Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Allosteric Binding Ligands To Modulate Serum Low Density Lipoprotein (LDL) Levels

Field Of Invention

This invention is related to the field of hypercholesterolemia. In particular, the invention provides compositions and methods to modulate circulating levels of low density lipoproteins by altering the conformation of the protein PCSK9 using a synthetic ligand and/or a synthetic ligand derivative having sequences of 3-8 amino acids ranging between 350 – 2,000 Da. Altering the conformation of PCSK9 affects the interaction between PCSK9 and an endogenous low density lipoprotein receptor, and can lead to reduced or increased levels of circulating LDL-cholesterol. High LDL-cholesterol levels are associated with increased risk for heart disease. Low LDL-cholesterol levels may be problematic in other conditions, such as liver dysfunction; thus, there is also utility for ligands which can raise LDL levels.

Background

Elevated plasma levels of low density lipoprotein cholesterol (LDL-C) represent the greatest risk factor for the development of coronary heart disease. Clearance of LDL-C from the plasma occurs primarily by the liver through the action of LDL receptors (LDLRs), which are cell surface glycoproteins that bind to apolipoprotein B100 (apoB100) on LDL particles with high affinity and mediate their endocytic uptake. Goldstein et al., *Annu. Rev. Cell Biol.* 1:1–39 (1985). Autosomal dominant hypercholesterolemia (ADH) is associated with mutations that reduce plasma LDL clearance that are found in genes encoding the LDLR (familial hypercholesterolemia (FH)) or apoB100 (familial defective apoB100). Hobbs et al., *Annu. Rev. Genet.* 24, 133–170 (1990); and Innerarity et al., *J. Lipid Res.* 31:1337–1349 (1990), respectively.

The low density lipoprotein (LDL) receptor (LDLR) mediates efficient endocytosis of VLDL, VLDL remnants, and LDL. As part of the endocytic process, the LDLR releases lipoproteins into hepatic endosomes.

One approach to modulating LDL-cholesterol levels would be to identify peptides which bind to PCSK9 and alter the kinetics of the interact between PCSK9 and the LDLR such that the rate of lipoprotein clearance by LDLR endocytosis is increased or decreased, as desired.

Summary Of The Invention

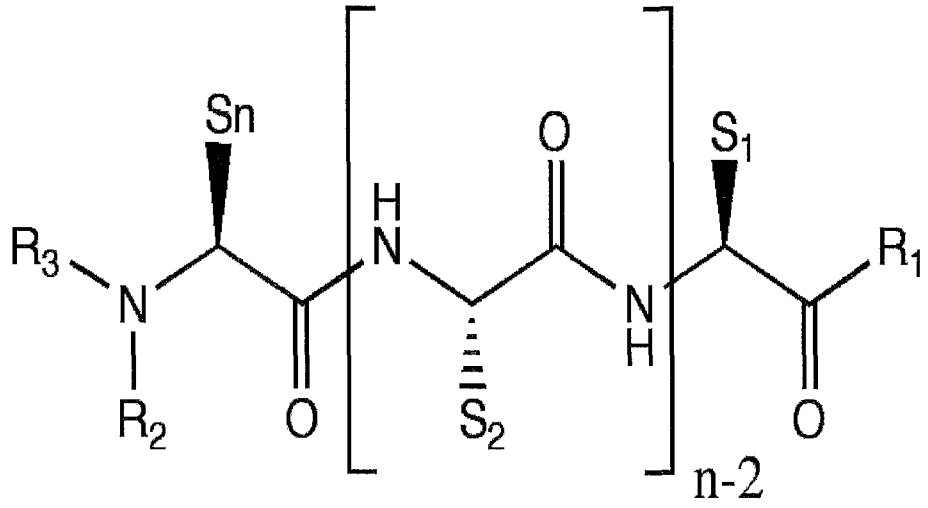
This invention is related to the field of hypercholesterolemia. In particular, the invention provides compositions and methods to modulate circulating levels of low density lipoproteins by altering the conformation of the protein PCSK9 using a synthetic ligand and/or a synthetic ligand derivative having sequences of 3-8 amino acids ranging between 350 – 2,000 Da. Altering the conformation of PCSK9 affects the interaction between PCSK9 and an endogenous low density lipoprotein receptor, and can lead to reduced or increased levels of circulating LDL-cholesterol. High LDL-cholesterol levels are associated with increased risk for heart disease. Low LDL-cholesterol levels may be problematic in other conditions, such as liver dysfunction; thus, there is also utility for ligands which can raise LDL levels.

In one embodiment, the present invention contemplates a method, comprising: a) providing; i) a PCSK9 protein, wherein said protein comprises a binding site that induces allosteric modulation and a low density lipoprotein receptor binding site; ii) a ligand capable of binding said binding site; iii) a plurality of hepatocyte cells comprising a low density lipoprotein receptor and low density lipoproteins; b) binding said synthetic ligand to said binding site, wherein said synthetic ligand induces a conformation shift of said protein; and c) modulating the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor by said conformational shift. In one embodiment, the binding site comprises His^{417}, Lys^{421}, Pro^{446}, Trp^{453}, Gln^{454}, Glu^{628}, Gly^{629}, Asn^{652}, and Thr^{653} of the PCSK9 protein. In one embodiment, the synthetic ligand is an allosteric inhibitor ligand wherein said modulating decreases the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor such that internalization of said low density lipoprotein by said plurality of hepatocytes is increased. In one embodiment, the synthetic ligand is an allosteric enhancer ligand said modulating increases the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor such that internalization of said low density lipoprotein by said plurality of hepatocytes is decreased. In one embodiment, the conformational shift of said protein is selected from the group consisting of an induced fit shift and a biomechanical shift. In one embodiment, the synthetic ligand is a synthetic peptide selected from the group consisting of VYVRFW, VLELYW and ISDLSY. In one embodiment, the allosteric inhibitor is a peptide is selected from the group consisting of SRX55, SRX56, SRX60, SRX61, SRX62, SRX63, SRX64, SRX65 and SRX66. In one

embodiment, the allosteric enhancer is a peptide is selected from the group consisting of SRX64, SRX67, SRX68, SRX69, SRX72 and SRX73. In one embodiment, the synthetic peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the synthetic peptide is six amino acids. In one embodiment, the synthetic peptide is less than 1,300 Da. In one embodiment, the synthetic peptide ranges between approximately 466-1067 Da. In one embodiment, the synthetic peptide ranges between approximately 175-1,000 Da.

In one embodiment, the present invention contemplates, a method, comprising: a) providing; i) a PCSK9 protein, wherein said protein comprises a binding site that induces allosteric modulation and a low density lipoprotein receptor binding site; ii) a synthetic ligand capable of binding said binding site; iii) a plurality of hepatocyte cells comprising a population of low density lipoprotein receptors; b) binding said synthetic ligand to said binding site, wherein said synthetic ligand induces a conformation shift of said protein; c) modulating said population of said low density lipoprotein receptors by said conformational shift. In one embodiment, the binding site comprises His^{417}, Lys^{421}, Pro^{446}, Trp^{453}, Gln^{454}, Glu^{628}, Gly^{629}, Asn^{652}, and Thr^{653} of the PCSK9 protein. In one embodiment, the synthetic ligand is an allosteric inhibitor ligand wherein said modulating increases said population of said low density lipoprotein receptors measurable on the cell surface of said plurality of hepatocytes. In one embodiment, the synthetic ligand is an allosteric enhancer ligand wherein said modulating decreases said population of said low density lipoprotein receptors measurable on the cell surface of said plurality of hepatocytes. In one embodiment, the conformational shift of said protein is selected from the group consisting of an induced fit shift and a biomechanical shift. In one embodiment, the ligand is a synthetic peptide selected from the group consisting of VYVRFW, VLELYW and ISDLSY. In one embodiment, the allosteric inhibitor peptide is selected from the group consisting of SRX55, SRX56, SRX60, SRX61, SRX62, SRX63, SRX64, SRX65 and SRX66. In one embodiment, the allosteric enhancer peptide is selected from the group consisting of SRX64, SRX67, SRX68, SRX69, SRX72 and SRX73. In one embodiment, the synthetic peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the synthetic peptide is six amino acids. In one embodiment, the synthetic peptide is less than 1,300 Da. In one embodiment, the synthetic peptide ranges between approximately 466-1067 Da. In one embodiment, the synthetic peptide ranges between approximately 50-1,000 Da.

In one embodiment, the present invention contemplates a compound of the formula:



wherein: i) n, the number of amino acid residues, is an integer in the range 3-8; ii) the constituent amino acids are single enantiomers of independently selected natural or unnatural amino acids; iii) R2 and R3, are independently selected from the group consisting of hydrogen, a lower alkyl, a branched alkyl, a hydroxyalkyl, a cycloalkyl, a heterocycle, aryl, heteroaryl, acyl, substituted or unsubstituted benzoyl, alkyl or aryl sulfonyl (e.g. mesyl or tosyl) and carbamoyl (e.g. BOC); iv) R1 is selected from the group consisting of –OH and -NR4-R5; v) R4 and R5, independently, are selected from the group consisting of hydrogen; a lower alkyl, an aryl, a cycloalkyl, an aromatic heterocycle, pyridine, tetrazole, alkoxy, cycloalkoxy; alternatively, R4 and R5 are joined as a heterocyle, such as piperidine; pyrrolidine; morpholine; piperazine; a substituted heterocycle, such as 4-methylpiperazine; or a fused heterocycle, such as dihydroquinoline or indoline and S1, S2 and S3 are side chains. In one embodiment, the compound further comprises a negatively charged polar group. In one embodiment, the negatively charged polar group is selected from at least one of the group consisting of O-phosphate, O-sulfate, 5-O-, and a 5-N- tetrazole incorporated in said side-chains S1, S2, or Sn. In one embodiment, the side chain selected from the group consisting of S1, S2 and Sn comprises a phosphoserine. In one embodiment, the side chain S1 comprises -CH2-NH-tetrazole. In one embodiment, the compound further comprises a glycine C-terminus. In one embodiment, the compound is selected from the group consisting of Val-Tyr-

Val-Arg-Phe-Trp, β-Ala-Phe(3-CH2NH2)-Val-D-Ser(p)-Phe-Trp, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p), Val-Leu-Glu-Leu-Tyr-Trp, Leu-Asp-Leu-Phe-Phe-Ser, Ile-Leu-Asp-Leu-Ser-Tyr, Ac-Trp-Ser-Ser(p), Ac-Trp-Ala-Ser(p), Ac-Trp(5-F)-Ala-Ser(p)-morpholine, Thr-Leu-Thr-Trp-Ser-Ser-Ser(p), Ac-Tyr-Trp-Gly, Phe(4-Ph)-Ala-Ser(p)-morpholine. In one embodiment, the compound comprises between approximately 3 – 8 amino acids. In one embodiment, the compound is six amino acids. In one embodiment, the compound is less than 1,300 Da. In one embodiment, the compound ranges between approximately 466-1067 Da. In one embodiment, the compound ranges between approximately 175-1,000 Da. In one embodiment, the compound comprises a synthetic peptide. In one embodiment, the compound is formulated as a pharmaceutical composition. In one embodiment, the pharmaceutical composition further comprises a pharmaceutical drug. In one embodiment, the pharmaceutical drug is selected from the group consisting of a statin, a cardiovascular drug, a metabolic drug, and an antihypertensive drug. In one embodiment, the pharmaceutical drug is selected from the group consisting of ezetimibe, amlodipine besylate, sitagliptin, metformin, atorvastatin, rosuvastatin and simvastatin. In one embodiment, the pharmaceutical composition is formulated as selected from the group consisting of a tablet, a liquid, a gel, a capsule, a sachet, a microparticle, a liposome, a nanoparticle, a salt, a transdermal patch, an ointment, a lotion, a cream, a gel, a drop, a strip, a suppository, a spray and a powder.

In one embodiment, the present invention contemplates a composition comprising a PCSK9 allosteric ligand ranging between approximately 350-1,500 Da. In one embodiment, the PCSK9 allosteric ligand is less than 1,300 Da. In one embodiment, the PCSK9 allosteric ligand comprises between approximately 3 – 6 amino acids. In one embodiment, the PCSK9 allosteric ligand ranges between approximately 550 – 1,000 Da. In one embodiment, the composition is a pharmaceutical composition. In one embodiment, the composition is a pharmaceutical composition. In one embodiment, said administering further comprises a delivery system selected from the group including, but not limited to, liposomes, microparticles and nanoparticles. In one embodiment, the pharmaceutical composition comprises an effective

dose of said ligand. In one embodiment, the pharmaceutical composition comprises salts. In one embodiment, the pharmaceutical composition is formulated for oral administration.

In one embodiment, the present invention contemplates a method, comprising: a) administering a PCSK9 allosteric inhibitor peptide to a subject, wherein said subject has at least one symptom of a cardiovascular disease; and b) reducing said at least one symptom of cardiovascular disease by said PCSK9 allosteric inhibitor peptide administration. In one embodiment, said at least one symptom is reduced between 10% - 85%. In one embodiment, said at least one symptom is reduced between 20% - 65%. In one embodiment, said at least one symptom is reduced between 30% - 55%. In one embodiment, the cardiovascular disease comprises a coronary disease. In one embodiment, the cardiovascular disease comprises hypertension. In one embodiment, the cardiovascular disease comprises hypercholesterolemia. In one embodiment, the cardiovascular disease comprises atherosclerosis. In one embodiment, the at least one symptom comprises reduced circulating high density lipoprotein. In one embodiment, the at least one symptom comprises elevated circulating cholesterol. In one embodiment, the at least one symptom comprises elevated circulating low density lipoprotein. In one embodiment, the at least one symptom comprises high blood pressure. In one embodiment, the administering comprises an effective dose of said PCSK9 allosteric inhibitor peptide. In one embodiment, said administering further comprises a delivery system selected from the group including, but not limited to, liposomes, microparticles and nanoparticles. In one embodiment, the effective dose comprises a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises salts. In one embodiment, the pharmaceutical composition is formulated for oral administration. In one embodiment, the allosteric inhibitor peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the allosteric inhibitor peptide is six amino acids. In one embodiment, the allosteric inhibitor peptide is less than 1,300 Da. In one embodiment, the allosteric inhibitor peptide ranges between approximately 466-1067 Da. In one embodiment, the allosteric inhibitor peptide ranges between approximately 175-1,000 Da.

