and Y;

X_4 is an amino acid selected from G, Q, D, N, K, H, E, and S; and/or

X_5 is an amino acid selected from F, S, and T.

11. The CKP of any one of claims 1-5, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), and wherein:

X_1 is an amino acid selected from P, Q, and R;

X_2 is an amino acid selected from T, L, and D;

X_3 is an amino acid selected from T, M and L;

X_4 is an amino acid selected from R, Q, and D;

X_5 is an amino acid selected from F, P, and V;

X_6 is an amino acid selected from K and F;

X_7 is an amino acid selected from Q and W; and

X_8 is an amino acid selected from Y, G, and D.

12. The CKP of any one of claims 1-7, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9X_{10} (SEQ ID NO: 6), and wherein:

X_1 is an amino acid selected from Q, R, T and V;

X_2 is an amino acid selected from T and D;

X_3 is P;

X_4 is an amino acid selected from T and W;

X_5 is an amino acid selected from F, E, P, and K;

X_6 is an amino acid selected from N and P;

X_7 is an amino acid selected from W and H; and

X_8 is an amino acid selected from A, D, E, and W;

X_9 is an amino acid selected from L and I; and

X_{10} is an amino acid selected from Y, T, M and N.

13. The CKP of claim 11 or claim 12, wherein in L5:

X_1 is an amino acid selected from G, H, and Q;

X_2 is an amino acid selected from P, M, W, and Y;

X_3 is an amino acid selected from N and Y;

X_4 is an amino acid selected from G, Q, and S; and

X_5 is an amino acid selected from F and S.

14. The CKP of any one of claims 1-5, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), and wherein:

X_1 is an amino acid selected from D, Q, N, and K;

X_2 is an amino acid selected from V, I, R, L, and P;

X_3 is an amino acid selected from L, S, M, T, and F;

X_4 is an amino acid selected from Q, L, and E;

X_5 is P;

X_6 is an amino acid selected from F, L, and Y;

X_7 is W; and

X_8 is G.

15. The CKP of claim 14, wherein in L5:

X_3 is Y;

X_5 is S; and

wherein X_1, X_2 and X_4 are each any amino acid, with the exception that X_1 is not G, X_2 is not P, X_4 is not G, and/or X_5 is not F.

16. The CKP of claim 14 or claim 15, wherein in L5:

X_1 is an amino acid selected from H, L, R, and Q;

X_2 is an amino acid selected from W, F, and Y;

X_3 is Y;

X_4 is an amino acid selected from Q, N, K, H, and E; and

X_5 is S.

17. The CKP of any one of claims 1-7, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ X_9 X_{10} (SEQ ID NO: 6), and wherein:

X_1 is an amino acid selected from K, Q, L, and R;

X_2 is an amino acid selected from N and D;

X_3 is an amino acid selected from P and L;

X_4 is an amino acid selected from L, T, S and K;

X_5 is an amino acid selected from F, V, I, and L;

X_6 is an amino acid selected from N and D;

X_7 is W;

X_8 is an amino acid selected from A and S;

X_9 is an amino acid selected from L, V, E and D; and

X_{10} is an amino acid selected from Y and F.

18. The CKP of claim 17, wherein in L5:

X_1 is Q;

X_2 is an amino acid selected from L, F, M, and H;

X_3 is an amino acid selected from F, Y, and H;

X_4 is an amino acid selected from D, Q, N, and K; and

X_5 is an amino acid selected from S and T.

19. The CKP of any one of claims 1-16, wherein in L2, X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D.

20. The CKP of any one of claim 1-4, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), and wherein:

X_5 is P;

X_7 is W;

X_8 is G; and

wherein X_1, X_2, X_3, X_4 and X_6 are each any amino acid, with the exception that X1 is not P, X_2 is not R, X_3 is not I, and/or X_6 is not R.

21. The CKP of any one of claims 1-5 and 18, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), and wherein:

X_1 is an amino acid selected from N and D;

X_2 is an amino acid selected from I and V;

X_3 is an amino acid selected from M and L;

X_4 is an amino acid selected from L, Q, D and K;

X_5 is P;

X_6 is an amino acid selected from F, Y, T, L, and M;

X_7 is W; and

X_8 is G.

22. The CKP of any one of claims 14-15 and 20-21, wherein in L5:

X_1 is an amino acid selected from Q, H, L, and R;

X_2 is an amino acid selected from Y and W;

X_3 is Y;

X_4 is an amino acid selected from Q and N; and

X_5 is S.

23. The CKP of claim 14-15 and 20-22, wherein in L5:

X_3 is Y;

X_5 is S; and

wherein X_1, X_2, and X_4 are each any amino acid, with the exception that:

X_1 is not G, X_2 is not P, and/or X_4 is not G.

24. The CKP of claim 20-23, wherein in L2:

X_1 is an amino acid selected from G or E;

X_2 is an amino acid selected from Q, L, P, R, E, and M;

X_3 is an amino acid selected from S, D, and N;

X_4 is an amino acid selected from F, Y, L, M, and I; and/or

X_5 is an amino acid selected from E, D, Q, L, and S.

25. The CKP of any of claims 1-22, wherein in L3, X_1 is L, X_2 is A, and X_3 is G.

26. The CKP of any of claims 1-23, wherein in L4, X1 is V or F.

27. The CKP of any one of claims 1-7, 11-12, 14, 17, 19-21, and 24-26, wherein in L5, each of X_1 – X_5 is any amino acid with the exception that X_2 is not proline (P).

28. The CKP of any one of claims 1-7, 11-12, 14, 17, 19-21, and 24-26 wherein in L5, each of X_1 – X_5 is any amino acid with the exception that X_4 is not glycine (G).

29. The CKP of any one of claims 1-7, 11-12, 14, 17, 19-21, and 24-28, wherein in L5:

X_1 is any amino acid except G;

X_2 is any amino acid except P;

X_3 is any amino acid except N;

X_4 is any amino acid except G; and/or

X_5 is any amino acid except F.

30. The CKP of any one of claims 1-5, wherein L1 has the structure $X_1X_2X_3X_4X_5X_6X_7X_8$ (SEQ ID NO: 4), and wherein:

X_1 is an amino acid selected from N, D, and X;

X_2 is an amino acid selected from I, V, and X;

X_3 is M or X;

X_4 is an amino acid selected from L, Q, and X;

X_5 is P or X;

X_6 is F, Y, or X;

X_7 is W or X; and

X_8 is G or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

31. The CKP of claim 28, wherein in L3, each of $X_1 - X_3$ is any amino acid or unnatural amino acid with the exception that X_1 is not Leucine (L), X_2 is not Alanine (A), and X_3 is not glycine (G), wherein the unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

32. The CKP of claim 28 or claim 29, wherein in L3:

X_1 is an amino acid selected from M, F, L V, and X;

X_2 is an amino acid selected from S, N, Q, I, Y, E, V, T, and X;

X_3 is an amino acid selected from D, Q, T, N, E, R, and X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-

L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

33. The CKP of any one of claims 28-30, wherein in L4, X_1 is any amino acid except V or an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

34. The CKP of any one of claims 28-31, wherein in L4, X_1 is I, L, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-

chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

35. The CKP of any one of claims 28-32, wherein in L5:

X_3 is Y or X;

X_5 is S or X; and

wherein X_1, X_2, and X_4 are each any amino acid or X, with the exception that X_1 is not G, X_2 is not P, and/or X_4 is not G, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine,

t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

36. The CKP of any one of claims 1-7, 11-12, 14, 17, 19-21, and 24-26, wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P).

37. The CKP of any one of claims 1-7, 11-12, 14, 17, 19-21, and 24-26 wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G).

38. The CKP of any one of claims 28-33, wherein in L5:

X_1 is an amino acid selected from Q, H, and X;

X_2 is an amino acid selected from Y, W, and X;

X_3 is Y or X;

X_4 is an amino acid selected from Q, N, or X;

X_5 is S or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine,

D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

39. The CKP of any one of claims 28-34, wherein in L2:

X_1 is G or X;

X_2 is R, P, or X;

X_3 is D or X;

X_4 is F, I, or X; and

X_5 is E, D, or X, wherein X is an unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide.

40. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECLQQCICQYYQSCG (SEQ ID NO: 103).

41. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECVERCICQYYQSCG (SEQ ID NO: 104).

42. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 105).

43. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECMNQCICQYYQSCG (SEQ ID NO: 106).

44. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECMQTCICQYYQSCG (SEQ ID NO: 107).

45. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECVYQCICQYYQSCG (SEQ ID NO: 108).

46. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECFINCICQYYQSCG (SEQ ID NO: 109).

47. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 110).

48. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECVTECICQYYQSCG (SEQ ID NO: 111).

49. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECFYECICQYYQSCG (SEQ ID NO: 112).

50. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113).

51. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114).

52. The CKP of any one of claims 28-35, comprising the amino acid sequence GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139).

53. The CKP of any one of claims 28-35, comprising the amino acid sequence GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140).

54. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142).

55. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle).

56. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle).

57. The CKP of any one of claims 28-35, comprising the amino acid sequence GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle).

58. The CKP of any one of claims 28-35, comprising the amino acid sequence GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine.

59. A non-naturally occurring cystine knot peptide (CKP) comprising an amino acid selected from the group consisting of: GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113), GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114), GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139), GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140), GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142), GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle), GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle), GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle), and GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine.

60. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIMLPFWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 113).

61. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIMLPFWGCGRDFECVYRCICQYYQSCG (SEQ ID NO: 114).

62. The CKP of claim 59, comprising the amino acid sequence set forth in GCDVMQPYWGCGPDIDCFVRCLCHWYNSCG (SEQ ID NO: 139).

63. The CKP of claim 59, comprising the amino acid sequence set forth in GCDVMQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 140).

64. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIMLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 142).

65. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIXLPFWGCGRDFECMSDCICQYYQSCG (SEQ ID NO: 144), wherein X is norleucine (Nle).

66. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIXLPFWGCGRDFECVSQCICQYYQSCG (SEQ ID NO: 145), wherein X is norleucine (Nle).

67. The CKP of claim 59, comprising the amino acid sequence set forth in GCNIXLPYWGCGRDFECMEQCICQYYQSCG (SEQ ID NO: 146), wherein X is norleucine (Nle).

68. The CKP of claim 59, comprising the amino acid sequence set forth in GCDVXQPYWGCGPDIDCLSNCICHWYNSCG (SEQ ID NO: 224), wherein X is norleucine.

69. The CKP of any one of claims 59-68, wherein the CKP binds VEGF-A.

70. A non-naturally occurring cystine knot peptide (CKP) comprising an amino acid selected from the group consisting of: GCDVX_1QPYWGCGPDI-D/E-CLS-N/K/X_2-CICHWYNSCG (SEQ ID NO: 534), GCDVX_1QPYWGCGPDI-N/K/X_2-CLS-D/E-CICHWYNSCG (SEQ ID NO: 535), GCNIX_1LPYWGCGRDF-D/E-CME-N/K/X_2-CICQYYQSCG (SEQ ID NO: 538), GCNIX_1LPYWGCGRDF-N/K/X_2-CME-D/E-CICQYYQSCG (SEQ ID NO: 539), GCNIX_1LPFWGCGRDF-D/E-CVS-N/K/X_2-CICQYYQSCG (SEQ ID NO: 540), and GCNIX_1LPFWGCGRDF-N/K/X_2-CVS-D/E-CICQYYQSCG (SEQ ID NO: 541), wherein X_1 is norleucine and X_2 is ornithine.

71. The CKP of claim 70, comprising the amino acid sequence set forth in GCDVX_1QPYWGCGPDI-D/E-CLS-N/K/X_2-CICHWYNSCG (SEQ ID NO: 534), wherein X_1 is norleucine and X_2 is ornithine.

72. The CKP of claim 71, comprising the amino acid sequence set forth in GCDVXQPYWGCGPDIDCLSKCICHWYNSCG (SEQ ID NO: 536), wherein X is norleucine.

73. The CKP of claim 71, comprising the amino acid sequence set forth in GCDVX_1QPYWGCGPDIDCLSX_2CICHWYNSCG (SEQ ID NO: 537), wherein X_1 is norleucine and X_2 is ornithine.

74. The CKP of claim 70, comprising the amino acid sequence set forth in GCDVX_1QPYWGCGPDI-N/K/X_2-CLS-D/E-CICHWYNSCG (SEQ ID NO: 535), wherein X_1 is norleucine and X_2 is ornithine.

75. The CKP of claim 70, comprising the amino acid sequence set forth in GCNIX_1LPYWGCGRDF-D/E-CME-N/K/X_2-CICQYYQSCG (SEQ ID NO: 538), wherein X_1 is norleucine and X_2 is ornithine.

76. The CKP of claim 75, comprising the amino acid sequence set forth in GCNIXLPYWGCGRDFECMEKCICQYYQSCG (SEQ ID NO: 543), wherein X is norleucine.

77. The CKP of claim 75, comprising the amino acid sequence set forth in GCNIX$_1$LPYWGCGRDFECMEX$_2$CICQYYQSCG (SEQ ID NO: 544), wherein X$_1$ is norleucine and X$_2$ is ornithine.

