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(54) Title: MODIFIED DDAH POLYPEPTIDES COMPRISING A PHARMACOKINETIC ENHANCING MOIETY, IMPROVED PHARMACOLOGY AND THEIR USES

(57) Abstract: Modified DDAH polypeptides and their uses thereof are provided. Exemplary embodiments provide DDAH polypeptides which include one or more amino acid substitutions, additions, or deletions with natural or non-naturally encoded amino acids, and/or linkage to other biologically active molecules including other DDAH polypeptides, as well as PKEM. Additionally, use of said DDAH polypeptides for treatment of disease, such as heart failure or renal disease, is also provided.

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**MODIFIED DDAH POLYPEPTIDES COMPRISING A PHARMACOKINETIC
ENHANCING MOIETY, IMPROVED PHARMACOLOGY AND THEIR USES**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of priority to U.S. Provisional Application No. 62/539,261, filed July 31, 2017, which is incorporated by reference herein in its entirety.

BACKGROUND

Asymmetric dimethyl arginine (ADMA) produced in the body as a result of degradation of argininemethylated proteins is an inhibitor of nitric oxide synthesis (NO).
10 During the disease states where protein degradation rates are high or the mechanisms of ADMA clearance are impaired, high levels of ADMA accumulate in the tissues and blood.

In some conditions such as kidney disease, there may be 3- to 9-fold increase in plasma levels of ADMA. High levels of ADMA is known to contribute to disease states by acting as a competitive inhibitor for nitric oxide generation by nitric oxide synthase (NOS) as
15 well as cationic amino acid transporter for NOS substrate arginine. ADMA also inhibits phosphorylation of endothelial NOS, thereby, reducing its activity. Deficiency of NO production which may be caused by ADMA is associated with a wide range of vascular diseases including, hypertension, heart failure, pulmonary arterial hypertension, erectile dysfunction, coronary and peripheral arterial disease, renal, disease, insulin resistance,
20 diabetes, atrial fibrillation, sickle cell disease, organ damage, sepsis, preeclampsia, and deficient wound healing, and tissue regeneration.

By reducing NO bioavailability, high levels of ADMA can promote endothelial dysfunction, vasoconstriction, pro-inflammatory and pro-thrombogenic state. In addition, high levels of ADMA can uncouple NOS causing it to produce oxygen free radical which
25 cause organ damage. Since vascular homeostasis plays a fundamental role in normal physiology and survival, a persistent dysfunction of vascular endothelium can lead to a variety of disease states and death. An association of high ADMA levels has been documented with vascular diseases such as retinal venous occlusive disease, early autosomal dominant polycystic kidney disease, proteinuria, erythropoietin resistance, secondary
30 amyloidosis and focal segmental glomerulosclerosis, pre-eclampsia, chronic thromboembolic pulmonary hypertension, diabetes, insulin resistance, obesity, pulmonary hypertension, lung injury, sickle cell disease, depression, congestive heart failure, Alzheimer's disease, cardio-renal syndrome, hyperhomocysteinaemia, hypertension, atherosclerosis and stroke.

A major pathway for reducing ADMA is through metabolism by the enzyme dimethylarginine dimethylamino hydrolase (DDAH) which eliminates more than 80% of ADMA. DDAH gene deletion and transgenic animal studies have shown that DDAH levels and activity regulate ADMA levels. Heterologous deletion of gene DDAH-1 increased ADMA level and impaired vascular responses. Conversely, transgenic expression of DDAH-1 reduced plasma ADMA, increased NO production, and decreased arterial blood pressure and systemic vascular resistance. Thus, ADMA levels in plasma can be modulated by the level of DDAH-1 gene expression.

In disease states where DDAH expression or activity is impaired, ADMA clearance is reduced leading to its accumulation in tissues and blood. For example, in pathological conditions such as diabetes, atherosclerosis, preeclampsia and inflammation, DDAH-1 gene expression is reduced and ADMA is increased. In lung disease such as pulmonary arterial hypertension (PAH), DDAH mRNA and protein expression are reduced and ADMA levels are increased. Therefore, methods that can increase enzyme levels in the body would reduce ADMA and produce therapeutic benefit in prevention or treatment of disease.

Two isoforms of DDAH are encoded by separate genes located on human chromosome 1 (DDAH-1) and 6 (DDAH-2). The two proteins share 63% amino acid homology but exhibit similar catalytic properties. Both enzymes metabolize ADMA into citrulline and dimethylamine. DDAH can hydrolase both the *NG*-monomethyl-L-arginine (L-NMMA) and ADMA, therefore it can reduce the inhibitory concentrations of the methylamines and allow more NO generation.

It has been found that high levels of ADMA are produced in response to ischemia reperfusion injury. Several human studies have reported a strong association of ADMA levels with cardiovascular events and mortality in patients with known coronary artery disease. ADMA levels were also an independent predictor of cardiovascular (CV) events in patients after coronary angioplasty. ADMA levels are strong predictor of mortality after AMI. ADMA levels are strongly associated with cardiovascular disease and all-cause mortality and to the cardiovascular events in patients undergoing hemodialysis. High levels of ADMA are associated with all-cause mortality, major cardiovascular and cerebrovascular events, and progression of renal disease, chronic heart failure, peripheral arterial disease, and type 2 diabetes. In patients with end-stage renal disease elevated ADMA levels were directly associated with carotid atherosclerosis and cardiovascular mortality.

High levels of ADMA are present in the blood of patients on dialysis and chronic kidney disease. Raising ADMA levels by infusion of ADMA decreases the effective renal

plasma flow. High level of ADMA is associated with erythropoietin resistance. Reducing plasma ADMA levels by DDAH therapy is expected to improve endothelial function and renal function in patients on dialysis and impaired kidney function.

5 Plasma ADMA is also elevated in patients with CAD and CKD and thus may play a role in cardio-renal syndrome. ADMA levels were significantly increased in patients suffering from pulmonary arterial hypertension (PAH) and idiopathic pulmonary arterial hypertension. In animal models of PAH, DDAH expression or activity is reduced. Reducing ADMA levels can improve endothelial function, reduce inflammation, fibrosis and reduce PAH and other inflammatory lung disease. Elevated ADMA levels have also been observed
10 in acute lung injury (ALI). Increased collagen deposition leads to impaired lung function in disease such as asthma and pulmonary fibrosis.

In critically ill patients, endothelial damage and microvascular oxidative stress and deficiency of nitric oxide leads to impaired organ perfusion, inflammation, infection and organ failure. High levels of ADMA have been reported in patients with sepsis, trauma and
15 major surgery. ADMA is also a predictor of mortality in ICU patients. ADMA levels are also elevated in hepatic failure. High ADMA could cause microvascular complications including endothelial dysfunction, microvascular constriction, inflammation, generation of oxygen free radical and thrombosis further contributing to the complications of organ dysfunction.

20 Deficient NO and endothelial dysfunction are associated with insulin resistance and type 2 diabetes. NO mediates insulin-induced skeletal muscle blood flow which promotes glucose uptake and thereby may regulate insulin sensitivity in the skeletal muscle. Plasma ADMA levels are elevated in patients with type 2 diabetes as well individual with metabolic syndrome. High ADMA levels are associated with glucose intolerance, insulin resistance and
25 diabetes. These studies support that high ADMA may promote insulin resistance and diabetes by reducing NO synthesis.

High level of ADMA and reduced DDAH are found in patients with preeclampsia which may contribute to hypertension, renal injury, reduced fetal growth and premature birth.

It is also well recognized that endothelial nitric oxide is diminished in severe malaria.
30 A recent study of Indonesian adults with malaria suggests that ADMA may contribute to mortality in severe malaria patients. In this prospective longitudinal study, patients with high ADMA levels showed almost 18-fold higher probability of death than those with lower ADMA.

SUMMARY

DDAH polypeptides and uses thereof are described herein. In exemplary embodiments, the DDAH polypeptides, such as human DDAH-1 and DDAH-2 polypeptides, can include one or more amino acid modifications and/or post-translational modifications that enhance or modulate pharmacokinetic, pharmacodynamic, or time-action properties of the DDAH polypeptide, including linkage to other biologically active molecules such as a half-life extending or pharmacokinetic enhancing moiety. Pharmaceutical compositions and medical use of such DDAH polypeptides are also described.

Described herein are methods and compositions that relate to the use of the DDAH enzyme dimethylarginine diamino hydrolase (DDAH) or a biologically active fragment or modified form of the DDAH enzyme, where the DDAH or fragment or modified form thereof is capable of hydrolyzing asymmetric dimethylarginine (ADMA) to citrulline and/or other breakdown products of ADMA. A cDNA encoding human DDAH protein has been made and used to express and produce recombinant biologically active human DDAH protein.

The DDAH or biologically active fragment or modified form thereof can be administered to a patient to lower plasma or tissue levels of ADMA. The DDAH or biologically active fragment thereof may be free in solution, or attached to a solid substrate or support, that is contacted with the blood or tissue of a patient. DDAH or biologically active fragment or modified form thereof can be particularly effective to reduce ADMA when utilized in conjunction with or as a part of hemodialysis or plasmaphoresis system components in order to extracorporeally treat a patient's blood to reduce levels of ADMA.

ADMA, along with a structurally related molecule N-monomethyl arginine (NMMA), are endogenous competitive inhibitors of nitric oxide synthase (NOS). The inhibition of NOS causes a decrease in the production of nitric oxide (NO). Increased ADMA therefore causes decreased NO production.

There is a direct correlation between renal failure and increased levels of ADMA in patient's blood along with decreased levels of NO. Elevated levels of ADMA have been found in patients with a wide variety of diseases and conditions such as renal disease, coronary artery disease, ischemic heart disease, congestive heart failure, hypertension, lung injury, pulmonary hypertension, preeclampsia, hypercholesterolemia, diabetes, atherosclerosis, sepsis, organ failure, surgical trauma, and in particular end stage renal failure and erythropoietin resistance. ADMA levels are also increased in patients with acute kidney injury and contrast induced renal injury. In addition, increased ADMA level is an indicator of risk for cardiovascular-related death.

Thus, there is an urgent need to develop a means to reduce ADMA concentration in the blood of patients, in particular patients with chronic kidney disease, organ failure and those who are receiving hemodialysis treatment for kidney related diseases. The ability to reduce ADMA from the blood of end stage renal disease patients in conjunction with hemodialysis treatment by administering DDAH or a biologically active fragment thereof may reduce ADMA-mediated morbidity and extend life. Described herein are DDAH polypeptides and modified DDAHs, as well as compositions and therapeutic uses thereof. In exemplary embodiments, the modified DDAHs exhibit enhanced or modulated pharmacokinetic, pharmacodynamic, or time-action properties, including an increased or enhanced in vivo half-life relative to wild-type DDAH, such as an in vivo half-life of at least 1, 2, 3, 4, 5, 6, 9, 10, 12, 15, 20, 25 hours, multiple days, or longer.

In embodiments, the DDAH polypeptide can comprises a wild-type or a modified human DDAH polypeptide or a wild-type or modified non-human DDAH polypeptide. Said DDAH or modified DDAH polypeptide may have at least 80% identity to the DDAH polypeptide of SEQ ID NO: 1, and/or the DDAH polypeptide of SEQ ID NO: 2 or the corresponding amino acids of SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; or SEQ ID NO 14.

In embodiments, the DDAH or modified DDAH can retain one or more properties of wild-type DDAH that are indicative of clinical efficacy, including hydrolyzing ADMA, in vitro or in vivo activity, and efficacy for treatment of cardiac diseases, heart failure, kidney diseases, lung disease, sepsis or in a model thereof.

In embodiments, the DDAH or modified DDAH can be linked to at least pharmacokinetic enhancing moiety (PKEM). Exemplary PKEM include acyl groups, alkyl groups, lipids, serum albumin, XTEN molecules, Fc molecules, adnectins, and albumin binding moieties. For example, the acyl group may comprise a C8-C30 acyl, such as a C12 acyl, C14 acyl, C16 acyl, C18 acyl, or C20 acyl.

The pharmacokinetic enhancing moiety can be linked to any amino acid residue of the DDAH or modified DDAH amino acid sequence, such as before position 1 (i.e. at the N-terminus), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and each amino acid position through 285, and after position 285 (i.e., at the carboxyl terminus of the protein), or the corresponding amino acids in SEQ ID NO: 2, or the corresponding amino acid position in SEQ ID NO: 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; or SEQ ID NO 14.

In further embodiments, the pharmacokinetic enhancing moiety can be linked to any single position in the DDAH amino acid sequence, or a combination of more than one of these sites, e.g., 2, 3, 4, or more sites, or at least one of these sites in combination with other sites. Positions in said polypeptide chain may be substituted with another amino acid, such as cysteine (Cys, C), e.g., in conjunction with linkage of the pharmacokinetic enhancing moiety to the DDAH polypeptide.

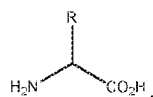
In embodiments, the DDAH or modified DDAH may include at least one non-naturally encoded amino acid. Said non-naturally encoded amino acid may be linked to the pharmacokinetic enhancing moiety, a linker, a biologically active molecule, or another DDAH polypeptide. For example, the DDAH or modified DDAH may include a pharmacokinetic enhancing moiety linked to a non-naturally encoded amino acid at any position of the DDAH polypeptide.

Also described are DDAH or modified DDAH polypeptides that comprise a substitution of a naturally encoded or non-naturally encoded amino acid substituted in the amino acid sequence, wherein: (a) the DDAH polypeptide comprises a DDAH polypeptide that has a sequence at least 80% identical to SEQ ID NO: 1, or at least 80% identical to SEQ ID NO: 2 or SEQ ID NO: 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; or SEQ ID NO 14; and (b) the substituted naturally encoded or non-naturally encoded amino acid is linked to a pharmacokinetic enhancing moiety.

In another aspect, the disclosure provides a DDAH or modified DDAH polypeptide comprising up to one, two, three, or four amino acid substitutions selected from substitution with naturally encoded or non-naturally encoded amino acids.

In another aspect, the disclosure provides a DDAH or modified DDAH polypeptide comprising at least one natural amino acid substitution and/or at least one non-naturally encoded amino acid substitution and substituted amino acid is linked to a linker, polymer, or biologically active molecule.

Said DDAH polypeptide may include a non-naturally encoded amino acid having the structure:



wherein the R group is any substituent other than the side chain found in alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine,

leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, pyrrolysine, or selenocysteine.

As stated above, in exemplary embodiments the DDAH or modified DDAH is linked to at least one pharmacokinetic enhancing moiety. The pharmacokinetic enhancing moiety can comprise an XTEN molecule. XTEN molecules are also referred to as unstructured recombinant polymers, unstructured recombinant polypeptides, or URPs, and are generally described in Schellenberger et al., *Nat Biotechnol.*, 2009 Dec;27(12):1186-90, U.S. Patent Application Publication No. 2012/0220011, U.S. Patent Application Publication No. 7,846,445, and International Publication No. WO/2012/162542, each of which is hereby incorporated by reference in its entirety. As disclosed therein, the half-life of the DDAH or modified DDAH polypeptide may be varied by varying the constitution of the XTEN molecule, e.g., by varying its size. For example, an XTEN molecule may be selected in order to achieve a desired half-life, such as in the range of 1 to 50 hours, such as at least 1, 2, 5, 10, 12, 15, 20, or 25 hours, or longer.

Exemplary XTEN molecules include a URP comprising at least 40 contiguous amino acids, wherein: (a) the URP comprises at least three different types of amino acids selected from the group consisting of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P) residues, wherein the sum of said group of amino acids contained in the URP constitutes more than about 80% of the total amino acids of the URP, and wherein said URP comprises more than one proline residue, and wherein said URP possesses reduced sensitivity to proteolytic degradation relative to a corresponding URP lacking said more than one proline residue; (b) at least 50% of the amino acids of said URP are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) the Tepitope score of said URP is less than -5. Additional exemplary XTEN molecules comprise an unstructured recombinant polymer (URP) comprising at least about 40 contiguous amino acids, and wherein (a) the sum of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P) residues contained in the URP, constitutes at least 80% of the total amino acids of the URP, and the remainder, when present, consists of arginine or lysine, and the remainder does not contain methionine, cysteine, asparagine, and glutamine, wherein said URP comprises at least three different types of amino acids selected from glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P); (b) at least 50% of the at least 40 contiguous amino acids in said URP are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) wherein the URP has a Tepitope score less than -4.

If desired, multiple DDAH polypeptide molecules can be linked to an XTEN molecule, e.g., up to 1, 2, 3, 4, 5, or more DDAH polypeptide molecules per XTEN molecule. For example, the DDAH polypeptide molecules can be linked to sites on differing portions of the XTEN molecule, e.g., near the N-terminus, near the C-terminus, or near the middle (mid-way between the N- and C-termini) thereof. In this context, the term “near” generally means linked to a site within a region of about 20%, about 15%, about 10%, or about 5% of the residues at the respective terminus or centered at the middle of the XTEN molecule.

Additional exemplary XTEN molecules include a hydrophobic residue (e.g., F, I, L, M, V, W or Y), a side chain amide-containing residue (e.g., N or Q) or a positively charged side chain residue (e.g., H, K or R). In some embodiments, the duration enhancing moiety includes A, E, G, P, S or T. In some embodiments, the XTEN includes glycine at 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-99%, or even glycine at 100%.

In some embodiments the PKEM molecule is linked to the N-terminal or C-terminal of the DDAH or modified DDAH polypeptide, or at another site. The attachment of a HELM to a DDAH polypeptide described herein is referred to herein as “PKEMylation”.

The DDAH or modified DDAH or modified DDAH polypeptide or polypeptides can be linked to the XTEN molecule through a dibenzylcyclooctyne (DBCO).

The XTEN molecule can be further linked to a polyethylene glycol.

In embodiments, the pharmacokinetic enhancing moiety can comprise an adnectin. Adnectins are disclosed, for example, in U.S. Patent Application Publication No. 2011/0305663, which is hereby incorporated by reference in its entirety. The adnectin can be based on a tenth fibronectin type III domain and can bind to serum albumin. The adnectin can comprise one or more of a BC loop, a DE loop, and an FG loop, or comprises a polypeptide selected from SEQ ID NO: 5, 6, 7, 8, 12, 16, 20, and 24-44 of U.S. Patent Application Publication No. 2011/0305663.

In embodiments, the pharmacokinetic enhancing moiety can comprise serum albumin, such as human serum albumin. For example, the DDAH or modified DDAH polypeptide can be linked to the Cys 34 residue of human serum albumin.

In embodiments, the DDAH or modified DDAH polypeptide can be linked to at least one pharmacokinetic enhancing moiety comprising an acyl group. An example acyl group can have from 6 to 40 carbon atoms, from 8 to 26 carbon atoms or from 14 to 22 carbon atoms, such as 16, 17, 18, 19, 20 carbon atoms, and may be branched or unbranched. In some embodiments, the acyl group can comprise $\text{CH}_3(\text{CH}_2)_r\text{CO}-$, wherein r is an integer from

4 to 38, such as an integer from 4 to 24, an integer from 6 to 20, or 10 or 12. For example, the acyl group can be $\text{CH}_3(\text{CH}_2)_6\text{CO}-$, $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$, or $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$. In some embodiments, the acyl group can comprise a group negatively charged at pH 7.4. In some embodiments, the acyl group can comprise a terminally attached acidic group. In some embodiments, the acyl group can comprise at least two acidic groups, wherein one of the acidic groups is terminally attached. For example, the acyl group may comprise a linear or branched lipophilic moiety containing 4-40 carbon atoms having a terminal acidic group. Additional exemplary acyl groups are disclosed in U.S. Patent Application Publication No. 2012/0295847, which is hereby incorporated by reference in its entirety.