In one embodiment, the present invention contemplates a method, comprising: a) administering a PCSK9 allosteric enhancer peptide to a subject, wherein said subject has at least one symptom of a cardiovascular disease; and b) reducing said at least one symptom of cardiovascular disease by said PCSK9 allosteric inhibitor peptide administration. In one

embodiment, the cardiovascular disease comprises hypercholesterolemia. In one embodiment, said at least one symptom comprises reduced circulating cholesterol. In one embodiment, said at least one symptom comprises elevated high density lipoprotein. In one embodiment, the at least one symptom comprises reduced low density lipoprotein. In one embodiment, the at least one symptom comprises low blood pressure. In one embodiment, said at least one symptom is reduced between 10% - 85%. In one embodiment, said at least one symptom is reduced between 20% - 65%. In one embodiment, said at least one symptom is reduced between 30% - 55%. In one embodiment, the administering comprises an effective dose of said PCSK9 allosteric inhibitor peptide. In one embodiment, said administering further comprises a delivery system selected from the group including, but not limited to, liposomes, microparticles and nanoparticles. In one embodiment, the effective dose comprises a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises salts. In one embodiment, the pharmaceutical composition is formulated for oral administration. In one embodiment, the allosteric enhancer peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the allosteric enhancer peptide is six amino acids. In one embodiment, the allosteric enhancer peptide is less than 1,300 Da. In one embodiment, the allosteric enhancer peptide ranges between approximately 466-1067 Da. In one embodiment, the allosteric enhancer peptide ranges between approximately 175-1,000 Da.

In one embodiment, the present invention contemplates a method, comprising: a) administering a PCSK9 allosteric synthetic peptide to a subject, wherein said subject has at least one symptom of a liver disease; and b) reducing said at least one symptom of liver disease by said PCSK9 allosteric peptide administration. In one embodiment, the at least one symptom comprises elevated low density lipoprotein receptor density. In one embodiment the at least one symptom comprises reduced low density lipoprotein receptor density. In one embodiment, said at least one symptom is reduced between 10% - 85%. In one embodiment, said at least one symptom is reduced between 20% - 65%. In one embodiment, said at least one symptom is reduced between 30% - 55%. In one embodiment, the PCSK9 allosteric synthetic peptide comprises a PCSK9 allosteric enhancer peptide. In one embodiment, the PCSK9 allosteric synthetic peptide comprises a PCSK9 allosteric inhibitor peptide. In one embodiment, the administering comprises an effective dose of said PCSK9 allosteric peptide. In one embodiment, said administering further comprises a delivery system selected from the group

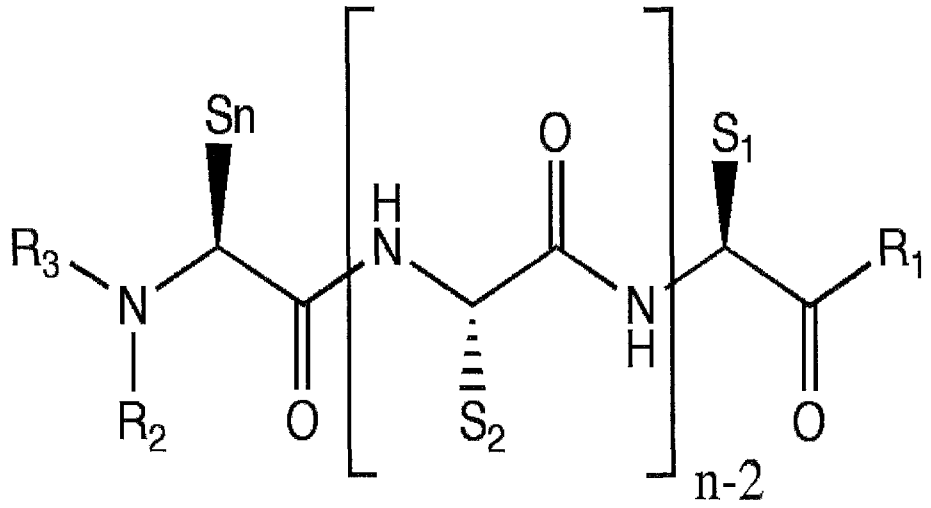
including, but not limited to, liposomes, microparticles and nanoparticles. In one embodiment, the effective dose comprises a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises salts. In one embodiment, the pharmaceutical composition is formulated for oral administration. In one embodiment, the allosteric synthetic peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the allosteric synthetic peptide is six amino acids. In one embodiment, the allosteric synthetic peptide is less than 1,300 Da. In one embodiment, the allosteric synthetic peptide ranges between approximately 466-1067 Da. In one embodiment, the allosteric synthetic peptide ranges between approximately 175-1,000 Da.

In one embodiment, the present invention contemplates a method comprising: a) providing; i) a PCSK9 protein, wherein said protein comprises an allosteric modulation site and an orthosteric low density lipoprotein receptor (LDLR) binding site; and ii) an allosteric synthetic peptide capable of binding said allosteric modulation site; b) binding said allosteric synthetic peptide to said allosteric modulation site, wherein said allosteric synthetic peptide induces a conformational shift of said orthosteric LDLR binding site. In one embodiment, said binding of said allosteric synthetic peptide to said allosteric modulation site, inhibits an induced fit conformational shift of said orthosteric LDLR binding site. In one embodiment, the binding induces a conformational shift of said PCSK9 protein. In one embodiment, the resulting PCSK9 conformational shift reduces the binding affinity of said orthosteric LDLR binding site interaction to a LDLR, wherein low density lipoprotein clearance is increased. In one embodiment, the conformational shift enhances dissociation of said orthosteric low density lipoprotein receptor binding site from a low density lipoprotein receptor. In one embodiment, the conformational shift reduces the orthosteric Cis-His Rich Domain (CHRD) binding site to a binding ligand (e.g., for example, to facilitate vesicle trafficking at low pH; DeVay et al., "Characterization of proprotein convertase subtilisin/kexin type 9 (PCSK9) trafficking reveals a novel lysosomal targeting mechanism via amyloid precursor-like protein 2 (APLP2)" *J Biol Chem.* 288(15):10805-10818 (2013). In one embodiment, the orthosteric low density lipoprotein receptor binding site conformational shift comprises an induced fit inhibition. In one embodiment, the binding of said allosteric synthetic peptide reduces the conformational shift required for the induced fit of the orthosteric LDLR binding site of PCSK9, inhibiting the binding affinity of said orthosteric LDLR interaction, wherein low density lipoprotein

clearance is increased. In one embodiment, the inducing of said orthosteric low density lipoprotein receptor binding site conformational shift is biomechanical. In one embodiment, the conformational shift results in biomechanical stiffening of the connecting loop between a PCSK9 catalytic domain and a PCSK9 C-terminal domain. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid alanine^{443} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid valine^{441} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid aspartic acid^{422} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid threonine^{162} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid proline^{445} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a translocational and/or rotational movement of amino acid proline ^{446} side chain and/or backbone. In one embodiment, the biomechanical conformational shift comprises a reorientation and translocation of histidine^{449}. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid alanine^{443} side chain and/or backbone. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid valine^{441} side chain and/or backbone. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid aspartic acid^{422} side chain and/or backbone. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid threonine^{162} side chain and/or backbone. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid proline^{445} side chain and/or backbone. In one embodiment, the biomechanical mechanism comprises the inhibition of the translocational and/or rotational movement of amino acid proline ^{446} side chain and/or backbone. In one embodiment, the biomechanical shift comprises the inhibition of the translocational and/or rotational movement of histidine^{449} side chain and/or backbone. In one embodiment, the allosteric synthetic peptide is VYVRFW. In one embodiment, the allosteric

synthetic peptide is VLELYW. In one embodiment, the allosteric synthetic peptide is ISDLSY. In one embodiment, the allosteric synthetic peptide comprises between approximately 3 – 8 amino acids. In one embodiment, the allosteric synthetic peptide is six amino acids. In one embodiment, the allosteric synthetic peptide is less than 1,300 Da. In one embodiment, the allosteric synthetic peptide ranges between approximately 466-1067 Da. In one embodiment, the allosteric synthetic peptide ranges between approximately 175-1,000 Da.

In one embodiment, the present invention contemplates a compound of the formula:



wherein: i) n, the number of amino acid residues, is an integer in the range 3-8; ii) the constituent amino acids are single enantiomers of independently selected natural or unnatural amino acids; iii) R2 and R3, are independently selected from the group consisting of hydrogen, a lower alkyl, a branched alkyl, a hydroxyalkyl, a cycloalkyl, a heterocycle, aryl, heteroaryl, acyl, substituted or unsubstituted benzoyl, alkyl or aryl sulfonyl (e.g. mesyl or tosyl) and carbamoyl (e.g. BOC); iv) R1 is selected from the group consisting of –OH and -NR4-R5; v) R4 and R5, independently, are selected from the group consisting of hydrogen; a lower alkyl, an aryl, a cycloalkyl, an aromatic heterocycle, pyridine, tetrazole; alternatively, R4 and R5 are joined as a heterocyle, such as piperidine; pyrrolidine; morpholine; piperazine; a substituted heterocycle, such as 4-methylpiperazine; or a fused heterocycle, such as dihydroquinoline or indoline. In one embodiment, the compound further comprises a negatively charged polar group. In one embodiment, the negatively charged polar group includes, but is not limited to,

O-phosphate, O-sulfate, or 5-O- or 5-N- tetrazole incorporated in the side-chain S1, S2, or S3. In one embodiment, the side chain S1, S2 or S3 comprises a phosphoserine. In one embodiment, the side chain S1 comprises -CH2-NH-tetrazole. In one embodiment, the C-terminus comprises a glycine. In one embodiment, the compound comprises between approximately 3 – 8 amino acids. In one embodiment, the compound is six amino acids. In one embodiment, the compound is less than 1,300 Da. In one embodiment, the compound ranges between approximately 466-1067 Da. In one embodiment, the compound ranges between approximately 175-1,000 Da. In one embodiment, the compound comprises a synthetic peptide.

In one embodiment, the present invention contemplates a compound of the formula: Val-Tyr-Val-Arg-Phe-Trp.

In one embodiment, the present invention contemplates a compound of the formula: β-Ala-Phe(3-CH2NH2)-Val-D-Ser(p)-Phe-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Val-Leu-Glu-Leu-Tyr-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Leu-Asp-Leu-Phe-Phe-Ser.

In one embodiment, the present invention contemplates a compound of the formula: Ile-Leu-Asp-Leu-Ser-Tyr.

In one embodiment, the present invention contemplates a compound of the formula: Ac-Trp-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Ac-Trp-Ala-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Ac-Trp(5-F)-Ala-Ser(p)-morpholine.

In one embodiment, the present invention contemplates a compound of the formula: Thr-Leu-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound of the formula: Ac-Tyr-Trp-Gly.

In one embodiment, the present invention contemplates a compound of the formula: Phe(4-Ph)-Ala-Ser(p)-morpholine.

In one embodiment, the present invention contemplates a compound including, but not limited to, Val-Tyr-Val-Arg-Phe-Trp-NH2, Ala-Phe(3-CH2NH2)-Val-D-Ser(p)-Phe-Trp-NH2, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p)-NH2, Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p)-NH2, Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p)-NH2, Val-Leu-Glu-Leu-Tyr-Trp-NH2, Leu-Asp-Leu-Phe-Phe-Ser-NH2, Ile-Leu-Asp-Leu-Ser-Tyr-NH2, Ac-Trp-Ser-Ser(p)-NH2, Ac-Trp-Ala-Ser(p)-NH2, Ac-Trp(5-F)-Ala-Ser(p)-NH2 and Thr-Leu-Thr-Trp-Ser-Ser-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Trp-Ser-Ser(p)-NHCH3, Ac-Trp-Ala-Ser(p)-NHCH3, Ac-Trp-Ala-Ser(p)-morpholine, Ac-Trp-Ala-Ser(p)-4-methylpiperizine, Ac-Trp-Ala-Ser(p)-piperidine, Ac-Trp-Ala-Ser(p)-pyrrolidine.

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p), Ac-Val-Leu-Glu-Leu-Tyr-Trp, Ac-Leu-Asp-Leu-Phe-Phe-Ser, Ac-Ile-Leu-Asp-Leu-Ser-Tyr and Ac-Thr-Leu-Thr-Trp-Ser-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ala-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ala-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ala-Ser(p), Thr-Leu-Hph-

Thr-Trp-Ser-Ala-Ser(p), Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ala-Ser(p) and Thr-Leu-Thr-Trp-Ser-Ala-Ser(p).

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Thr-Leu-Hph-Thr-Trp-Ser-Ala-Ser(p)-NH2, Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ala-Ser(p)-NH2 and Thr-Leu-Thr-Trp-Ser-Ala-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ala-Ser(p), Ac-Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ala-Ser(p), Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ala-Ser(p), Ac-Thr-Leu-Hph-Thr-Trp-Ser-Ala-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ala-Ser(p) and Ac-Thr-Leu-Thr-Trp-Ser-Ala-Ser(p).

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Ac-Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ala-Ser(p)-NH2, Ac-Thr-Leu-Hph-Thr-Trp-Ser-Ala-Ser(p)-NH2, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ala-Ser(p)-NH2, Ac-Thr-Leu-Thr-Trp-Ser-Ala-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser(p), Thr-Leu-Hph-Thr-Trp-Ser-Ser(p), Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser(p) and Thr-Leu-Thr-Trp-Ser-Ser(p).

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser(p)-NH2, Ac-Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser(p)-NH2, Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser(p)-NH2, Ac-Thr-Leu-Hph-Thr-Trp-Ser-Ser(p)-NH2, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser(p)-NH2 and Ac-Thr-Leu-Thr-Trp-Ser-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ala-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ala-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ala-Ser(p), Thr-Leu-Hph-Thr-Trp-Ala-Ser(p), Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ala-Ser(p) and Thr-Leu-Thr-Trp-Ala-Ser(p).