78. The CKP of claim 70, comprising the amino acid sequence set forth in GCNIX$_1$LPYWGCGRDF-N/K/X$_2$-CME-D/E.CICQYYQSCG (SEQ ID NO: 539), wherein X$_1$ is norleucine and X$_2$ is ornithine.

79. The CKP of claim 70, comprising the amino acid sequence set forth in GCNIX$_1$LPFWGCGRDF-D/E-CVS-N/K/X$_2$-CICQYYQSCG (SEQ ID NO: 540), wherein X$_1$ is norleucine and X$_2$ is ornithine.

80. The CKP of claim 79, comprising the amino acid sequence set forth in GCNIXLPFWGCGRDFECVSKCICQYYQSCG (SEQ ID NO: 545), wherein X is norleucine.

81. The CKP of claim 79, comprising the amino acid sequence set forth in GCNIX$_1$LPFWGCGRDFECVSX$_2$CICQYYQSCG (SEQ ID NO: 546), wherein X$_1$ is norleucine and X$_2$ is ornithine.

82. The CKP of claim 70, comprising the amino acid sequence set forth in GCNIX$_1$LPFWGCGRDF-N/K/X$_2$-CVS-D/E-CICQYYQSCG (SEQ ID NO: 541), wherein X$_1$ is norleucine and X$_2$ is ornithine.

83. The CKP of any one of claims 70-82, wherein the CKP binds VEGF-A.

84. A non-naturally occurring cystine knot peptide (CKP) that binds to VEGF-A, wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2$$

wherein:

Z$_1$ and Z$_2$ are any amino acid, more than one amino acid, or an unnatural amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid or an unnatural amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of X_1 – X_3 is any amino acid or an unnatural amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid or an unnatural amino acid; L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid or an unnatural amino acid;

wherein the unnatural amino acid selected from the group consisting of L-propargylglycine-PEG_6-, L-sulfotyrosine, L-norleucine, L-1-naphthylalanine, L-2-naphthylalanine, L-2-chlorotryptophan, L-3-fluorotyrosine, L-4-fluorophenylalanine, gamma-benzyl-L-proline, gamma-(4-fluoro-benzyl)-L-proline, 4-OH-L-proline, 4-fluoro-L-proline, 4-[4-(trifluoromethyl)-benzyl]-L-proline, 3,4-difluoro-L-phenylalanine, 3,4-dichloro-L-phenylalanine, 4-chloro-L-phenylalanine, 3-F,4-Cl-L-phenylalanine, 2-pyridone(NH para)-L-alanine, pyridone(NH meta)-L-alanine, 3-(1-N-methyl indole)-L-alanine, 3-(1-N-ethyl indole)-L-alanine, 3-(1-N-isopropyl indole)-L-alanine, 3-(5-aza-indole)-L-alanine, 4-methyl-L-phenylalanine, 2-naphthyl-L-alanine, L-4,4'-biphenylalanine, 3-(3-quinolinyl)-L-alanine, 3-(2-quinolinyl)-L-alanine, 3-(2-quinoxalinyl)-L-alanine, 4-methyl-2-pyridyl-alanine, 4-ethyl-2-pyridyl-L-alanine, benzothiazole-L-alanine, benzothiophene-L-alanine, 3-isoquinolinyl-L-alanine, t-butyl-L-alanine (also known as L-Nepentyl glycine), 3-cyclobutyl-L-alanine, cyclopentyl-L-alanine, 5,5,5-Trifluoro-L-leucine, t-butyl-L-glycine (also known as L-tert-Leucine), L-cyclopentylglycine, L-cyclobutylglycine, 3,4-hydroxy-L-phenylalanine, 3,4-fluoro-L-phenylalanine, 3-fluoro,4-OH-L-phenylalanine, 2-chloro-L-tyrosine, 2-methyl-L-tyrosine, 2-ethyl-L-tyrosine, 4-(naphthalen-1-ol)-L-alanine, D-serine, L-beta-homoserine, L-beta-alanine, N-alpha-methyl glycine, glycine amide, glycine ester of glycerol, glycine ester of glycol, glycine ester of oxetane-3-yl, and glycine morpholine amide, and

wherein the CKP has an altered disulfide bond connectivity with reference to a wild-type *Ecballium elaterium* trypsin inhibitor EETI-II protein having the amino acid sequence set forth in SEQ ID NO: 1; wherein the altered disulfide bond connectivity is C1-C4, C2-C3 and C5-C6; and wherein the CKP has a percent alpha helix content of at least 20%.

85. The CKP of claim 84, wherein the CKP binds to VEGF-A with an affinity of 500 pM or less.

86. The CKP of claims 1-58 and 84-85, wherein the binding affinity is determined via surface plasmon resonance.

87. The CKP of any one of claims 84-86, wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_2 is not proline (P).

88. The CKP of any one of claims 84-86, wherein in L5, each of $X_1 - X_5$ is any amino acid with the exception that X_4 is not glycine (G).

89. The CKP of any one of claims 1-88, wherein:

(a) the C-terminal carboxyl group of the peptide is capped;

(b) the N-terminal amine of the peptide (CKP) is capped; or

(c) the C-terminal carboxyl group and the N-terminal amine of the peptide (CKP) is capped.

90. The CKP of claim 89, wherein:

(a) the C-terminal carboxyl group of the peptide is amidated;

(b) the N-terminal amine of the peptide (CKP) is acetylated; or

(c) the C-terminal carboxyl group of the peptide is amidated and the N-terminal amine of the peptide (CKP) is acetylated.

91. The CKP of any one of claims 1-90, wherein the CKP inhibits VEGF-A activity.

92. The CKP of claims 1-91, wherein the CKP inhibits VEGF-A activity with and IC_{50} between about 0.5 nM and about 1.0 nM.

93. The CKP of any one of claims 1-92, wherein the non-naturally occurring EETI-II scaffold protein binds human VEGF-A, mouse VEGF-A, and rat VEGF-A.

94. A non-naturally occurring CKP that competes with the antibody G6.31 for binding to VEGF-A.

95. A non-naturally occurring CKP that competes with the CKP of any one of claims 1-94 for binding to VEGF-A.

96. A non-naturally occurring CKP that binds to an epitope on VEGF-A comprising at least one of the amino acid residues selected from the group consisting of: V14, V15, F17, D19,Y21, Q22, Y25, I46, K48, N62, D63, L66, M81, I83, K84, P85, H86, G88, Q89, I91, C104, R105, and P106.

97. The CKP of claim 96, wherein the residues are selected from the group consisting of: K48, N62, and D63.

98. The CKP of claim 96, wherein the residues are selected from the group consisting of:
Y21, Y25, and P106.

99. The CKP of claim 96, wherein the residues are selected from the group consisting of: H86 and Q89.

100. The CKP of claim 96, wherein the residues are selected from the group consisting of: M81, D19, and Q22.

101. The CKP of claim 96, wherein the residues are selected from the group consisting of: F17, M81, and I91.

102. The CKP of claim 96, wherein the residues are selected from the group consisting of: V14, F17, D19, Q22, M81, and I91.

103. The CKP of claim 96, wherein the residues are selected from the group consisting of: Y25.

104. The CKP of any one of claims 1-103 conjugated to a therapeutic agent.

105. The CKP of any one of claims 1-103 conjugated to a label.

106. The CKP of claim 105, wherein the label is selected from the group consisting of a radioisotope, a fluorescent dye, and an enzyme.

107. An isolated nucleic acid encoding the CKP of any one of claims 1-103.

108. An expression vector encoding the nucleic acid molecule of claim 107.

109. A cell comprising the expression vector of claim 108.

110. A method of producing the CKP of any one of claims 1-103 comprising culturing the cell of claim 109 and recovering the CKP from the cell culture.

111. A method of producing the CKP of any one of claims 1-103, comprising chemically synthesizing the CKP.

112. A composition comprising the CKP of any one of claims 1-104 and a pharmaceutically acceptable carrier.

113. A method of treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject, comprising administering an effective amount of the CKP of any one of claims 1-104 or the composition of claim 112 to the subject.

114. The method of claim 113, wherein the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP).

115. The method of any one of claims 113 or 114, wherein the CKP or the composition is administered to the subject via an implantable device.

116. The method of claim 115, wherein the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

117. A composition comprising the CKP of any one of claims 1-104, for use in treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject.

118. The composition for use according to claim 117, wherein the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP).

119. The composition for use according to claim 117 or 118, wherein the composition is administered to the subject via an implantable device.

120. The composition for use according to claim 119, wherein the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

121. Use of a composition comprising the CKP of any one of claims 1-104 for treating an ocular disease characterized by angiogenesis and/or vascular permeability or leakage in a subject.

122. The use according to claim 121, wherein the ocular disease is an intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and

other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization, and retinopathy of prematurity (ROP).

123. The use according to claim 121 or 122, wherein the medicament is administered to the subject via an implantable device.

124. The use according to claim 123, wherein the implantable device selected from the group consisting of: an ocular insert, a slow-release depot, an ocular plug/reservoir, an non-biodegradable ocular implant or a biodegradable ocular implant.

125. The CKP of any one of claims 1-104, wherein the CKP is formulated for long acting delivery.

126. A formulation comprising the CKP of any of claims 1-104 and PLGA.

127. The formulation of claim 126, wherein the PLGA is a PLGA rod.

128. A non-naturally occurring cystine knot peptide (CKP) that binds to human low density lipoprotein receptor-related protein 6 (LRP6), wherein the CKP comprises the cystine scaffold structure:

$$Z_1C_1L1C_2L2C_3L3C_4L4C_5L5C_6Z_2;$$

wherein:

Z_1 and Z_2 are any amino acid;

L1 is Loop 1 and has a structure selected from the group consisting of: $X_1X_2X_3X_4X_5X_6$, $X_1X_2X_3X_4X_5X_6X_7$, $X_1X_2X_3X_4X_5X_6X_7X_8$, $X_1X_2X_3X_4X_5X_6X_7X_8X_9$, and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}$, wherein each of X_1 - X_{10} is any amino acid;

L2 is Loop 2 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of X_1 – X_5 is any amino acid;

L3 is Loop 3 and has the structure: $X_1X_2X_3$ wherein each of X_1 – X_3 is any amino acid;

L4 is Loop 4 and has the structure: X_1, wherein X_1 is any amino acid; and

L5 is Loop 5 and has the structure: $X_1X_2X_3X_4X_5$, wherein each of $X_1 - X_5$ is any amino acid.

129. The CKP of claim 128, wherein Z_1 and/or Z_2 is G.

130. The CKP of any one of claims 128 or 129, wherein in L1:
X_1 is an amino acid selected from R, V, M, A, G, N, S, and E;
X_2 is an amino acid selected from T, N, S, G, R, and A;
X_3 is an amino acid selected from N, R, H, V, K, S, G, I, and Y;
X_4 is an amino acid selected from R, V, N, I, K, S, and T;
X_5 is an amino acid selected from V, R, K, I, T, S, L, and N; and
X_6 is an amino acid selected from K, G, A, I, R, N, S, and V.

131. The CKP of any one of claims 128-130, wherein in L1:
X_7 is an amino acid selected from G, R, K, E, P, and T.

132. The CKP of any one of claims 128-131, wherein in L1:
X_8 is an amino acid selected from G, R, K, Q, A, and S.

133. The CKP of any one of claims 128-132, wherein in L1:
X_9 is an amino acid selected from R or G.

134. The CKP of any one of claims 128-133, wherein in L1:
X_{10} is an amino acid selected from E, W, and G.

135. The CKP of any one of claims 128-134, wherein in L5:
X_1 is an amino acid selected from G, S, N, Y, A, and R;
X_2 is an amino acid selected from P, G, S, V, E, R, F, and D;
X_3 is an amino acid selected from N, G, S, E, P, K, H, and R;
X_4 is an amino acid selected from G, R, H, S, Q, V, and D; and
X_5 is an amino acid selected from F, D, N, R, G, Y, S, and T.