In one embodiment, the albumin binding moiety can comprise a carboxylic acid group, such as $\text{HOOC}(\text{CH}_2)_s\text{CO}-$, wherein s is an integer from 12 to 22, such as 10, 12, 16 or 18.

In embodiments, the pharmacokinetic enhancing moiety can be linked to DDAH or modified DDAH polypeptide through a linker (e.g., a bivalent linker). For example, the linker can comprise one or two amino acids which are bound at one end to the pharmacokinetic enhancing moiety (e.g., an albumin binding moiety) and are bound at the other end to any available position on the polypeptide backbone. Other example linkers include hydrophilic linker, such as a chemical moiety which comprises at least 5 non-hydrogen atoms where 30-50% of these are either N or O (e.g., oligo- or polyalkylene oxides, such as oligoethylene glycols and polyethylene glycols). Other linkers which can link the pharmacokinetic enhancing moiety to the DDAH or modified DDAH are disclosed in U.S. Patent Application Publication No. 2012/0295847 and International Publication No. WO/2012/168430, each of which is hereby incorporated by reference in its entirety.

In some embodiments, in addition to said pharmacokinetic enhancing moiety, DDAH or modified DDAH polypeptide can also include a polyethylene glycol, optionally which may have a molecular weight of from about 2 kDa to 100 kDa (e.g., from 2 kDa to 100 kDa). Said polyethylene glycol may be linked to the DDAH at any suitable position in the amino acid sequence of the polypeptide.

In embodiments, the DDAH or modified DDAH polypeptide can be a fusion protein comprising DDAH and a proteinaceous pharmacokinetic enhancing moiety (e.g., albumin, an Fc chain, certain XTEN molecules, or a PKE adnectin), which can be fused to any suitable amino acid of the DDAH polypeptide and may be fused to the N- or C-terminus thereof.

The pharmacokinetic enhancing moiety can be covalently linked to DDAH or modified DDAH polypeptide (e.g., covalently linked to a naturally encoded or a non-naturally encoded amino acid). For example, the DDAH or modified DDAH polypeptide can comprise a pharmacokinetic enhancing moiety linked to a cysteine via a thiol linkage.

5 Optionally, multiple DDAH or modified DDAH polypeptides may be joined by a linker polypeptide, wherein said linker polypeptide optionally is 1, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, and longer in length, wherein optionally the carboxy terminus of one DDAH polypeptide is fused to the amino-terminus of the linker polypeptide and the carboxy terminus of the linker polypeptide is fused to the amino terminus of another DDAH
10 polypeptide. Further exemplary linker polypeptides which may be utilized are disclosed in International Publication No. WO/2013/004607, which is hereby incorporated by reference in its entirety.

In another embodiment, two DDAH polypeptides are linked to form a homodimer of DDAH, or a homodimer of a modified DDAH, or a heterodimer of DDAH and a modified
15 DDAH, or a heterodimer of different modified DDAH polypeptides, or any combination of DDAH polypeptides. The DDAH dimer may be formed by chemical linking of their respective N-termini. It is understood that linking two DDAH polypeptides each having a molecular weight of about 30kDa, in a head-to-head fashion each at their N-terminus, may result in an enzymatically active molecule that would have an overall molecular weight of
20 about 60kDa. A polypeptide of this size can have significantly reduced kidney clearance from the bloodstream, which may in turn result in a significantly increased circulating half-life after administration to a patient. Therefore, a dimer of DDAH polypeptides may have the same half-life extending effect compared to monomeric DDAH, as observed using a single DDAH polypeptide chemically linked to a non-DDAH pharmacokinetic enhancing moiety.

25 In embodiments, a pharmacokinetic enhancing moiety is linked to a naturally encoded amino acid or a non-naturally encoded amino acid, which can comprise a functional group which reacts and thereby forms a covalent bond with functional group on the pharmacokinetic enhancing moiety.

Also described are polynucleotides encoding the DDAH. The DDAH-coding
30 polynucleotides may be contained in different molecules or in the same molecules, e.g., joined in any order and optionally joined via a nucleotide sequence that encodes a connecting peptide. Said polynucleotide may be isolated. Said polynucleotide may be contained in one or more vectors, plasmids, etc., such as an expression vector. Additional exemplary embodiments provide a composition for translation of said polynucleotides, such as a cell or

in vitro translation system comprising said polynucleotides. Further exemplary embodiments provide a host cells, such as a prokaryotic cell (e.g., *E. coli*) or eukaryotic cell (e.g., a yeast or mammalian cell) comprising said polynucleotide and optionally further comprising said orthogonal tRNA. Additional exemplary embodiments provide a method of producing a DDAH or modified DDAH, comprising causing a cell or in vitro translation system to

5 translate said polynucleotide or an mRNA transcribed therefrom.

Optionally, the DDAH or modified DDAH polypeptide can include one or more natural variant sequences of human DDAH1, such as DDAH2, or the DDAH1 isoform 2. Additional natural variant sequences which can be present include sequences in the nucleic

10 acid that encode the secretion signal sequence.

In embodiments, the DDAH or modified DDAH polypeptide has one or more biological activities of DDAH, such as the hydrolysis of ADMA to citrulline and/or other breakdown products of ADMA.

Also provided are pharmaceutical compositions or medicaments comprising DDAH

15 or the modified DDAH polypeptides described herein. A further embodiment provides the use of said DDAH or a modified DDAH polypeptide described herein for the treatment of a variety of diseases that are associated with high ADMA levels, including but not limited to heart failure, or renal diseases. The compositions described herein may be used for reducing the concentration of ADMA, and/or increasing the levels of citrulline, and/or increasing the

20 levels of NO in patients in need of such treatment. Further provided is the use of the compositions described herein for the prophylaxis and/or treatment of renal disease, sepsis, sickle cell crisis, severe malaria, Mediterranean fever, trauma, ICU patients, acute kidney injury contrast induced kidney injury, decompensated heart failure, diurectic resistant heart failure, cardiac failure and cardiac insufficiency thromboembolic disorders, reperfusion

25 damage following ischemia, micro- and macrovascular lesions (vasculitis), arterial and venous thromboses, edemas, ischemias such as myocardial infarction, stroke and transient ischemic attacks, for cardio protection in connection with coronary artery bypass operations (coronary artery bypass graft, CABG), primary percutaneous transluminal coronary angioplasties (PTCAs), PTCAs after thrombolysis, rescue PTCA, heart transplants and open-

30 heart operations, and for organ protection in connection with transplants, bypass operations, catheter examinations and other surgical procedures. Also provided are methods of using DDAH or the modified DDAH polypeptide described herein for the prophylaxis and/or treatment of respiratory disorders, such as, for example, chronic obstructive pulmonary disease (chronic bronchitis, COPD), asthma, pulmonary emphysema, bronchiectases, lung

injury, cystic fibrosis (mucoviscidosis) and pulmonary hypertension, in particular pulmonary arterial hypertension, preeclampsia and erythropoietin resistance.

Also provided are methods of using DDAH or a modified DDAH polypeptide described herein as a medicament for the prophylaxis and/or treatment of kidney diseases, especially of acute and chronic kidney diseases and acute and chronic renal insufficiencies, as well as acute and chronic renal failure, including acute and chronic stages of renal failure with or without the requirement of dialysis, as well as the underlying or related kidney diseases such as renal hypoperfusion, dialysis induced hypotension, glomerulopathies, glomerular and tubular proteinuria, renal edema, hematuria, primary, secondary, as well as acute and chronic glomerulonephritis, membranous and membranoproliferative glomerulonephritis, Alport-Syndrome, glomerulosclerosis, interstitial tubular diseases, nephropathic diseases, such as primary and inborn kidney diseases, renal inflammation, immunological renal diseases like renal transplant rejection, immune complex induced renal diseases, as well as intoxication induced nephropathic diseases, diabetic and non-diabetic renal diseases, pyelonephritis, cystic kidneys, nephrosclerosis, hypertensive nephrosclerosis, nephrotic syndrome, that are characterized and diagnostically associated with an abnormal reduction in creatinine clearance and/or water excretion, abnormal increased blood concentrations of urea, nitrogen, potassium and/or creatinine, alteration in the activity of renal enzymes, such as glutamylsynthetase, urine osmolarity and urine volume, increased microalbuminuria, macroalbuminuria, glomerular and arteriolar lesions, tubular dilation, hyperphosphatemia and /or the requirement of dialysis.

The DDAH or a modified DDAH polypeptide described herein can be used as a medicament for the prophylaxis and/or treatment of renal carcinomas, after incomplete resection of the kidney, dehydration after overuse of diuretics, uncontrolled blood pressure increase with malignant hypertension, urinary tract obstruction and infection, amyloidosis, as well as systemic diseases associated with glomerular damage, such as Lupus erythematoses, and rheumatic immunological systemic diseases, as well as renal artery stenosis, renal artery thrombosis, renal vein thrombosis, analgetics induced nephropathy and renal tubular acidosis, gastric cancer.

The DDAH or a modified DDAH polypeptide described herein can be used as a medicament for the prophylaxis and/or treatment of contrast medium induced and drug induced acute and chronic interstitial kidney diseases, metabolic syndrome and insulin resistance. The DDAH or a modified DDAH polypeptide described herein can be used as a medicament for the prophylaxis and/or treatment of aftereffects associated with acute and/or

chronic kidney diseases, such as diabetic nephropathy, pulmonary edema, heart failure, uremia, anemia, electrolyte disturbances (e.g. hyperkalemia, hyponatremia), as well as bony and carbohydrate metabolism.

Also provided are pharmaceutical compositions comprising DDAH or a modified
5 DDAH polypeptide in a pharmacologically acceptable vehicle. The DDAH or modified DDAH polypeptides can be administered systemically or locally. Example modes of administration include, but are not limited to, intravenous, intraperitoneal, intraarterial, intranasal, by inhalation, oral, subcutaneous administration, transdermal, by local injection or in form of a surgical implant. Administration can be accomplished orally or parenterally.
10 Example methods of parenteral delivery include topical, transdermal, intra-arterial, intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration. In certain embodiments, administration may be performed subcutaneously.

Also provided are pharmaceutical compositions comprising a modified DDAH
15 polypeptide, alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. In one embodiment, the pharmaceutically acceptable carrier may be pharmaceutically inert. Any of these peptides can be administered to a patient alone, or in combination with other agents, drugs or
20 hormones, in pharmaceutical compositions where it is mixed with excipient(s) or pharmaceutically acceptable carriers. In this context, the term combination encompasses any means of concurrent administration, whether or not the DDAH or modified DDAH polypeptide and the other agent are contained in the same composition or administered separately, which administration may be through the same or different modes of
25 administration.

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for
30 ingestion by the patient.

Pharmaceutical formulations for parenteral administration include aqueous solutions of active compounds. For injection, the pharmaceutical compositions may be formulated in aqueous solutions, for example in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may

contain substances that increase viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles may include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Suitable liposomes include, but are not limited to, the phospholipid vesicles described in Geho, W., et.al., J Diabetes Sci Technol, Vol 3, Issue 6, November 2009, which is incorporated by reference herein. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

The DDAH or modified DDAH polypeptides described herein can be used alone or in combination with other active compounds. Accordingly, also provided are medicaments comprising at least one DDAH or modified DDAH polypeptide described herein and one or more further active ingredients, in particular for the treatment and/or prevention of the disorders mentioned above. Suitable active ingredients for combination may include, by way of example: active ingredients which modulate lipid metabolism, antidiabetics, hypotensive agents, perfusion-enhancing and/or antithrombotic agents, antioxidants, chemokine receptor antagonists, p38-kinase inhibitors, NPY agonists, orexin agonists, anorectics, PAF-AH inhibitors, antiphlogistics (COX inhibitors, LTB₄-receptor antagonists), analgesics for example aspirin, antidepressants and other psychopharmaceuticals.

Also described are combinations of at least one modified DDAH polypeptide with at least one lipid metabolism-altering active ingredient, antidiabetic, blood pressure reducing active ingredient and/or agent having antithrombotic effects. The DDAH or modified DDAH polypeptide can be combined with one or more lipid metabolism-modulating active ingredients, by way of example from the group of the HMG-CoA reductase inhibitors, inhibitors of HMG-CoA reductase expression, squalene synthesis inhibitors, ACAT inhibitors, LDL receptor inducers, cholesterol absorption inhibitors, polymeric bile acid adsorbers, bile acid reabsorption inhibitors, MTP inhibitors, lipase inhibitors, LpL activators, fibrates, niacin, CETP inhibitors, PPAR- α , PPAR- γ and/or PPAR- δ agonists, RXR modulators, FXR modulators, LXR modulators, thyroid hormones and/or thyroid mimetics, ATP citrate lyase inhibitors, Lp(a) antagonists, cannabinoid receptor 1 antagonists, leptin receptor agonists, bombesin receptor agonists, histamine receptor agonists and the antioxidants/radical scavengers, antidiabetics mentioned in the Rote Liste 2004/11, chapter 12, and also, by way of example, those from the group of the sulfonylureas, biguanides, meglitinide derivatives, glucosidase inhibitors, inhibitors of dipeptidyl-peptidase IV (DPP-IV

inhibitors), oxadiazolidinones, thiazolidinediones, GLP 1 receptor agonists, glucagon antagonists, insulin sensitizers, CCK 1 receptor agonists, leptin receptor agonists, inhibitors of liver enzymes involved in the stimulation of gluconeogenesis and/or glycogenolysis, modulators of glucose uptake and also potassium channel openers, such as, for example, those disclosed in WO 97/26265 and WO 99/03861; hypotensive active ingredients, by way of example from the group of the calcium antagonists, angiotensin II antagonists, ACE inhibitors, renin inhibitors, beta-receptor blockers, alpha-receptor blockers, aldosterone antagonists, mineralocorticoid receptor antagonists, ECE inhibitors, ACE/NEP inhibitors and the vasopeptidase inhibitors; and/or antithrombotic agents, by way of example from the group of the platelet aggregation inhibitors or the anticoagulants; diuretics; vasopressin receptor antagonists; organic nitrates and NO donors; compounds with positive inotropic activity; compounds which inhibit the degradation of cyclic guanosine monophosphate (cGMP) and/or cyclic adenosine monophosphate (cAMP), such as, for example, inhibitors of phosphodiesterases (PDE) 1, 2, 3, 4 and/or 5, in particular PDE 5 inhibitors, such as sildenafil, vardenafil and tadalafil, and also PDE 3 inhibitors, such as milrinone; natriuretic peptides, such as, for example, "atrial natriuretic peptide" (ANP, anaritide), "B-type natriuretic peptide" or "brain natriuretic peptide" (BNP, nesiritide), "C-type natriuretic peptide" (CNP) and also urodilatin; agonists of the prostacyclin receptor (IP receptor), such as, by way of example, iloprost, beraprost, cicaprost; inhibitors of the If (funny channel) channel, such as, by way of example, ivabradine; calcium sensitizers, such as, by way of example, levosimendan; potassium supplements; NO-independent, but heme-dependent stimulators of guanylate cyclase, such as, in particular, the compounds described in WO 00/06568, WO 00/06569, WO 02/42301 and WO 03/095451; NO- and heme-independent activators of guanylate cyclase, such as, in particular, the compounds described in WO 01/19355, WO 01/19776, WO 01/19778, WO 01/19780, WO 02/070462 and WO 02/070510; inhibitors of human neutrophil elastase (HNE), such as, for example, sivelestat and DX-890 (Reltran); compounds which inhibit the signal transduction cascade, such as, for example, tyrosine-kinase inhibitors, in particular sorafenib, imatinib, gefitinib and erlotinib; and/or compounds which modulate the energy metabolism of the heart, such as, for example, etomoxir, dichloroacetate, ranolazine and trimetazidine.

In an embodiment, a DDAH or modified DDAH polypeptide can be administered in combination with an HMG-CoA reductase inhibitor from the class of the statins, such as, by way of example, lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin, cerivastatin, or pitavastatin; a thyroid hormone and/or thyroid mimetic, such as, by way of

example, D-thyroxine or 3,5,3'-triiodothyronine (T3); an agonist of the niacin receptor, such as, by way of example, niacin, acipimox, acifran or radecol; a PPAR- γ agonist, for example from the class of the thiazolidinediones, such as, by way of example, pioglitazone or rosiglitazone.

5 In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with insulin and modified insulins. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a sulfonylurea, such as, by way of example, tolbutamide, glibenclamide, glimepiride, glipizide or gliclazide. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a biguanide, such as, by way of example, metformin. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a meglitinide derivative, such as, by way of example, repaglinide or nateglinide. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a glucosidase inhibitor, such as, by way of example, miglitol or acarbose. In an embodiment, 10 the DDAH or modified DDAH polypeptide can be administered in combination with a DPP-IV inhibitor, such as, by way of example, sitagliptin and vildagliptin.

In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a calcium antagonist, such as, by way of example, nifedipine, amlodipine, verapamil or diltiazem. In an embodiment, the DDAH or modified DDAH polypeptide can 20 be administered in combination with an ACE inhibitor, such as, by way of example, enalapril, captopril, lisinopril, ramipril, delapril, fosinopril, quinopril, perindopril ortrandopril. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a beta-receptor blocker, such as, by way of example, propranolol, atenolol, timolol, pindolol, alprenolol, oxprenolol, penbutolol, bupranolol, metipranolol, nadolol, mepindolol, 25 carazalol, sotalol, metoprolol, betaxolol, celiprolol, bisoprolol, carteolol, esmolol, labetalol, carvedilol, adaprolol, landiolol, nebivolol, epanolol or bucindolol. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with an alpha-receptor blocker, such as, by way of example, prazosin. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a diuretic, such as, by 30 way of example, furosemide, bumetanide, torsemide, bendroflumethiazide, chlorothiazide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide, polythiazide, trichloromethiazide, chlorothalidone, indapamide, metolazone, quinethazone, acetazolamide, dichlorophenamide, methazolamide, glycerol, isosorbide, mannitol, amiloride or triamteren.

In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with an aldosterone or mineralocorticoid receptor antagonist, such as, by way of example, spironolactone or eplerenone.

5 In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a vasopressin receptor antagonist, such as, by way of example, conivaptan, tolvaptan, lixivaptan or SR-121463.

In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with an organic nitrate or NO donor, such as, by way of example, sodium nitroprusside, nitroglycerol, isosorbide mononitrate, isosorbide dinitrate, molsidomin or SIN-1, or in combination with inhalative NO.

10 In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a positive- inotropic compound, such as, by way of example, cardiac glycosides (digoxin), beta-adrenergic and dopaminergic agonists, such as isoproterenol, adrenaline, noradrenaline, dopamine or dobutamine.

15 In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with antisympathotonics, such as reserpine, clonidine or alpha-methyldopa, or in combination with potassium channel agonists, such as minoxidil, diazoxide, dihydralazine or hydralazine, or with substances which release nitrogen oxide, such as glycerol nitrate or sodium nitroprusside.

20 In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with endothelin receptor antagonists such as ambrisentan, bosentan, mecitantan, etc.