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ala-Ser(p)-NH2, Ac-Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ala-Ser(p)-NH2, Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ala-Ser(p)-NH2, Ac-Thr-Leu-Hph-Thr-Trp-Ala-Ser(p)-NH2, Ac-Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ala-Ser(p)-NH2 and Ac-Thr-Leu-Thr-Trp-Ala-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Ala-Trp-Ser-Ser-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Ala-Trp-Ser-Ser-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Ala-Trp-Ser-Ser-Ser(p), Thr-Leu-Hph-Ala-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Ala-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Asp(NHCH2Ph)-Ala-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Ala-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Hph-Ala-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Ala-Trp-Ser-Ser-Ser(p)-NH2, Ac-Thr-Leu-Asp(NHCH2Ph)-Ala-Trp-Ser-Ser-Ser(p)-NH2, Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Ala-Trp-Ser-Ser-Ser(p)-NH2 and Ac-Thr-Leu-Hph-Ala-Trp-Ser-Ser-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH2-Ph)-Ser-Trp-Ser-Ser-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Ser-Trp-Ser-Ser-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Ser-Trp-Ser-Ser-Ser(p), Thr-Leu-Hph-Ser-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Ser-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Asp(NHCH2Ph)-Ser-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Ser-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Hph-Ser-Trp-Ser-Ser-Ser(p), Ac-Thr-Leu-Cys(CH2-Ph)-Ser-Trp-Ser-Ser-Ser(p)-NH2, Ac-Thr-Leu-Asp(NHCH2Ph)-Ser-Trp-Ser-Ser-Ser(p)-NH2, Ac-Thr-Leu-Gly(CH2CH2cyclohexyl)-Ser-Trp-Ser-Ser-Ser(p)-NH2 and Ac-Thr-Leu-Hph-Ser-Trp-Ser-Ser-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound including, but not limited to, Ac-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), Ac-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p), Ac-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), Ac-Hph-Thr-Trp-Ser-Ser-Ser(p), Ac-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, Ac-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, Ac-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p)-NH2 and Ac-Hph-Thr-Trp-Ser-Ser-Ser(p)-NH2.

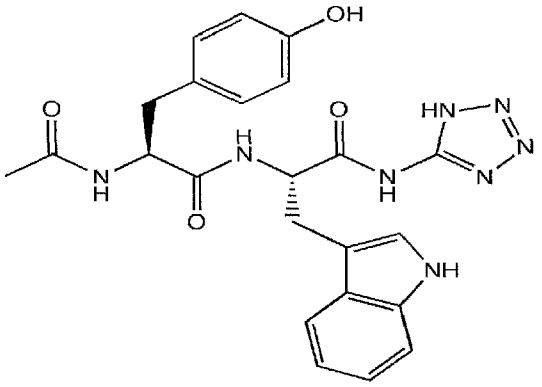
In one embodiment, the present invention contemplates a compound including, but not limited to, BOC-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), BOC-Asp(NHCH2Ph)-Thr-Trp-Ser-

Ser-Ser(p), BOC-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), BOC-Hph-Thr-Trp-Ser-Ser-Ser(p), BOC-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, BOC-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2, BOC-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p)-NH2 and BOC-Hph-Thr-Trp-Ser-Ser-Ser(p)-NH2.

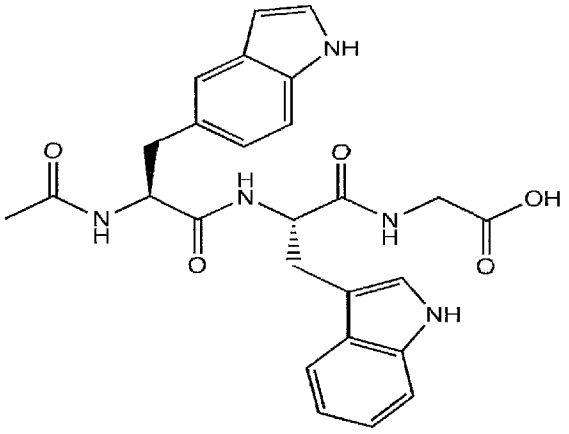
In one embodiment, the present invention contemplates a compound including, but not limited to, Thr-Leu-Cys(CH3)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH(CH3)2)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH2-3,4-difluorophenyl)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH2-3-hydroxyphenyl)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH2-3-methyphenyl)-Thr-Trp-Ser-Ser-Ser(p) and Ac-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p)-NH2.

In one embodiment, the present invention contemplates a compound having the formula of Phe(4-Ph)-Gly(Et)-Ser(p)-morpholine.

In one embodiment, the present invention contemplates a compound selected from the group consisting of Ac-Tyr-Trp(6-OMe)-Gly, Ac-Tyr(3-F)-Trp-Gly, pivaloyl-Tyr-Trp-Gly, mesyl-Tyr-Trp-Gly, BOC-Tyr-Trp-Gly,

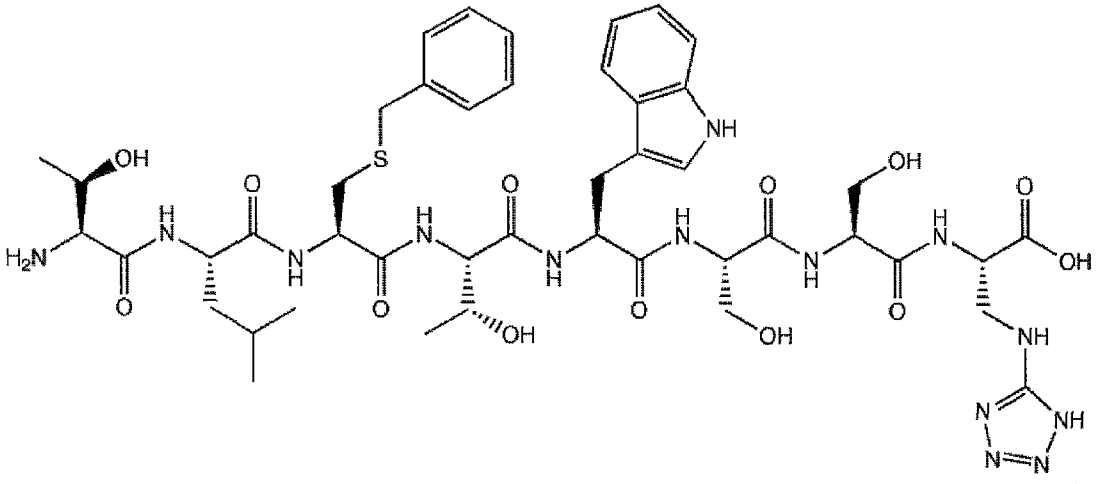


,



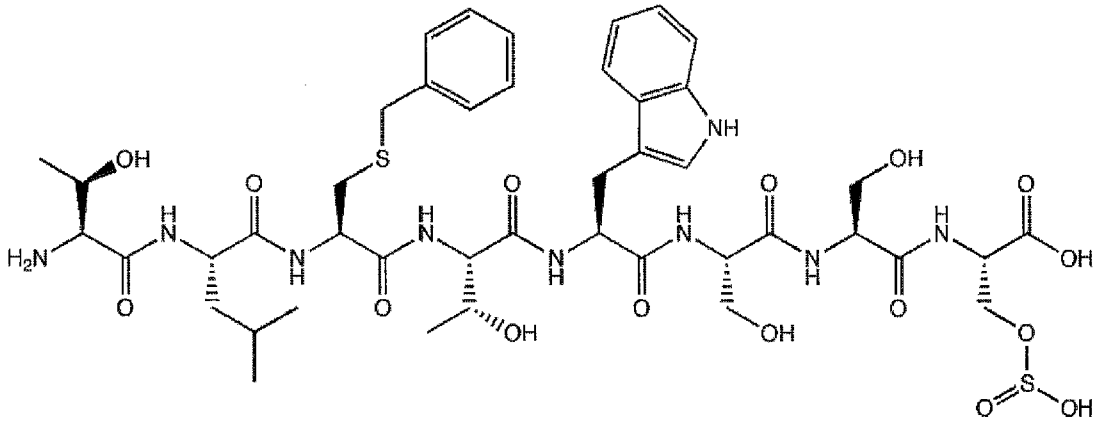
,

5



, and

10



In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Trp-D-Phe(3CF3)-D-Arg-NH2.

In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Trp-D-Phe(3Cl)-D-Arg-NH2.

In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Trp-D-Phe-D-Arg-NH2.

In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Trp-D-Phe-D-Arg.

In one embodiment, the present invention contemplates a compound of the formula: NAc-NMe-D-Arg-D-Phe(3OH)-D-Trp-NH2.

In one embodiment, the present invention contemplates a compound of the formula: Ac-Arg-Phe(3CF3)-Gly.

In one embodiment, the present invention contemplates a compound of the formula: Ac-Ala-Val-Arg-N(Me)(Ph3CF3).

In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Arg-D-Phe(3OH)-D-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Ac-D-Arg-D-Phe(3OH)-D-Trp-NH2.

In one embodiment, the present invention contemplates a compound of the formula: Propionyl-D-Arg-D-Phe(3OH)-D-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Ac-Val-Arg-Phe-Trp.

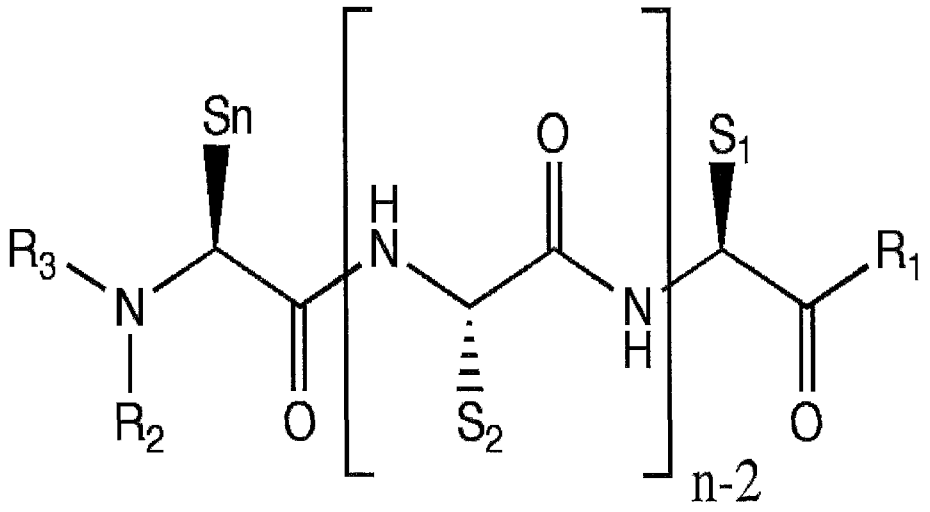
In one embodiment, the present invention contemplates a compound of the formula: Ac-Tyr-Val-Arg-Phe-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Val-Tyr-Asp-Arg-Phe-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Val-Tyr-Glu-Arg-Phe-Trp.

In one embodiment, the present invention contemplates a compound of the formula: Val-Tyr-Val-Cit-Phe-W (Cit = Citrulline).

In one embodiment, the present invention contemplates a pharmaceutical composition comprising a compound of the formula:



and a carrier, wherein: i) n, the number of amino acid residues, is an integer in the range 3-8; ii) the constituent amino acids are single enantiomers of independently selected natural or unnatural amino acids; iii) R2 and R3, are independently selected from the group consisting of

hydrogen, a lower alkyl, a branched alkyl, a hydroxyalkyl, a cycloalkyl, a heterocycle, aryl, heteroaryl, acyl, substituted or unsubstituted benzoyl, alkyl or aryl sulfonyl (e.g. mesyl or tosyl) and carbamoyl (e.g. BOC); iv) R1 is selected from the group consisting of –OH and -NR4-R5; v) R4 and R5, independently, are selected from the group consisting of hydrogen; a lower alkyl, an aryl, a cycloalkyl, an aromatic heterocycle, pyridine, tetrazole; alternatively, R4 and R5 are joined as a heterocyle, such as piperidine; pyrrolidine; morpholine; piperazine; a substituted heterocycle, such as 4-methylpiperazine; or a fused heterocycle, such as dihydroquinoline or indoline. In one embodiment, the pharmaceutical composition further comprises a negatively charged polar group. In one embodiment, said negatively charged polar group is selected from at least one of the group consisting of O-phosphate, O-sulfate, or 5-O- or 5-N- tetrazole incorporated in the side-chain S1, S2, or S3. In one embodiment, the side chain S1, S2 or S3 comprises a phosphoserine. In one embodiment, the side chain S1 comprises -CH2-NH-tetrazole. In one embodiment, the C-terminus comprises a glycine. In one embodiment, the pharmaceutical composition further comprises a statin. In one embodiment, the statin includes, but is not limited to, atorvastatin, rosuvastatin and/or simvastatin. In one embodiment, the pharmaceutical composition comprises an anti-diabetic drug. In one embodiment, the pharmaceutical composition comprises a cardiovascular drug. In one embodiment, the pharmaceutical composition comprises ezetimibe (Zetia®). In one embodiment, the pharmaceutical composition comprises an anti-hypertensive including, but not limited to, amlodipine besylate (Norvasc®). In one embodiment the anti-diabetic drug includes, but not limited to, sitagliptin (Januvia®) and/or metformin. In one embodiment, the compound comprises between approximately 3 – 8 amino acids. In one embodiment, the compound is six amino acids. In one embodiment, the compound is less than 1,300 Da. In one embodiment, the compound ranges between approximately 466-1067 Da. In one embodiment, the compound ranges between approximately 175-1,000 Da. In one embodiment, the compound comprises a synthetic peptide.

Definitions

The term "compound" or "ligand" as used herein, refers to any exogenous molecule comprising natural amino acids capable of interacting with (i.e., for example, attaching, binding etc.) to a binding partner thereby altering the biological function of the binding partner.