136. The CKP of any one of claims 128-135, wherein in L2, X_1 is K, X_2 is Q, X_3 is D, X_4 is S, and X_5 is D.

137. The CKP of any one of claims 128-136, wherein in L3, X_1 is L, X_2 is A, and X_3 is G.

138. The CKP of any one of claims 128-137, wherein in L4, X_1 is V.

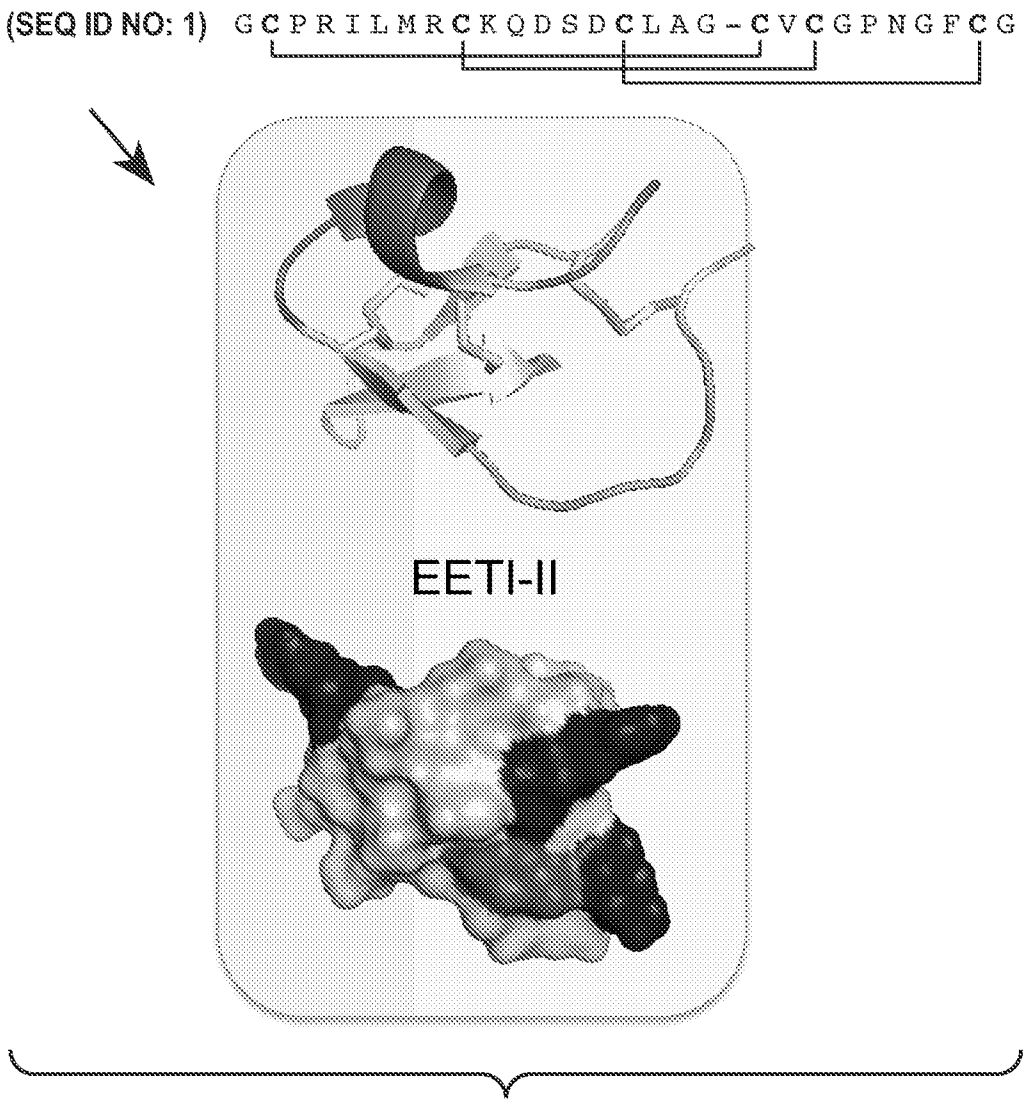


FIG. 1

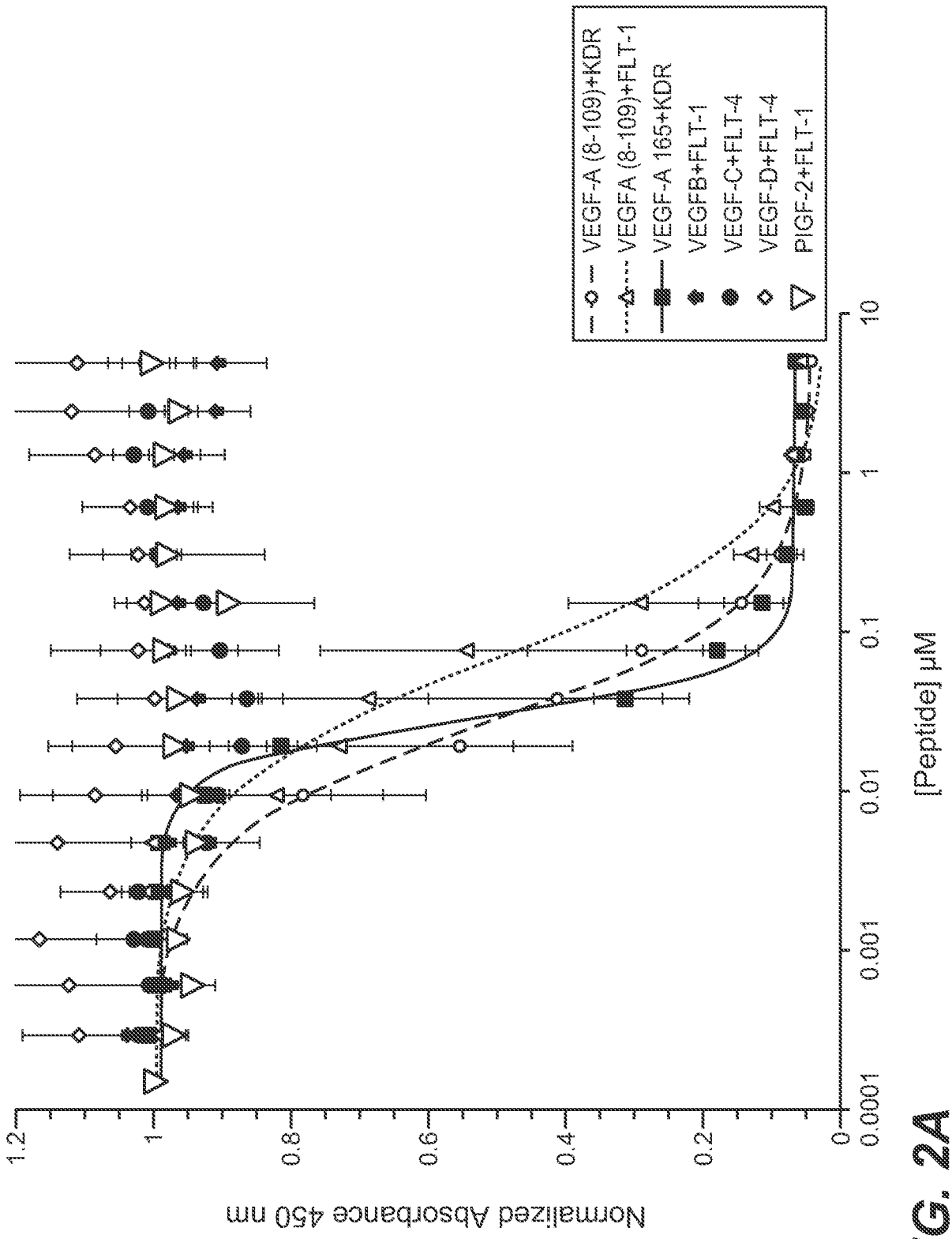

VEGF-A (8-109)+KDR
VEGFA (8-109)+FLT-1
VEGF-A 165+KDR
VEGFB+FLT-1
VEGF-C+FLT-4
VEGF-D+FLT-4
PlGF-2+FLT-1
Normalized Absorbance 450 nm
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
10
[Peptide] µM

FIG. 2A

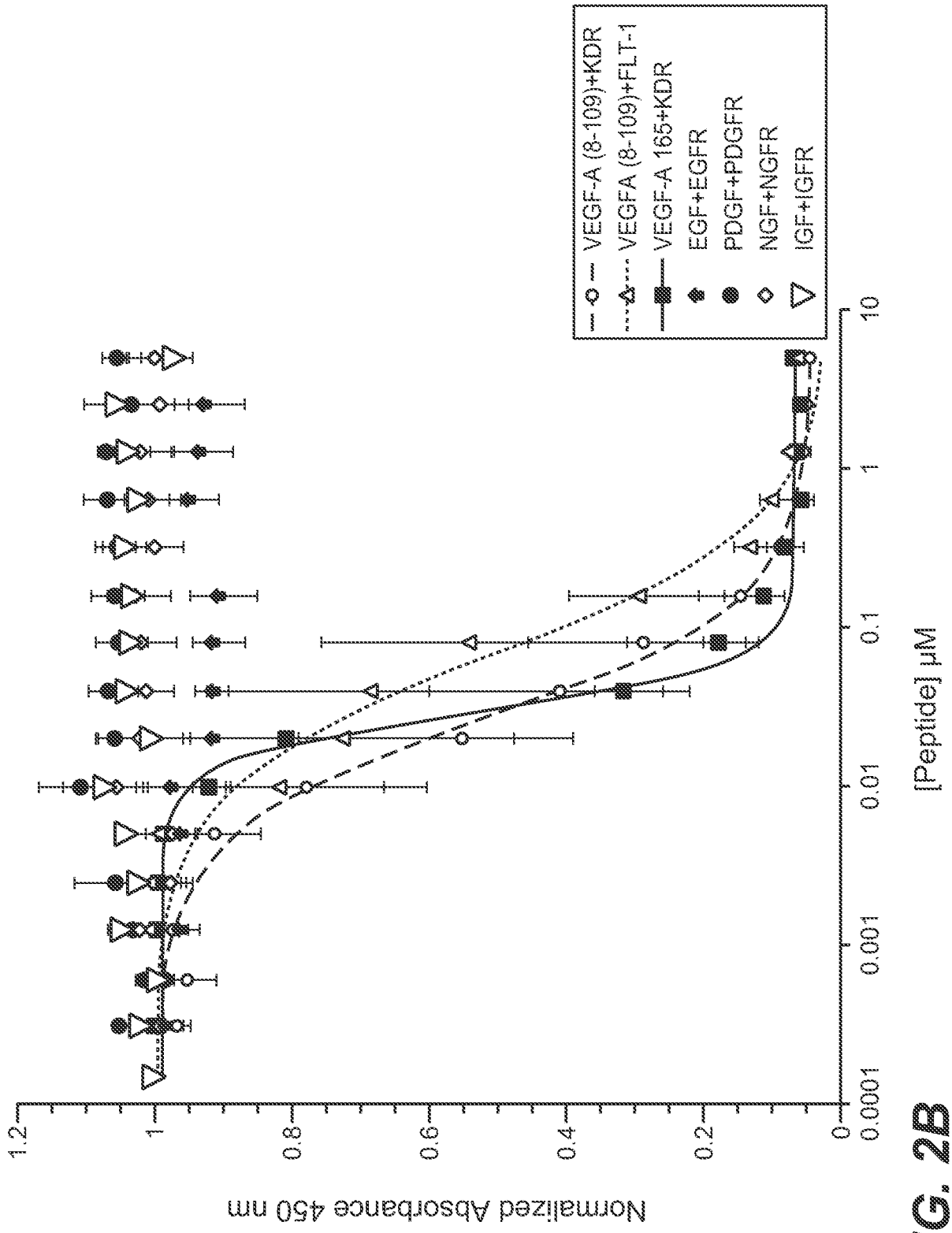

VEGF-A (8-109)+KDR
VEGFA (8-109)+FLT-1
VEGF-A 165+KDR
EGF+EGFR
PDGF+PDGFR
NGF+NGFR
IGF+IGFR
Normalized Absorbance 450 nm
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.001
0.01
0.1
1
10
[Peptide] µM

FIG. 2B

CKP	Loop1	Loop2	Loop5	
WT EETI	GCPRILMR	CKQDSDCLAGCVCGPNGFCG		SEQ ID NO: 1
E9	GCQLMQPFWGCKQDSDCLAGCVCHWYQSCG			SEQ ID NO: 23
EM54	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG			SEQ ID NO: 52
V_L2.9.54.90	GCNIMLPFWGCGRDFECLAGCVCQYYQSCG			SEQ ID NO: 102