Antithrombotics are to be understood as meaning, for example, compounds from the group of the platelet aggregation inhibitors or the anticoagulants. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a platelet aggregation inhibitor, such as, by way of example, aspirin, clopidogrel, ticlopidine or dipyridamole. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a thrombin inhibitor, such as, by way of example, ximelagatran, melagatran, dabigatran, bivalirudin or clexane. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a GPIIb/IIIa antagonist, such as, by way of example, tirofiban or abciximab. In an embodiment, the DDAH or modified DDAH polypeptide can be administered in combination with a factor Xa inhibitor, such as, by way of example, rivaroxaban (BAY 59-7939), DU-176b, apixaban, otamixaban, fidexaban, razaxaban, fondaparinux, idraparinux, PMD-3112, YM-150, KFA-

1982, EMD-503982, MCM-17, MLN-1021, DX 9065a, DPC 906, JTV 803, SSR-126512 or SSR-128428.

A therapeutically effective dose refers to that amount of a DDAH or modified DDAH that ameliorates the symptoms or condition, the form employed, sensitivity of the patient, and the route of administration. Normal dosage amounts may vary from 0.1 to 1000 milligrams total dose, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature. See U.S. Pat. No. 4,657,760; 5,206,344; or 5,225,212. Those skilled in the art will employ different formulations for polynucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

In another aspect, the disclosure provides a DDAH or modified DDAH polypeptide comprising: at least 80% identity to SEQ ID NO: 1, 2 or 5, and at least one pharmacokinetic enhancing moiety linked to the DDAH polypeptide, which pharmacokinetic enhancing moiety is optionally linked to at least one amino acid contained in said DDAH or modified DDAH, wherein said DDAH polypeptide is biologically active, and wherein said pharmacokinetic enhancing moiety optionally comprises at least one acyl group, lipid, alkyl group, carbohydrate, polypeptide, polynucleotide, polysaccharide, antibody or antibody fragment, sialic acid(s), a prodrug, serum albumin, XTEN molecule, Fc molecule, adnectin, fibronectin, a biologically active molecule, or a combination thereof.

The DDAH or modified DDAH polypeptide sequence may be at least 90% identical to SEQ ID NO: 1, 2, or 5. Said DDAH or modified DDAH may comprise zero, one, two, three, or four amino acid substitutions, insertions, or deletions, wherein said substitutions are with natural or non-naturally encoded amino acids. Said pharmacokinetic enhancing moiety may be linked to said naturally encoded or non-naturally encoded amino acid that is substituted in the DDAH amino acid sequence.

A naturally encoded or non-naturally encoded amino acid that is incorporated into a modified DDAH polypeptide may comprise a first functional group and the pharmacokinetic enhancing moiety may comprise a second functional group, wherein the first functional group and second functional group are not identical and each comprise a carbonyl group, an aminooxy group, a hydrazide group, a hydrazine group, a semicarbazide group, an azide group, or an alkyne group.

The pharmacokinetic enhancing moiety can comprise at least one acyl group, lipid, alkyl group, serum albumin, XTEN molecule, Fc molecule, adnectin, or a combination thereof.

In some embodiments, the pharmacokinetic enhancing moiety may comprise at least one acyl group. Said acyl group may comprise a branched or unbranched C8-C30 acyl. Said acyl group may comprise a branched or unbranched C14 acyl, C16 acyl, C18 acyl, or C20 acyl. Said acyl group may be of the formula: $\text{CH}_3(\text{CH}_2)_{12}\text{C}(=\text{O})-$ or $\text{CH}_3(\text{CH}_2)_{14}\text{C}(=\text{O})-$. Said
5 acyl group may be of the formula: $\text{CH}_3(\text{CH}_2)_{16}\text{C}(=\text{O})-$ or $\text{CH}_3(\text{CH}_2)_{18}\text{C}(=\text{O})-$.

The pharmacokinetic enhancing moiety can comprise at least one alkyl group. Said alkyl group may be branched or unbranched. Said alkyl group may be a C8-C30 alkyl group. Said alkyl group may be a C14, C16, C18, or C20 alkyl group.

The pharmacokinetic enhancing moiety can comprise at least one serum albumin.
10 Said serum albumin may comprise human serum albumin. For example, the DDAH or modified DDAH polypeptide may be linked to the Cys 34 residue of said human serum albumin.

The pharmacokinetic enhancing moiety can comprise at least one XTEN molecule. Said XTEN molecule may be linked to a single modified DDAH polypeptide molecule. The
15 DDAH or modified DDAH polypeptide may be linked to a site at or near the N-terminus of said XTEN molecule. Said XTEN molecule may be linked to multiple modified DDAH polypeptide molecules. Each said XTEN molecule may be linked to one, two, three, four, or five modified DDAH polypeptide molecules. Each said XTEN molecule may be linked to three modified DDAH polypeptide molecules. Said three modified DDAH polypeptide
20 molecules are linked to the XTEN molecule at or near the N-terminus, C-terminus, and middle of the XTEN molecule, respectively. Said XTEN molecule may comprise an unstructured recombinant polymer (URP) comprising at least 40 contiguous amino acids, wherein: (a) the URP comprises at least three different types of amino acids selected from the group consisting of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T),
25 glutamate (E) and proline (P) residues, wherein the sum of said group of amino acids contained in the URP constitutes more than about 80% of the total amino acids of the URP, and wherein said URP comprises more than one proline residue, and wherein said URP possesses reduced sensitivity to proteolytic degradation relative to a corresponding URP lacking said more than one proline residue; (b) at least 50% of the amino acids of said URP
30 are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) the Tepitope score of said URP is less than -5. Said XTEN molecule may comprise an unstructured recombinant polymer (URP) comprising at least about 40 contiguous amino acids, and wherein (a) the sum of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P) residues contained in the URP, constitutes at least 80% of

the total amino acids of the URP, and the remainder, when present, consists of arginine or lysine, and the remainder does not contain methionine, cysteine, asparagine, and glutamine, wherein said URP comprises at least three different types of amino acids selected from glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P); (b) at least 50% of the at least 40 contiguous amino acids in said URP are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) wherein the URP has a Tepitope score less than -4. Each modified DDAH polypeptide may be linked to said XTEN molecule through a dibenzylcyclooctyne (DBCO). Said XTEN molecule may be further linked to a polyethylene glycol molecule.

10 The pharmacokinetic enhancing moiety can comprise at least one adnectin. Said adnectin may comprise one or more of a BC loop, a DE loop, and an FG loop.

 The pharmacokinetic enhancing moiety can comprise one or more additional DDAH polypeptides or modified DDAH polypeptides in combination, linked to form a dimer, homodimer, heterodimer, multimer, homomultimer, heteromultimer, or other combinations of DDAH polypeptides and/or modified DDAH polypeptides. Each of these may also be linked to a pharmacokinetic enhancing moiety, such as those disclosed herein, other than a DDAH polypeptide or modified DDAH polypeptide.

 The pharmacokinetic enhancing moiety can comprise at least one lipid. Said lipid may comprise a fat-soluble vitamin, fat, wax, sterol, monoglyceride, diglyceride, triglyceride, or phospholipid.

 The DDAH or modified DDAH polypeptide can exhibit an *in vivo* half-life of at least 1, 2, 5, 10, 12, 15, 20, 25 hours, or multiple days. Said *in vivo* half-life may be determined in human, mouse, rat, dog, cynomolgus monkey, rabbit, horse, cattle, cat, hamster, or rhesus macaque. Said *in vivo* half-life may be determined following subcutaneous or intravenous administration of said DDAH or modified DDAH polypeptide.

 The DDAH or modified DDAH polypeptide can be attached to another biologically active moiety.

 Multiple DDAH polypeptides can be joined by a linker polypeptide, wherein said linker polypeptide optionally is 6-14, 7-13, 8-12, 7-11, 9-11, or 9 amino acids in length.

30 Other linkers include but are not limited to small polymers such as PEG, which may be multi-armed allowing for multiple DDAH molecules to be linked together. Multiple DDAH polypeptides and modified DDAH polypeptides may be linked to each other via their N-termini in a head-to-head configuration through the use of such a linker or by direct chemical bonding between the respective N-terminus of each polypeptide. For example, two DDAH

polypeptides may be linked to form a dimer by chemical bonding between their N-terminal amino groups or modified N-terminal amino groups. Also, a linking molecule that is designed to comprise multiple chemical functional groups for bonding with the N-terminus of each DDAH polypeptide may be used to join multiple DDAH polypeptides each at their
5 respective N-terminus. In addition, multiple DDAH polypeptides may be linked through bonding between amino acids other than the N-terminal amino acid or C-terminal amino acid. An example of covalent bonds that may be utilized to form the dimmers and multimers of DDAH that are described herein include, but are not limited to disulphide or sulfhydryl or thiol bonds. In addition, certain enzymes, such as sortase, may be used to form covalent
10 bonds between the DDAH polypeptides and the linker, including at the N-termini of the DDAH polypeptides.

In another aspect, the disclosure provides a DDAH or modified DDAH polypeptide or composition containing a DDAH or modified DDAH polypeptide as herein described, wherein said DDAH polypeptide may be conjugated to at least one substance including but
15 not limited to a label, a dye, a polymer, a water-soluble polymer, a derivative of polyethylene glycol, a photocrosslinker, a radionuclide, a cytotoxic compound, a drug, an affinity label, a photoaffinity label, a reactive compound, a resin, another polypeptide or protein, a polypeptide analog, an antibody, an antibody fragment, a metal chelator, a cofactor, a fatty acid, a carbohydrate, a polynucleotide, a DNA, a RNA, an antisense polynucleotide, a
20 saccharide, a water-soluble dendrimer, a cyclodextrin, an inhibitory ribonucleic acid, a biomaterial, a nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a photoisomerizable moiety, biotin, a derivative of biotin, a biotin analogue, a moiety
25 incorporating a heavy atom, a chemically cleavable group, a photocleavable group, an elongated side chain, a carbon-linked sugar, a redox-active agent, an amino thioacid, a toxic moiety, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, a detectable label, a small
30 molecule, a quantum dot, a nanotransmitter, a radionucleotide, a radiotransmitter, a neutron-capture agent, or any combination of the above.

In another aspect, the disclosure provides an isolated cell, vector, plasmid, prokaryotic cell, eukaryotic cell, virus, prokaryotic cell, eukaryotic cell, mammalian cell, yeast, bacterium, or cell-free translation system comprising one or more polynucleotides that

encode the DDAH or modified DDAH to express the DDAH or modified DDAH polypeptide. The method of expression may produce any DDAH or modified DDAH polypeptide as herein described.

In another aspect, the disclosure provides a method of producing any DDAH or modified DDAH polypeptide as herein described, comprising chemically synthesizing said DDAH or modified DDAH polypeptide.

In further embodiments, the DDAH may be administered daily, in an injectable form, an orally-available formulation, as a sustained release formulation, as a prodrug formulation, or as a continuous infusion.

Also provided are methods of increasing renal vasodilation and hyperfiltration, generally comprising administering a formulation comprising an amount of DDAH or modified DDAH polypeptide. These methods are useful in treating a variety of renal pathologies. Accordingly, also provided are methods of treating a renal pathology related to the effects of ADMA.

Also provided are methods of reducing pulmonary hypertension, generally comprising administering a formulation comprising an amount of DDAH or modified DDAH polypeptide.

In some embodiments, the DDAH polypeptide comprises one or more post-translational modifications. In some embodiments, the DDAH polypeptide is linked to a linker, polymer, or biologically active molecule. In some embodiments, the DDAH polypeptide is linked to a bifunctional polymer, bifunctional linker, or at least one additional DDAH polypeptide.

In some embodiments, the DDAH or modified DDAH is linked to a pharmacokinetic enhancing moiety. In some embodiments, the DDAH is linked to the pharmacokinetic enhancing moiety with a linker or is bonded to the pharmacokinetic enhancing moiety. In some embodiments, the pharmacokinetic enhancing moiety is a bifunctional molecule. In some embodiments, the bifunctional molecule is linked to a second polypeptide. In some embodiments, the second polypeptide is a DDAH polypeptide.

In some embodiments, the DDAH polypeptide comprises at least two amino acids linked to a pharmacokinetic enhancing moiety. In some embodiments, at least one amino acid is a non-naturally encoded amino acid.

In some embodiments, one or more naturally encoded or non-naturally encoded amino acids are incorporated in one or more of the following positions in any of the DDAH polypeptide, or proDDAH polypeptides, DDAH analogs, proDDAH, or modified DDAH

polypeptide amino acid sequence before position 1 (i.e. at the N-terminus), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, and at each individual amino acid position up to and including position 285, after position 285 (i.e., at the carboxyl terminus of the protein of SEQ ID NO: 1, the corresponding position in SEQ ID NO: 2, or SEQ ID NO:5; SEQ ID NO 6;SEQ ID NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ ID NO 13;or SEQ ID NO 14).

The sites selected for incorporation, deletion, addition, or substitution of a naturally encoded or of a non-naturally encoded amino acid that enhance or modulate pharmacokinetic, pharmacodynamic, or time-action properties of the DDAH polypeptide, and/or for linkage to a pharmacokinetic enhancing moiety or other biologically active molecule may be selected based upon a variety of factors which may be predicted to influence the activity and half-life of the resulting modified DDAH polypeptide.. Another factor to consider is the residue's participation in forming multimers including homodimerization, binding of DDAH modulators such as zinc, and substrate binding, or proximity to residues involved in the above activities, wherein modification of that residue can enhance or modulate pharmacokinetic, pharmacodynamic, or time-action properties of the DDAH polypeptide, and/or for linkage to a pharmacokinetic enhancing moiety or other biologically active molecule might interfere with activity. Yet another factor to consider is proximity to residues which may interact with the pharmacokinetic enhancing moiety (PKEM). For example, where the PKEM is a hydrophobic molecule that binds to serum albumin, proximity to surface-exposed hydrophobic residues may cause the PKEM to bind to the DDAH or modified DDAH polypeptide and potentially interfere with the ability of the PKEM to bind to serum albumin effectively increase half-life. Likewise, a hydrophilic PKEM could interact with proximate hydrophilic residues and therefore decrease the ability of the PKEM to improve half-life.

Also provided are formulations. The formulations can comprise a mixture of two or more of a DDAH, a DDAH dimer, a DDAH multimer, a DDAH variant, a DDAH analog, an acylated DDAH, a HELMylated or acylated or PEGylated DDAH analog. In another embodiment, the formulations containing a mixture of two or more of DDAH, a DDAH analog, an acylated DDAH, or acylated DDAH analog also includes at least one pharmacokinetic enhancing moiety attached to at least one of the DDAH polypeptides.

Also described are heterogeneous mixtures wherein DDAH polypeptides and DDAH analogs are prepared by the methods disclosed herein, and are then mixed so that a formulation may be administered to a patient in need thereof which contains, for example, various percentages of different forms of DDAH polypeptides which have been coupled to a particular pharmacokinetic enhancing moiety, and the remainder consisting of DDAH polypeptide having a different or no PKEM. All different mixtures of different percentage amounts of DDAH polypeptide variants wherein the DDAH polypeptides include a variety (1) with differently sized PKEM, or (2) PKEM are included at different positions in the sequence. In embodiments, the DDAH polypeptide variants to include in the formulation mixture can be chosen by their varying dissociation times so that the formulation may provide a sustained release of DDAH for a patient in need thereof, or the formulation may provide immediate or fast acting DDAH as well as longer acting DDAH molecules including one or more PKEMs.

Also described are formulation for inhalation. A DDAH analogs with increased pharmacokinetic and pharmacodynamic properties for patient use via administration to the lung, resulting in elevated blood levels of DDAH that are sustained for at least 6 hours, and more typically for at least 8, 10, 12, 14, 18, 24 hours or greater post-administration. Another embodiment allows for mixtures of DDAH analogs for therapeutic formulations designed to be administered to patients as an inhalant.

Also described are embodiments useful for introducing additional, customized sites within the DDAH molecule, for example, for forming a DDAH or modified DDAH having improved resistance to enzymatic degradation. Such an approach provides greater flexibility in the design of an optimized DDAH conjugate having the desired balance of activity, stability, solubility, and pharmacological properties. Mutations can be carried out, i.e., by site specific mutagenesis, at any number of positions within the DDAH molecule. Typically, a pharmacokinetic enhancing moiety is activated with a suitable activating group appropriate for coupling a desired site or sites on the DDAH molecule. An activated pharmacokinetic enhancing moiety may possess a reactive group at a terminus for reaction with DDAH.

Branched HELMs such as PEGs can include those described in International Patent Publication WO 96/21469. Generally, branched PKEMs can be represented by the formula $R(\text{PKEM--OH})_n$, where R represents the central "core" molecule and n represents the number of arms. Branched PKEMs have a central core from which extend 2 or more "PKEM" arms. In a branched configuration, the branched polymer core possesses a single reactive site for attachment to DDAH. Branched PKEMs can comprise fewer than 4 PKEM arms, and more

preferably, will comprise fewer than 3 PKEM arms. Branched PKEMs offer the advantage of having a single reactive site, coupled with a larger, denser polymer cloud than their linear PKEM counterparts. One particular type of branched PKEM can be represented as (MeO-PKEM-)_p R--X, where p equals 2 or 3, R is a central core structure such as lysine or glycerol having 2 or 3 PKEM arms attached thereto, and X represents any suitable functional group that is or that can be activated for coupling to DDAH. One particularly preferred branched PEG is mPEG2-NHS (Shearwater Corporation, Alabama) having the structure mPEG2-lysine-succinimide.

In yet another branched architecture, "pendant PKEM" has reactive groups for protein coupling positioned along the PKEM backbone rather than at the end of PKEM chains. The reactive groups extending from the PKEM backbone for coupling to DDAH may be the same or different. Pendant PKEM structures may be useful but are generally less preferred, particularly for compositions for inhalation.

Alternatively, the PKEM may possess a forked structure having a branched moiety at one end of the polymer chain and two free reactive groups (or any multiple of 2) linked to the branched moiety for attachment to DDAH. The forked polyethylene glycol may optionally include an alkyl or "R" group at the opposing end of the polymer chain. More specifically, a forked PKEM-DDAH conjugate in accordance with embodiments described herein has the formula: R-PKEM-L(Y-DDAH)_n where R is alkyl, L is a hydrolytically stable branch point and Y is a linking group that provides chemical linkage of the forked polymer to DDAH, and n is a multiple of 2. L may represent a single "core" group, such as "--CH--", or may comprise a longer chain of atoms. Exemplary L groups include lysine, glycerol, pentaerythritol, or sorbitol. Typically, the particular branch atom within the branching moiety is carbon.

In one embodiment, the linkage of the forked PKEM to the DDAH molecule, (Y), is hydrolytically stable. In a preferred embodiment, n is 2. Suitable Y moieties, prior to conjugation with a reactive site on DDAH, include but are not limited to active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, and iodacetamides. Selection of a suitable activating group will depend upon the intended site of attachment on the DDAH molecule and can be readily determined by one of skill in the art. The corresponding Y group in the resulting PKEM-DDAH conjugate is that which results from reaction of the activated forked polymer with a suitable reactive site on DDAH. For example, if the reactive forked PKEM contains an activated ester, such as a succinimide or maleimide ester, conjugation via an

amine site on DDAH will result in formation of the corresponding amide linkage. These particular forked polymers are particularly attractive since they provide conjugates having a molar ratio of DDAH to PKEM of 2:1 or greater. Such conjugates may be less likely to block the DDAH substrate binding or other binding site, while still providing the flexibility in design to protect the DDAH against enzymatic degradation, e.g., by DDAH degrading enzyme.