Compounds/ligands may include, but are not limited to, an amino acid chain comprising at least two peptide bonds, antibodies, proteins, peptides, and/or tripeptides. Such compounds/ligands may be derivatized by substitutents including, but not limited to, hydroxyls, sulfurs, amines, amides, ethers, esters, aliphatic chains, aromatic rings, aliphatic rings, subtituted aromatic rings and/or substituted aliphatic rings. Such compounds/ligands may be an inhibitor compound/ligand, or an enhancer compound/ligand. A compound/ligand may also include a "drug", thereby referring to any pharmacologically active substance capable of being administered, which achieves a desired effect. Drugs or compounds/ligands can be synthetic or naturally occurring

The term "synthetic ligand" as used herein, refers to a molecule comprising amino acids which is a ligand, and was designed ex vivo and is subsequently synthesized using *in vitro*, *in vivo*, or a combination of *in vitro* and *in vivo* means to produce a molecule of pre-specified characteristics (e.g., charge, shape, molecular weight) and is bound by another naturally occurring biomolecule to form a complex. Preferably these synthetic ligands are smaller than a target natural biomolecule, more preferably these synthetic ligands are less than 1,300 Da, and more preferably are between 350 and 1,250 Da.

The term "synthetic peptide" as used herein, refers to non-natural amino acid sequence of approximately 3-8 amino acids and ranging between approximately 350-1,500 Da. Preferably a non-natural amino acid sequence of approximately 4-5 amino acids and ranging between approximately 550 – 1,000 Da. For example, a synthetic peptide is six amino acids and less than 1,300 Da, for example, ranging between approximately 466-1067 Da. Preferably, a synthetic peptide is made in accordance with Example V.

The term "allosteric site" as used herein, refers to a ligand binding site, other than the native chemically active/receptor binding site that, when bound to an exogenous ligand, changes the shape and activity of a protein (as an enzyme). For example, an "allosteric enhancer peptide" refers to a ligand binding to an allosteric site that may increase the native activity and/or respective affinity(ies) of the protein (e.g., for example, a PCSK9 allosteric enhancer peptide). Alternatively, an "allosteric inhibitor peptide" refers to a ligand binding to an allosteric site that may decrease the native activity and/or respective affinity(ies) of the protein (e.g., for example, a PCSK9 allosteric inhibitor peptide). For example, the binding site

comprises His^{417}, Lys^{421}, Pro^{446}, Trp^{453}, Gln^{454}, Glu^{628}, Gly^{629}, Asn^{652}, and Thr^{653} of the PCSK9 protein.

The term "orthosteric site" as used herein, refers to a primary, unmodulated binding site of a ligand (e.g., for example, a peptide) to a receptor, binding and/or a catalytic site.

The term "conformation" as used herein, refers to a three-dimensional stereochemical configuration of an amino acid sequence. For example, any specific conformation results from a thermodynamic balance between steric interactions, hydrophobic interactions, hydrogen bonding, electrochemical bonding and/or salt bridge interactions between individual amino acids in an amino acid sequence.

The term "conformational shift" as used herein, refers to the introduction of an exogenous force or molecule (e.g., an inhibitor peptide) that may alter a first thermodynamic balance (conformation 1) into a second thermodynamic balance (conformation 2), or enhances the dynamic range and/or the type and/or the number of metastable folding states within a lone protein, and/or a protein-ligand complex, and/or a protein-protein complex. Furthermore, a conformation shift may be predominantly exhibited under certain specific external conditions (pH, temperature, etc.) and/or during specific periods within the lifetime of a lone protein or multi-part complex, including but not limited to conditions preferential for molecular recognition, initial binding interaction, induced fit interaction, functional activity, and/or dissociation.

The term "EGFA" as used herein, refers to the most amino EGF-like domain of the low density lipoprotein receptor. For example, the EGF-like domain may comprise an extracellular portion of the LDLR receptor.

The term "LDL-R" and "LDLR" as used herein, refers to an abbreviation for the low density lipoprotein receptor. The abbreviation may be in reference to the entire LDL-R receptor protein or any portion thereof. LDL-Rs reside on a cell surface and can bind to low density lipoproteins such that the LDL-R/LDL complex become internalized within a cell (i.e., for example, a hepatocyte), wherein the LDL is released and the LDL-R is recycled back to the cell surface.

The term, "binding interface" as used herein, refers to any collection of attractive interactions (i.e., for example, hydrogen bonding, electrostatic interactions, hydrophobic interactions, etc) between the functional groups (i.e., for example, hydroxyl, amide, amine,

carboxyl, amidine, guanidine, hydrocarbon, sulfonyl etc.) of at least two different molecules. The collection of attractive forces forms a stable molecular plane thereby forming a 'binding interface' between the at least two molecules.

The term "induced fit" as used herein, refers to any acceptance of a peptide requiring a change in receptor conformation. Such a conformation may be facilitated by a translational / rotational movement of amino acid side chains and flexible loops, thereby rearranging the electrostatic and/or hydrophobic fields.

The term "complex" or "composition" as used herein, refers to any chemical association of two or more ions or molecules joined usually by weak electrostatic bonds rather than by covalent bonds. For example, a complex or composition may be formed between a peptide as described herein and a PCSK9 amino acid sequence, thereby creating a peptide/PCSK9 amino acid sequence complex or composition. Optionally, such complexes or compositions may also include, but are not limited to, an LDLR amino acid sequence or any portion thereof, including but not limited to the EGFA region.

The term "hydrogen bond" as used herein, an electrostatic attraction between a hydrogen atom in one polar molecule (as of water) and a small electronegative atom (as of oxygen, nitrogen, or fluorine) in usually another molecule of the same or a different polar substance.

The term "salt bridge" as used herein, refers to any interaction or a combinations of interactions, such as hydrogen bonding and/or electrostatic interactions, which align cationic and anionic chemical structures in such a way that the charged moieties overlap.

The term "interaction" as used herein, refers to any effect that one molecule and/or functional group may have on another molecule and/or functional group. Such effects may include, but are not limited to, steric (i.e., for example, physical), electrostatic (i.e., for example, electrical attraction or repulsion), electromagnetic, hydrophilic, or hydrophobic effects.

The term "overlap" as used herein, refers to any positioning of molecules in such a way that the electronic structure of one molecule is on top of, and extending past the border of another molecule, or be positioned in this way.

The term "hypercholesterolemia" as used herein, refers to any medical condition wherein blood cholesterol levels are elevated above the clinically recommended levels. For

example, if cholesterol is measured using low density lipoproteins (LDLs), hypercholesterolemia may exist if the measured LDL levels are above, for example, approximately 70 mg/dl. Alternatively, if cholesterol is measured using free plasma cholesterol, hypercholesterolemia may exist if the measured free cholesterol levels are above, for example, approximately 200-220 mg/dl.

The term "at risk for" as used herein, refers to a medical condition or set of medical conditions exhibited by a patient which may predispose the patient to a particular disease or affliction. For example, these conditions may result from influences that include, but are not limited to, behavioral, emotional, chemical, biochemical, or environmental influences.

The term "effective amount" as used herein, refers to a particular amount of a pharmaceutical composition comprising a therapeutic agent that achieves a clinically beneficial result (i.e., for example, a reduction of symptoms). Toxicity and therapeutic efficacy of such compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and additional animal studies can be used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The term "symptom", as used herein, refers to any subjective or objective evidence of disease or physical disturbance observed by the patient. For example, subjective evidence is usually based upon patient self-reporting and may include, but is not limited to, pain, headache, visual disturbances, nausea and/or vomiting. Alternatively, objective evidence is usually a result of medical testing including, but not limited to, body temperature, complete blood count, lipid panels, thyroid panels, blood pressure, heart rate, electrocardiogram, tissue and/or body imaging scans.

The term "disease" and/or "disorder", as used herein, refers to any impairment of the normal state of the living animal or plant body or one of its parts that interrupts or modifies the performance of the vital functions. Typically manifested by distinguishing signs and symptoms, it is usually a response to: i) environmental factors (as malnutrition, industrial hazards, or climate); ii) specific infective agents (as worms, bacteria, or viruses); iii) inherent defects of the organism (as genetic anomalies); and/or iv) combinations of these factors

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," "prevent" and grammatical equivalents (including "lower," "smaller," etc.) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity and/or magnitude of the symptoms in the untreated subject.

The terms "increase," "enhance," "elevate," and grammatical equivalents (including "higher," "larger," etc.) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is greater than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10% greater than, at least 25% greater than, at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity and/or magnitude of the symptoms in the untreated subject.

The term "attached" as used herein, refers to any interaction between a medium (or carrier) and a drug. Attachment may be reversible or irreversible. Such attachment includes, but is not limited to, covalent bonding, ionic bonding, Van der Waals forces or friction, and the like. A drug is attached to a medium (or carrier) if it is impregnated, incorporated, coated, in suspension with, in emulsion with, in solution with, mixed with, *etc.*

The term "administered" or "administering", as used herein, refers to any method of providing a composition to a patient such that the composition has its intended effect on the patient. An exemplary method of administering is by a direct mechanism such as, local tissue administration (*i.e.,* for example, extravascular placement), oral ingestion, transdermal patch, topical, inhalation, suppository *etc.*

The term "patient" or "subject", as used herein, is a human or animal and need not be hospitalized. For example, out-patients, persons in nursing homes are "patients." A patient may comprise any age of a human or non-human animal and therefore includes both adult and juveniles (*i.e.,* children). It is not intended that the term "patient" connote a need for medical treatment, therefore, a patient may voluntarily or involuntarily be part of experimentation whether clinical or in support of basic science studies.

The term "affinity" as used herein, refers to the measure of the thermodynamic tendency of two or more molecules to assemble to form a multi-part complex and to remain assembled in said complex. For example, the SRX55 ligand has a high affinity for PCSK9 and is thermodynamically favored to form a complex. It is understood that a change in conditions (e.g., pH during the receptor internalization process) For example, a decrease in the LDL affinity for LDLR and the two molecules may dissociate, or separate, from one another.

The term "derived from" as used herein, refers to the source of a compound or amino acid sequence. In one respect, a compound or amino acid sequence may be derived from an organism or particular species. In another respect, a compound or amino acid sequence may be derived from a larger complex or sequence. In another respect, a compound or sequence may be derived by chemical modification of part or all of an amino acid sequence found in nature.

The term "protein" as used herein, refers to any of numerous naturally occurring extremely complex substances (as an enzyme or antibody) that consist of amino acid residues joined by peptide bonds, contain the elements carbon, hydrogen, nitrogen, oxygen, usually sulfur. In general, a protein comprises amino acids having an order of magnitude within the hundreds.

The term "peptide" as used herein, refers to any of various amides that are derived from three or more amino acids by combination of the amino group of one acid with the carboxyl

group of another and are usually obtained by partial hydrolysis of proteins. In general, a peptide comprises amino acids having an order of magnitude within the tens or smaller.

The term "pharmaceutically" or "pharmacologically acceptable", as used herein, refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human.

The term, "pharmaceutically acceptable carrier", as used herein, includes any and all solvents, or a dispersion medium including, but not limited to, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and dimethylsulfoxide, vegetable oils, coatings, isotonic and absorption delaying agents, liposome, commercially available cleansers, and the like. Supplementary bioactive ingredients also can be incorporated into such carriers.

The term, "purified" or "isolated", as used herein, may refer to a peptide composition that has been subjected to treatment (i.e., for example, fractionation) to remove various other components, and which composition substantially retains its expressed biological activity.

Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the composition (i.e., for example, weight/weight and/or weight/volume). The term "purified to homogeneity" is used to include compositions that have been purified to 'apparent homogeneity" such that there is single protein species (i.e., for example, based upon SDS-PAGE or HPLC analysis). A purified composition is not intended to mean that some trace impurities may remain.

As used herein, the term "substantially purified" refers to molecules, such as amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and more preferably 90% free from other components with which they are naturally associated. An "isolated polypeptide" is therefore a substantially purified polypeptide.

The term "biocompatible", as used herein, refers to any material does not elicit a substantial detrimental response in the host. There is always concern, when a foreign object is introduced into a living body, that the object will induce an immune reaction, such as an inflammatory response that will have negative effects on the host. In the context of this

invention, biocompatibility is evaluated according to the application for which it was designed: for example; a bandage is regarded a biocompatible with the skin, whereas an implanted medical device is regarded as biocompatible with the internal tissues of the body. Preferably, biocompatible materials include, but are not limited to, biodegradable and biostable materials.

The terms "amino acid sequence" and "polypeptide sequence" as used herein, are interchangeable and to refer to a sequence of amino acids.

A "variant" of a protein is defined as an amino acid sequence which differs by one or more amino acids from a polypeptide sequence or any homolog of the polypeptide sequence. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions (i.e., additions), or both.

A "deletion" is defined as a change in amino acid sequence in which one or more amino acid residues, respectively, are absent.

An "insertion" or "addition" is that change in an amino acid sequence which has resulted in the addition of one or more amino acid residues.

The term "derivative" as used herein, refers to any chemical modification of an amino acid. Illustrative of such modifications would include, but are not limited to, replacement of hydrogen by an alkyl, aryl, hydroxyl, sulfhydryl, sulfoxyl, sulfonyl, acyl, phosphoryl, alkoxyl, amino or amino heterocyclic group. For example, tyrosine is a 4-hydroxyl amino acid derivative of phenylalanine, and phosphoserine is an O-phosphoric derivative of serine. Other possible chemical modifications might include, but are not limited to, C-terminal amides, and acyl or sulfonyl N-terminal modifications.

The term "bind" as used herein, includes any physical attachment or close association, which may be permanent or temporary. Generally, an interaction of hydrogen bonding, hydrophobic forces, van der Waals forces, covalent and ionic bonding etc., facilitates physical attachment between the molecule of interest and the analyte/target being measuring/affected. The "binding" interaction may be brief as in the situation where binding causes a chemical reaction to occur. That is typical when the binding component is an enzyme and the analyte/target is a substrate for the enzyme. Reactions resulting from contact between the

binding agent and the analyte/target are also within the definition of binding for the purposes of the present invention.