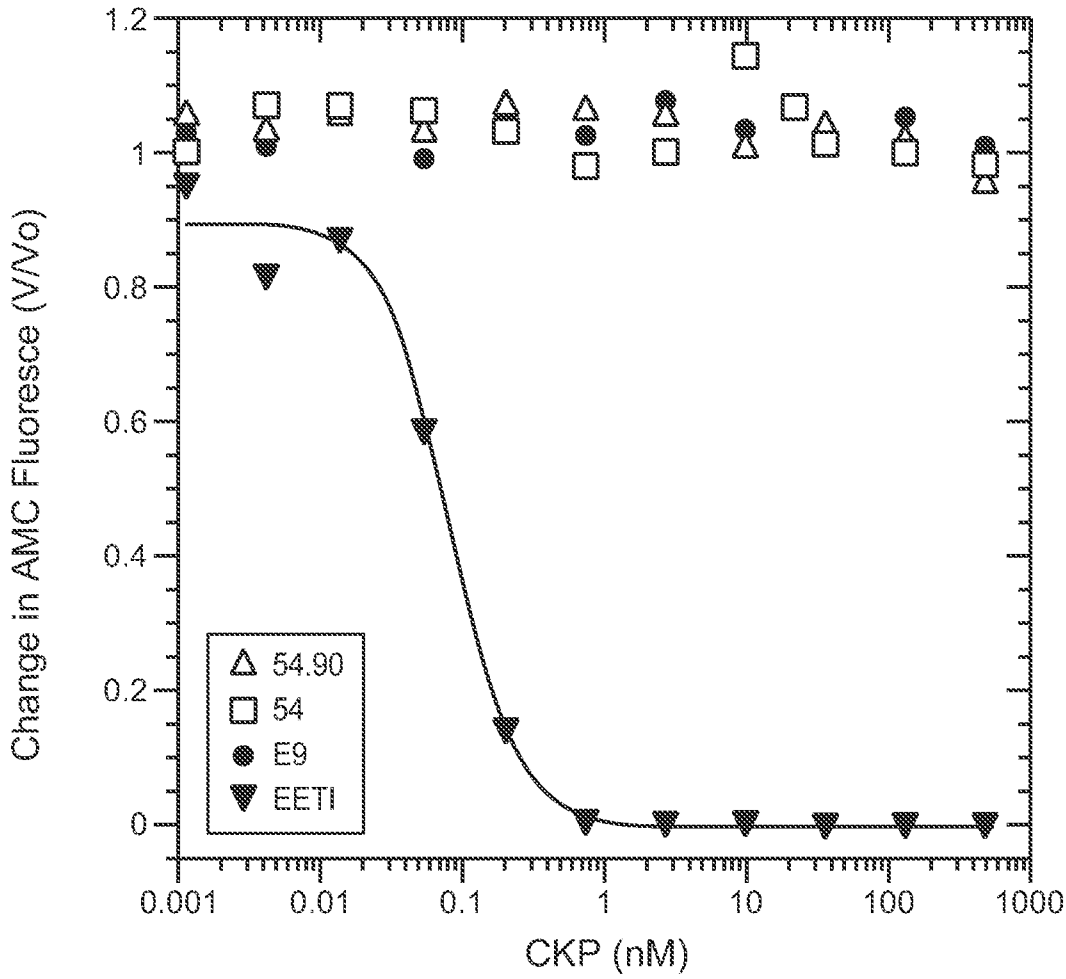
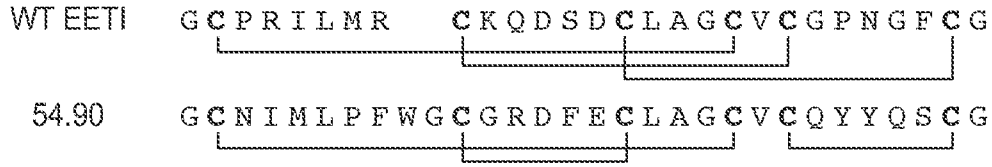


FIG. 3



	CKP	Loop1	Loop2	Loop5	
	E9	GCQLMQPFWGCKQDSDCLAGCVCHWYQSCG			SEQ ID NO: 23
	EM54	GCNIMLPFWGCKQDSDCLAGCVCQYYQSCG			SEQ ID NO: 52
*	V_L2.9.54.1	GCNIMLPFWGCGQSFECLAGCVCQYYQSCG			SEQ ID NO: 99
	V_L2.9.54.90	GCNIMLPFWGCGRDFECLAGCVCQYYQSCG			SEQ ID NO: 102
	EM63	GCDVMQPYWGCKQDSDCLAGCVCHWYNSCG			SEQ ID NO: 55
*	V_L2.9.63.1	GCDVMQPYWGCGENFLCLAGCVCHWYNSCG			SEQ ID NO: 122
*	V_L2.9.63.44	GCDVMQPYWGCEMDFDCLAGCVCHWYNSCG			SEQ ID NO: 125
*	V_L2.9.63.12	GCDVMQPYWGCGPDIDCLAGCVCHWYNSCG			SEQ ID NO: 123

* : No trypsin cutting sites

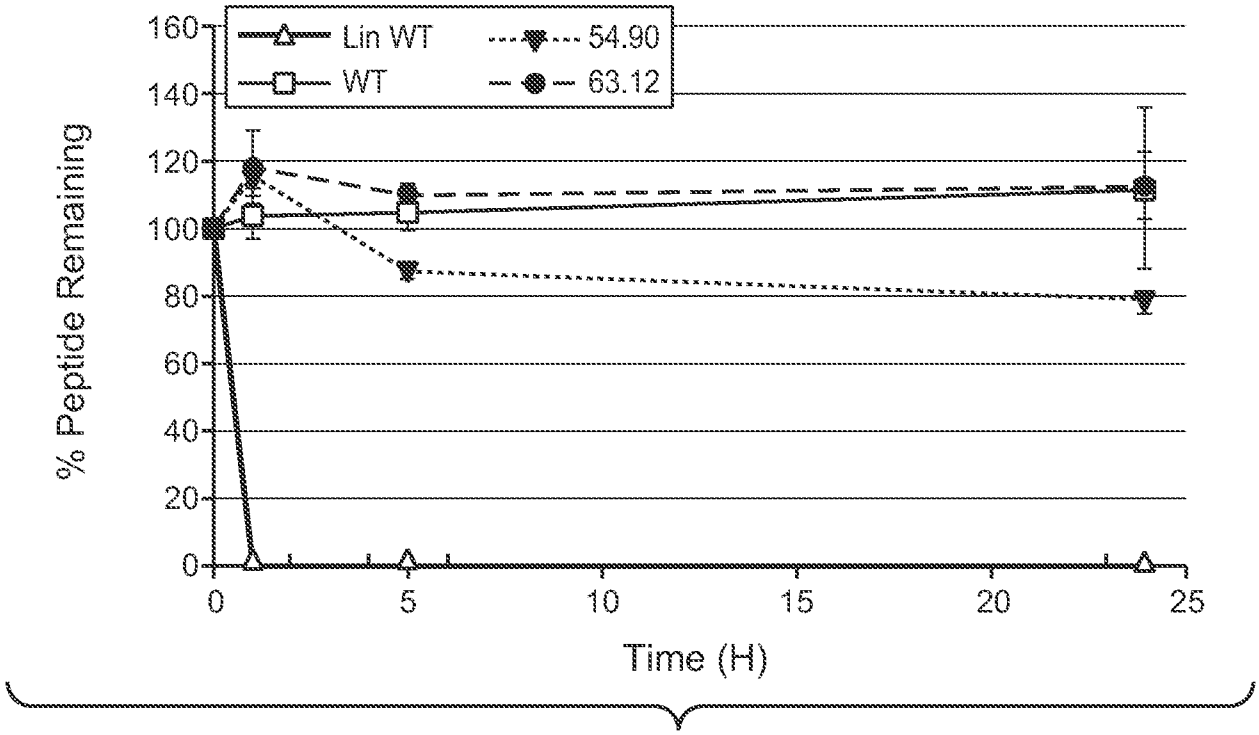


FIG. 4

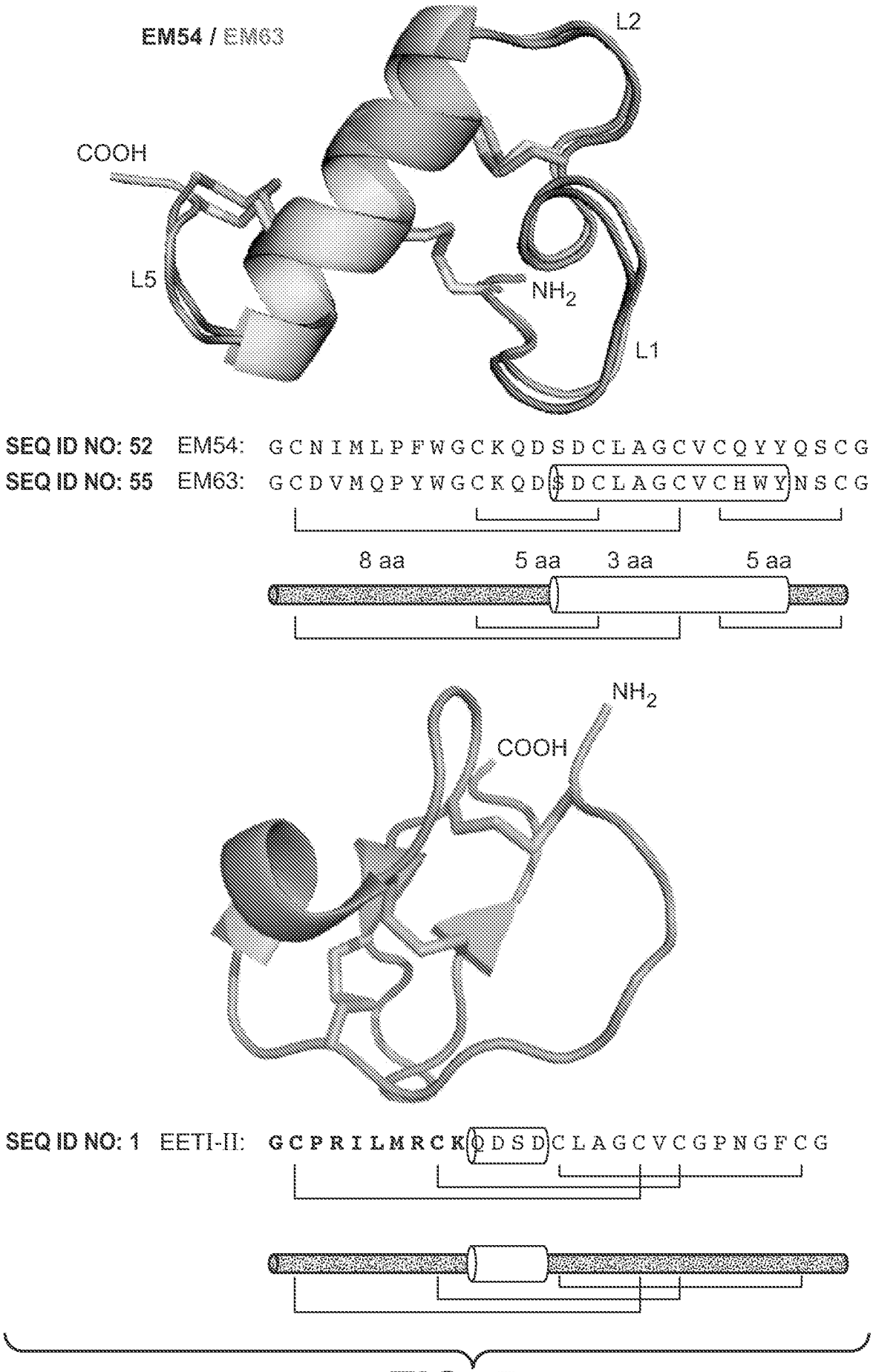

EM54 / EM63
L2
COOH
L5
NH2
L1
SEQ ID NO: 52 EM54: G C N I M L P F W G C K Q D S D C L A G C V C Q Y Y Q S C G
SEQ ID NO: 55 EM63: G C D V M Q P Y W G C K Q D $ D C L A G C V C H W Y N S C G
8 aa
5 aa
3 aa
5 aa
NH2
COOH
SEQ ID NO: 1 EETI-II: G C P R I L M R C K Q D S D C L A G C V C G P N G F C G