In one embodiment, the forked PKEM-DDAH conjugate can be represented by the formula: $R-[PKEM-L(Y-DDAH)_2]_n$. In this instance R represents a natural or non-naturally encoded amino acid having attached thereto at least one PKEM-di-DDAH conjugate.

Specifically, in some embodiments the forked polymers can be those where n is selected from the group consisting of 1,2,3,4,5,and 6. In an alternative embodiment, the chemical linkage between the non-natural amino acid within DDAH, DDAH polypeptide, or DDAH analog and the polymer branch point may be degradable (i.e., hydrolytically unstable). Alternatively, one or more degradable linkages may be contained in the polymer backbone to allow generation in vivo of a PKEM-DDAH conjugate having a smaller PKEM chain than in the initially administered conjugate. For example, a large and relatively inert conjugate (i.e., having one or more high molecular weight PKEM chains attached thereto, e.g., one or more PKEM chains having a molecular weight greater than about 10,000, wherein the conjugate possesses essentially no bioactivity) may be administered, which then either in the lung or in the bloodstream, is hydrolyzed to generate a bioactive conjugate possessing a portion of the originally present PKEM chain. Upon in-vivo cleavage of the hydrolytically degradable linkage, either free DDAH (depending upon the position of the degradable linkage) or DDAH having a small polyethylene tag attached thereto, is then released and more readily absorbed through the lung and/or circulated in the blood.

In some embodiments, the poly(ethylene glycol) molecule has a molecular weight of between about 0.1 kDa and about 100 kDa. In some embodiments, the poly(ethylene glycol) molecule has a molecular weight of between 0.1 kDa and 50 kDa.

In some embodiments, the poly(ethylene glycol) molecule is a branched polymer. In some embodiments, each branch of the poly(ethylene glycol) branched polymer has a molecular weight of between 1 kDa and 100 kDa, or between 1 kDa and 50 kDa.

In some cases, a PKEM can terminate on one end with hydroxy or methoxy, i.e., X is H or CH₃ ("methoxy PEG"). Alternatively, the PKEM can terminate with a reactive group, thereby forming a bifunctional polymer. Typical reactive groups can include those reactive groups that are commonly used to react with the functional groups found in the 20 common

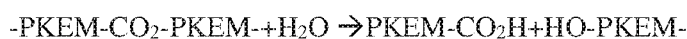
amino acids (including but not limited to, maleimide groups, activated carbonates (including but not limited to, p-nitrophenyl ester), activated esters (including but not limited to, N-hydroxysuccinimide, p-nitrophenyl ester) and aldehydes) as well as functional groups that are inert to the 20 common amino acids but that react specifically with complementary functional groups (including but not limited to, azide groups, alkyne groups). It is noted that the other end of the PKEM, which is shown in the above formula by Y, will attach either directly or indirectly to a DDAH polypeptide via a naturally-occurring or non-naturally encoded amino acid. For instance, Y may be an amide, carbamate or urea linkage to an amine group (including but not limited to, the epsilon amine of lysine or the *N*-terminus) of the polypeptide. Alternatively, Y may be a maleimide linkage to a thiol group (including but not limited to, the thiol group of cysteine). Alternatively, Y may be a linkage to a residue not commonly accessible via the 20 common amino acids. For example, an azide group on the PKEM can be reacted with an alkyne group on the DDAH polypeptide to form a Huisgen [3+2] cycloaddition product. Alternatively, an alkyne group on the PKEM can be reacted with an azide group present in a DDAH polypeptide to form a similar product. In some embodiments, a strong nucleophile (including but not limited to, hydrazine, hydrazide, hydroxylamine, semicarbazide) can be reacted with an aldehyde or ketone group present in a DDAH polypeptide to form a hydrazone, oxime or semicarbazone, as applicable, which in some cases can be further reduced by treatment with an appropriate reducing agent. Alternatively, the strong nucleophile can be incorporated into the DDAH polypeptide via a non-naturally encoded amino acid and used to react preferentially with a ketone or aldehyde group present in the water-soluble polymer.

Any molecular mass for a PKEM can be used as practically desired, including but not limited to, from about 100 Daltons (Da) to 100,000 Da or more as desired (including but not limited to, sometimes 0.1-50 kDa or 10-40 kDa). The molecular weight of PKEM may be of a wide range, including but not limited to, between about 100 Da and about 100,000 Da or more. PKEM may be between about 100 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, 1,000 Da, 900 Da, 800 Da, 700 Da, 600 Da, 500 Da, 400 Da, 300 Da, 200 Da, and 100 Da. In some embodiments, PKEM is between about 100 Da and about 50,000 Da. Branched chain PKEMs, including but not limited to, PKEM molecules with each chain having a MW ranging from 1-100 kDa (including but not limited to, 1-50

kDa or 5-20 kDa) can also be used. The molecular weight of each chain of the branched chain PKEM may be, including but not limited to, between about 1,000 Da and about 100,000 Da or more. The molecular weight of each chain of the branched chain PKEM may be between about 1,000 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, and 1,000 Da. In some embodiments, the molecular weight of each chain of the branched chain PKEM is between about 1,000 Da and about 50,000 Da. In some embodiments, the molecular weight of each chain of the branched chain PKEM is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of each chain of the branched chain PKEM is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of each chain of the branched chain PKEM is between about 5,000 Da and about 20,000 Da. A wide range of PKEM molecules are described in, including but not limited to, the Shearwater Polymers, Inc. catalog, Nektar Therapeutics catalog, incorporated herein by reference.

Also provided are azide- and acetylene-containing polymer derivatives comprising a water soluble polymer backbone having an average molecular weight from about 800 Da to about 100,000 Da. The polymer backbone of the water-soluble polymer can be poly(ethylene glycol). However, it should be understood that a wide variety of water soluble polymers including but not limited to poly(ethylene)glycol and other related polymers, including poly(dextran) and poly(propylene glycol), are also suitable. The use of the term PEG or poly(ethylene glycol) is intended to encompass and include all such molecules. The term PEG includes, but is not limited to, poly(ethylene glycol) in any of its forms, including bifunctional PEG, multiarmed PEG, derivatized PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

In addition to these forms of PKEM, the polymer can also be prepared with weak or degradable linkages in the backbone. For example, PKEM can be prepared with ester linkages in the polymer backbone that are subject to hydrolysis. As shown below, this hydrolysis results in cleavage of the polymer into fragments of lower molecular weight:



In some embodiments, polymer backbones that are water-soluble, with from 2 to about 300 termini. Examples of suitable polymers include, but are not limited to, other poly(alkylene

glycols), such as poly(propylene glycol) ("PPG"), copolymers thereof (including but not limited to copolymers of ethylene glycol and propylene glycol), terpolymers thereof, mixtures thereof, and the like. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 800 Da to about 100,000 Da, often from about 6,000 Da to about 80,000 Da. The molecular weight of each chain of the polymer backbone may be between about 100 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, 1,000 Da, 900 Da, 800 Da, 700 Da, 600 Da, 500 Da, 400 Da, 300 Da, 200 Da, and 100 Da. In some embodiments, the molecular weight of each chain of the polymer backbone is between about 100 Da and about 50,000 Da. In some embodiments, the molecular weight of each chain of the polymer backbone is between about 100 Da and about 40,000 Da. In some embodiments, the molecular weight of each chain of the polymer backbone is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of each chain of the polymer backbone is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of each chain of the polymer backbone is between about 10,000 Da and about 40,000 Da.

Appropriate physiologically cleavable linkages include but are not limited to ester, carbonate ester, carbamate, sulfate, phosphate, acyloxyalkyl ether, acetal, and ketal. Such conjugates should possess a physiologically cleavable bond that is stable upon storage and upon administration. For instance, a DDAH or modified DDAH linked to a pharmacokinetic enhancing moiety should maintain its integrity upon manufacturing of the final pharmaceutical composition, upon dissolution in an appropriate delivery vehicle, if employed, and upon administration irrespective of route.

In some embodiments, the polypeptide described herein can comprise one or more naturally encoded or non-naturally encoded amino acid substitution, addition, or deletion in the signal sequence. In some embodiments, the polypeptides can comprise one or more naturally encoded or non-naturally encoded amino acid substitution, addition, or deletion in the signal sequence for DDAH or any of the DDAH analogs or polypeptides disclosed within this specification. In some embodiments, the polypeptides comprise one or more naturally encoded amino acid substitution, addition, or deletion in the signal sequence as well as one or more non-naturally encoded amino acid substitutions, additions, or deletions in the signal sequence for DDAH or any of the DDAH analogs or polypeptides disclosed within this

specification. In some embodiments, one or more non-natural amino acids are incorporated in the leader or signal sequence for DDAH or any of the DDAH analogs or polypeptides described herein.

In some embodiments, the DDAH polypeptide comprises a substitution, addition or
5 deletion that modulates affinity of the DDAH polypeptide for ADMA or other binding partner, including but not limited to, a protein, polypeptide, small molecule, or nucleic acid. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases the stability of the DDAH polypeptide when compared with the stability of the corresponding DDAH without the substitution, addition, or deletion. Stability and/or
10 solubility may be measured using a number of different assays known to those of ordinary skill in the art. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates the immunogenicity of the DDAH polypeptide when compared with the immunogenicity of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a
15 substitution, addition, or deletion that modulates serum half-life or circulation time of the DDAH polypeptide when compared with the serum half-life or circulation time of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates the enzymatic activity of the DDAH polypeptide when compared with the enzymatic activity of
20 the corresponding DDAH without the substitution, addition, or deletion.

In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases the aqueous solubility of the DDAH polypeptide when compared to aqueous solubility of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion
25 that increases the solubility of the DDAH polypeptide produced in a host cell when compared to the solubility of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases the expression of the DDAH polypeptide in a host cell or increases synthesis in vitro when compared to the expression or synthesis of the corresponding DDAH without the
30 substitution, addition, or deletion. The DDAH polypeptide comprising this substitution retains enzymatic activity and retains or improves expression levels in a host cell. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases protease resistance of the DDAH polypeptide during manufacturing processes when compared to the protease resistance of the corresponding DDAH without the substitution,

addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates DDAH homodimerization, modulator binding such as zinc binding, ADMA hydrolyzing activity, and substrate binding activity of the DDAH polypeptide during manufacturing processes when compared with the activity of the DDAH polypeptide without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates its binding to another molecule such as a substrate or modulator or other DDAH polypeptide when compared to the binding of the corresponding DDAH polypeptide without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates its enzymatic activity compared to the enzymatic activity of the corresponding DDAH polypeptide without the substitution, addition, or deletion.

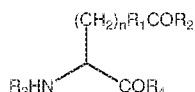
In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that modulates the stability of the DDAH polypeptide when compared to stability of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases the stability of the DDAH polypeptide produced in a host cell when compared to the stability of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases the half-life of enzymatically active circulating DDAH after administration to a patient when compared to the corresponding DDAH without the substitution, addition, or deletion. The DDAH polypeptide comprising this substitution retains enzymatic activity and yet is resistant to deactivation, destabilization, or destruction caused, for example, by proteases or other substances that affect the structural integrity or enzymatic activity of the DDAH polypeptides. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases protease resistance of the DDAH polypeptide when compared to the protease resistance of the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that increases DDAH homodimerization, changes modulator binding such as zinc binding, increases ADMA hydrolyzing activity, and increases substrate binding activity of the DDAH polypeptide when compared with the activity of the DDAH polypeptide without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that decreases the half-life of enzymatically active circulating DDAH after administration to a patient when compared to

the corresponding DDAH without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that decreases its binding to another molecule such as a substrate or modulator or other DDAH polypeptide when compared to the binding of the corresponding DDAH polypeptide without the substitution, addition, or deletion. In some embodiments, the DDAH polypeptide comprises a substitution, addition, or deletion that decreases its enzymatic activity compared to the enzymatic activity of the corresponding DDAH polypeptide without the substitution, addition, or deletion.

In some embodiments, one or more amino acid substitutions in the DDAH polypeptide may be with one or more naturally occurring or non-naturally encoded amino acids. In some embodiments the amino acid substitutions in the DDAH polypeptide may be with naturally occurring or non-naturally encoded amino acids, provided that at least one substitution is with a non-naturally encoded amino acid. In some embodiments, one or more amino acid substitutions in the DDAH polypeptide may be with one or more naturally occurring amino acids, and additionally at least one substitution is with a non-naturally encoded amino acid.

In some embodiments, the non-naturally encoded amino acid comprises a carbonyl group, an acetyl group, an aminooxy group, a hydrazine group, a hydrazide group, a semicarbazide group, an azide group, or an alkyne group.

In some embodiments, the non-naturally encoded amino acid comprises a carbonyl group. In some embodiments, the non-naturally encoded amino acid has the structure:



wherein n is 0-10; R1 is an alkyl, aryl, substituted alkyl, or substituted aryl; R2 is H, an alkyl, aryl, substituted alkyl, and substituted aryl; and R3 is H, an amino acid, a polypeptide, or an amino terminus modification group, and R4 is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

Also provided are isolated nucleic acids comprising a polynucleotide that hybridizes under stringent conditions nucleic acids that encode DDAH polypeptides of SEQ ID NOs: 3, and 4. Also provided are isolated nucleic acids comprising a polynucleotide that hybridizes under stringent conditions to nucleic acids that encode DDAH polypeptides of SEQ ID NOs: 3 and 4. Also provided are isolated nucleic acids comprising a polynucleotide that encodes the polypeptides shown as SEQ ID NOs.: 1, 2, 5, 6, 7, 9, 10, 11, 12, 13, and 14.

Azide- and acetylene-containing amino acids may also be incorporated site-selectively into proteins such as DDAH using the methods known in the art. Thereafter said azide- and acetylene-containing amino acids may be linked to a pharmacokinetic enhancing moiety using methods known in the art.

5 In a further aspect, also provided are recombinant nucleic acids encoding the DDAH proteins, expression vectors containing the variant nucleic acids, host cells comprising the variant nucleic acids and/or expression vectors, and methods for producing the variant proteins. In an additional aspect, also provided are methods of treating a DDAH responsive disorder by administering to a patient a variant protein, usually with a pharmaceutical carrier,
10 in a therapeutically effective amount. Also provided are methods for modulating immunogenicity (particularly reducing immunogenicity) of DDAH polypeptides by altering MHC Class II epitopes.

In therapeutic applications, compositions containing the modified non-natural amino acid polypeptide can be administered to a patient already suffering from a disease, condition
15 or disorder, in an amount sufficient to cure or at least partially arrest the symptoms of the disease, disorder or condition. Such an amount is defined to be a "therapeutically effective amount," and will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician

20 In prophylactic applications, compositions containing the DDAH polypeptide are administered to a patient susceptible to or otherwise at risk of a particular disease, disorder or condition. In this use, the precise amounts also depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art to determine such prophylactically effective amounts by routine experimentation (e.g., in clinical trial).

25 DDAH polypeptides described herein can be used to modulate the concentration of ADMA in a patient. In one embodiment, a patient in need thereof receives a therapeutic amount of a DDAH polypeptide described herein that would decrease the patient's ADMA concentration over the baseline of their seeking treatment by 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, more than
30 100%, 150%, more than 150%, 200%, more than 200%. In another embodiment, provided are methods of treatment of a patient in need thereof to increase the patient's NO production by administering a therapeutically effective amount of DDAH polypeptide to increase NO production by 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%,

75%, 80%, 85%, 90%, 95%, 100%, more than 100%, 150%, more than 150%, 200%, more than 200%.

DDAH polypeptides described herein can have up to a 10-fold or more increase in AUC as compared to a wild type DDAH 15-fold increase; more than 15-fold increase; 20-fold increase; more than 20-fold increase; 25 fold increase; more than 25-fold increase; 30-fold increase; more than 30-fold increase; 35-fold increase; more than 35-fold increase; 40-fold increase; more than 40-fold increase; 45-fold increase; more than 45-fold increase; 50-fold increase; more than 50-fold increase; 55-fold increase; more than 55-fold increase; 60-fold increase; more than 60-fold increase; 65-fold increase; more than 65-fold increase; 70-fold increase; more than 70-fold increase; 75-fold increase; more than 75-fold increase; 80-fold increase; more than 80-fold increase; 85-fold increase; more than 85-fold increase; 90-fold increase; more than 90-fold increase; 95-fold increase; more than 95-fold increase; 100-fold increase; more than 100-fold increase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the structure of plasmid containing human DDAH or Pa-DDAH.

Figure 2 shows the enzymatic activity and purity of rDDAH. DDAH activity was determined using a colorimetric assay measuring citrulline generation from ADMA. Purity of the rDDAH inset lane 2 was determined by SDS gel electrophoresis, lane 1 is MW markers.

Figures 3A and 3B show that rDDAH lowered ADMA in plasma and blood in vitro. Different concentrations of rDDAH was added to whole blood (Figure 3A) or human plasma with 3uM exogenous ADMA (Figure 3B). Samples were incubated at 37C for 30 min, ADMA concentration was then determined using LC-MS.

Figures 4A-4C show the effect of PEGylation on the activity of a DDAH polypeptide. Figure 4A shows the activity of rPa-DDAH before (solid line) and after PEGylation (M-DDAH) (dotted line). rPa-DDAH was PEGylated using 10Kd N-hydroxysuccinimide. PEGylation was determined using SDS-PAGE as shown in Figure 4B (Lane 1 – molecular weight markers; Lane 2 - rPa-DDAH; Lane 3 - PEGylated rPa-DDAH (M-DDAH)). Figure 4C shows the DDAH activity of a control and M-DDAH. As shown in Figure 4C, the half life was extended by PEGylation. Briefly, rPa-DDAH (solid line) or M-DDAH 9 (dotted line) was administered to rats (1mg/kg). Blood samples were collected in heparin tubes at the indicated time points and centrifuged to prepare plasma. DDAH activity in plasma samples was determined as described under DDAH assay.

Figure 5 shows the efficacy of M-DDAH as determined in a rat acute kidney injury model. Figure 5 shows that M-DDAH significantly attenuated the loss of renal function as indicated by the reduced serum creatinine at 24 hours of injury-reperfusion.

DETAILED DESCRIPTION

5 Definitions

The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

As used herein and in the appended claims, the singular forms “a,” “an,” and “the”
10 include plural reference unless the context clearly indicates otherwise. Thus, for example, reference to a “DDAH” or “DDAH polypeptide” and various hyphenated and unhyphenated forms is a reference to one or more such proteins and includes equivalents thereof known to those of ordinary skill in the art, and so forth.

The term “DDAH” as used herein, refers to human and non-human DDAH,
15 specifically human or non-human DDAH1 or human DDAH 2, unless the context indicates otherwise, whose amino acid sequence and spatial structure are well-known, including but not limited to the amino acid sequences set forth in SEQ ID NO: 1; SEQ ID NO:2; SEQ ID NO:5; SEQ ID NO 6;SEQ ID NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ ID NO 13;or SEQ ID NO 14. Human DDAH is comprised of a 285 amino acid
20 long polypeptide chain that contains no intra-chain disulfide bonds. DDAH is also known to naturally form homodimers (Murray-Rust, J., et.al., Nature Structural Biology, Volume 8, Number 8, August 2001). In addition, DDAH enzymatic activity is known to be modulated by the binding of zinc to a particular site on the DDAH polypeptide such that when zinc is bound to the DDAH polypeptide the enzymatic activity of the DDAH is reduced (Knipp, M.,
25 et.al., JBC, Volume 276, Number 44, pp 40449-40456, 2001).