Brief Description Of The Figures

Figure 1 shows exemplary data of WT PCSK9 inhibition as measured by FACS in HuH7 cells. HuH7 cells were incubated for 18h in the absence (Cnt) or presence of 0.75 μg/ml PCSK9-WT protein alone (WT) or mixed with 100 μM of various SRX peptides. The level of LDLR at the cell surface was measured by FACS using anti human LDLR Ab and a suitable secondary Ab labeled with Alexa 647. Cell surface LDLR is reported relative to Cnt. % inhibition of activity was calculated as [SRX – WT] / [Cnt – WT] x 100.

Figure 2 shows exemplary data of WT PCSK9 activity by numerous PCSK9 allosteric modulation peptides. HuH7 cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence of 1.0 μg/ml PCSK9-WT protein alone (WT) or mixed with 100 uM of various SRX peptides. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells.

Figure 3 shows exemplary data of a mutated PCSK9 protein ("gain of function" (GOF)-D374Y) modulation by numerous PCSK9 allosteric modulation peptides. HuH7 cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence of 0.5 μg/ml PCSK9-D374Y protein alone (DY) or mixed with 100 uM of various SRX peptides. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells.

Figure 4 shows exemplary data of a mutated PCSK9 protein ('gain of function" GOF-D374Y) modulation showing dose dependent inhibition by SRX55, as measured by dil-LDL uptake in HuH7 cells. HuH7 cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence of 0.5 μg/ml PCSK9 GOF-D374Y protein alone (DY) or mixed with increasing concentrations of various SRX peptides. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells.

Figure 5 shows exemplary data of a mutated PCSK9 protein ("gain of function" GOF-D374Y) modulation showing dose-dependent inhibition by SRX55, as measured by dil-LDL uptake in HepG2 cells. HepG2 cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence of 2 μg/ml PCSK9 GOF-D374Y protein alone (DY) or mixed with increasing concentrations of SRX55 peptide. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells.

Figure 6 shows exemplary data of HepG2 cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence PCSK9 protein alone (D374Y: 0.6 ug/ml; WT: 1.2 ug/ml) or mixed with increasing concentrations of SRX55 peptide. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells. The PCSK9 and -/+ SRX55 mixtures were pre-incubated for 3 hrs at 37C prior to addition to the cells.

Figure 7 shows exemplary data of FL-83B cells were incubated in a 96-well plate for a total of 20h in the absence (Cnt) or presence PCSK9 protein alone (D374Y: 0.6 ug/ml; WT: 1.2 ug/ml) or mixed with increasing concentrations of SRX55 peptide. After 16h, dil-LDL (5 ug/ml) was added to the incubation mixtures. After 4h, fluorescence was measured (Ex: 520 nm/ Em: 575nm; cutoff: 550nm). Dil-LDL uptake is calculated as RFU corrected for the number of cells. The PCSK9 and -/+ SRX55 mixtures were pre-incubated for 3 hrs at 37C prior to addition to the cells.

Figure 8 presents an illustrative embodiment showing the binding of an allosteric modulatory synthetic peptide (e.g., SRX55) to a PCSK9 protein. The prodomain is shown in light blue. The two halves of the PCSK9 "catalytic" domain are shown as yellow and dark blue, respectively. The EGF-A binding site is shown as blue and yellow spacefill. SRX55 (green) is shown binding to the allosteric ligand binding site. The N-terminal helix is shown in white.

Detailed Description Of The Invention

This invention is related to the field of hypercholesterolemia. In particular, the invention provides compositions and methods to modulate circulating levels of low density

lipoproteins by altering the conformation of the protein PCSK9 using a synthetic peptide and/or a synthetic peptide derivative sequences of 3-8 amino acids ranging between 350 – 2,000 Da. Altering the conformation of PCSK9 affects the interaction between PCSK9 and an endogenous low density lipoprotein receptor, and can lead to reduced or increased levels of circulating LDL-cholesterol. High LDL-cholesterol levels are associated with increased risk for heart disease. Low LDL-cholesterol levels may be problematic in other conditions, such as liver dysfunction; thus, there is also utility for peptides which can raise LDL levels.

I. Physiological Role Of Native PCSK9

Proprotein convertase subtilisin/kexin type 9, also known as PCSK9, is an enzyme that in humans is encoded by the PCSK9 gene. Seidah et al., "The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation" *Proc. Natl. Acad. Sci. U.S.A.* 100 (3): 928–933 (2003). Similar genes (orthologs) are found across many species. Many enzymes, including PSCK9, are inactive when they are first synthesized, because they have a section of peptide chains that blocks their activity; proprotein convertases remove that section to activate the enzyme.

An illustrative embodiment shows the binding of an allosteric modulatory synthetic peptide (e.g., SRX55) to a PCSK9 protein. See, Figure 8. The prodomain is shown in light blue. The two halves of the PCSK9 "catalytic" domain are shown as yellow and dark blue, respectively. The EGF-A binding site is shown as blue and yellow spacefill. SRX55 (green) is shown binding to the allosteric ligand binding site. The N-terminal helix is shown in white.

The PSCK9 gene encodes a proprotein convertase belonging to the proteinase K subfamily of the secretory subtilase family. The encoded protein is synthesized as a soluble zymogen that undergoes autocatalytic intramolecular processing in the endoplasmic reticulum. The protein may function as a proprotein convertase. For example, a human PCSK9 amino acid sequence is:

001 mgtvssrrsw wplpllllll lllgpagara qededgdyee lvlalrseed glaeapehgt
061 tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp
121 gflvkmsgdl lelalklphv dyieedssvf aqsipwnler itppryrade yqppdggslv
181 evylldtsiq sdhreiegrv mvtdfenvpe edgtrfhrqa skcdshgthl agvvsgrdag

241 vakgasmrsl rvlncqgkgt vsgtliglef irksqlvqpv gplvvllpla ggysrvlnaa
301 cqrlaragvv lvtaagnfrd daclyspasa pevitvgatn aqdqpvtlgt lgtnfgrcvd
361 lfapgediig assdcstcfv sqsgtsqaaa hvagiaamml saepeltlae lrqrlihfsa
421 kdvineawfp edqrvltpnl vaalppsthg agwqlfcrtv wsahsgptrm atavarcapd
481 eellscssfs rsgkrrgerm eaqggklvcr ahnafggegv yaiarcdllp qancsvhtap
541 paeasmgtrv hchqqghvlt gcsshweved lgthkppvlr prgqpnqcvg hreasihasc
601 chapgleckv kehgipapqe qvtvaceegw tltgcsalpg tshvlgayav dntcvvrsrd
661 vsttgstseg avtavaiccr srhlaqasqe lq (Accession No. NP_777596).

PSCK9 is believed to play a regulatory role in cholesterol homeostasis. For example, PCSK9 can bind to the epidermal growth factor-like repeat A (EGF-A) domain of the low-density lipoprotein receptor (LDL-R) resulting in LDL-R internalization and degradation. Clearly, it would be expected that reduced LDL-R levels result in decreased metabolism of LDL-C, which could lead to hypercholesterolemia.

As it is estimated that approximately 9 million Americans have a high or very high risk for heart-related problems that could benefit from PCSK9 inhibitors (especially when in combination with statins). PCSK9 inhibitors could result in such widespread usage having the potential to replace statins in certain conditions. PCSK9 has medical significance because it acts in cholesterol homeostasis. Drugs that block PCSK9 biological actions are believed to lower circulating low-density lipoprotein cholesterol (LDL-C) levels (i.e., for example, by increasing the availability of LDL-Rs and, consequently, LDL-C clearance). Such drugs are beginning Phase III clinical trials to assess their safety and efficacy in humans, and to determine if they can improve outcomes in heart disease.

Drugs that inhibit LDL-R/PCSK9 complex formation have been suggested to lower cholesterol much more than conventionally available cholesterol-lowering drugs (i.e., for example, statins). It is biologically plausible that this would also lower heart attacks and other diseases caused by raised cholesterol. Studies with humans, including phase III clinical trials now underway, are focused as to whether PCSK9 inhibition actually does lower cardiovascular disease, with acceptable side effects. Lopez D., "Inhibition of PCSK9 as a novel strategy for the treatment of hypercholesterolemia" *Drug News Perspect.* 21(6): 323–e30 (2008); Steinberg et al., "Inhibition of PCSK9: a powerful weapon for achieving ideal LDL cholesterol levels"

Proc. Natl. Acad. Sci. U.S.A. 106(24): 9546–9547 (2009); Mayer, "Annexin A2 is a C-terminal PCSK9-binding protein that regulates endogenous low density lipoprotein receptor levels" *J. Biol. Chem.* 283(46): 31791–31801 ((2008); and Anonomyous, "Bristol-Myers Squibb selects Isis drug targeting PCSK9 as development candidate for prevention and treatment of cardiovascular disease" *Press Release. FierceBiotech.* 2008-04-08.

Currently, it has been reported that PCSK9 antibody drugs are in clinical trials (e.g., for example, Sanofi/Regeneron, Amgen, Pfizer, Novartis, Roche). However, one disadvantage of antibody therapy is that the administration is performed by subcutaneous or intravenous injection. A number of monoclonal antibodies that bind to PCSK9 near the catalytic domain that interact with the LDL-R and hence inhibit LDL-R/PCSK9 complex formation are currently in clinical trials. These antibodies include AMG145 (Amgen), 1D05-IgG2 (Merck & Co.), and SAR236553/REGN727 (Aventis/Regeneron). Lambert et al., "The PCSK9 decade" *J. Lipid Res.* 53(12): 2515–2524 (2012).

Peptides that mimic the EGF-A domain of the LDL-R have been developed to inhibit LDL-R/PCSK9 complex formation. Shan et al., "PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide". *Biochem. Biophys. Res. Commun.* 375(1): 69–73 (2008). Peptidic PCSK9 inhibitors of the EGF-A binding site were identified by screening both linear and disulfide-constrained phage-displayed peptide libraries. This approach identified a 13-amino acid peptide (Pep2-8) that includes structural mimicry of the natural binding domain of LDL receptor. The peptide inhibitor binding site was determined to largely overlap with that of the EGF(A) domain; therefore, Pep2-8 acts a competitive inhibitor of LDL receptor binding. This is akin to the inhibition mechanism of anti-PCSK9 monoclonal antibodies, which also disrupt the interaction of the LDL receptor-EGF(A) domain with PCSK9. Zhang et al., "Identification of a Small Peptide That Inhibits PCSK9 Protein Binding to the Low Density Lipoprotein Receptor' *J Biol Chem 289:942–955 (2014).*

PCSK9 antisense oligonucleotides (Isis Pharmaceuticals) have been shown to increase expression of the LDL-R and decrease circulating total cholesterol levels in mice. Graham et al., "Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice" *J. Lipid Res.* 48(4): 763–767 (2007). It has also been reported that a locked nucleic acid (Santaris Pharma) reduced PCSK9 mRNA levels in mice. Gupta et al., "A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR

expression in vitro and in vivo" *PLoS ONE* 5(5): e10682 (2010); and Lindholm et al., "PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates". *Mol. Ther.* 20(2):376–381 (2012). Initial clinical trials of an RNAi (ALN-PCS, Alnylam Pharmaceuticals) has shown positive results as an effective means of inhibiting LDL-R/PCSK9 complex formation. Frank-Kamenetsky et al., "Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates" *Proc. Natl. Acad. Sci. U.S.A.* 105(33): 11915–11920 (2008).

II. PCSK9 Allosteric Site Modulation Peptides

Variants of PCSK9 can reduce or increase circulating cholesterol. Abifadel et al., "Mutations in PCSK9 cause autosomal dominant hypercholesterolemia" *Nat. Genet.* 34 (2): 154–156 (2003). LDL-C is normally removed from the blood when it binds to an LDL-R on the surface of liver cells, and is internalized within the hepatocyte as a receptor-ligand complex. However, when PCSK9 binds to an LDL-R, the LDL-R is concomitantly degraded along with the complexed LDL particle. However, if a PCSK9 is not bound to an LDL-R, the LDL-R is recycled after internalization thereby returning to the surface of the cell for removal of more cholesterol.

In some embodiments, the invention relates to synthetic peptide sequences of 3-8 amino acids in length, and less than approximately 1,300 Da, having a modulation effect on PCSK9's ability to form an LDL-R/PCSK9 complex. In some embodiments, the synthetic peptides comprise a lipophilic N-terminal amino acid (e.g., phenylalanine). In some embodiments, the present invention contemplate the use of peptides that bind to a PCSK9 allosteric site. In some embodiments, the peptides decrease LDL-R/PCSK9 complex formation and are thereby useful to treat various diseases comprising lipid dysregulation. In some embodiments, the peptides increase LDL-R/PCSK9 complex formation and are thereby useful in research and development of therapies relevant to LDL dysregulation.

Although it is not necessary to understand the mechanism of an invention, it is believed that "gain-of-function" (GOF) PCSK9 mutants may result in conditions including, but not limited to, hypercholesterolemia. For example, peptides (e.g., synthetic peptides and/or synthetic peptide derivatives) that bind to a PCSK9 allosteric site and increase the affinity of PCSK9's low density lipoprotein receptor for a low density lipoprotein receptor on the surface

of a cell (e.g., a hepatocyte) would be expected to increase the symptoms of hypercholesterolemia by increasing low density lipoprotein receptor internalization and degradation.

Although it is not necessary to understand the mechanism of an invention, it is believed that "loss-of-function" (LOF) PCSK9 mutants may result in conditions comprising reduced low density lipoproteins and would be expected to result in hypocholesterolemia thereby reducing the risk of cardiovascular diseases, including but not limited to, coronary heart disease. For example, peptides that bind to a PCSK9 allosteric site that decrease the affinity of PCSK9's low density lipoprotein receptor binding site for a low density lipoprotein receptor on the surface of a cell (e.g., a hepatocyte) would be expected to reduce the symptoms of hypercholesterolemia by promoting low density lipoprotein internalization and clearance due to concomitant recycling of the low density lipoprotein receptor.