FIG. 5

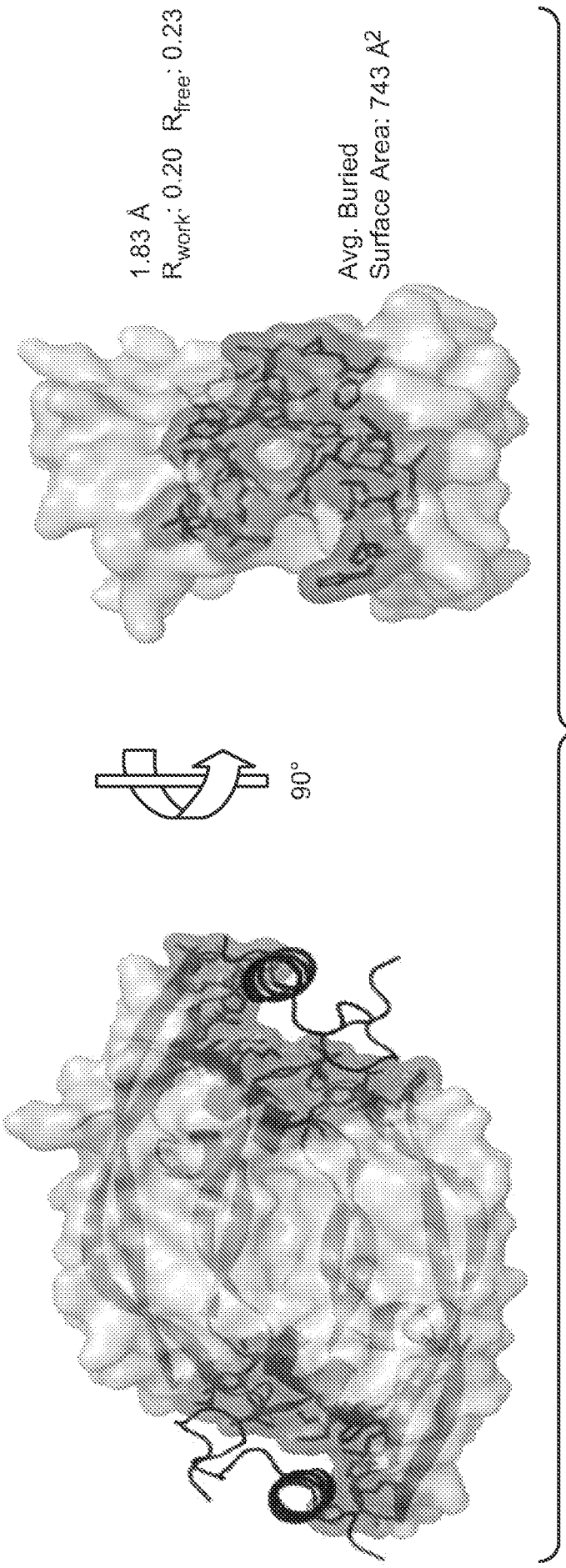


FIG. 6

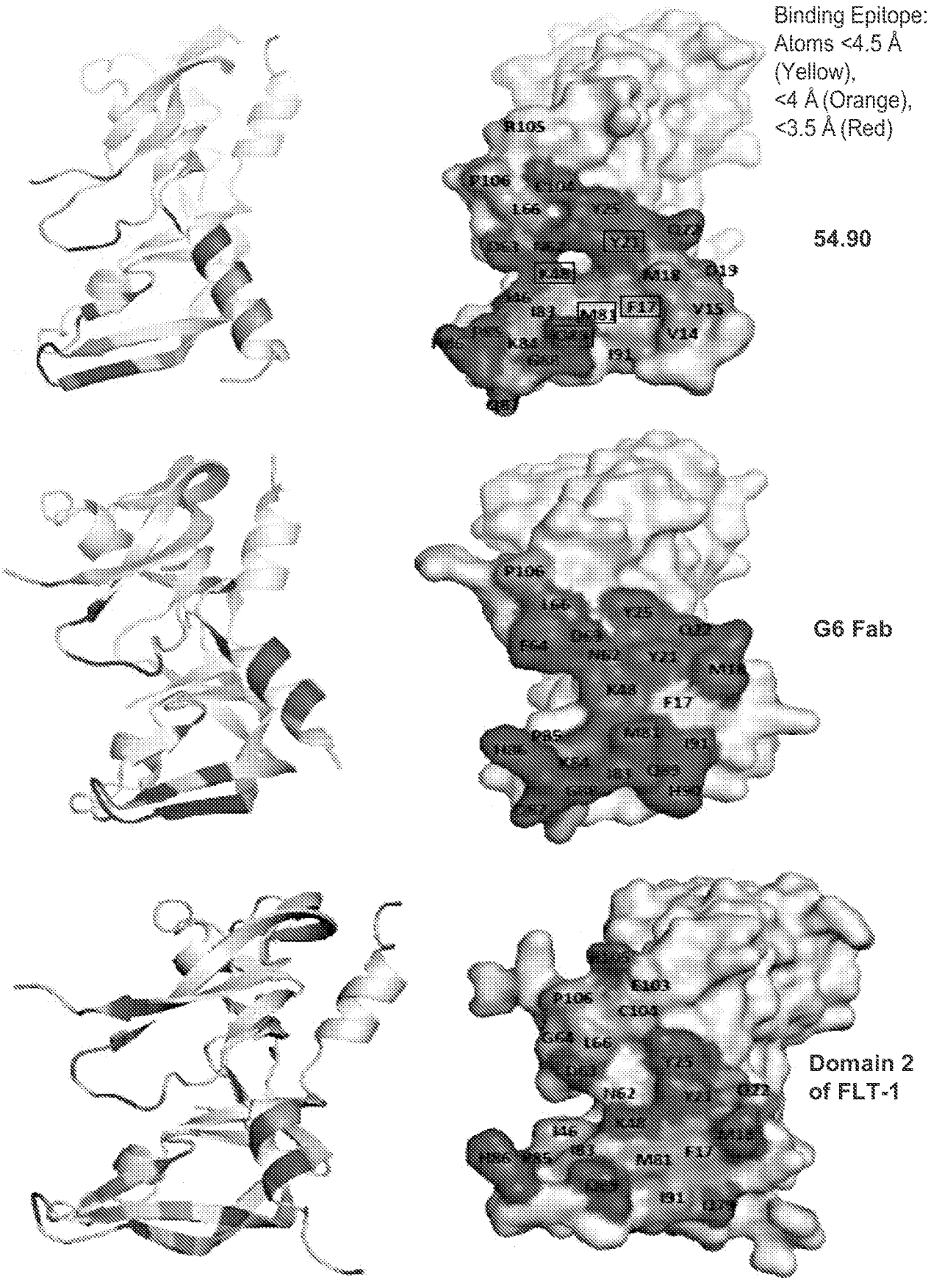

Binding Epitope:
Atoms <4.5 Å
(Yellow),
<4 Å (Orange),
<3.5 Å (Red)
54.90
G6 Fab
Domain 2
of FLT-1

FIG. 7

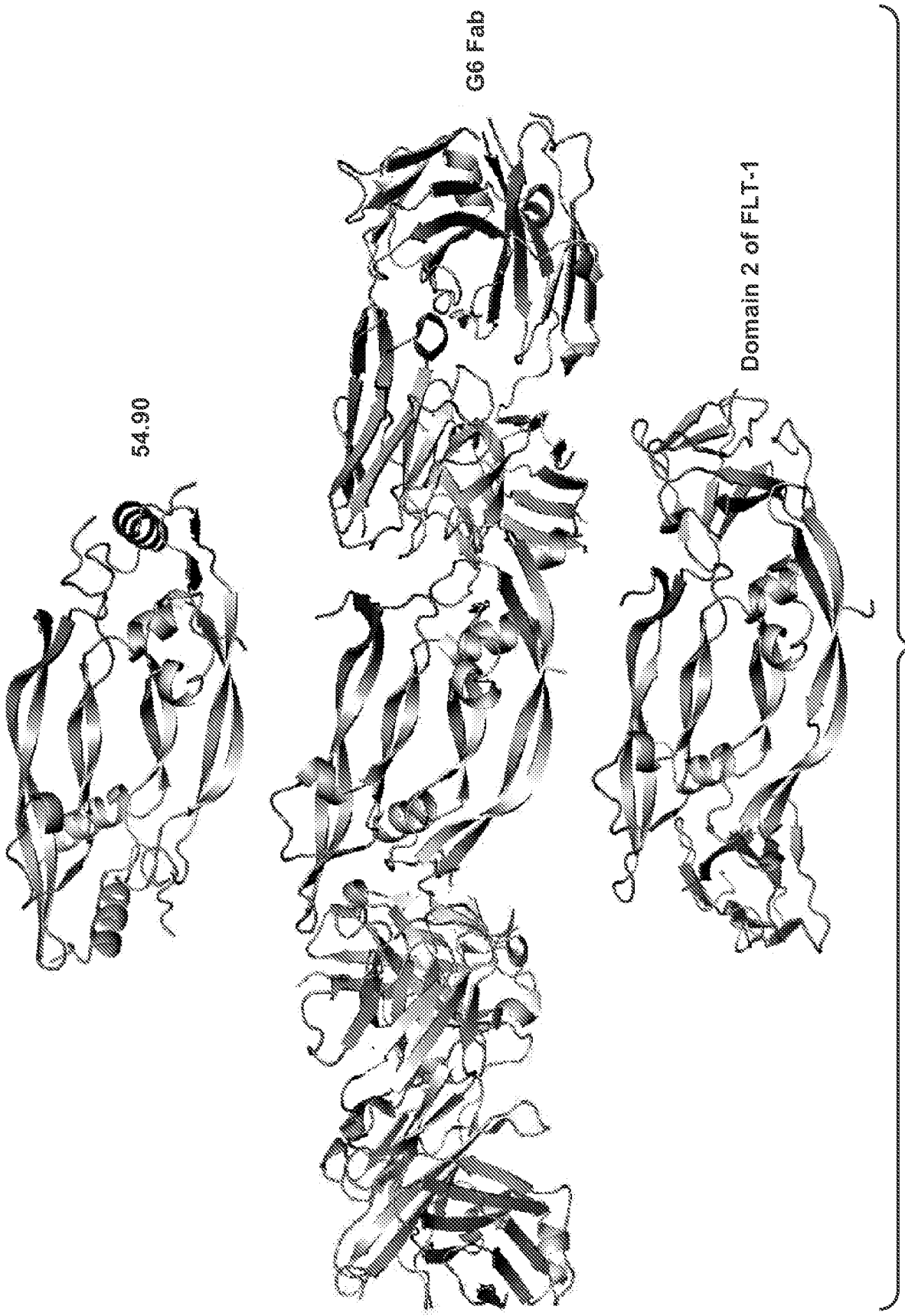


FIG. 8

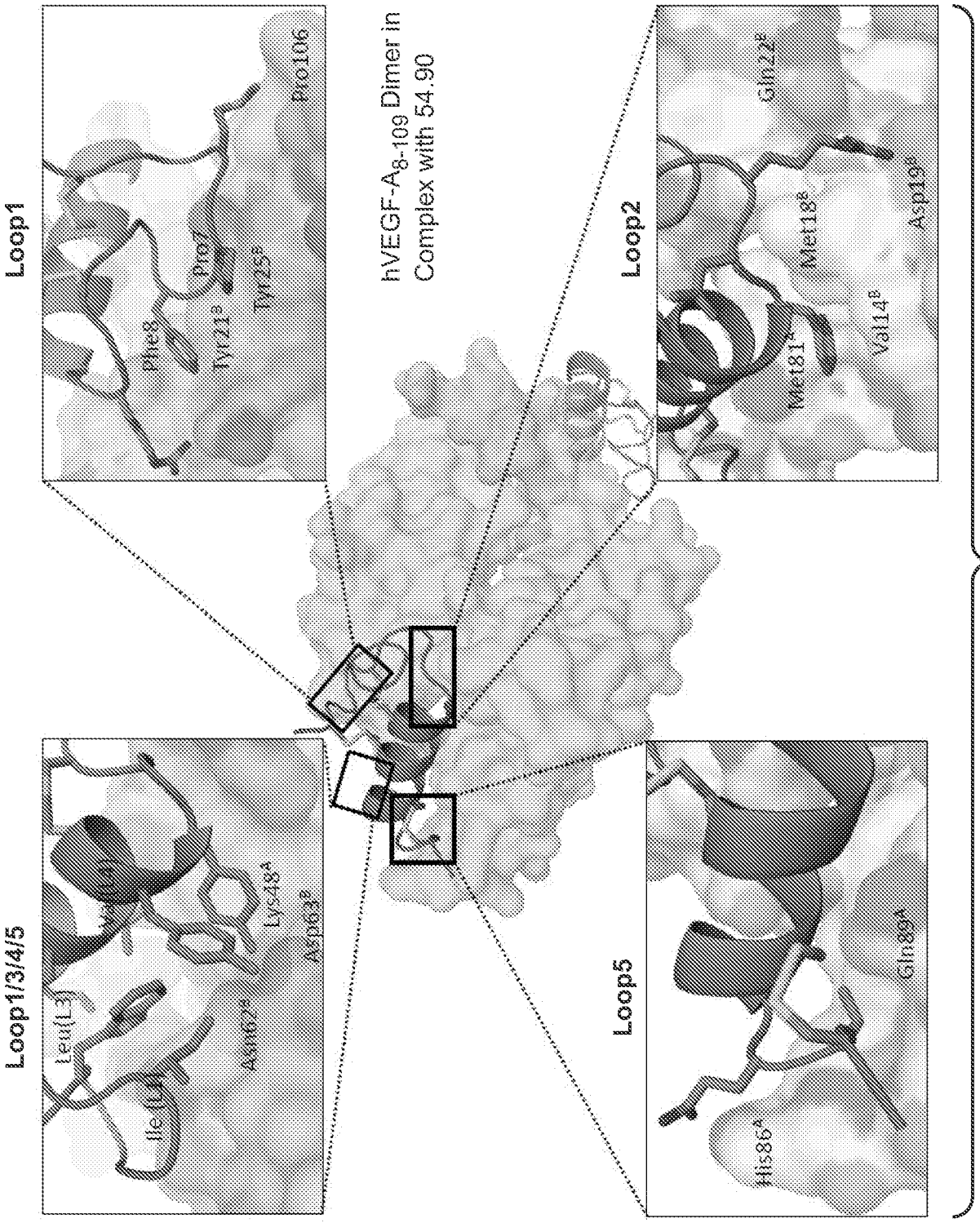

Loop1
Phe8
Pro7
Tyr21B
Tyr25B
Pro106
hVEGF-A8-109 Dimer in Complex with 54.90
Loop2
Gln22E
Met18A
Met81A
Val14B
Asp19E
Loop1/3/4/5
Leu(L3)
Ile(L4)
Asn62A
Lys48A
Asp63B
Loop5
His86A
Gln89A