The terms “wild-type DDAH,” “WT DDAH,” and “wt rhDDAH” refer to a human DDAH polypeptide having the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or the corresponding amino acid in SEQ ID NO:5. The terms “wild-type DDAH,” “WT DDAH,” and “wt rDDAH” also refer to a non-human DDAH polypeptide having the amino
30 acid sequence SEQ ID NO 6;SEQ ID NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ ID NO 13;or SEQ ID NO 14. Human WT DDAH 1and human WT DDAH 2 mature polypeptides in monomeric form each have a predicted molecular weight of about 30kDa.

The term "DDAH analog" as used herein is a protein exhibiting the enzymatic activity of hydrolyzing ADMA to citrulline and other ADMA breakdown products.

For example, the term "DDAH analog" includes a protein that differs from the wild-type DDAH by having one or more amino acid deletions, one or more amino acid

5 replacements, and/or one or more amino acid additions that do not destroy the DDAH activity of the DDAH analog. Other examples of DDAH analogs include but are not limited to amino acid differences that enhance or modulate pharmacokinetic, pharmacodynamic, or time-action properties of the DDAH polypeptide, and/or are useful for linkage to a pharmacokinetic enhancing moiety or other biologically active molecule.

10 By "DDAH polypeptide" as used herein is meant a compound having a molecular structure similar to that of human DDAH, and which have DDAH enzymatic activity. As used herein, "DDAH1" or "DDAH2" shall include those polypeptides and proteins that have at least one biological activity of a human DDAH enzyme, as well as DDAH analogs, DDAH isoforms, DDAH mimetics, DDAH fragments, hybrid DDAH proteins, fusion proteins

15 oligomers and multimers, homologues, glycosylation pattern variants, and muteins, thereof, regardless of the biological activity of same, and further regardless of the method of synthesis or manufacture thereof including, but not limited to, recombinant (whether produced from cDNA, genomic DNA, synthetic DNA or other form of nucleic acid), synthetic, transgenic, and gene activated methods. As used herein, the term "DDAH unit", or DDAH "enzymatic unit", [U], refers to that amount of enzyme (DDAH) which causes the production of 1mmol of L-citrulline per minute under the conditions described in the reference "Markus Knipp and Milan Vasak, Analytical Biochem., 286, 257 (2000)". The amino acid sequence and polynucleotide sequence for DDAH1 and DDAH2 are shown in Tables 2, 3, 4, 5, 6, and 7

herein. Fusions comprising additional amino acids at the amino terminus, carboxyl terminus, 25 or both, are encompassed by the term "DDAH polypeptide." Exemplary fusions include, but are not limited to, e.g., methionyl DDAH in which a methionine is linked to the N-terminus of DDAH resulting from the recombinant expression of the mature form of DDAH lacking the leader or signal peptide or portion thereof (a methionine is linked to the N-terminus of DDAH resulting from the recombinant expression), fusions for the purpose of purification 30 (including, but not limited to, to poly-histidine or affinity epitopes), fusions with serum albumin binding peptides and fusions with serum proteins such as serum albumin. Chimeric molecules comprising DDAH and one or more other molecules are also included. The chimeric molecule can contain specific regions or fragments of one or both of the DDAH and the other molecule(s). Any such fragments can be prepared from the proteins by standard

biochemical methods, or by expressing a polynucleotide encoding the fragment. DDAH, or a fragment thereof, can be produced as a fusion protein comprising human serum albumin (HSA), Fc, or a portion thereof. Such fusion constructs are suitable for enhancing expression of the DDAH, or fragment thereof, in an eukaryotic host cell. Exemplary HSA portions
5 include the N-terminal polypeptide (amino acids 1-369, 1-419, and intermediate lengths starting with amino acid 1), as disclosed in U.S. Pat. No. 5,766,883, and publication WO 97/24445, which are incorporated by reference herein. Other chimeric polypeptides can include a HSA protein with DDAH, or fragments thereof, attached to each of the C-terminal and N-terminal ends of the HSA. Such HSA constructs are disclosed in U.S. Pat. No.
10 5,876,969, which is incorporated by reference herein. Other fusions may be created by fusion of DDAH with a) the Fc portion of an immunoglobulin; b) an analog of the Fc portion of an immunoglobulin; and c) fragments of the Fc portion of an immunoglobulin.

The term "DDAH polypeptide" includes glycosylated DDAH, such as but not limited to, polypeptides glycosylated at any amino acid, N-linked or O-linked glycosylated forms of
15 the polypeptide. Variants containing single nucleotide changes are also considered as biologically active variants of DDAH polypeptide. In addition, splice variants are also included. The term "DDAH polypeptide" also includes DDAH polypeptide heterodimers, homodimers, heteromultimers, or homomultimers of any one or more DDAH polypeptides or any other polypeptide, protein, carbohydrate, polymer, small molecule, linker, ligand, or
20 other biologically active molecule of any type, linked by chemical means or expressed as a fusion protein, as well as polypeptide analogues containing, for example, specific deletions or other modifications yet maintain biological activity.

The term "DDAH polypeptide" or "DDAH" encompasses DDAH polypeptides comprising one or more amino acid substitutions, additions or deletions. DDAH
25 polypeptides modification may be comprised of one or more natural amino acids in conjunction with one or more non-natural amino acid modification. Exemplary substitutions in a wide variety of amino acid positions in naturally-occurring DDAH polypeptides, including but not limited to substitutions that modulate pharmaceutical stability, that modulate one or more of the biological activities of the DDAH polypeptide, such as but not
30 limited to, increase or decrease enzymatic activity, increase or decrease solubility of the DDAH polypeptide, increase or decrease protease susceptibility, increase or decrease homodimerization, increase or decrease zinc binding, increase or decrease stability of the DDAH polypeptide, etc. and are encompassed by the term "DDAH polypeptide." In some embodiments, the DDAH polypeptide is linked to a pharmacokinetic enhancing moiety or

other biologically active molecule, present in a substrate, activity modulator such as zinc, protease cleavage site, or other DDAH polypeptide binding region of the DDAH molecule.

In some embodiments, the DDAH polypeptides further comprise an addition, substitution or deletion that modulates biological activity of the DDAH polypeptide. For example, the additions, substitutions or deletions may modulate one or more properties or activities of DDAH. For example, the additions, substitutions or deletions may modulate affinity for the DDAH substrate, modulate circulating half-life, modulate therapeutic half-life, modulate stability of the polypeptide, modulate cleavage by proteases, modulate dimerization of DDAH, modulate dose, modulate release or bio-availability, facilitate purification, or improve or alter a particular route of administration. Similarly, DDAH polypeptides may comprise protease cleavage sequences, reactive groups, antibody-binding domains (including but not limited to, FLAG or poly-His) or other affinity-based sequences (including but not limited to, FLAG, poly-His, GST, etc.) or linked molecules (including but not limited to, biotin) that improve detection (including but not limited to, GFP), purification or other traits of the polypeptide.

The term "DDAH polypeptide" also encompasses homodimers, heterodimers, homomultimers, and heteromultimers that are linked, including but not limited to those linked directly via the N-termini, the C-termini, a naturally encoded or non-naturally encoded amino acid side chains, either to the same or different naturally encoded or non-naturally encoded amino acid side chains, to naturally-encoded amino acid side chains, or indirectly via a linker. Exemplary linkers including but are not limited to, small organic compounds, PKEM.

The term "pharmacokinetic enhancing moiety" (also referred to herein as "PKEM") refers to a pharmaceutically acceptable moiety, domain, or "vehicle" covalently linked ("conjugated") to the DDAH polypeptide directly or via a linker, that prevents or mitigates in vivo proteolytic degradation or other activity- diminishing chemical modification of the DDAH polypeptide, increases half-life or other pharmacokinetic properties such as but not limited to increasing the rate of absorption, reduces toxicity, improves solubility, increases biological activity, catalytic efficiency and/or target selectivity of the DDAH polypeptide, increases manufacturability, and/or reduces immunogenicity of the DDAH polypeptide, compared to an unconjugated form of the DDAH polypeptide. The term "pharmacokinetic enhancing moiety" includes non-proteinaceous, PKEM, such as polyethylene glycol (PEG) or hydroxyethyl starch (HES), and proteinaceous PKEM, such as serum albumin, transferrin, adnectins (e.g., PKE adnectins), or Fc domain.

The term "albumin binding moiety" as used herein refers to any chemical group capable of binding to albumin, i.e. has albumin binding affinity. In one embodiment the albumin binding moiety is an acyl group.

5 A "non-naturally encoded amino acid" refers to an amino acid that is not one of the 20 common amino acids or pyrrolysine or selenocysteine.

The term "substantially purified" refers to a DDAH polypeptide that may be substantially or essentially free of components that normally accompany or interact with the protein as found in its naturally occurring environment, includes protein having less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating protein. When the DDAH polypeptide or variant thereof is recombinantly produced by the host cells, the protein may be present at about 30%, about 25%, about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1% or less of the dry weight of the cells. When the DDAH polypeptide or variant thereof is recombinantly produced by the host cells, the protein may be present in the culture medium at about 5g/L, about 4g/L, about 3g/L, about 2g/L, about 1g/L, about 750mg/L, about 500mg/L, about 250mg/L, about 100mg/L, about 50mg/L, about 10mg/L, or about 1mg/L or less of the dry weight of the cells. Thus, "substantially purified" DDAH polypeptide as produced by the methods described herein may have a purity level of at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, specifically, a purity level of at least about 75%, 80%, 85%, and more specifically, a purity level of at least about 90%, a purity level of at least about 95%, a purity level of at least about 99% or greater as determined by appropriate methods such as SDS/PAGE analysis, RP-HPLC, SEC, and capillary electrophoresis.

A "recombinant host cell" or "host cell" refers to a cell that includes an exogenous polynucleotide, regardless of the method used for insertion. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

30 As to amino acid sequences, one of ordinary skill in the art will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the deletion of an amino acid, addition of an amino acid, or an amino acid

with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are known to those of ordinary skill in the art. The following eight groups each contain amino acids that are conservative substitutions for one another:

1)	Alanine (A), Glycine (G);
2)	Aspartic acid (D), Glutamic acid (E);
3)	Asparagine (N), Glutamine (Q);
4)	Arginine (R), Lysine (K);
5)	Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6)	Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7)	Serine (S), Threonine (T); and
8)	Cysteine (C), Methionine (M)

5 The term “linkage” or “linker” is used herein to refer to groups or bonds that normally are formed as the result of a chemical reaction and typically are covalent linkages. “Hydrolytically stable linkages” refer to linkages are substantially stable in water and do not react with water at useful pH values, including but not limited to, under physiological conditions for an extended period of time, perhaps even indefinitely. Hydrolytically unstable or degradable linkages mean that the linkages are degradable in water or in aqueous solutions, including for example, blood. Enzymatically unstable or degradable linkages mean that the linkage can be degraded by one or more enzymes. Hydrolytically degradable linkages include, but are not limited to, carbonate linkages; imine linkages resulted from reaction of an amine and an aldehyde; phosphate ester linkages formed by reacting an alcohol with a phosphate group; hydrazone linkages which are reaction product of a hydrazide and an aldehyde; acetal linkages that are the reaction product of an aldehyde and an alcohol; orthoester linkages that are the reaction product of a formate and an alcohol; peptide linkages formed by an amine group, and a carboxyl group of a peptide; and oligonucleotide linkages formed by a phosphoramidite group, including but not limited to, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide. Linkers include but are not limited to short linear, branched, multi-armed, or dendrimeric molecules such as polymers.

Table 1 provides various starting electrophiles and nucleophiles which may be combined to create a desired functional group. The information provided is meant to be illustrative and not limiting to the synthetic techniques described herein.

Table 1: Examples of Covalent Linkages and Precursors Thereof

Covalent Linkage Product	Electrophile	Nucleophile
Carboxamides	Activated esters	amines/anilines
Carboxamides	acyl azides	amines/anilines
Carboxamides	acyl halides	amines/anilines
Esters	acyl halides	alcohols/phenols
Esters	acyl nitriles	alcohols/phenols
Carboxamides	acyl nitriles	amines/anilines
Imines	Aldehydes	amines/anilines
Hydrazones	aldehydes or ketones	Hydrazines
Oximes	aldehydes or ketones	Hydroxylamines
Alkyl amines	alkyl halides	amines/anilines
Esters	alkyl halides	carboxylic acids
Thioethers	alkyl halides	Thiols
Ethers	alkyl halides	alcohols/phenols
Thioethers	alkyl sulfonates	Thiols
Esters	alkyl sulfonates	carboxylic acids
Ethers	alkyl sulfonates	alcohols/phenols
Esters	Anhydrides	alcohols/phenols
Carboxamides	Anhydrides	amines/anilines
Thiophenols	aryl halides	Thiols
Aryl amines	aryl halides	Amines
Thioethers	Azidines	Thiols
Boronate esters	Boronates	Glycols
Carboxamides	carboxylic acids	amines/anilines
Esters	carboxylic acids	Alcohols
hydrazines	Hydrazides	carboxylic acids
<i>N</i> -acylureas or Anhydrides	Carbodiimides	carboxylic acids
Esters	Diazoalkanes	carboxylic acids
Thioethers	Epoxides	Thiols
Thioethers	Haloacetamides	Thiols
Ammotriazines	Halotriazines	amines/anilines
Triazinyl ethers	Halotriazines	alcohols/phenols
Amidines	imido esters	amines/anilines
Ureas	Isocyanates	amines/anilines
Urethanes	Isocyanates	alcohols/phenols
Thioureas	Isothiocyanates	amines/anilines
Thioethers	Maleimides	Thiols
Phosphite esters	Phosphoramidites	Alcohols
Silyl ethers	silyl halides	Alcohols
Alkyl amines	sulfonate esters	amines/anilines
Thioethers	sulfonate esters	Thiols
Esters	sulfonate esters	carboxylic acids
Ethers	sulfonate esters	Alcohols
Sulfonamides	sulfonyl halides	amines/anilines
Sulfonate esters	sulfonyl halides	phenols/alcohols

The terms "chemically coupled" and "chemically couple" and grammatical variations thereof refer to the covalent and noncovalent bonding of molecules and include specifically, but not exclusively, covalent bonding, electrostatic bonding, hydrogen bonding and van der
 5 Waals' bonding. The terms encompass both indirect and direct bonding of molecules. Thus, if a first compound is chemically coupled to a second compound, that connection may be through a direct chemical bond, or through an indirect chemical bond via other compounds, linkers or connectors.

The term "substituents" includes but is not limited to "non-interfering substituents".
 10 "Non-interfering substituents" are those groups that yield stable compounds. Suitable non-interfering substituents or radicals include, but are not limited to, halo, C1 -C10 alkyl, C2-C10 alkenyl, C2-C10 alkynyl, C1-C10 alkoxy, C1-C12 aralkyl, C1-C12 alkaryl, C3-C12 cycloalkyl, C3-C12 cycloalkenyl, phenyl, substituted phenyl, toluoyl, xylenyl, biphenyl, C2-C12 alkoxyalkyl, C2-C12 alkoxyaryl, C7-C12 aryloxyalkyl, C7-C12 oxyaryl, C1-C6
 15 alkylsulfinyl, C1-C10 alkylsulfonyl, $-(CH_2)_m-O-(C1-C10\text{ alkyl})$ wherein m is from 1 to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, $--NO_2$, $--CN$, $--NRC(O)-(C1-C10\text{ alkyl})$, $--C(O)-(C1-C10\text{ alkyl})$, C2-C10 alkyl thioalkyl, $--C(O)O-(C1-C10\text{ alkyl})$, $--OH$, $--SO_2$, $=S$, $--COOH$, $--NR_2$, carbonyl, $--C(O)-(C1-C10\text{ alkyl})-CF_3$, $--C(O)-CF_3$, $--C(O)NR_2$, $-(C1-C10\text{ aryl})-S-(C6-C10\text{ aryl})$, $--C(O)-(C1-C10\text{ aryl})$, $-(CH_2)_m-O-(CH_2)_m-O-(C1-C10\text{ alkyl})$ wherein each
 20 m is from 1 to 8, $--C(O)NR_2$, $--C(S)NR_2$, $--SO_2NR_2$, $--NRC(O)NR_2$, $--NRC(S)NR_2$, salts thereof, and the like. Each R as used herein is H, alkyl or substituted alkyl, aryl or substituted aryl, aralkyl, or alkaryl.

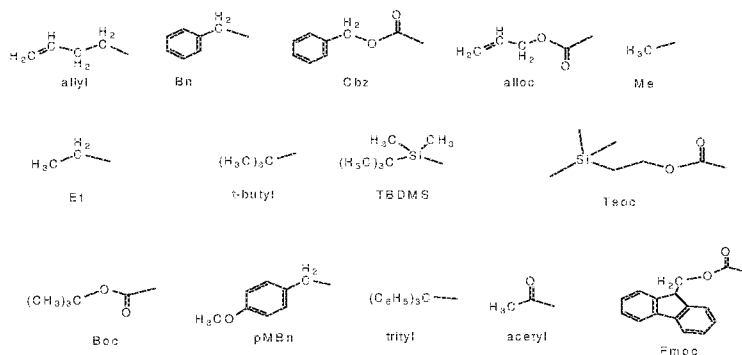
The term "modulated therapeutic half-life" as used herein means the positive or
 25 negative change in the half-life of the therapeutically effective amount of DDAH, relative to its non-modified form.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if
 30 necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

The term “modified,” as used herein refers to any changes made to a given polypeptide, such as changes to the length of the polypeptide, the amino acid sequence, chemical structure, co-translational modification, or post-translational modification of a polypeptide. The form “(modified)” term means that the polypeptides being discussed are optionally modified, that is, the polypeptides under discussion can be modified or unmodified.

The term “post-translationally modified” refers to any modification of a natural or non-natural amino acid that occurs to such an amino acid after it has been incorporated into a polypeptide chain. The term encompasses, by way of example only, co-translational in vivo modifications, co-translational in vitro modifications (such as in a cell-free translation system), post-translational in vivo modifications, and post-translational in vitro modifications.

The term “protected” refers to the presence of a “protecting group” or moiety that prevents reaction of the chemically reactive functional group under certain reaction conditions. protecting groups known in the art may also be used in or with the methods and compositions described herein, including photolabile groups such as Nvoc and MeNvoc. Other protecting groups known in the art may also be used in or with the methods and compositions described herein. By way of example only, blocking/protecting groups may be selected from:



Other protecting groups are described in Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999.

DDAH polypeptides presented herein may include isotopically-labelled compounds with one or more atoms replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into the present compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, fluorine and chlorine, such as 2H , 3H , 13C , 14C , 15N , 18O , 17O , 35S , 18F , 36Cl ,

respectively. Certain isotopically-labelled compounds described herein, for example those into which radioactive isotopes such as ^3H and ^{14}C are incorporated, may be useful in drug and/or substrate tissue distribution assays. Further, substitution with isotopes such as deuterium, i.e., ^2H , can afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements.