The presently disclosed embodiments of PCSK9 allosteric peptides have several advantages over current therapeutic strategies to control LDL discussed above. For example, small PCSK9 allosteric peptides, as contemplated herein, have the advantage that these peptides can be administered orally without immunological reactions seen with antibody administration, or systemic degradation problems as seen with nucleic acid administration (i.e., antisense or locked nucleic acids). Nonetheless, as these small peptides have long half-lives, encapsulation drug delivery systems, such as liposomes or other biodegradable protective compositions, will lengthen these half-lives to a greater extent than either antibodies or nucleic acids.

The data presented herein exemplifies sixteen (16) synthetic peptides having various effects on PCSK9's ability to bind to LDL-R mediated by binding to a PCSK9 allosteric site. For example, three synthetic peptides were able to increase cell surface expression of LDL-R by 60-95%, by preventing WT PCSK9 /LDL-R complex formation, as measured by FACS in HuH7 cells. In particular, one synthetic peptide (SRX55) was able to increase cell surface expression of LDL-R by 100%, by changing WT PCSK9/LDL-R complex affinity. See, Figure 1. These same three synthetic peptides were determined to increase LDL internalization by 30-50 %, as measured by dil-LDL uptake in HuH7 cells. In another study, one peptide was able to inhibit the activity of GOF PCSK9-D374Y by 100%, as measured by diI-LDL uptake in

HepG2 cells, and four peptides showed a 20-30 %, as measured by dil-LDL uptake in HuH7 cells. Some peptides also show inhibitory activity in mouse hepatocyte dil-LDL uptake.

In particular, the present data shows an ability of PSCK9 allosteric synthetic peptides to modulate LDLR cell surface levels by binding a peptide to PCSK9. See, Figure 1. In that experiment, the LDLR levels of a hepatocyte culture model (HuH7 cells) were measured by fluorescence activated cell sorting (FACS) in accordance with Example III. Cell surface LDLR is reported as a percentage of Basal levels of LDLR, indicated by the Cnt_Amm.Bic, Cnt_Ac.Acid, and Cnt bars in the top graph. See, Figure 1 (top panel). LDLR levels in the presence of exogenous PCSK9 is indicated as WT_Amm.Bic, WT-Ac.Acid, and WT, and Exogenous PCSK9 in combination with a tested peptide is indicated as SRX##. The measured LDLR levels are reported as % versus basal controls (Cnt) of the respective group. Examples of peptides (e.g., an allosteric synthetic inhibitor peptide) which positively modulate (increase) LDLR cell surface level include SRX55, SRX56, SRX60, and SRX62, and exemplary peptides (e.g., an allosteric synthetic enhancer peptide) which negatively modulate (decrease) LDLR cell surface levels include SRX69, SRX72, and SRX73. This was further shown a percent inhibition (% inhibition was calculated as [SRX - WT] / [Cnt-WT] x 100%) where positive modulation (increase) of LDLR level is reported as positive % inhibition, and negative modulation (decrease) of LDLR level is reported as negative % inhibition. See, Figure 1 (bottom panel).

The ability to modulate hepatocyte LDL internalization by the binding of a ligand to the PCSK9:LDLR complex is demonstrated in Figures 2 through 7. LDL internalization was measured by uptake of a fluorescently tagged LDL molecule (dil-LDL) in the absence of exogenous PCSK9 (Cnt), in the presence of exogenous PCSK9 (normal PCSK9 = WT, D374Y mutant PCSK9 = DY), or in the presence of PCSK9 and a tested peptide (indicated as SRX##, or SRX if a single peptide results is shown in a graph).

LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be modulated in a model hepatocyte cell line (HuH7) in the presence of the tested SRX peptides. See, Figure 2 (top panel). LDL internalization was shown to be positively modulated (increased) by allosteric synthetic inhibitor peptides such as SRX55, SRX 56, SRX60, and SRX67. LDL internalization can be negatively modulated (decreased) by allosteric synthetic enhancer peptides such SRX36, SRX61, SRX64, SRX65, SRX66, and SRX73. The percent inhibition is

shown, where positive modulation (increase) in LDL internalization is reported as >0% inhibition, and negative modulation (decrease) in LDL internalization is reported as <0% inhibition. See, Figure 2 (bottom panel).

LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be modulated in a model hepatocyte cell line (HuH7) by the presence of the tested SRX peptides in combination with a clinically relevant pathologic gain-of-function D374Y exogenous PCSK9 (DY). See, Figure 3 (top panel). LDL internalization was shown to be positively modulated (increased) by allosteric synthetic inhibitor peptides such as SRX55, SRX 56, SRX60, SRX63, SRX64, and SRX66. LDL internalization can be negatively modulated (decreased) by allosteric synthetic enhancer peptides such SRX36, SRX71, SRX72, and SRX73. The percent inhibition is shown, where positive modulation (increase) in LDL internalization is reported as >0% inhibition, and negative modulation (decrease) in LDL internalization is reported as <0% inhibition. See, Figure 3 (bottom panel).

LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be positively modulated (increased) by the presence of allosteric synthetic inhibitor peptides (SRX55, SRX 60, SRX66 and SRX56) in combination with a clinically relevant pathologic gain-of-function D374Y PCSK9 (DY). SRX55 was shown to have a positive modulation in a dose dependent manner. See, Figure 4 (top panel). The percent inhibition is shown, where positive modulation (increase) in LDL internalization is reported as >0% inhibition, note that SRX55 at 11.1 uM is within sampling noise of 0%. See, Figure 4 (bottom panel).

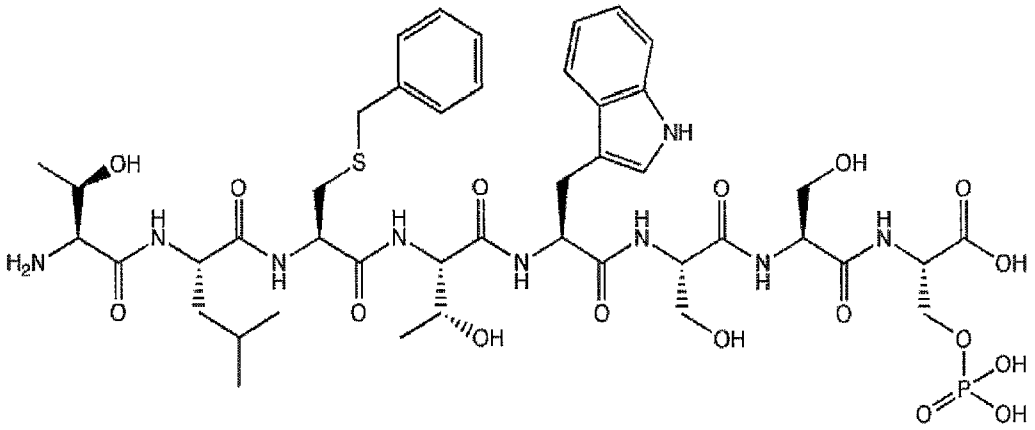
LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be positively modulated (increased) in a second model hepatocyte cell line (HepG2) in combination with a clinically relevant pathologic gain-of-function D374Y PCSK9 (DY) in a dose dependent manner with SRX55. See, Figure 5 (top panel). This positive modulation is further shown as a percent inhibition, where positive modulation (increase) in LDL internalization is reported as >0% inhibition. See, Figure 5 (bottom panel).

LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be positively modulated (increased) in a second hepatocyte cell line (HepG2) when pre incubated in combination with a clinically relevant pathologic gain-of-function D374Y PCSK9 (DY) or normal PCSK9 (WT) in a dose dependent manner with SRX55. See, Figure 6 (top panel).

This positive modulation is further shown as percent inhibition, where positive modulation (increase) in LDL internalization is reported as >0% inhibition. See, Figure 6 (bottom panel).

LDL internalization, as reported by dil-LDL % uptake vs Cnt, can be positively modulated (increased) in a third hepatocyte cell line (FL83B) when pre incubated in combination with a clinically relevant pathologic gain-of-function D374Y PCSK9 (DY) or normal PCSK9 (WT) in a dose dependent manner with SRX55. See, Figure 7 (top panel). This positive modulation is further shown as percent inhibition, where positive modulation (increase) in LDL internalization is reported as >0% inhibition. See, Figure 7 (bottom panel).

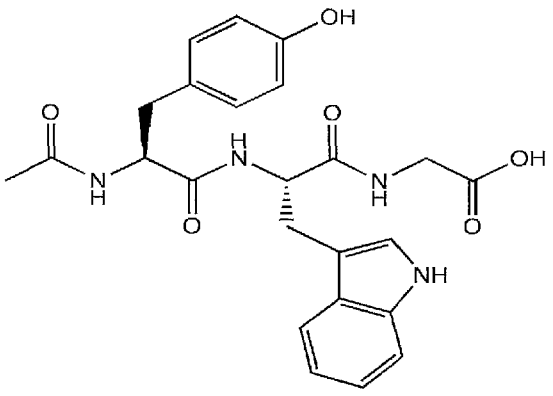
The most efficacious peptides (e.g., for example, SRX55; Compound 1) performed in consistent order across all assays and PCSK9 phenotypes. Improved peptides were then designed that were expected to have better drug-like properties, as they were designed based upon an analysis of the preliminary results. Typically, the design of these improved peptides have at least one of the first three amino acids from the C-terminus incorporated with a negatively charged polar group, such as a phosphate, a sulfate, a tetrazole or a carboxylic acid. For example, in Compound 3, the polar group comprises a phosphate group:



Compound 3: Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p).

Alternatively, in Compound 14, the C-terminal glycine comprises a polar group:

Compound 14: Ac-Tyr-Trp-Gly.



The constituent amino acids may be of defined stereochemistry, usually the natural "L" enantiomer and may have naturally occurring or synthetic side chains. The peptide "N" terminus may be free, alkylated, sulfonated, or acylated. The "C" terminus may be the carboxylic acid or an amide.

Various natural and unnatural amino acids may be contemplated. Tryptophan indole side chains may be substituted with alkyl, alkoxy, halo, carboxy, etc. to form other analogs. Phenyalanine, tyrosine, and homophenylalanine phenyl moieties may have additional phenyl substitution, such as alkyl, alkoxy, halo, carboxy, etc. Serine may be substituted in some examples by alanine. Threonine may be substituted by serine or alanine. Valine, leucine, and isoleucine may be interchanged in some analogs. Amino acids with carboxylic acid side chains, such as aspartic acid, may have the side chain derivatized as an amide.

III. Clinical Therapeutics

In some embodiments, the present invention contemplates the administration of a PCSK9 allosteric inhibitor peptide to a subject having a symptom of a cardiovascular disease. In one embodiment, the cardiovascular disease comprises hypercholesterolemia. In one embodiment, the cardiovascular disease comprises hypertension. In one embodiment, the hypercholesterolemia comprises elevated low density lipoprotein levels.

In some embodiments, the present invention contemplates the administration of a PCSK9 allosteric inhibitor peptide to a subject having a symptom of a metabolic disease. In one embodiment, the metabolic disease comprises diabetes.

Although it is not necessary to understand the mechanism of an invention, it is believed that the administration of a PCSK9 allosteric inhibitor synthetic peptide (i.e., for example, SRX55) induces a conformational shift of the PCSK9 protein such that the affinity of the low density lipoprotein binding site for a low density lipoprotein receptor is decreased, wherein PCSK9/LDL-R complex formation is decreased. The decrease in PCSK9/LDL-R complex formation results in an increase in the bioavailability of LDL-R receptors for binding to circulating LDL, thereby increasing the internalization and clearance of LDL by LDL-R. It is further believed that PCSK9 allosteric inhibitor peptides result in increased bioavailability of hepatocyte cell LDL-Rs.

A. Hypercholesterolemia

Hypercholesterolemia (also spelled hypercholesterolaemia) is the presence of high levels of cholesterol in the blood. It is a form of "hyperlipidemia" (elevated levels of lipids in the blood) and "hyperlipoproteinemia" (elevated levels of lipoproteins in the blood). Durrington, P "Dyslipidaemia" *The Lancet* 362(9385):717–731. Hypercholesterolemia is typically due to a combination of environmental and genetic factors. Environmental factors include obesity and dietary choices. Genetic contributions are usually due to the additive effects of multiple genes, though occasionally may be due to a single gene defect such as in the case of familial hypercholesterolaemia. A number of secondary causes exist including: diabetes mellitus type 2, obesity, alcohol, monoclonal gammopathy, dialysis, nephrotic syndrome, obstructive jaundice, hypothyroidism, Cushing's syndrome, anorexia nervosa, medications (thiazide diuretics, ciclosporin, glucocorticoids, beta blockers, retinoic acid). Bhatnagar et al., (2008) "Hypercholesterolaemia and its management" *BMJ* 337: a993. Genetic abnormalities are in some cases completely responsible for hypercholesterolemia, such as in familial hypercholesterolemia where there is one or more genetic mutations in the autosomal dominant APOB gene, the autosomal recessive LDLRAP1 gene, autosomal dominant familial hypercholesterolemia (HCHOLA3) variant of the PCSK9 gene, or the LDL receptor gene. "Hypercholesterolemia" *Genetics Home Reference* U.S. National Institutes of Health, ghr.nlm.nih.gov/condition=hypercholesterolemia. Even when there is no single mutation

responsible for hypercholesterolemia, genetic predisposition still plays a major role in combination with sedentary lifestyle, obesity, or an atherogenic diet. Citkowitz et al., (2010) "Polygenic Hypercholesterolemia". *eMedicine Medscape*, emedicine.medscape.com/article/121424-overview.