FIG. 9

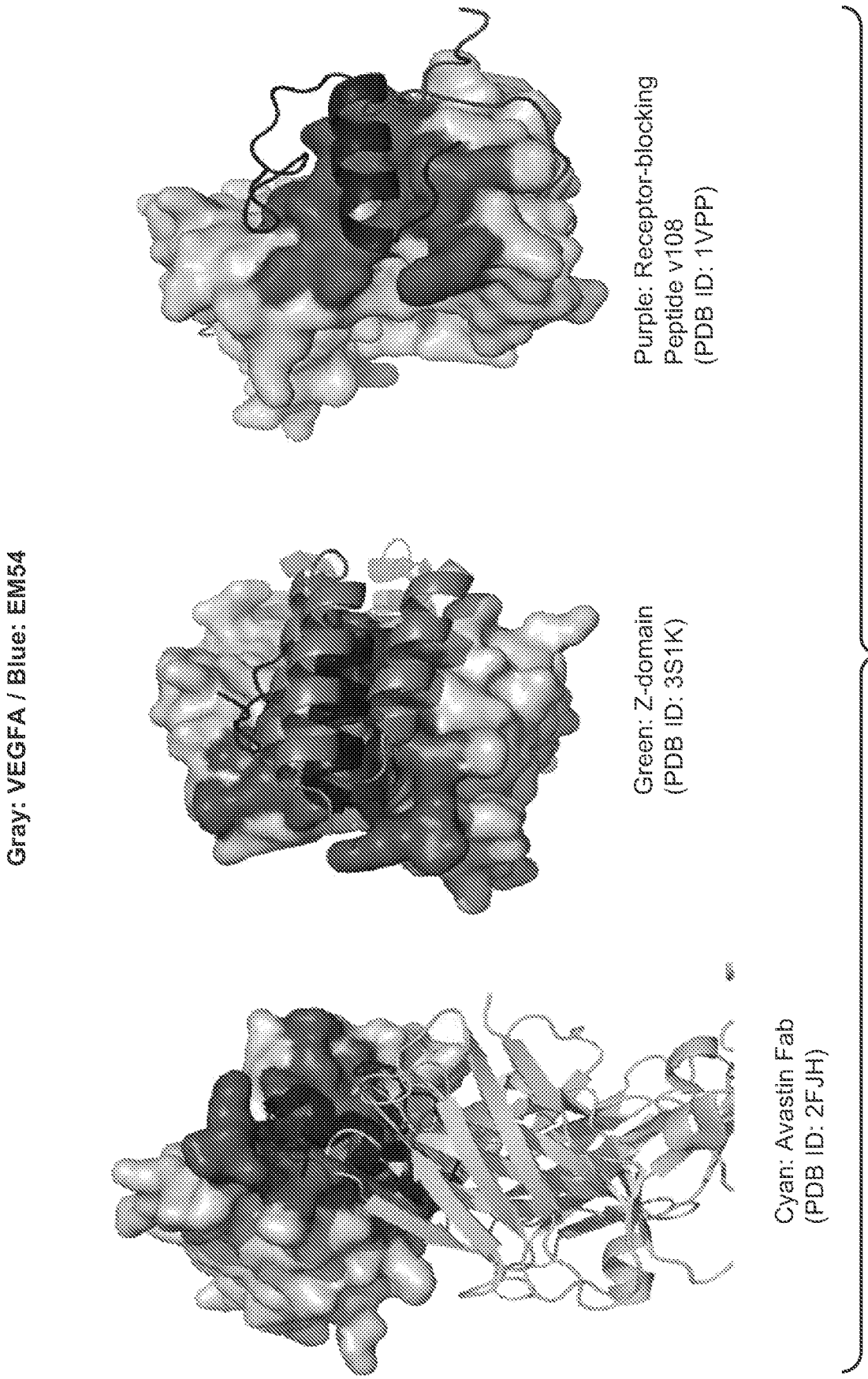

Gray: VEGFA / Blue: EM54
Purple: Receptor-blocking Peptide v108 (PDB ID: 1VPP)
Green: Z-domain (PDB ID: 3S1K)
Cyan: Avastin Fab (PDB ID: 2FJH)

FIG. 10

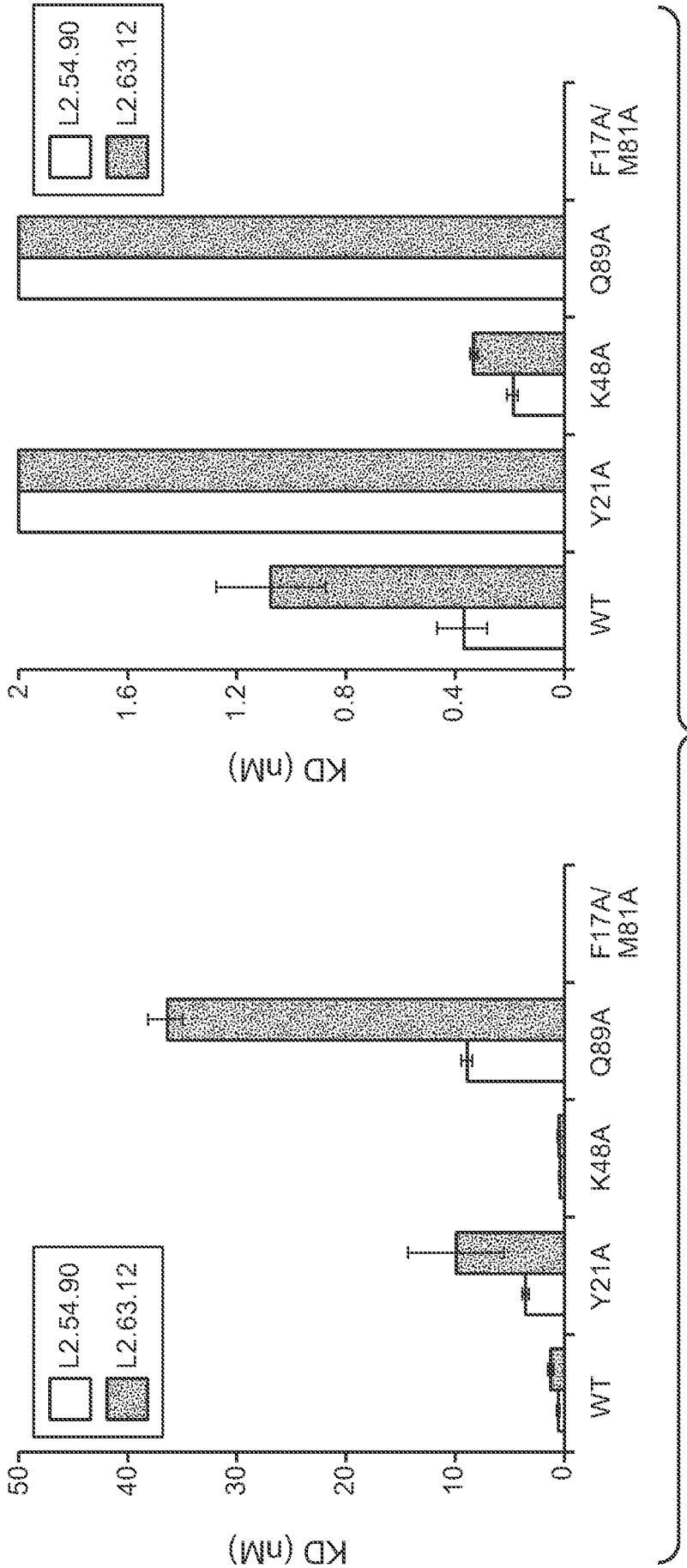

L2.54.90
L2.63.12
KD (nM)
0
10
20
30
40
50
WT
Y21A
K48A
Q89A
F17A/
M81A
L2.54.90
L2.63.12
KD (nM)
0
0.4
0.8
1.2
1.6
2
WT
Y21A
K48A
Q89A
F17A/
M81A

FIG. 11

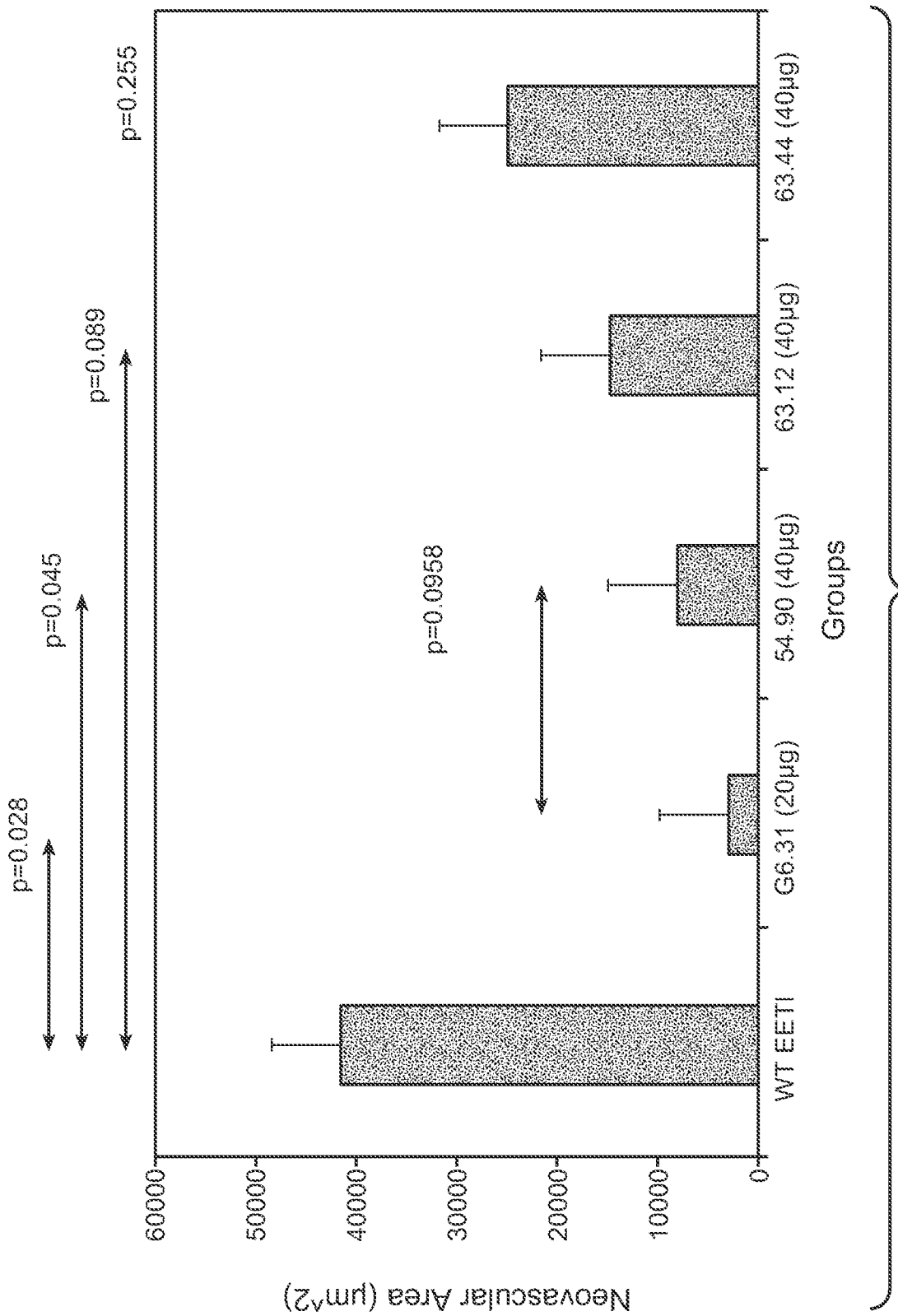

Neovascular Area (μm^2)
60000
50000
40000
30000
20000
10000
0
WT EETI
G6.31 (20μg)
54.90 (40μg)
63.12 (40μg)
63.44 (40μg)
Groups
p=0.028
p=0.045
p=0.089
p=0.255
p=0.0958