DDAH polypeptides including DDAH polypeptides comprising at least one amino acid substitution, addition, deletion or insertion are provided. In certain embodiments, the DDAH polypeptide includes at least one post-translational modification. In one embodiment, the at least one post-translational modification comprises attachment of a molecule including but not limited to, a pharmacokinetic enhancing moiety, a label, a dye, a polymer, a water-soluble polymer, a derivative of polyethylene glycol, a photocrosslinker, a radionuclide, a cytotoxic compound, a drug, an affinity label, a photoaffinity label, a reactive compound, a resin, a second protein or polypeptide or polypeptide analog, an antibody or antibody fragment, a metal chelator, a cofactor, a fatty acid, a carbohydrate, a polynucleotide, a DNA, a RNA, an antisense polynucleotide, a saccharide, a water-soluble dendrimer, a cyclodextrin, an inhibitory ribonucleic acid, a biomaterial, a nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a novel functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a photoisomerizable moiety, biotin, a derivative of biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, an elongated side chain, a carbon-linked sugar, a redox-active agent, an amino thioacid, a toxic moiety, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, a detectable label, a small molecule, a quantum dot, a nanotransmitter, a radionucleotide, a radiotransmitter, a neutron-capture agent, or any combination of the above or any other desirable compound or substance, comprising a second reactive group to at least one amino acid comprising a first reactive group utilizing chemistry methodology that is known to one of ordinary skill in the art to be suitable for the particular reactive groups. In certain embodiments, the post-translational modification is made in vivo in a eukaryotic cell or in a non-eukaryotic cell. A linker, polymer, pharmacokinetic enhancing moiety, or other molecule may attach the molecule to the polypeptide. The molecule may be linked directly to the polypeptide.

In certain embodiments, the protein includes at least one post-translational modification that is made in vivo by one host cell, where the post-translational modification is not normally made by another host cell type. In certain embodiments, the protein includes at least one post-translational modification that is made in vivo by a eukaryotic cell, where
 5 the post-translational modification is not normally made by a non-eukaryotic cell. Examples of post-translational modifications include, but are not limited to, glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, glycolipid-linkage modification, and the like.

In some embodiments, the DDAH polypeptide comprises one or more post-
 10 translational modification including but not limited to glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, or glycolipid-linkage modification of the polypeptide. In one embodiment, the post-translational modification comprises attachment of an oligosaccharide to an asparagine by a GlcNAc-asparagine linkage (including but not limited to, where the oligosaccharide comprises (GlcNAc-Man)₂-Man-
 15 GlcNAc-GlcNAc, and the like). In another embodiment, the post-translational modification comprises attachment of an oligosaccharide (including but not limited to, Gal-GalNAc, Gal-GlcNAc, etc.) to a serine or threonine by a GalNAc-serine, a GalNAc-threonine, a GlcNAc-serine, or a GlcNAc-threonine linkage. In certain embodiments, a protein or polypeptide described herein can comprise a secretion or localization sequence, an epitope tag, a FLAG
 20 tag, a polyhistidine tag, a GST fusion, and/or the like. Examples of secretion signal sequences include, but are not limited to, a prokaryotic secretion signal sequence, a eukaryotic secretion signal sequence, a eukaryotic secretion signal sequence 5'-optimized for bacterial expression, a novel secretion signal sequence, pectate lyase secretion signal sequence, Omp A secretion signal sequence, and a phage secretion signal sequence.
 25 Examples of secretion signal sequences, include, but are not limited to, STII (prokaryotic), Fd GIII and M13 (phage), Bgl2 (yeast), and the signal sequence bla derived from a transposon. Any such sequence may be modified to provide a desired result with the polypeptide, including but not limited to, substituting one signal sequence with a different signal sequence, substituting a leader sequence with a different leader sequence, etc.

30 Provided herein are conjugates of substances having a wide variety of functional groups, substituents or moieties, with other substances including but not limited to a pharmacokinetic enhancing moiety; a label; a dye; a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a photocrosslinker; a radionuclide; a cytotoxic compound; a drug; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein

or polypeptide or polypeptide analog; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide; a water-soluble dendrimer; a cyclodextrin; an inhibitory ribonucleic acid; a biomaterial; a nanoparticle; a spin label; a fluorophore, a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a photoisomerizable moiety; biotin; a derivative of biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; a toxic moiety; an isotopically labeled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent; a detectable label; a small molecule; a quantum dot; a nanotransmitter; a radionucleotide; a radiotransmitter; a neutron-capture agent; or any combination of the above, or any other desirable compound or substance. Also provided are conjugates of substances having azide or acetylene moieties with pharmacokinetic enhancing moiety derivatives having the corresponding acetylene or azide moieties. For example, a pharmacokinetic enhancing moiety containing an azide moiety can be coupled to a biologically active molecule at a position in the protein that contains a non-genetically encoded amino acid bearing an acetylene functionality.

In some embodiments, provided herein are DDAH polypeptides coupled to another molecule having the formula DDAH-L-M, wherein L is a linking group or a chemical bond, and M is any other molecule. In some embodiments, L is stable in vivo. In some embodiments, L is hydrolyzable in vivo. In some embodiments, L is metastable in vivo.

DDAH and M can be linked together through L using standard linking agents and procedures known to those skilled in the art. In some aspects, DDAH and M are fused directly and L is a bond. In other aspects, DDAH and M are fused through a linking group L. For example, in some embodiments, DDAH and M are linked together via a peptide bond, optionally through a peptide or amino acid spacer. In some embodiments, DDAH and M are linked together through chemical conjugation, optionally through a linking group (L). In some embodiments, L is directly conjugated to each of DDAH and M.

Chemical conjugation can occur by reacting a nucleophilic reactive group of one compound to an electrophilic reactive group of another compound. In some embodiments when L is a bond, DDAH is conjugated to M either by reacting a nucleophilic reactive moiety

on DDAH with an electrophilic reactive moiety on Y, or by reacting an electrophilic reactive moiety on DDAH with a nucleophilic reactive moiety on M. In embodiments when L is a group that links DDAH and M together, DDAH and/or M can be conjugated to L either by reacting a nucleophilic reactive moiety on DDAH and/or M with an electrophilic reactive moiety on L, or by reacting an electrophilic reactive moiety on DDAH and/or M with a nucleophilic reactive moiety on L. Nonlimiting examples of nucleophilic reactive groups include amino, thiol, and hydroxyl. Nonlimiting examples of electrophilic reactive groups include carboxyl, acyl chloride, anhydride, ester, succinimide ester, alkyl halide, sulfonate ester, maleimido, haloacetyl, and isocyanate. In embodiments where DDAH and M are conjugated together by reacting a carboxylic acid with an amine, an activating agent can be used to form an activated ester of the carboxylic acid.

The activated ester of the carboxylic acid can be, for example, N-hydroxysuccinimide (NHS), tosylate (Tos), mesylate, triflate, a carbodiimide, or a hexafluorophosphate. In some embodiments, the carbodiimide is 1,3-dicyclohexylcarbodiimide (DCC), 1,1'-carbonyldiimidazole (CDI), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), or 1,3-diisopropylcarbodiimide (DICD). In some embodiments, the hexafluorophosphate is selected from a group consisting of hexafluorophosphate benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU), and o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU).

In some embodiments, DDAH comprises a nucleophilic reactive group (e.g. the amino group, thiol group, or hydroxyl group of the side chain of lysine, cysteine or serine) that is capable of conjugating to an electrophilic reactive group on M or L. In some embodiments, DDAH comprises an electrophilic reactive group (e.g. the carboxylate group of the side chain of Asp or Glu) that is capable of conjugating to a nucleophilic reactive group on M or L. In some embodiments, DDAH is chemically modified to comprise a reactive group that is capable of conjugating directly to M or to L. In some embodiments, DDAH is modified at the C-terminal to comprise a natural or nonnatural amino acid with a nucleophilic side chain. In exemplary embodiments, the C-terminal amino acid of DDAH is selected from the group consisting of lysine, ornithine, serine, cysteine, and homocysteine. For example, the C-terminal amino acid of DDAH can be modified to comprise a lysine residue. In some embodiments, DDAH is modified at the C-terminal amino acid to comprise a natural or nonnatural amino acid with an electrophilic side chain such as, for example, Asp and Glu. In

some embodiments, an internal amino acid of DDAH is substituted with a natural or nonnatural amino acid having a nucleophilic side chain, as previously described herein. In exemplary embodiments, the internal amino acid of DDAH that is substituted is selected from the group consisting of lysine, ornithine, serine, cysteine, and homocysteine. For example, an internal amino acid of DDAH can be substituted with a lysine residue. In some embodiments, an internal amino acid of DDAH is substituted with a natural or nonnatural amino acid with an electrophilic side chain, such as, for example, Asp and Glu.

In some embodiments, M comprises a reactive group that is capable of conjugating directly to DDAH or to L. In some embodiments, M comprises a nucleophilic reactive group (e.g. amine, thiol, hydroxyl) that is capable of conjugating to an electrophilic reactive group on DDAH or L. In some embodiments, M comprises electrophilic reactive group (e.g. carboxyl group, activated form of a carboxyl group, compound with a leaving group) that is capable of conjugating to a nucleophilic reactive group on DDAH or L. In some embodiments, M is chemically modified to comprise either a nucleophilic reactive group that is capable of conjugating to an electrophilic reactive group on DDAH or L. In some embodiments, M is chemically modified to comprise an electrophilic reactive group that is capable of conjugating to a nucleophilic reactive group on DDAH or L.

In some embodiments, conjugation can be carried out through organosilanes, e.g., aminosilane treated with glutaraldehyde; carbonyldiimidazole (CDI) activation of silanol groups; or utilization of dendrimers. A variety of dendrimers are known in the art and include poly (amidoamine) (PAMAM) dendrimers, which are synthesized by the divergent method starting from ammonia or ethylenediamine initiator core reagents; a sub-class of PAMAM dendrimers based on a tris-aminoethylene-imine core; radially layered poly(amidoamine-organosilicon) dendrimers (PAMAMOS), which are inverted unimolecular micelles that consist of hydrophilic, nucleophilic polyamidoamine (PAMAM) interiors and hydrophobic organosilicon (OS) exteriors; Poly (Propylene Imine) (PPI) dendrimers, which are generally poly-alkyl amines having primary amines as end groups, while the dendrimer interior consists of numerous of tertiary tris-propylene amines; Poly (Propylene Amine) (POPAM) dendrimers; Diaminobutane (DAB) dendrimers; amphiphilic dendrimers; micellar dendrimers which are unimolecular micelles of water soluble hyper branched polyphenylenes; polylysine dendrimers; and dendrimers based on poly-benzyl ether hyper branched skeleton.

Indirect conjugation via high affinity specific binding partners, e.g. streptavidin/biotin or avidin/biotin or lectin/carbohydrate is also contemplated.

In some embodiments, DDAH and/or M are functionalized to comprise a nucleophilic reactive group or an electrophilic reactive group with an organic derivatizing agent. This derivatizing agent is capable of reacting with selected side chains or the N- or C-terminal residues of targeted amino acids on DDAH and functional groups on M. Reactive groups on DDAH and/or M include, e.g., aldehyde, amino, ester, thiol, α -haloacetyl, maleimido or hydrazino group. Derivatizing agents include, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art. Alternatively, DDAH and/or M can be linked to each other indirectly through intermediate carriers, such as polysaccharide or polypeptide carriers. Examples of polysaccharide carriers include aminodextran. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier.

Cysteinyll residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyll residues can be derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyll and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyll residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyll residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline

conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) can be selectively modified by reaction with carbodiimides ($R-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

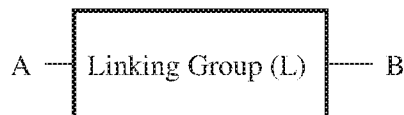
Another type of covalent modification involves chemically or enzymatically coupling glycosides to the peptide at arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of tyrosine, or tryptophan, or (f) the amide group of glutamine, as described in WO87/05330 published 11 Sep. 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

In some embodiments, L is a bond. In these embodiments, DDAH and M are conjugated together by reacting a nucleophilic reactive moiety on DDAH with and electrophilic reactive moiety on M. In alternative embodiments, DDAH and M are conjugated together by reacting an electrophilic reactive moiety on DDAH with a nucleophilic moiety on M. In exemplary embodiments, L is an amide bond that forms upon reaction of an amine on DDAH (e.g. an ϵ -amine of a lysine residue) with a carboxyl group on M. In alternative embodiments, DDAH and/or M are derivatized with a derivatizing agent before conjugation.

In some embodiments, L is linking group, a bifunctional linker and comprises only two reactive groups before conjugation to DDAH and M. In embodiments where both DDAH and M have electrophilic reactive groups, L comprises two of the same or two different nucleophilic groups (e.g. amine, hydroxyl, thiol) before conjugation to DDAH and M. In embodiments where both DDAH and M have nucleophilic reactive groups, L comprises two of the same or two different electrophilic groups (e.g. carboxyl group, activated form of a carboxyl group, compound with a leaving group) before conjugation to DDAH and M. In embodiments where one of DDAH or M has a nucleophilic reactive group

and the other of DDAH or M has an electrophilic reactive group, L comprises one nucleophilic reactive group and one electrophilic group before conjugation to DDAH and M.

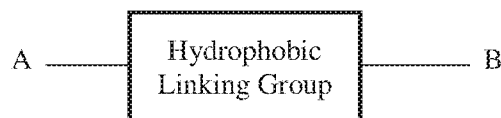
L can be any molecule with at least two reactive groups (before conjugation to DDAH and M) capable of reacting with each of DDAH and M. In some embodiments L has only two reactive groups and is bifunctional. L (before conjugation to the peptides) can be represented by the formula below:



wherein A and B are independently nucleophilic or electrophilic reactive groups. In some embodiments A and B are either both nucleophilic groups or both electrophilic groups. In some embodiments one of A or B is a nucleophilic group and the other of A or B is an electrophilic group. Nonlimiting combinations of A and B are described below.

In some embodiments, A and B may include alkene and/or alkyne functional groups that are suitable for olefin metathesis reactions. In some embodiments, A and B include moieties that are suitable for click chemistry (e.g. alkene, alkynes, nitriles, azides). Other nonlimiting examples of reactive groups (A and B) include pyridyldithiol, aryl azide, diazirine, carbodiimide, and hydrazide.

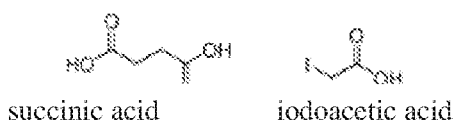
In some embodiments, L can be hydrophobic. Hydrophobic linkers are known in the art. See, e.g., *Bioconjugate Techniques*, G. T. Hermanson (Academic Press, San Diego, CA, 1996), which is incorporated by reference in its entirety. Suitable hydrophobic linking groups known in the art include, for example, 8-hydroxy octanoic acid and 8-mercaptooctanoic acid. Before conjugation to the peptides of the composition, the hydrophobic linking group comprises at least two reactive groups (A and B), as described herein and as shown below:



In some embodiments, the hydrophobic linking group comprises either a maleimido or an iodoacetyl group and either a carboxylic acid or an activated carboxylic acid (e.g. NHS ester) as the reactive groups. In these embodiments, the maleimido or iodoacetyl group can be coupled to a thiol moiety on DDAH or M and the carboxylic acid or activated carboxylic acid can be coupled to an amine on DDAH or M with or without the use of a coupling reagent. Any coupling agent known to one skilled in the art can be used to couple the

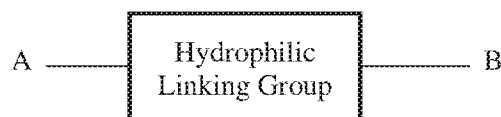
carboxylic acid with the free amine such as, for example, DCC, DIC, HATU, HBTU, TBTU, and other activating agents described herein. In specific embodiments, the hydrophilic linking group comprises an aliphatic chain of 2 to 100 methylene groups wherein A and B are carboxyl groups or derivatives thereof (e.g. succinic acid). In other specific embodiments the

5 L is iodoacetic acid.



In some embodiments, the linking group is hydrophilic such as, for example, polyalkylene glycol. Before conjugation to the peptides of the composition, the hydrophilic

10 linking group comprises at least two reactive groups (A and B), as described herein and as shown below:



In specific embodiments, the linking group is polyethylene glycol (PEG). The PEG in

15 certain embodiments has a molecular weight (MW) of about 100 Daltons to about 10,000 Daltons, e.g. about 500 Daltons to about 5000 Daltons. The PEG in some embodiments has a MW of about 10,000 Daltons to about 40,000 Daltons.

In some embodiments, the hydrophilic linking group comprises either a maleimido or an iodoacetyl group and either a carboxylic acid or an activated carboxylic acid (e.g. NHS ester) as the reactive groups. In these embodiments, the maleimido or iodoacetyl group can

20 be coupled to a thiol moiety on DDAH or M and the carboxylic acid or activated carboxylic acid can be coupled to an amine on DDAH or M with or without the use of a coupling reagent. Any appropriate coupling agent known to one skilled in the art can be used to couple the carboxylic acid with the amine such as, for example, DCC, DIC, HATU, HBTU,

25 TBTU, and other activating agents described herein. In some embodiments, the linking group is maleimido-PKEM(20 kDa)-COOH, iodoacetyl-PKEM(20 kDa)-COOH, maleimido-PKEM(20 kDa)-NHS, or iodoacetyl-PKEM(20 kDa)-NHS.

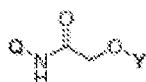
In some embodiments, the linking group is comprised of an amino acid, a dipeptide, a tripeptide, or a polypeptide, wherein the amino acid, dipeptide, tripeptide, or polypeptide

30 comprises at least two activating groups, as described herein. In some embodiments, the

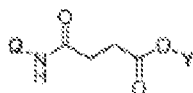
linking group (L) comprises a moiety selected from the group consisting of: amino, ether, thioether, maleimido, disulfide, amide, ester, thioester, alkene, cycloalkene, alkyne, trizoyl, carbamate, carbonate, cathepsin B-cleavable, and hydrazone.

In some embodiments, L comprises a chain of atoms from 1 to about 60, or 1 to 30 atoms or longer, 2 to 5 atoms, 2 to 10 atoms, 5 to 10 atoms, or 10 to 20 atoms long. In some embodiments, the chain atoms are all carbon atoms. In some embodiments, the chain atoms in the backbone of the linker are selected from the group consisting of C, O, N, and S. Chain atoms and linkers may be selected according to their expected solubility (hydrophilicity) so as to provide a more soluble conjugate. In some embodiments, L provides a functional group that is subject to cleavage by an enzyme or other catalyst or hydrolytic conditions found in the target tissue or organ or cell. In some embodiments, the length of L is long enough to reduce the potential for steric hindrance.

In some embodiments, L is stable in biological fluids such as blood or blood fractions. In some embodiments, L is stable in blood serum for at least 5 minutes, e.g. less than 25%, 20%, 15%, 10% or 5% of the conjugate is cleaved when incubated in serum for a period of 5 minutes. In other embodiments, L is stable in blood serum for at least 10, or 20, or 25, or 30, or 60, or 90, or 120 minutes, or 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 or 24 hours. In these embodiments, L does not comprise a functional group that is capable of undergoing hydrolysis *in vivo*. In some exemplary embodiments, L is stable in blood serum for at least about 72 hours. Nonlimiting examples of functional groups that are not capable of undergoing significant hydrolysis *in vivo* include amides, ethers, and thioethers. For example, the following compound does not undergoing significant hydrolysis *in vivo*:



In some embodiments, L is hydrolyzable *in vivo*. In these embodiments, L comprises a functional group that is capable of undergoing hydrolysis *in vivo*. Nonlimiting examples of functional groups that are capable of undergoing hydrolysis *in vivo* include esters, anhydrides, and thioesters. For example the following compound is capable of undergoing hydrolysis *in vivo* because it comprises an ester group:



In some embodiments, L is labile and undergoes substantial hydrolysis within 3 hours in blood plasma at 37 °C, with complete hydrolysis within 6 hours. In some exemplary embodiments, L is not labile.