Cholesterol is a sterol. It is one of three major classes of lipids which all animal cells utilize to construct their membranes and is thus manufactured by all animal cells. Plant cells do not manufacture cholesterol. It is also the precursor of the steroid hormones, bile acids and vitamin D. Since cholesterol is insoluble in water, it is transported in the blood plasma within protein particles (lipoproteins). Lipoproteins are classified by their density: very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Biggerstaff et al., (2004). "Understanding lipoproteins as transporters of cholesterol and other lipids" *Adv Physiol Educ* 28 (1–4): 105–6. All the lipoproteins carry cholesterol, but elevated levels of the lipoproteins other than HDL (termed non-HDL cholesterol), particularly LDL-cholesterol are associated with an increased risk of atherosclerosis and coronary heart disease. Carmena et al., (2004) "Atherogenic lipoprotein particles in atherosclerosis" *Circulation* 109(23 Suppl 1): III2–7. In contrast, higher levels of HDL cholesterol are protective. Kontush et al., (2006) "Antiatherogenic small, dense HDL--guardian angel of the arterial wall?" *Nat Clin Pract Cardiovasc Med* 3(3):144–153. Elevated levels of non-HDL cholesterol and LDL in the blood may be a consequence of diet, obesity, inherited (genetic) diseases (such as LDL receptor mutations in familial hypercholesterolemia), or the presence of other diseases such as diabetes and an underactive thyroid. Total cholesterol is the amount of all of the fats in your blood. These fats are called lipids. There are different types of lipid that make up your total cholesterol. The two most important types are: low density lipoprotein (LDL) -"bad" cholesterol and high density lipoprotein (HDL) -"good" cholesterol. High cholesterol, especially "bad" cholesterol (LDL), can clog your arteries. This may reduce blood flow to your heart. It can lead to heart disease, stroke, or heart attack. Cholesterol is measured in milligrams per deciliter (mg/dL). In conditions such as heart disease or diabetes, LDL cholesterol should stay below 100 mg/dL. If there is a risk for heart disease, LDL cholesterol should be lower than 130 mg/dL. In general, LDL cholesterol should be lower than 160 - 190 mg/dL. Alternative, HDL "good" cholesterol should be high. For

example, HDL levels in men should be above 40 mg/dL, while HDL levels should be above 50 mg/dL for women.

One symptom of hypercholesterolemia comprises a longstanding elevation of serum cholesterol that can lead to atherosclerosis. Bhatnagar et al., (2008) "Hypercholesterolaemia and its management" *BMJ* 337: a993. Over a period of decades, chronically elevated serum cholesterol contributes to formation of atheromatous plaques in the arteries. This can lead to progressive stenosis (narrowing) or even complete occlusion (blockage) of the involved arteries. Alternatively smaller plaques may rupture and cause a clot to form and obstruct blood flow. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R (July 2010). "Concept of vulnerable/unstable plaque" *Arterioscler. Thromb. Vasc. Biol.* 30(7): 1282–1292. A sudden occlusion of a coronary artery results in a myocardial infarction or heart attack. An occlusion of an artery supplying the brain can cause a stroke. If the development of the stenosis or occlusion is gradual blood supply to the tissues and organs slowly diminishes until organ function becomes impaired. At this point that tissue ischemia (restriction in blood supply) may manifest as specific symptoms including, but not limited to, temporary ischemia of the brain (commonly referred to as a transient ischemic attack) may manifest as temporary loss of vision, dizziness and impairment of balance, aphasia (difficulty speaking), paresis (weakness) and paresthesia (numbness or tingling), usually on one side of the body. Insufficient blood supply to the heart may manifest as chest pain, and ischemia of the eye may manifest as transient visual loss in one eye. Insufficient blood supply to the legs may manifest as calf pain when walking, while in the intestines it may present as abdominal pain after eating a meal. Grundy et al., (1998) "Primary prevention of coronary heart disease: guidance from Framingham: a statement for healthcare professionals from the AHA Task Force on Risk Reduction. American Heart Association" *Circulation* 97(18):1876–1887.

B. Hypocholesterolemia

Hypocholesterolemia is the presence of abnormally low (hypo-) levels of cholesterol in the blood (-emia). Although the presence of high total cholesterol (hyper-cholesterolemia) correlates with cardiovascular disease, a defect in the body's production of cholesterol can lead to adverse consequences as well. Cholesterol is an essential component of mammalian cell membranes and is required to establish proper membrane permeability and fluidity. It is not

clear if a lower than average cholesterol level is directly harmful; it is often encountered in particular illnesses.

Possible causes of low cholesterol include, but are not limited to, statins, hyperthyroidism, or an overactive thyroid gland, adrenal insufficiency, liver disease, malabsorption (inadequate absorption of nutrients from the intestines), such as in celiac disease, malnutrition, abetalipoproteinemia (a genetic disease that causes cholesterol readings below 50 mg/dl), hypobetalipoproteinemia (a genetic disease that causes cholesterol readings below 50 mg/dl, manganese deficiency, Smith-Lemli-Opitz syndrome, Marfan syndrome, leukemias and other hematological diseases.

Demographic studies suggest that low cholesterol is associated with increased mortality, mainly due to depression, cancer, hemorrhagic stroke, aortic dissection and respiratory diseases. Jacobs et al., (1992). "Report of the Conference on Low Blood Cholesterol: Mortality Associations" *Circulation* 86 (3): 1046–1060; and Suarez E.C., (1999) "Relations of trait depression and anxiety to low lipid and lipoprotein concentrations in healthy young adult women". *Psychosom Med* 61(3): 273–279. It is also possible that whatever causes the low cholesterol level also causes mortality, and that the low cholesterol is simply a marker of poor health.

C. Diabetes

Diabetes affects more than 20 million Americans. Over 40 million Americans have pre-diabetes (which often develops before type 2 diabetes).Diabetes is usually a lifelong (chronic) disease in which there is a high level of sugar in the blood. Insulin is a hormone produced by the pancreas to control blood sugar. Diabetes can be caused by too little insulin, resistance to insulin, or both. To understand diabetes, it is important to first understand the normal process by which food is broken down and used by the body for energy.

Several things happen when food is digested. A sugar called glucose enters the bloodstream. Glucose is a source of fuel for the body. An organ called the pancreas makes insulin. The role of insulin is to move glucose from the bloodstream into muscle, fat, and liver cells, where it can be used as fuel.

People with diabetes have high blood sugar because their body cannot move sugar into fat, liver, and muscle cells to be stored for energy. This is because either their pancreas does not make enough insulin or their cells do not respond to insulin normally.

There are two major types of diabetes. The causes and risk factors are different for each type. Type 1 diabetes can occur at any age, but it is most often diagnosed in children, teens, or young adults. In this disease, the body makes little or no insulin. Daily injections of insulin are needed. The exact cause is unknown. Type 2 diabetes makes up most diabetes cases. It most often occurs in adulthood. But because of high obesity rates, teens and young adults are now being diagnosed with it. Many people with type 2 diabetes do not know they have it.

Gestational diabetes is high blood sugar that develops at any time during pregnancy in a woman who does not have diabetes.

Diabetes symptoms may result from high blood sugar level and include, but are not limited to, blurry vision, excess thirst, fatigue, hunger, urinating often and weight loss.

IV. Pharmaceutical Compositions

The present invention further provides pharmaceutical compositions (e.g., comprising the peptides described above). The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, (e.g., intrathecal or intraventricular), administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions and formulations for oral, sublingual or buccal administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, gels,

drops, strips, gums, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

In some embodiment, the pharmaceutical compositions may further comprise other drugs, hormones, and/or peptides. For example, the pharmaceutical composition may further comprise a statin drug. Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a role in the production of cholesterol in the liver. Increased cholesterol levels have been associated with cardiovascular diseases, and statins are therefore used in the prevention of these diseases. Lewington et al., "Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths" *Lancet* 370(9602): 1829–1839 (2007). Research has found that statins are most effective for treating cardiovascular disease (CVD) as a secondary prevention strategy, with questionable benefit in those with elevated cholesterol levels but without previous CVD. Taylor et al. "Statins for the primary prevention of cardiovascular disease". In: Taylor, Fiona. Cochrane Database Syst Rev (1) (2011). Statins have rare but severe adverse effects, particularly muscle damage.

Specific examples of statins include, but are not limited to, atorvastatin (Lipitor® and Torvast®), fluvastatin (Lescol®), lovastatin (Mevacor®, Altocor®, Altoprev®), pitavastatin (Livalo®, Pitava®), pravastatin (Pravachol®, Selektine®, Lipostat®), rosuvastatin (Crestor®) and simvastatin (Zocor®, Lipex®). Several combination preparations of a statin and another agent, such as ezetimibe/simvastatin, are also available.

Specific examples of cardiovascular drugs include, but are not limited to, propranolol, digitalis, amlodipine besylate, and nifedipine.

Specific examples of other pharmaceutical compositions may further include, but are not limited to, exetimibe (Zetia®), amlodipine besylate (Norvasc®), niacin, sitagliptin (Januvia®), metformin or orlistat (Alli®/Xenical®).

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances

and the like which do not deleteriously interact with the active pharmaceutical ingredient(s) of the formulation.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in in vitro and in vivo animal models or based on the peptides described herein. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the subject undergo maintenance therapy to prevent the recurrence of the disease state, wherein the peptide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

Experimental

Example I

Cell Culture And Transfections

HepG2/shPCSK9 or HuH7/shPCSK9 cells (1) lacking endogenous PCSK9 were seeded at 1 x 10^5cells/well in a 12 well microplate (Greiner Bio-One). These cells were then incubated for 4h or overnight with 0.7 μg/ml of either V5-tagged PCSK9 or its gain-of-function PCSK9-D374Y pre-incubated, or not, for 4h with each peptide at 50 μM (or less if needed for the most active peptides). The cells were then lysed in 1x RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0), containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with 1x complete protease inhibitor mixture (Roche Applied Science), and analyzed by Western blot.

Example II

Western Blot Analyses

Proteins in the cell lysates were resolved by 10% Tris-Glycine SDS-PAGE. The gels were blotted onto polyvinylidene difluoride (PVDF, Perkin Elmer Life Sciences) membranes (GE Healthcare), blocked for 1h in TBS-T (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween 20) containing 5% nonfat milk and immunoblotted with a homemade polyclonal human PCSK9 antibody (1:1000) (13), human LDLR antibody (1:1000, R&D Systems), beta-Actin (1:5000; Sigma) and monoclonal antibody (mAb) V5-HRP (1:5000; Sigma). Appropriate horseradish peroxidase-conjugated secondary antibodies (1:10000, Sigma) were used for detection with enhanced chemiluminescence using the ECL Plus kit (GE Healthcare). Quantitation of protein bands was obtained using Image J software.

Example III

FACS Analysis

HuH7/shPCSK9 cells were incubated at 37°C for 4h as above with PCSK9 pre-incubated, or not, with each of the exemplified peptides used at 50 μM (or less if needed for the most active peptides). Benjannet et al., "Effects of the prosegment and pH on the activity of

PCSK9: evidence for additional processing events" *J Biol Chem.* 285(52): 40965-40978 (2010).

The cells were then washed 3x with solution A (calcium/magnesium-free Dulbecco's PBS (Invitrogen) containing 0.5% bovine serum albumin (Sigma) and 1g/liter glucose)). The cells were then incubated for 10 min at room temperature with 1x Versene solution (Invitrogen) followed by the addition of 5 ml of solution A. The cells were then incubated for 40 min in solution A containing a human LDLR mAb-C7 (1:100; Santa Cruz Biotechnology). Following washes, the cells were then incubated for 20 min in solution A containing a secondary antibody (Alexa Fluor 647 donkey anti-mouse antibody; 1:250; Molecular Probes).

Following suspension in PBS containing 0.2% of propidium iodide, the cells were analyzed by FACS for both propidium iodide (dead cells) and LDLR in live cells with Alexa Fluor 647 using the FACS BD LSR (BD Biosciences).

Cell Activity of Exemplified Peptides

Compound 1	+
Compound 2	+
Compound 3	+
Compound 4	+
Compound 5	+
Compound 6	-
Compound 7	-
Compound 8	+
Compound 9	+
Compound 10	-
Compound 11	-
Compound 12	(+)
Compound 13	-
Compound 14	(+)
Compound 15	-
Compound 16	(+)

+ implies > 30% inhibition above control at 100 uM
- implies inhibition within error range
(+)implies inhibition >30% *below* control at 100 uM

Example IV

Cellular diI-LDL Uptake Assay

Cells, such as HepG2, HuH7, FL83B or a cell line transfected with a short-hairpin PCSK9 knockdown sequence such as HepG2/shPCSK9, HuH7/shPCSK9, FL83B/shPCSK9, were seeded at 2 x 10^4 cells/well in a 96-well plate and cultured at 37 degC in RPMI + 10% FBS. After approximately 24 hours, the cell media was aspirated off and replaced with RPMI + 3-5 mg/mL LPDS (Lipoprotein Deficient Serum, Millipore) media for further experimentation. Benjannet et al., "Effects of the prosegment and pH on the activity of PCSK9: evidence for additional processing events" *J Biol Chem.* 285(52): 40965-40978 (2010).

Peptide activity was assessed by culturing cells with: i) no SRX peptide/PCSK9 protein complex (control, Cnt); ii) PCSK9 protein; and iii) SRX peptide/PCSK9 complex. Various permutations of these experimental conditions were also used, including: i) the addition of wild type PCSK9 (WT); ii) a mutant PCSK9 (e.g., D374Y mutant PCSK9, DY); iii) various SRX peptides and/or PCSK9 at the same concentration and/or combinations; iv) various SRX peptides and/or PCSK9 at different concentrations and/or combinations; v) the use of different cells, as mentioned above, with or without a transfected short-hairpin sequence; vi) a pre-incubation of the PCSK9 and SRX peptide (e.g., 1, hour, 2 hours, 3 hours, 4 hours etc.); vii) various temperatures including, but not limited to, body temperature (e.g., 37 °C), supraphysiologic temperature (e.g., 39 °C); and viii) with/without agitation (e.g., shaker or gentle periodic vortexing).

Cells were cultured using one of the combinations of conditions described in the preceding paragraph for 16 hours. After 16 hours, a quantity of diI-LDL (Low density lipoprotein coupled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) needed to bring the media concentration to 5 ug/mL of diI-LDL was added to the culture well and cells continued to be cultured under these new conditions for 4 additional hours. At the end of the 4-hour incubation period (20 total hours of cell culture), the cellular uptake was halted with the addition of 4% formaldehyde in 10 uM Hoechst 33342 in a solvent such as deionized autoclaved water or PBS, and specimens were incubated at 20 °C for 20 minutes.