FIG. 12

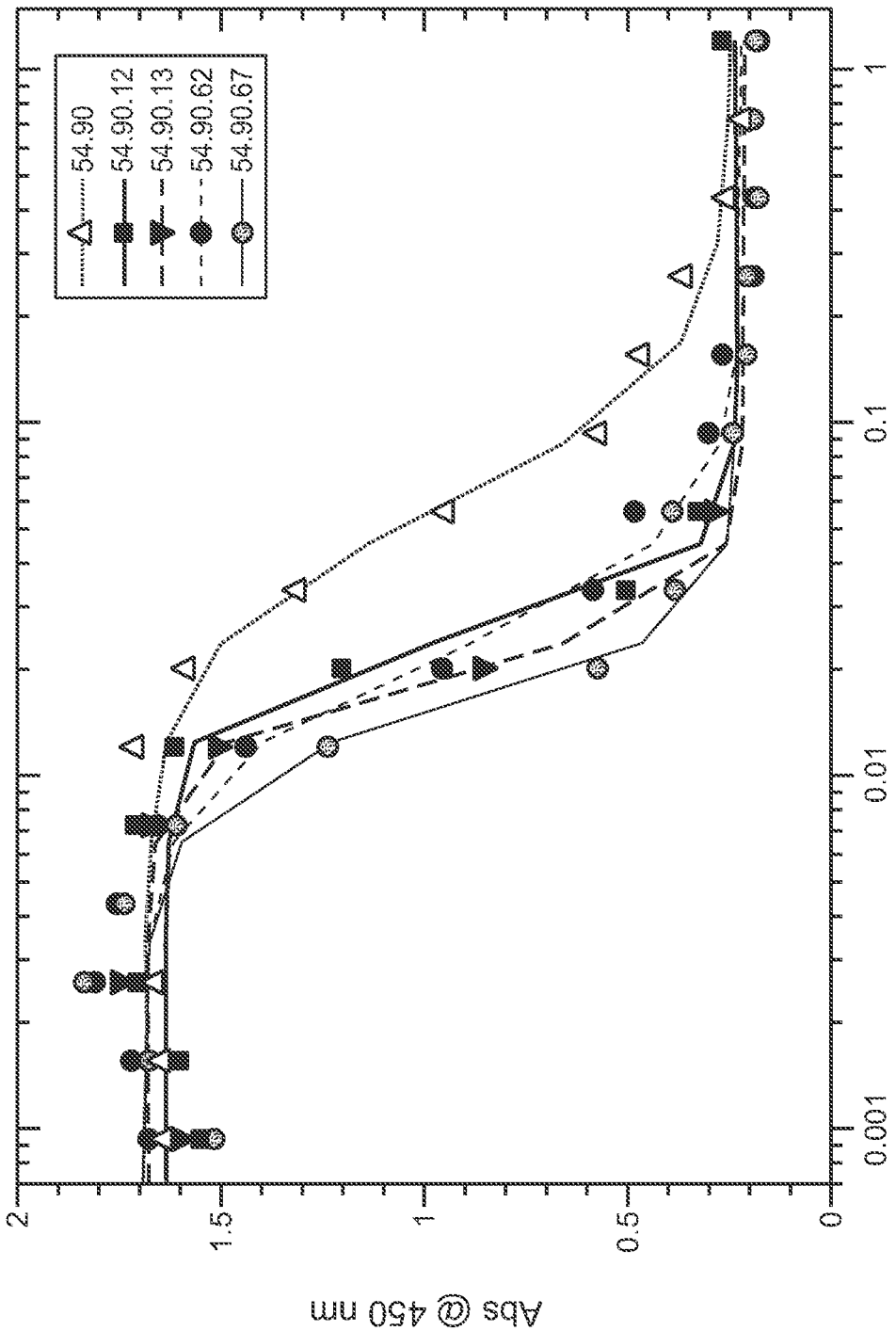

54.90
54.90.12
54.90.13
54.90.62
54.90.67
Abs @ 450 nm
0
0.5
1
1.5
2
0.001
0.01
0.1
1
[Peptide], μM

FIG. 13

Syn	Clone	N-term	Loop1	Loop2	Loop3	4	Loop5	
	WT	G	C P R I L M R	C K Q D S D	C L A G	C V	C G P N G F	C G
E9	C9	G	C Q L M Q P F W G	C	C	C	C H W Y Q S	C G
EM54	C54	G	C N I M L P F W G	C	C	C	C Q Y Y Q S	C G
EM63	C63	G	C D V M Q P Y W G	C	C	C	C H W Y N S	C G

	SEQ ID NO:
	SEQ ID NO: 1
E9	SEQ ID NO: 23
EM54	SEQ ID NO: 52
EM63	SEQ ID NO: 55

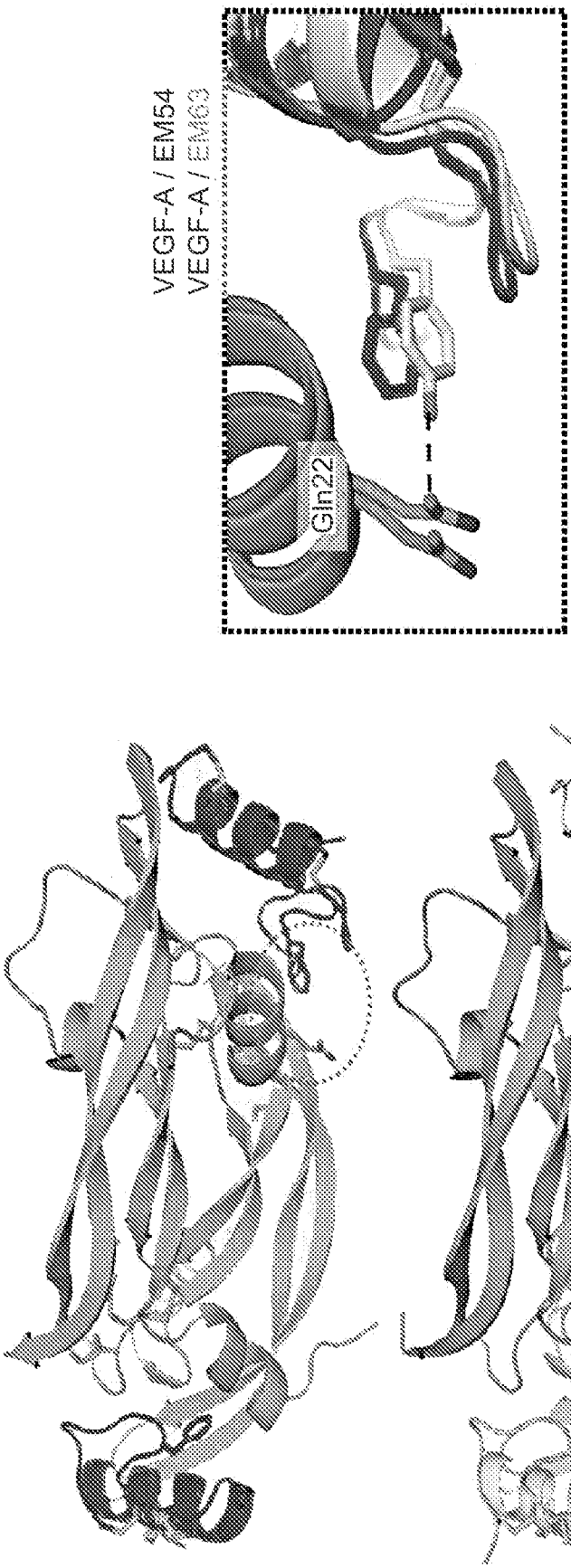


FIG. 14

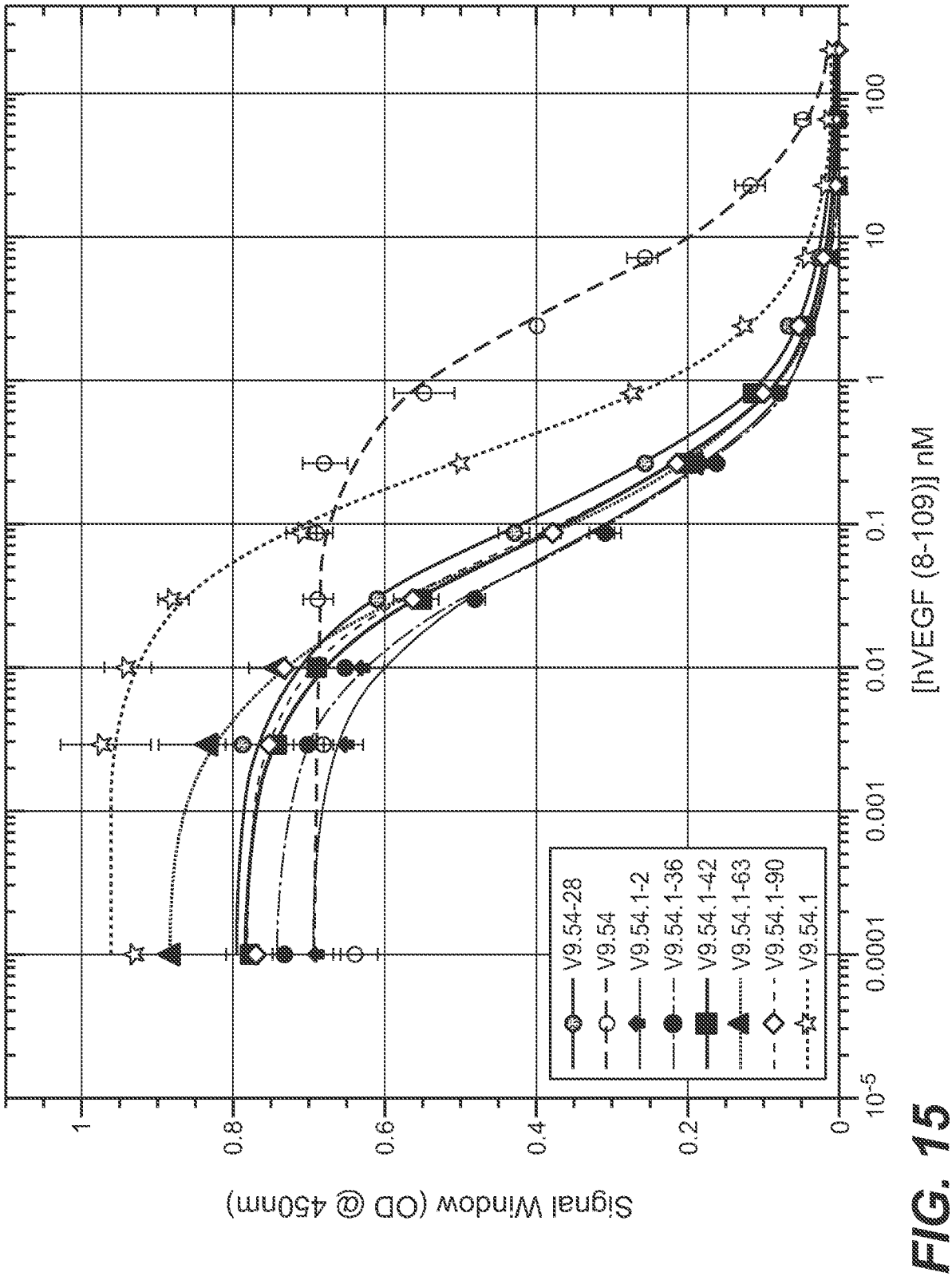

Signal Window (OD @ 450nm)
0
0.2
0.4
0.6
0.8
1
10-5
0.0001
0.001
0.01
0.1
1
10
100
[hVEGF (8-109)] nM
V9.54-28
V9.54
V9.54.1-2
V9.54.1-36
V9.54.1-42
V9.54.1-63
V9.54.1-90
V9.54.1

FIG. 15

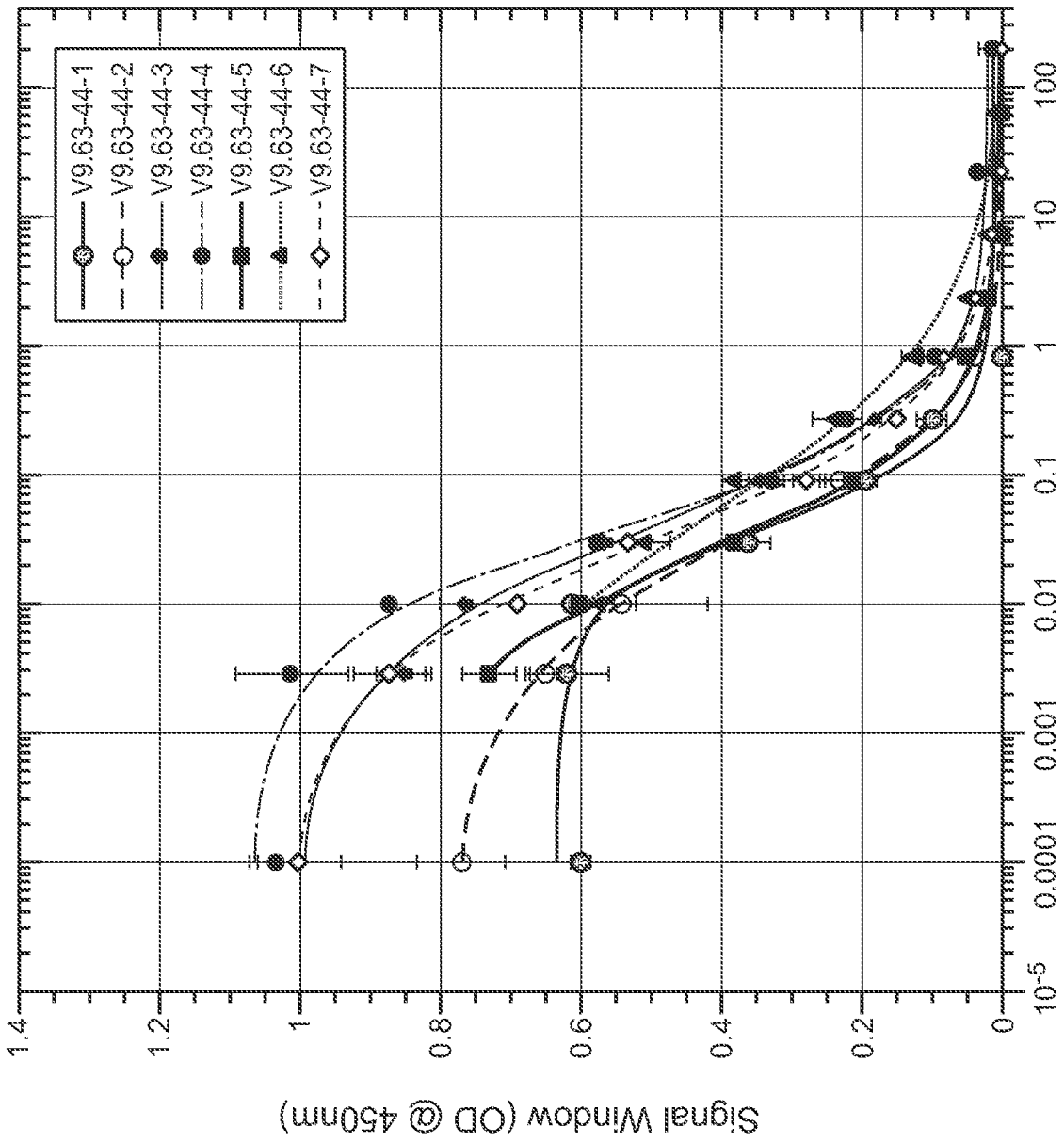

Signal Window (OD @ 450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10-5
0.0001
0.001
0.01
0.1
1
10
100
[hVEGF (8-109)] nM
V9.63-44-1
V9.63-44-2
V9.63-44-3
V9.63-44-4
V9.63-44-5
V9.63-44-6
V9.63-44-7