In some embodiments, L is metastable in vivo. In these embodiments, L comprises a functional group that is capable of being chemically or enzymatically cleaved in vivo (e.g., an acid-labile, reduction-labile, or enzyme-labile functional group), optionally over a period of time. In these embodiments, L can comprise, for example, a hydrazone moiety, a disulfide moiety, or a cathepsin-cleavable moiety. When L is metastable, and without intending to be bound by any particular theory, the DDAH-L-M conjugate is stable in an extracellular environment, e.g., stable in blood serum for the time periods described above, but labile in the intracellular environment or conditions that mimic the intracellular environment, so that it cleaves upon entry into a cell. In some embodiments when L is metastable, L is stable in blood serum for at least about 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 42, or 48 hours, for example, at least about 48, 54, 60, 66, or 72 hours, or about 24-48, 48-72, 24-60, 36-48, 36-72, or 48-72 hours.

General Recombinant Nucleic Acid Methods

In some embodiments, nucleic acids encoding a DDAH polypeptide of interest will be isolated, cloned and often altered using recombinant methods. Such embodiments are used, including but not limited to, for protein expression or during the generation of variants, derivatives, expression cassettes, or other sequences derived from a DDAH polypeptide. In some embodiments, the sequences encoding the polypeptides described herein are operably linked to a heterologous promoter.

A nucleotide sequence encoding a DDAH polypeptide include but not limited to, having the amino acid sequence shown in SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO:5; SEQ ID NO 6;SEQ ID NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ ID NO 13;or SEQ ID NO 14 and then changing the nucleotide sequence so as to effect introduction (i.e., incorporation or substitution) or removal (i.e., deletion or substitution) of the relevant amino acid residue(s). The nucleotide sequence may be conveniently modified by site-directed mutagenesis in accordance with conventional methods.

Various types of mutagenesis are used for a variety of purposes, including but not limited to, to produce novel DDAH polypeptides of interest. They include but are not limited to site-directed, random point mutagenesis, homologous recombination, DNA shuffling or

other recursive mutagenesis methods, chimeric construction, mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like, PCT-mediated mutagenesis, or any combination thereof. Additional suitable methods include point mismatch repair, 5 mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, including but not limited to, involving chimeric constructs, are also possible. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, 10 including but not limited to, sequence, sequence comparisons, physical properties, secondary, tertiary, or quaternary structure, crystal structure or the like.

Also provided are eukaryotic, non-eukaryotic host cells, and organisms for the in vivo incorporation of an unnatural amino acid via orthogonal tRNA/RS pairs. Host cells are genetically engineered (including but not limited to, transformed, transduced or transfected) 15 with the polynucleotides described herein or constructs which include a polynucleotide described herein, including but not limited to, a vector which can be, for example, a cloning vector or an expression vector.

Also described are post-translation modifications includes proteolytic processing of precursors (including but not limited to, proDDAH or a variant or analog thereof), assembly 20 into a multisubunit protein or macromolecular assembly, translation to another site in the cell (including but not limited to, to organelles, such as the endoplasmic reticulum, the Golgi apparatus, the nucleus, lysosomes, peroxisomes, mitochondria, chloroplasts, vacuoles, etc., or through the secretory pathway). In certain embodiments, the protein comprises a secretion or localization sequence, an epitope tag, a FLAG tag, a polyhistidine tag, a GST fusion, or the 25 like.

Location of modifications in DDAH polypeptides

The substitution, addition, deletion, or incorporation of one or more naturally encoded or non-naturally-occurring amino acids into DDAH polypeptides are also described. One or 30 more of these modifications may be incorporated at a particular position which does not disrupt activity of the polypeptide. This can be achieved by making "conservative" substitutions, including but not limited to, substituting hydrophobic amino acids with hydrophobic amino acids, bulky amino acids for bulky amino acids, hydrophilic amino acids for hydrophilic amino acids and/or inserting the non-naturally-occurring amino acid in a

location that is not required for activity. It is also known that Cys273, His172 and Asp126 of the mammalian DDAH polypeptide are important for enzymatic activity. Therefore, the remaining cysteine residues other than Cys273 may individually, or in combination, or entirely, be substituted with another amino acid, such as but not limited to serine or alanine, in order to remove them from the DDAH polypeptide and test the biological properties of the resulting DDAH polypeptide.

Residues other than those identified as critical to biological activity by alanine or homolog scanning mutagenesis may be good candidates for substitution, deletion, or insertion depending on the desired activity sought for the polypeptide. Alternatively, the sites identified as critical to biological activity may also be good candidates for substitution, insertion or deletion, again depending on the desired activity sought for the polypeptide. Another alternative would be to simply make serial substitutions in each position on the polypeptide chain with a non-naturally encoded amino acid and observe the effect on the activities of the polypeptide. It is readily apparent to those of ordinary skill in the art that any means, technique, or method for selecting a position for substitution with a non-natural amino acid into any polypeptide described herein.

In some embodiments, the DDAH polypeptides described herein can comprise one or more addition or deletion of amino acids, or of substitution of naturally encoded or non-naturally encoded amino acids positioned in a region of the protein that does not disrupt the structure of the polypeptide.

One of ordinary skill in the art recognizes that such analysis of DDAH enables the determination of which amino acid residues are surface exposed compared to amino acid residues that are buried within the tertiary structure of the protein. Therefore, it is an embodiment to substitute, insert or delete one or more amino acid for an amino acid that is a surface exposed residue.

An examination of the crystal structure of DDAH and its interaction with the DDAH substrate, modulator, or another DDAH molecule can indicate which certain amino acid residues have side chains that are fully or partially accessible to solvent. The side chain of an amino acid at these positions may point away from the protein surface and out into the solvent.

In one embodiment, the method further includes incorporating into the protein the unnatural amino acid, where the unnatural amino acid comprises a first reactive group; and contacting the protein with a molecule (including but not limited to, a pharmacokinetic enhancing moiety, a water-soluble polymer, a derivative of polyethylene glycol, a

photocrosslinker, a radionuclide, a cytotoxic compound, a drug, an affinity label, a photoaffinity label, a reactive compound, a resin, a second protein or polypeptide or polypeptide analog, an antibody or antibody fragment, a metal chelator, a cofactor, a fatty acid, biotin, a derivative of biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, an elongated side chain, a carbon-linked sugar, a redox-active agent, an amino thioacid, a toxic moiety, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electron dense group. The first reactive group reacts with the second reactive group to attach the molecule to the unnatural amino acid through a [3+2] cycloaddition. In one embodiment, the first reactive group is an alkynyl or azido moiety and the second reactive group is an azido or alkynyl moiety. For example, the first reactive group is the alkynyl moiety (including but not limited to, in unnatural amino acid p-propargyloxyphenylalanine) and the second reactive group is the azido moiety. In another example, the first reactive group is the azido moiety (including but not limited to, in the unnatural amino acid p-azido-L-phenylalanine) and the second reactive group is the alkynyl moiety.

In some cases, the naturally encoded or non-naturally encoded amino acid substitution(s) will be combined with other additions, substitutions or deletions within the DDAH polypeptide to affect other biological traits of the DDAH polypeptide. In some cases, the other additions, substitutions or deletions may increase the stability (including but not limited to, resistance to proteolytic degradation) of the DDAH polypeptide or increase affinity of the DDAH polypeptide for its substrate, activity modulator, or other DDAH polypeptide. In some cases, the other additions, substitutions or deletions may increase the pharmaceutical stability of the DDAH polypeptide. In some cases, the other additions, substitutions or deletions may enhance the activity/efficacy of the DDAH polypeptide. In some cases, the other additions, substitutions or deletions may increase the solubility (including but not limited to, when expressed in *E. coli* or other host cells) of the DDAH polypeptide. In some embodiments additions, substitutions or deletions may increase the DDAH polypeptide solubility following expression in *E. coli* or other recombinant host cells. In some embodiments sites are selected for substitution with a naturally encoded or non-natural amino acid in addition to another site for incorporation of a non-natural amino acid that results in increasing the polypeptide solubility following expression in *E. coli* or other recombinant host cells. In some embodiments, the DDAH polypeptides comprise another addition, substitution or deletion that modulates affinity for the DDAH polypeptide substrate, modulator such as zinc, binding proteins, or associated ligand, modulates DDAH activity,

modulates circulating half-life, modulates release or bio-availability, facilitates purification, or improves or alters a particular route of administration. In some embodiments, the DDAH polypeptides comprise an addition, substitution or deletion that increases the affinity of the DDAH variant for its substrate, modulator, or other DDAH polypeptides. Similarly, DDAH polypeptides can comprise chemical or enzyme cleavage sequences, protease cleavage sequences, reactive groups, antibody-binding domains (including but not limited to, FLAG or poly-His) or other affinity based sequences (including, but not limited to, FLAG, poly-His, GST, etc.) or linked molecules (including, but not limited to, biotin) that improve detection (including, but not limited to, GFP), purification, transport through tissues or cell membranes, prodrug release or activation, DDAH size reduction, or other traits of the polypeptide.

In some embodiments, the substitution of a naturally encoded or non-naturally encoded amino acid generates a DDAH polypeptide that has decreased enzymatic activity but has greater stability when compared to unmodified DDAH. Increasing stability may result in a DDAH polypeptide that has, for example, an increased circulation time after administration to a patient even though the DDAH polypeptide has a decreased enzymatic activity, which may in certain cases be more desirable than the wild type DDAH. In some embodiments, a naturally encoded or non-naturally encoded amino acid is substituted or added in a region involved with substrate, modulator, or DDAH binding. In some embodiments, the modified DDAH polypeptide comprises at least one substitution that causes the DDAH to act as an antagonist of DDAH which may be useful to modulate the activity of a DDAH polypeptide that has been administered to a patient.

In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more amino acids are substituted with one or more naturally encoded or non-naturally-encoded amino acids. In some cases, the DDAH polypeptide further includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions of one or more non-naturally encoded amino acids for naturally-occurring amino acids. For example, in some embodiments, one or more residues in DDAH are substituted with one or more non-naturally encoded amino acids. In some cases, the one or more non-naturally encoded residues are linked to one or more lower molecular weight PKEM, thereby enhancing binding affinity and comparable serum half-life relative to the species attached to a single, higher molecular weight pharmacokinetic enhancing moiety.

Expression in Non-eukaryotes and Eukaryotes

To obtain high level expression of a cloned DDAH polynucleotide, one typically subclones polynucleotides encoding a DDAH polypeptide described herein into an expression

vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are known to those of ordinary skill in the art and are also commercially available. In cases where orthogonal tRNAs and aminoacyl tRNA synthetases (described above) are used to express the DDAH polypeptides described herein, host cells for expression are selected based on their ability to use the orthogonal components. Exemplary host cells include Gram-positive bacteria (including but not limited to *B. brevis*, *B. subtilis*, or *Streptomyces*) and Gram-negative bacteria (*E. coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas putida*), as well as yeast and other eukaryotic cells. Cells comprising O-tRNA/O-RS pairs can be used as described herein.

A eukaryotic host cell or non-eukaryotic host cell can provide the ability to biosynthesize proteins that comprise natural or unnatural amino acids in large useful quantities. For example, proteins comprising an unnatural amino acid can be produced at a concentration of, including but not limited to, at least 10 µg/liter, at least 50 µg/liter, at least 75 µg/liter, at least 100 µg/liter, at least 200 µg/liter, at least 250 µg/liter, or at least 500 µg/liter, at least 1mg/liter, at least 2mg/liter, at least 3 mg/liter, at least 4 mg/liter, at least 5 mg/liter, at least 6 mg/liter, at least 7 mg/liter, at least 8 mg/liter, at least 9 mg/liter, at least 10 mg/liter, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 mg/liter, 1 g/liter, 5 g/liter, 10 g/liter or more of protein in a cell extract, cell lysate, culture medium, a buffer, and/or the like.

Useful expression vectors for eukaryotic hosts, include but are not limited to, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Such vectors include pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jolla, Calif., USA). Bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a, wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages may be used. The 2µ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373 which is incorporated by reference), the pJSO37 vector described in (Okkels, Ann. New York Acad. Sci. 782, 202 207, 1996) and pPICZ A, B or C (Invitrogen) may be used with yeast host cells. For insect cells, the vectors include but are not limited to, pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance and

Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (Invitrogen, Carlsbad, CA).

The nucleotide sequence encoding a DDAH polypeptide may or may not also include sequence that encodes a signal peptid, coding sequence, such as:

5 MPRLFFFHLLGVCLLLNQFSRAVA .

Examples of suitable mammalian host cells may be Chinese hamster ovary (CHO) cells, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cells (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC
10 CRL-1573)), as well as plant cells in tissue culture.

Expression Systems, Culture, and Isolation

DDAH polypeptides may be expressed in any number of suitable expression systems including, for example, yeast, insect cells, mammalian cells, and bacteria.

15 Yeasts: Various yeasts include, but are not limited to, ascosporogenous yeasts (Endomycetales), basidiosporogenous yeasts and yeasts belonging to the Fungi imperfecti (Blastomycetes) group. The ascosporogenous yeasts are divided into two families, Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (e.g., genus Schizosaccharomyces), Nadsonioideae,
20 Lipomycoideae and Saccharomycoideae (e.g., genera Pichia, Kluyveromyces and Saccharomyces). The basidiosporogenous yeasts include the genera Leucosporidium, Rhodosporidium, Sporidiobolus, Filobasidium, and Filobasidiella. Yeasts belonging to the Fungi Imperfecti (Blastomycetes) group are divided into two families, Sporobolomycetaceae (e.g., genera Sporobolomyces and Bullera) and Cryptococcaceae (e.g., genus Candida). In
25 some cases, the yeast can be a species within the genera Pichia, Kluyveromyces, Saccharomyces, Schizosaccharomyces, Hansenula, Torulopsis, and Candida, including, but not limited to, *P. pastoris*, *P. guilliermondii*, *S. cerevisiae*, *S. carlsbergensis*, *S. diastaticus*, *S. douglasii*, *S. kluyveri*, *S. norbensis*, *S. oviformis*, *K. lactis*, *K. fragilis*, *C. albicans*, *C. maltosa*, or *H. polymorpha*.

30 Yeast enhancers also may be used with yeast promoters. In addition, synthetic promoters may also function as yeast promoters. For example, the upstream activating sequences (UAS) of a yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation

region. See U.S. Patent Nos. 4,880,734 and 4,876,197, which are incorporated by reference herein. Other examples of hybrid promoters include promoters that consist of the regulatory sequences of the ADH2, GAL4, GAL10, or PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK. See EP 0 164 556.

- 5 Furthermore, a yeast promoter may include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription.

Other control elements that may comprise part of the yeast expression vectors include terminators, for example, from GAPDH or the enolase genes (Holland et al., J. BIOL. CHEM. (1981) 256:1385). In addition, the origin of replication from the 2 μ plasmid origin is
10 suitable for yeast. Methods of introducing exogenous DNA into yeast include, but are not limited to, either the transformation of spheroplasts or of intact yeast host cells treated with alkali cations.

Baculovirus-Infected Insect Cells: Baculovirus expression of DDAH polypeptides is useful and the use of rDNA technology, polypeptides or precursors thereof because DDAH
15 may be biosynthesized in any number of host cells including bacteria, mammalian cells, insect cells, yeast or fungi. An embodiment includes biosynthesis of DDAH, modified DDAH, DDAH polypeptides, or DDAH analogs in bacteria, yeast or mammalian cells. Another embodiment involves biosynthesis done in *E. coli* or a yeast.

E. coli, *Pseudomonas* species, and other Prokaryotes: In selecting bacterial hosts for
20 expression, suitable hosts may include those shown to have, inter alia, good inclusion body formation capacity, low proteolytic activity, and overall robustness. Industrial/pharmaceutical fermentation generally use bacterial derived from K strains (e.g. W3110) or from bacteria derived from B strains (e.g. BL21). Other examples of suitable *E. coli* hosts include, but are not limited to, strains of BL21, DH10B, or derivatives thereof. In another embodiment of
25 the methods described herein, the *E. coli* host is a protease minus strain including, but not limited to, OMP- and LON-. The host cell strain may be a species of *Pseudomonas*, including but not limited to, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. *Pseudomonas fluorescens* biovar 1, designated strain MB101, is known to be useful for recombinant production and is available for therapeutic protein production
30 processes. Examples of a *Pseudomonas* expression system include the system available from The Dow Chemical Company (Midland, MI) as a host strain.

Once a recombinant the expression construct has been introduced into the host cell and host cells with the proper expression construct are isolated, the recombinant host cell strain is cultured under conditions appropriate for production of DDAH polypeptides.

The DDAH polypeptides described herein are normally purified after expression in recombinant systems. In embodiments, amino acid substitutions may readily be made in the DDAH polypeptide that are selected for the purpose of increasing the solubility of the recombinantly produced protein utilizing the methods disclosed herein as well as those known in the art. In the case of insoluble protein, the protein may be collected from host cell lysates by centrifugation and may further be followed by homogenization of the cells. In the case of poorly soluble protein, compounds including, but not limited to, polyethylene imine (PEI) may be added to induce the precipitation of partially soluble protein.

Insoluble or precipitated DDAH polypeptide may then be solubilized using any suitable solubilization agents known to the art such as urea or guanidine hydrochloride.

When DDAH polypeptide is produced as a fusion protein, the removal of a fusion sequence may be accomplished by enzymatic or chemical cleavage. Enzymatic removal of fusion sequences may be accomplished using methods known to those of ordinary skill in the art. conditions will be specified by the choice of enzyme e.g. TEV or ULP-1 as will be apparent to one of ordinary skill in the art. Methods for purification may include, but are not limited to, size-exclusion chromatography, hydrophobic interaction chromatography, ion-exchange chromatography or dialysis or any combination thereof.

Any of the following exemplary procedures can be employed for purification of DDAH polypeptides: affinity chromatography; anion- or cation-exchange chromatography (using, including but not limited to, DEAE SEPHAROSE); chromatography on silica; high performance liquid chromatography (HPLC); reverse phase HPLC; gel filtration (using, including but not limited to, SEPHADEX G-75); hydrophobic interaction chromatography; size-exclusion chromatography; metal-chelate chromatography; ultrafiltration/diafiltration; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; displacement chromatography; electrophoretic procedures (including but not limited to preparative isoelectric focusing), differential solubility (including but not limited to ammonium sulfate precipitation), SDS-PAGE, or extraction.

In the case of prokaryotic production of DDAH polypeptide, the DDAH polypeptide thus produced may be misfolded and thus lacks or has reduced biological activity. The bioactivity of the protein may be restored by "refolding by solubilizing (where the DDAH polypeptide is also insoluble), unfolding and reducing the polypeptide chain using, for example, one or more chaotropic agents (e.g. urea and/or guanidine) and a reducing agent capable of reducing disulfide bonds (e.g. dithiothreitol, DTT or 2-mercaptoethanol, 2-ME). At a moderate concentration of chaotrope, an oxidizing agent is then added (e.g., oxygen,

cystine or cystamine), which allows the reformation of disulfide bonds. DDAH polypeptide may be refolded using standard methods known in the art, such as those described in U.S. Pat. Nos. 4,511,502, 4,511,503, and 4,512,922, which are incorporated by reference herein. The DDAH polypeptide may also be cofolded with other proteins to form heterodimers or heteromultimers.