Cell specimens were rinsed twice with PBS and then fluorescence measured with excitation at 360 nm and emission detected at 460 nm to measure DNA content. Cell specimens were then be incubated with a 0.1% SDS in a 0.1 N NaOH solution while being

shaken for 10 minutes. Fluorescence of the diI-LDL in the specimens were quantified using excitation at 530 nm and resulting emission at 580 nm.

Fluorescence measurements of diI-LDLR were normalized to estimated cell numbers, determined from the Hoechst fluorescence. Data was analyzed for the different experimental conditions and reported as percentage relative fluorescence units (RFU) of the Cnt specimen. Percent inhibition was calculated as the difference in RFU of a peptide exposed specimen to the RFU of PCSK9-no peptide, divided by the RFU difference in PCSK9-no peptide to RFU of Cnt specimen, also expressed as [(SRX:RFU) – (PCSK9-no peptide:RFU)] / [(PCSK9-no peptide:RFU) – (Cnt:RFU)] x 100.

Example V

Methods Of Making PCSK9 Allosteric Inhibitor Peptides

This example presents several methods of identifying and synthesizing peptides of the present invention. R. B. Merrifield (1963). "Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide". J. Am. Chem. Soc. 85 (14): 2149–2154; Albericio, F. (2000). Solid-Phase Synthesis: A Practical Guide (1 ed.). Boca Raton: CRC Press. p. 848. ISBN 0-8247-0359-6; and Albericio F, Carpino LA., "Coupling reagents and activation" Methods Enzymol. 1997;289:104-126.

All peptides were manufactured using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry (21st Century Biochemicals, 260 Cedar Hill St., Marlboro, MA 01752). In brief, the peptides are made using a polystyrene resin, functionalized with an appropriate linker, and the peptides are then manufactured using an Intavis RS Peptide Synthesizer (Germany) or manufactured by hand using glass peptide synthesis vessels fitted with coarse glass frits for removing reactants by vacuum (Chemglass).

In either case, the amino acids are added sequentially as follows: the amino acids are dissolved in either NMP (N-Methyl-2- pyrrolidone) or DMF (Dimethylformamide); these solvents are also used for washing the resin following each step. The Fmoc-protected amino acid to be added is activated using either HATU (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) or HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate); for a 4-fold stochiometric to be added (relative to the resin), a 3.95-fold excess of HATU or HCTU is used to create the active ester.

Along with an 8-fold excess of DIPEA (N,N-Diisopropylethylamine) as the base, these reagents catalyze the addition of the next amino acid. Once the amino acid is coupled (each cycle includes a double coupling cycle to insure efficient coupling) the resin is exposed to 20% acetic anhydride to terminate ("cap-off") any peptide chains that have no added the next amino acid. The resin is then washed using DMF (3X), Methanol (MeOH, 2X) and DMF again, 2X. Piperidine is used to remove the Fmoc group at the end of each coupling cycle which exposes the N-terminal amine and allows the next amino acid to be added.

Once synthesis of each step is completed, the peptides (on resin) were dried using MeOH (3X) and DCM (3X), cleaved and deprotected using 92% TFA, 2% water, 2% triisopropylsilane, 2% thioanisole and 2% ethanedithiol for 3-4h at room temperature. Peptides were precipitated in cold diethyl ether, centrifuged (2,000 RPM) and the pellets washed 2X with cold ether. After drying the peptides were solubilized in water containing 0.1% TFA (buffer A) and subjected to RP-HPLC using C18 columns (buffer B = 95% acetonitrile/0.1% TFA).

Some PCSK9 allosteric synthetic peptides, and their physical characteristics, are listed below:

Compound 1 (SRX-55): Val-Tyr-Val-Arg-Phe-Trp, Calc'd m/z: 868.46; Obs.: 869.00

Compound 2: (SRX-56) β-Ala-Phe(3-CH2NH2)-Val-D-Ser(p)-Phe-Trp, Calc'd m/z: 864.36; Obs.: 864.80

Compound 3 (SRX-60): Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), Calc'd m/z: 1053.39; Obs.: 1053.80

Compound 4 (SRX-61): Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p), Calc'd m/z: 1064.42; Obs.: 1064.90

Compound 5: (SRX-62) Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), Calc'd m/z: 1027.46; Obs.:

Compound 6: (SRX-63) Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p), Calc'd m/z: 1021.42; Obs.: 1022.30

Compound 7: (SRX-64) Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p), Calc'd m/z: 1067.40; Obs.: 1067.80

Compound 8: (SRX-65) Val-Leu-Glu-Leu-Tyr-Trp, Calc'd m/z: 821.43; Obs.: 821.90

Compound 9: (SRX-66) Leu-Asp-Leu-Phe-Phe-Ser, Calc'd m/z: 740.37; Obs.: 740.80

Compound 10: (SRX-67) Ile-Leu-Asp-Leu-Ser-Tyr, Calc'd m/z: 722.39; Obs.: 722.80

Compound 11: (SRX-68) Ac-Trp-Ser-Ser(p), Calc'd m/z: 500.13; Obs.: 500.15

Compound 12: (SRX-69) Ac-Trp-Ala-Ser(p), Calc'd m/z: 484.14; Obs.: 484.40

Compound 13: (SRX-70) Ac-Trp(5-F)-Ala-Ser(p)-morpholine, Calc'd m/z: 571.18; Obs.: 571.00

Compound 14: (SRX-72) Ac-Tyr-Trp-Gly, Calc'd m/z: 466.19; Obs.: 466.47

Compound 15: (SRX-36) Thr-Leu-Thr-Trp-Ser-Ser-Ser(p), Calc'd m/z: 860.33; Obs.: 860.00

Compound 16: (SRX-73) Phe(4-Ph)-Ala-Ser(p)-morpholine, Calc'd m/z: 548.20; Obs.: 548.00

Claims

We claim:

1. A method, comprising:

a) providing;

i) a PCSK9 protein, wherein said protein comprises a binding site that induces allosteric modulation and a low density lipoprotein receptor binding site;

ii) a synthetic ligand capable of binding to said binding site ;

iii) a plurality of hepatocyte cells comprising a low density lipoprotein receptor and low density lipoproteins;

b) binding said synthetic ligand to said binding site , wherein said synthetic ligand induces a conformation shift of said protein; and

c) modulating the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor by said conformational shift.

2. The method of Claim 1, wherein said synthetic ligand is an allosteric inhibitor ligand wherein said modulating decreases the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor such that internalization of said low density lipoprotein by said plurality of hepatocytes is increased.

3. The method of Claim 1, wherein synthetic peptide is an allosteric enhancer ligand said modulating increases the affinity of said low density lipoprotein receptor binding site for said low density lipoprotein receptor such that internalization of said low density lipoprotein by said plurality of hepatocytes is decreased.

4. The method of Claim 1, wherein said conformational shift of said protein is selected from the group consisting of an induced fit shift and a biomechanical shift.

5. The method of Claim 1, wherein said synthetic ligand is a synthetic peptide selected from the group consisting of VYVRFW, VLELYW and ISDLSY.

6. The method of Claim 2, wherein said allosteric inhibitor is a peptide is selected from the group consisting of SRX55, SRX56, SRX60, SRX61, SRX62, SRX63, SRX64, SRX65 and SRX66.

7. The method of Claim 3, wherein said allosteric enhancer peptide is selected from the group consisting of SRX64, SRX67, SRX68, SRX69, SRX72 and SRX73.

8. A method, comprising:

a) providing;

i) a PCSK9 protein, wherein said protein comprises a binding site that induces allosteric modulation and a low density lipoprotein receptor binding site;

ii) a synthetic ligand capable of binding said binding site;

iii) a plurality of hepatocyte cells comprising a population of low density lipoprotein receptors;

b) binding said synthetic ligand to said binding site , wherein said synthetic ligand induces a conformation shift of said protein;

c) modulating said population of said low density lipoprotein receptors by said conformational shift.

9. The method of Claim 8, wherein said synthetic ligand is an allosteric inhibitor ligand wherein said modulating increases said population of said low density lipoprotein receptors measurable on the cell surface of hepatocytes.

10. The method of Claim 8, wherein said synthetic ligand is an allosteric enhancer ligand wherein said modulating decreases said population of said low density lipoprotein receptors measurable on the cell surface of hepatocytes.

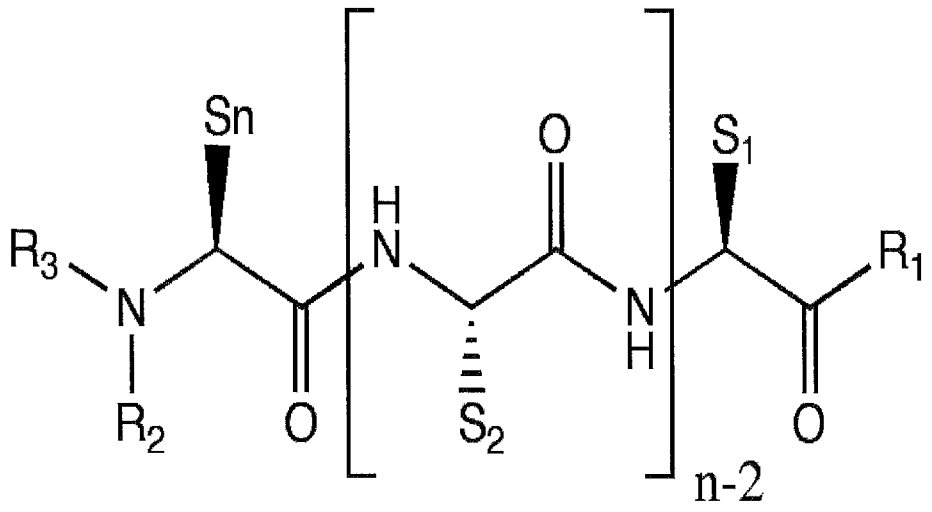
11. The method of Claim 8, wherein said conformational shift of said protein is selected from the group consisting of an induced fit shift and a biomechanical shift.

12. The method of Claim 8, wherein said ligand is a synthetic peptide is selected from the group consisting of VYVRFW, VLELYW and ISDLSY.

13. The method of Claim 9, wherein said allosteric inhibitor is a peptide is selected from the group consisting of SRX55, SRX56, SRX60, SRX61, SRX62, SRX63, SRX64, SRX65 and SRX66.

14. The method of Claim 10, wherein said allosteric enhancer is a peptide is selected from the group consisting of SRX64, SRX67, SRX68, SRX69, SRX72 and SRX73.

15. A compound of the formula:



wherein: i) n, the number of amino acid residues, is an integer in the range 3-8; ii) the constituent amino acids are single enantiomers of independently selected natural or unnatural aminoacids; iii) R2 and R3, are independently selected from the group consisting of hydrogen, a lower alkyl, a branched alkyl, a hydroxyalkyl, a cycloalkyl, a heterocycle, aryl, heteroaryl, acyl, substituted or unsubstituted benzoyl, alkyl or aryl

sulfonyl (e.g. mesyl or tosyl) and carbamoyl (e.g. BOC); iv) R1 is selected from the group consisting of –OH and -NR4-R5; v) R4 and R5, independently, are selected from the group consisting of hydrogen; a lower alkyl, an aryl, a cycloalkyl, an aromatic heterocycle, pyridine, tetrazole, alkoxy, cycloalkoxy; alternatively, R4 and R5 are joined as a heterocyle, such as piperidine; pyrrolidine; morpholine; piperazine; a substituted heterocycle, such as 4-methylpiperazine; or a fused heterocycle, such as dihydroquinoline or indoline and S1, S2 and Sn are side chains.

16. The compound of Claim 15, further comprising a negatively charged polar group.

17. The compound of Claim 16, wherein said negatively charged polar group is selected from at least one of the group consisting of O-phosphate, O-sulfate, 5-O-, and a 5-N-tetrazole incorporated in said side-chains S1, S2, or Sn.

18. The compound of Claim 15, wherein said a side chain selected from the group consisting of S1, S2 and Sn comprises a phosphoserine.

19. The compound of Claim 15, wherein said side chain S1 comprises -CH2-NH-tetrazole.

20. The compound of Claim 12, further comprising a glycine C-terminus.

21. The compound of Claim 15, wherein said compound is selected from the group consisting of Val-Tyr-Val-Arg-Phe-Trp, β-Ala-Phe(3-CH2NH2)-Val-D-Ser(p)-Phe-Trp, Thr-Leu-Cys(CH2-Ph)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Asp(NHCH2Ph)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Gly(CH2CH2cyclohexyl)-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Hph-Thr-Trp-Ser-Ser-Ser(p), Thr-Leu-Cys(CH2-Ph)-Thr-Trp(3-Me)-Ser-Ser-Ser(p), Val-Leu-Glu-Leu-Tyr-Trp, Leu-Asp-Leu-Phe-Phe-Ser, Ile-Leu-Asp-Leu-Ser-Tyr, Ac-Trp-Ser-Ser(p), Ac-Trp-Ala-Ser(p), Ac-Trp(5-F)-Ala-Ser(p)-morpholine, Thr-Leu-Thr-Trp-Ser-Ser-Ser(p), Ac-Tyr-Trp-Gly, Phe(4-Ph)-Ala-Ser(p)-morpholine.

22. The compound of Claim 15, wherein said compound is formulated as a pharmaceutical composition.

23. The compound of Claim 22, wherein said pharmaceutical composition further comprises a pharmaceutical drug.

24. The compound of Claim 23, wherein said pharmaceutical drug is selected from the group consisting of a statin, a cardiovascular drug, a metabolic drug, and an antihypertensive drug.

25. The compound of Claim 23, wherein said pharmaceutical drug is selected from the group consisting of ezetimibe, amlodipine besylate, sitagliptin, metformin, atorvastatin, rosuvastatin and simvastatin.

26. The compound of Claim 23, wherein said pharmaceutical composition is formulated as selected from the group consisting of a tablet, a liquid, a gel, a capsule, a sachet, a microparticle, a liposome, a nanoparticle, a salt, a transdermal patche, an ointment, a lotion, a cream, a gel, a drop, a strip, a suppository, a spray and a powder.