FIG. 16A

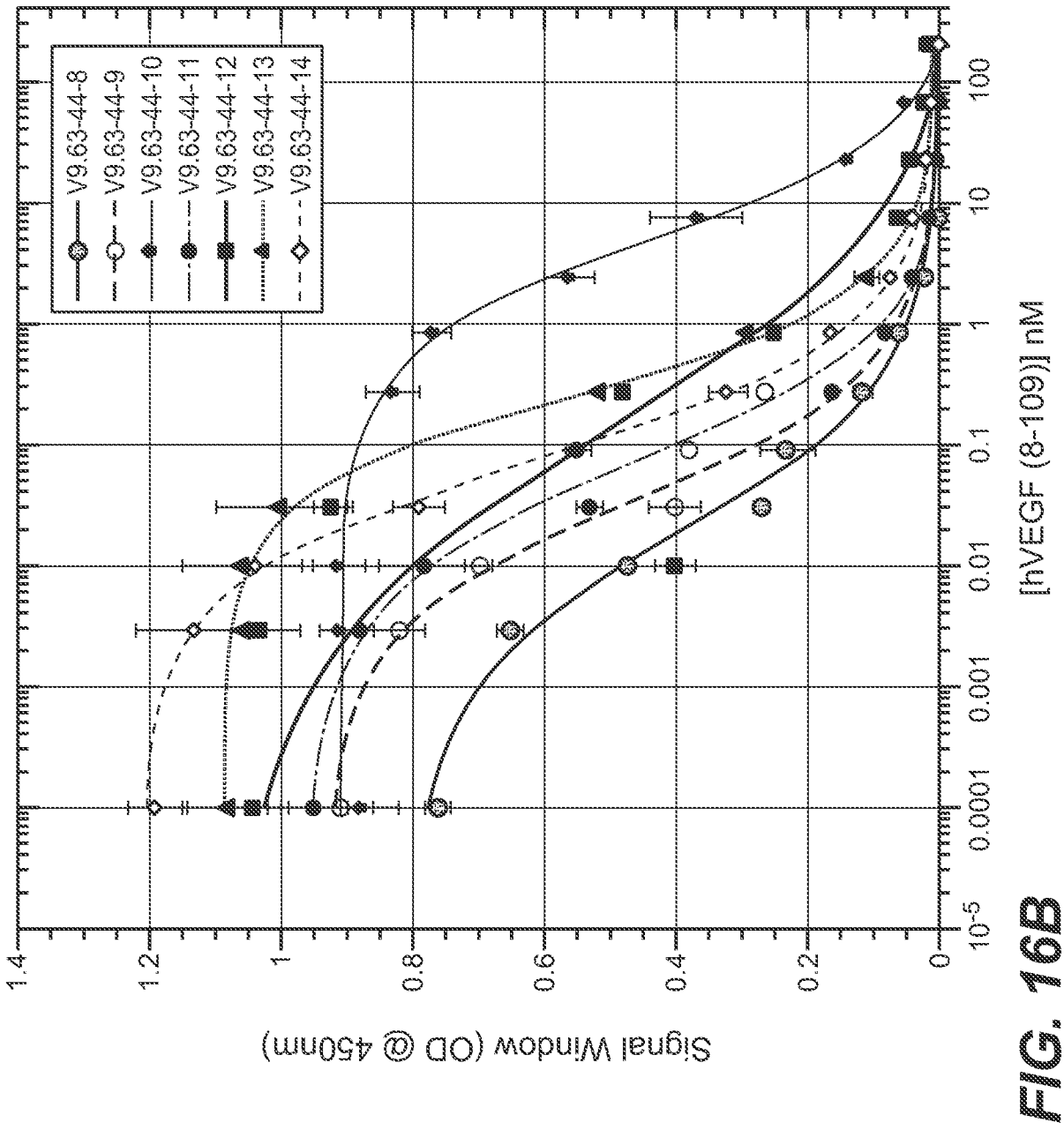

Signal Window (OD @ 450nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10-5
0.0001
0.001
0.01
0.1
1
10
100
[hVEGF (8-109)] nM
V9.63-44-8
V9.63-44-9
V9.63-44-10
V9.63-44-11
V9.63-44-12
V9.63-44-13
V9.63-44-14

FIG. 16B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/052012

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/81 A61K38/00 C07K14/415 C07K14/47
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/136740 A1 (COCHRAN JENNIFER R [US] ET AL) 9 June 2011 (2011-06-09) the whole document figures 14,15	1,3-5, 10,19, 25,26, 84-86, 91-127
X	WO 2012/064658 A1 (UNIV LELAND STANFORD JUNIOR [US]; COCHRAN JENNIFER R [US]; JONES DOUGL) 18 May 2012 (2012-05-18) the whole document EA7.08 mutant	1,3-5, 8-10,19, 25-29, 36,37, 84-88, 91-127

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 2016	13/02/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilat, Daniel

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/052012

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/038619 A2 (AMUNIX INC [US]; STEMMER WILLEM P C [US]; SCHELLENBERGER VOLKER [US];) 5 April 2007 (2007-04-05) the whole document page 84, line 16 - page 85, line 1	1,3-5, 10,19, 26, 84-86, 91-127
X	KRÄTZNER RALPH ET AL: "Structure of Ecballium elaterium trypsin inhibitor II (EETI-II): a rigid molecular scaffold", ACTA CRYSTALLOGRAPHICA SECTION D: BIOLOGICAL CRYSTALLOGRAPHY, MUNKSGAARD PUBLISHERS LTD. COPENHAGEN, DK, vol. D61, no. Pt 9, 1 September 2005 (2005-09-01), pages 1255-1262, XP002562802, ISSN: 0907-4449, DOI: 10.1107/S0907444905021207 figure 1	1,3-5, 8-10,19, 25-29, 36,37, 84-88, 91-127
X	SUNITHI GUNASEKERA ET AL: "Engineering Stabilized Vascular Endothelial Growth Factor-A Antagonists: Synthesis, Structural Characterization, and Bioactivity of Grafted Analogues of Cyclotides", JOURNAL OF MEDICINAL CHEMISTRY, vol. 51, no. 24, 25 December 2008 (2008-12-25), pages 7697-7704, XP055204929, ISSN: 0022-2623, DOI: 10.1021/jm800704e the whole document	1-39, 84-95
A	WO 2008/045252 A2 (UNIV LELAND STANFORD JUNIOR [US]; COCHRAN JENNIFER R [US]; KIMURA RICH) 17 April 2008 (2008-04-17) abstract; claims 1-3	1-127
A	MICHAEL REINWARTH ET AL: "Chemical Synthesis, Backbone Cyclization and Oxidative Folding of Cystine-knot Peptides - Promising Scaffolds for Applications in Drug Design", MOLECULES, vol. 17, no. 12, 24 October 2012 (2012-10-24), pages 12533-12552, XP055321534, DOI: 10.3390/molecules171112533 abstract	1-127

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/052012

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CEMAZAR M ET AL: "The Structure of a Two-Disulfide Intermediate Assists in Elucidating the Oxidative Folding Pathway of a Cyclic Cystine Knot Protein", STRUCTURE, ELSEVIER, AMSTERDAM, NL, vol. 16, no. 6, 11 June 2008 (2008-06-11), pages 842-851, XP022708577, ISSN: 0969-2126, DOI: 10.1016/J.STR.2008.02.023 [retrieved on 2008-06-10] abstract -----	1-127
A	WO 2014/033184 A1 (NOVARTIS AG [CH]; OSBORNE AARON [CH]) 6 March 2014 (2014-03-06) cited in the application abstract; claim -----	107-127

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/052012

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/052012

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-127

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-127

a non-naturally occurring cystine knot peptide (CKP) that binds to vascular endothelial growth factor A (VEGF-A), wherein the CKP comprises the cystine scaffold structure: Z 1 C1 L1C2 L2C3 L3C4 L4 C5 L5C6 Z2 wherein: Z1 and Z2 are any amino acid; L1 is Loop 1 and has a structure selected from the group consisting of: X1 X2 X3 X4 X5 X6 (SEQ ID NO: 2), X1 X2 X3 X4 X5 X6 X7 (SEQ ID NO: 3), X1 X2 X3 X4 X5 X6 X7 X8 (SEQ ID NO: 4), X1 X2 X3 X4 X5 X6 X7 X8 X9 (SEQ ID NO: 5), and X1 X2 X3 X4 X5 X6 X7 X8 X9 X10 (SEQ ID NO: 6), wherein each of X1 - X10 is any amino acid; L2 is Loop 2 and has the structure: X1 X2 X3 X4 X5 (SEQ ID NO: 7), wherein each of X1 - X5 is any amino acid or an unnatural amino acid;
L3 is Loop 3 and has the structure: X1 X2 X3 wherein each of X1 - X3 is any amino acid or an unnatural amino acid;
L4 is Loop 4 and has the structure: X1 , wherein X1 is any amino acid or an unnatural amino acid;
L5 is Loop 5 and has the structure: X1 X2 X3 X4 X5 (SEQ ID NO: 7), wherein each of X1 - X5 is any amino acid or an unnatural amino acid;
wherein the unnatural amino acid is selected from the group given in claim 1, and embodiments thereof

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2. claims: 128-138

a non-naturally occurring cystine knot peptide (CKP) that binds to human low density lipoprotein receptor-related protein 6 (LRP6), wherein the CKP comprises the cystine scaffold structure:
Z1 C1 L1C2 L2C3 L3C4 L4C5 L5C6 Z2 ;
wherein:
Zi and Z2 are any amino acid;
L1 is Loop 1 and has a structure selected from the group consisting of:
X1 X2 X3 X4 X5 X6 , X1 X2 X3 X4 X5 X6 X7 , X1 X2 X3 X4 X5 X6 X7 X8 , X1 X2 X3 X4 X5 X6 X7 X8 X9 ,and X1 X2 X3 X4 X5 X6 X7 X8 X9 X10 ,
wherein each of X1 - X10 is any amino acid;
L2 is Loop 2 and has the structure: X1 X2 X3 X4 X5 ,
wherein each of X1 - X5 is any amino acid; L3 is Loop 3 and has the structure: X1 X2 X3 wherein each of X1 - X3 is any amino acid; L4 is Loop 4 and has the structure X1 ,
wherein X1 is any amino acid; and
L5 is Loop 5 and has the structure: X1 X2 X3 X4 X5 ,
wherein each of X1 - X5 is any amino acid; and embodiments thereof.
The unifying concept underlying the group of inventions 1 and 2 is that they refer to a cystine knot peptide (CKP) that binds an epitope wherein the CKP comprises the cystine

scaffold structure: Z 1 C1 L1C2 L2C3 L3C4 L4 C5 L5C6 Z2
wherein:
Z1 and Z2 are any amino acid;
L1 is Loop 1 and has a structure selected from the group consisting of:
X1 X2 X3 X4 X5 X6 (SEQ ID NO: 2), X1 X2 X3 X4 X5 X6 X7 (SEQ ID NO: 3), X1 X2 X3 X4 X5 X6 X7 X8 (SEQ ID NO: 4), X1 X2 X3 X4 X5 X6 X7 X8 X9 (SEQ ID NO: 5), and X1 X2 X3 X4 X5 X6 X7 X8 X9 X10 (SEQ ID NO: 6), wherein each of X1 - X10 is any amino acid;
L2 is Loop 2 and has the structure: X1 X2 X3 X4 X5 (SEQ ID NO: 7), wherein each of X1 - X5 is any amino acid or an unnatural amino acid;
L3 is Loop 3 and has the structure: X1 X2 X3 wherein each of X1 - X3 is any amino acid or an unnatural amino acid;
L4 is Loop 4 and has the structure: X1 , wherein X1 is any amino acid or an unnatural amino acid;
L5 is Loop 5 and has the structure: X1 X2 X3 X4 X5 (SEQ ID NO: 7), wherein each of X1 - X5 is any amino acid or an unnatural amino acid;

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/052012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2015297675 A1	22-10-2015
		WO 2014033184 A1	06-03-2014