The purified DDAH may be at least 90% pure (as measured by reverse phase high performance liquid chromatography, RP-HPLC, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) or at least 95% pure, or at least 98% pure, or at least 99% or greater pure. Regardless of the exact numerical value of the purity of the DDAH, the DDAH is sufficiently pure for use as a pharmaceutical product or for further processing, such as conjugation with a pharmacokinetic enhancing moiety.

Certain DDAH molecules may be used as therapeutic agents in the absence of other active ingredients or proteins (other than excipients, carriers, and stabilizers, serum albumin and the like), or they may be complexed with another protein or a polymer.

In one embodiment, for example, the DDAH polypeptide may be reduced and denatured by first denaturing the resultant purified DDAH polypeptide in urea, followed by dilution into TRIS buffer containing a reducing agent (such as DTT) at a suitable pH. In another embodiment, the DDAH polypeptide is denatured in urea in a concentration range of between about 2 M to about 9 M, followed by dilution in TRIS buffer at a pH in the range of about 5.0 to about 8.0. The refolding mixture of this embodiment may then be incubated. In one embodiment, the refolding mixture is incubated at room temperature for four to twenty-four hours. The reduced and denatured DDAH polypeptide mixture may then be further isolated or purified.

Ion Exchange Chromatography. In one embodiment, and as an optional, additional step, ion exchange chromatography may be performed on the first DDAH polypeptide mixture. Anion or cation exchange column chromatography may be performed on the DDAH polypeptide at any stage of the purification process to isolate substantially purified DDAH polypeptide.

Reverse-Phase Chromatography. RP-HPLC may be performed to purify proteins following suitable protocols that are known to those of ordinary skill in the art.

Hydrophobic Interaction Chromatography Purification Techniques. Hydrophobic interaction chromatography (HIC) may be performed on the DDAH polypeptide.

Other Purification Techniques. Yet another isolation step using, for example, gel filtration.

In some embodiments, the yield of DDAH after each purification step may be at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.9%, or at least about 99.99%, of the DDAH in the starting material for each purification step.

Purity may be determined using standard techniques, such as SDS-PAGE, or by measuring DDAH polypeptide using Western blot and ELISA assays.

Additional methods that may be employed include, but are not limited to, steps to remove endotoxins.

Previously, it has been shown that unnatural amino acids can be site-specifically incorporated into proteins in vitro by the addition of chemically aminoacylated suppressor tRNAs to protein synthesis reactions programmed with a gene containing a desired amber nonsense mutation. Using these approaches, one can substitute a number of the common twenty amino acids with close structural homologues, e.g., fluorophenylalanine for phenylalanine, using strains auxotrophic for a particular amino acid.

A tRNA may be aminoacylated with a desired amino acid by any method or technique, including but not limited to, chemical or enzymatic aminoacylation.

The ability to incorporate unnatural amino acids directly into proteins in vivo offers a wide variety of advantages including but not limited to, high yields of mutant proteins, technical ease, the potential to study the mutant proteins in cells or possibly in living organisms and the use of these mutant proteins in therapeutic treatments and diagnostic uses.

In one attempt to site-specifically incorporate para-F-Phe, a yeast amber suppressor tRNA^{Phe}CUA /phenylalanyl-tRNA synthetase pair was used in a p-F-Phe resistant, Phe auxotrophic *Escherichia coli* strain. See, e.g., R. Furter, *Protein Sci.* 7:419 (1998).

Macromolecular Polymers Coupled to DDAH Polypeptides

Various modifications to the amino acid polypeptides can be effected using the compositions, methods, techniques and strategies including incorporation of further functionality onto the amino acid component of the polypeptide, including but not limited to, a pharmacokinetic enhancing moiety, a label; a dye; a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a photocrosslinker; a radionuclide; a cytotoxic compound; a drug; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein

or polypeptide or polypeptide analog; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide; a water-soluble dendrimer; a cyclodextrin; an inhibitory ribonucleic acid; a biomaterial; a nanoparticle; a spin label; a fluorophore, a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a photoisomerizable moiety; biotin; a derivative of biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; a toxic moiety; an isotopically labeled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent; a detectable label; a small molecule; a quantum dot; a nanotransmitter; a radionucleotide; a radiotransmitter; a neutron-capture agent; or any combination of the above, or any other desirable compound or substance. As an illustrative, non-limiting example of the compositions, methods, techniques and strategies described herein, the following description will focus on adding macromolecular polymers to the non-natural amino acid polypeptide with the understanding that the compositions, methods, techniques and strategies described thereto are also applicable (with appropriate modifications, if necessary and for which one of skill in the art could make with the disclosures herein) to adding other functionalities, including but not limited to those listed above.

A wide variety of macromolecular polymers and other molecules can be linked to DDAH polypeptides described herein to modulate biological properties of the DDAH polypeptide, and/or provide new biological properties to the DDAH molecule. These macromolecular polymers can be linked to the DDAH polypeptide via a naturally encoded amino acid, via a non-naturally encoded amino acid, or any functional substituent of a natural or non-natural amino acid, or any substituent or functional group added to a natural or non-natural amino acid. The molecular weight of the polymer may be of a wide range, including but not limited to, between about 100 Da and about 100,000 Da or more. The molecular weight of the polymer may be between about 100 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, 1,000 Da, 900 Da, 800 Da, 700 Da, 600 Da, 500

Da, 400 Da, 300 Da, 200 Da, and 100 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and about 40,000 Da.

The polymer selected may be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer may be branched or unbranched. For therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

Examples of polymers include but are not limited to certain half-life extending moieties, polyalkyl ethers and alkoxy-capped analogs thereof (e.g., polyoxyethylene glycol, polyoxyethylene/propylene glycol, and methoxy or ethoxy-capped analogs thereof, especially polyoxyethylene glycol, the latter is also known as polyethyleneglycol or PEG); polyvinylpyrrolidones; polyvinylalkyl ethers; polyoxazolines, polyalkyl oxazolines and polyhydroxyalkyl oxazolines; polyacrylamides, polyalkyl acrylamides, and polyhydroxyalkyl acrylamides (e.g., polyhydroxypropylmethacrylamide and derivatives thereof); polyhydroxyalkyl acrylates; polysialic acids and analogs thereof; hydrophilic peptide sequences; polysaccharides and their derivatives, including dextran and dextran derivatives, e.g., carboxymethyldextran, dextran sulfates, aminodextran; cellulose and its derivatives, e.g., carboxymethyl cellulose, hydroxyalkyl celluloses; chitin and its derivatives, e.g., chitosan, succinyl chitosan, carboxymethylchitin, carboxymethylchitosan; hyaluronic acid and its derivatives; starches; alginates; chondroitin sulfate; albumin; pullulan and carboxymethyl pullulan; polyaminoacids and derivatives thereof, e.g., polyglutamic acids, polylysines, polyaspartic acids, polyaspartamides; maleic anhydride copolymers such as: styrene maleic anhydride copolymer, divinylethyl ether maleic anhydride copolymer; polyvinyl alcohols; copolymers thereof; terpolymers thereof; mixtures thereof; and derivatives of the foregoing.

Those of ordinary skill in the art will recognize that the foregoing list for substantially water-soluble backbones is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated as being suitable.

In some embodiments, the polymer derivatives can be “multi-functional”, backbone with at least two termini, and possibly as many as about 300 termini, functionalized or activated with a functional group. Multifunctional polymer derivatives include, but are not

limited to, linear polymers having two termini, each terminus being bonded to a functional group which may be the same or different.

In one embodiment, the polymer derivative has the structure:



5 wherein:

N=N=N is an azide moiety;

B is a linking moiety, which may be present or absent;

POLY is a water-soluble non-antigenic polymer;

A is a linking moiety, which may be present or absent and may be the same as B or
10 different; and

X is a second functional group.

Examples of a linking moiety for A and B include, but are not limited to, a multiply-functionalized alkyl group containing up to 18, and may contain between 1-10 carbon atoms. A heteroatom such as nitrogen, oxygen or sulfur may be included with the alkyl chain. The
15 alkyl chain may also be branched at a heteroatom. Other examples of a linking moiety for A and B include, but are not limited to, a multiply functionalized aryl group, containing up to 10 and may contain 5-6 carbon atoms. The aryl group may be substituted with one more carbon atoms, nitrogen, oxygen or sulfur atoms. Other examples of suitable linking groups include those linking groups described in U.S. Pat. Nos. 5,932,462; 5,643,575; and U.S. Pat.
20 Appl. Publication 2003/0143596, each of which is incorporated by reference herein. Those of ordinary skill in the art will recognize that the foregoing list for linking moieties is by no means exhaustive and is merely illustrative, and that all linking moieties having the qualities described above are contemplated to be suitable for use.

Examples of suitable functional groups for use as X include, but are not limited to,
25 hydroxyl, protected hydroxyl, alkoxyl, active ester, such as N-hydroxysuccinimidyl esters and 1-benzotriazolyl esters, active carbonate, such as N-hydroxysuccinimidyl carbonates and 1-benzotriazolyl carbonates, acetal, aldehyde, aldehyde hydrates, alkenyl, acrylate, methacrylate, acrylamide, active sulfone, amine, aminooxy, protected amine, hydrazide, protected hydrazide, protected thiol, carboxylic acid, protected carboxylic acid, isocyanate,
30 isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxals, diones, mesylates, tosylates, tresylate, alkene, ketone, and azide. As is understood by those of ordinary skill in the art, the selected X moiety should be compatible with the azide group so that reaction with the azide group does not occur. The azide-containing polymer derivatives may be homobifunctional, meaning that the second functional

group (i.e., X) is also an azide moiety, or heterobifunctional, meaning that the second functional group is a different functional group.

The term “protected” refers to the presence of a protecting group or moiety that prevents reaction of the chemically reactive functional group under certain reaction conditions. The protecting group will vary depending on the type of chemically reactive group being protected. For example, if the chemically reactive group is an amine or a hydrazide, the protecting group can be selected from the group of tert-butyloxycarbonyl (t-Boc) and 9-fluorenylmethoxycarbonyl (Fmoc). If the chemically reactive group is a thiol, the protecting group can be orthopyridyldisulfide. If the chemically reactive group is a carboxylic acid, such as butanoic or propionic acid, or a hydroxyl group, the protecting group can be benzyl or an alkyl group such as methyl, ethyl, or tert-butyl. Other protecting groups known in the art may also be used.

In certain embodiments, the polymer derivatives described herein comprise a polymer backbone having the structure:



wherein:

X is a functional group as described above; and

n is about 20 to about 4000.

In another embodiment, the polymer derivatives described herein comprise a polymer backbone having the structure:



wherein:

W is an aliphatic or aromatic linker moiety comprising between 1-10 carbon atoms;

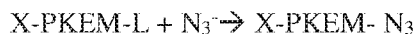
n is about 20 to about 4000

m is between 1 and 10; and

X is a functional group as described above.

The azide-containing PKEM derivatives described herein can be prepared by a variety of methods known in the art and/or disclosed herein. In one method, shown below, a water-soluble polymer backbone having an average molecular weight from about 800 Da to about 100,000 Da, the polymer backbone having a first terminus bonded to a first functional group and a second terminus bonded to a suitable leaving group, is reacted with an azide anion (which may be paired with any of a number of suitable counter-ions, including sodium, potassium, tert-butylammonium and so forth). The leaving group undergoes a nucleophilic

displacement and is replaced by the azide moiety, affording the desired azide-containing PKEM polymer.



As shown, a suitable polymer backbone can have the formula X-PKEM-L, wherein
 5 PKEM is poly(ethylene glycol) and X is a functional group which does not react with azide groups and L is a suitable leaving group. Examples of suitable functional groups include, but are not limited to, hydroxyl, protected hydroxyl, acetal, alkenyl, amine, aminooxy, protected amine, protected hydrazide, protected thiol, carboxylic acid, protected carboxylic acid, maleimide, dithiopyridine, and vinylpyridine, and ketone. Examples of suitable leaving
 10 groups include, but are not limited to, chloride, bromide, iodide, mesylate, tresylate, and tosylate.

In another method for preparation of the azide-containing polymer derivatives described herein, a linking agent bearing an azide functionality is contacted with a water
 15 soluble polymer backbone having an average molecular weight from about 800 Da to about 100,000 Da, wherein the linking agent bears a chemical functionality that will react selectively with a chemical functionality on the PKEM polymer, to form an azide-containing polymer derivative product wherein the azide is separated from the polymer backbone by a linking group.

An exemplary reaction scheme is shown below:



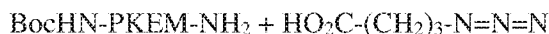
wherein:

PKEM is poly(ethylene glycol) and X is a capping group such as alkoxy or a functional group as described above; and

M is a functional group that is not reactive with the azide functionality but that will
 25 react efficiently and selectively with the N functional group.

Examples of suitable functional groups include, but are not limited to, M being a carboxylic acid, carbonate or active ester if N is an amine; M being a ketone if N is a hydrazide or aminooxy moiety; M being a leaving group if N is a nucleophile.

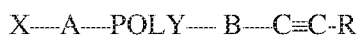
A more specific example is shown below in the case of PKEM diamine, in which one
 30 of the amines is protected by a protecting group moiety such as tert-butyl-Boc and the resulting mono-protected PKEM diamine is reacted with a linking moiety that bears the azide functionality:



In this instance, the amine group can be coupled to the carboxylic acid group using a variety of activating agents such as thionyl chloride or carbodiimide reagents and N-hydroxysuccinimide or N-hydroxybenzotriazole to create an amide bond between the monoamine PKEM derivative and the azide-bearing linker moiety. After successful
 5 formation of the amide bond, the resulting N-tert-butyl-Boc-protected azide-containing derivative can be used directly to modify bioactive molecules or it can be further elaborated to install other useful functional groups. For instance, the N-t-Boc group can be hydrolyzed by treatment with strong acid to generate an omega-amino-PKEM-azide. The resulting amine can be used as a synthetic handle to install other useful functionality such as
 10 maleimide groups, activated disulfides, activated esters and so forth for the creation of valuable heterobifunctional reagents.

Heterobifunctional derivatives are particularly useful when it is desired to attach different molecules to each terminus of the polymer. For example, the omega-N-amino-N-azido PKEM would allow the attachment of a molecule having an activated electrophilic
 15 group, such as an aldehyde, ketone, activated ester, activated carbonate and so forth, to one terminus of the PKEM and a molecule having an acetylene group to the other terminus of the PKEM.

In another embodiment, the polymer derivative has the structure:



20 wherein:

R can be either H or an alkyl, alkene, alkoxy, or aryl or substituted aryl group;

B is a linking moiety, which may be present or absent;

POLY is a water-soluble non-antigenic polymer;

A as linking moiety, may be present or absent and may be the same as B or different;

25 and

X is a second functional group.

Examples of a linking moiety for A and B include, but are not limited to, a multiply-functionalized alkyl group containing up to 18, and may contain between 1-10 carbon atoms. A heteroatom such as nitrogen, oxygen or sulfur may be included with the alkyl chain. The
 30 alkyl chain may also be branched at a heteroatom. Other examples of a linking moiety for A and B include, but are not limited to, a multiply functionalized aryl group, containing up to 10 and may contain 5-6 carbon atoms. The aryl group may be substituted with one more carbon atoms, nitrogen, oxygen, or sulfur atoms. Other examples of suitable linking groups

include those linking groups described in U.S. Pat. Nos. 5,932,462 and 5,643,575 and U.S. Pat. Appl. Publication 2003/0143596, each of which is incorporated by reference herein. Those of ordinary skill in the art will recognize that the foregoing list for linking moieties is by no means exhaustive and is intended to be merely illustrative, and that a wide variety of

5 linking moieties having the qualities described above are contemplated to be useful.

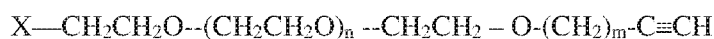
Examples of suitable functional groups for use as X include hydroxyl, protected hydroxyl, alkoxyl, active ester, such as N-hydroxysuccinimidyl esters and 1-benzotriazolyl esters, active carbonate, such as N-hydroxysuccinimidyl carbonates and 1-benzotriazolyl carbonates, acetal, aldehyde, aldehyde hydrates, alkenyl, acrylate, methacrylate, acrylamide,

10 active sulfone, amine, aminooxy, protected amine, hydrazide, protected hydrazide, protected thiol, carboxylic acid, protected carboxylic acid, isocyanate, isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxals, diones, mesylates, tosylates, and tresylate, alkene, ketone, and acetylene. As would be understood, the selected X moiety should be compatible with the acetylene group so that reaction with the

15 acetylene group does not occur. The acetylene-containing polymer derivatives may be homobifunctional, meaning that the second functional group (i.e., X) is also an acetylene moiety, or heterobifunctional, meaning that the second functional group is a different functional group.

In another embodiment, the polymer derivatives comprise a polymer backbone having

20 the structure:



wherein:

- X is a functional group as described above;
- n is about 20 to about 4000; and
- 25 m is between 1 and 10.

Specific examples of each of the heterobifunctional PKEM polymers are shown below.

The acetylene-containing PKEM derivatives described herein be prepared using methods known to those of ordinary skill in the art and/or disclosed herein. In one method, a water-soluble polymer backbone having an average molecular weight from about 800 Da to

30 about 100,000 Da, the polymer backbone having a first terminus bonded to a first functional group and a second terminus bonded to a suitable nucleophilic group, is reacted with a compound that bears both an acetylene functionality and a leaving group that is suitable for reaction with the nucleophilic group on the PKEM. When the PKEM polymer bearing the

nucleophilic moiety and the molecule bearing the leaving group are combined, the leaving group undergoes a nucleophilic displacement and is replaced by the nucleophilic moiety, affording the desired acetylene-containing polymer.



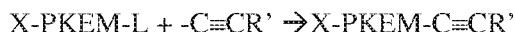
5 As shown, a preferred polymer backbone for use in the reaction has the formula X-PKEM-Nu, wherein PKEM is poly(ethylene glycol), Nu is a nucleophilic moiety and X is a functional group that does not react with Nu, L or the acetylene functionality.

Examples of Nu include, but are not limited to, amine, alkoxy, aryloxy, sulfhydryl, imino, carboxylate, hydrazide, aminoxy groups that would react primarily via a SN2-type mechanism. Additional examples of Nu groups include those functional groups that would
10 react primarily via a nucleophilic addition reaction. Examples of L groups include chloride, bromide, iodide, mesylate, tresylate, and tosylate and other groups expected to undergo nucleophilic displacement as well as ketones, aldehydes, thioesters, olefins, alpha-beta unsaturated carbonyl groups, carbonates and other electrophilic groups expected to undergo
15 addition by nucleophiles.

In another embodiment, A is an aliphatic linker of between 1-10 carbon atoms or a substituted aryl ring of between 6-14 carbon atoms. X is a functional group which does not react with azide groups and L is a suitable leaving group.

In another method for preparation of the acetylene-containing polymer derivatives, a
20 PKEM polymer having an average molecular weight from about 800 Da to about 100,000 Da, bearing either a protected functional group or a capping agent at one terminus and a suitable leaving group at the other terminus is contacted by an acetylene anion.

An exemplary reaction scheme is shown below:



25 wherein:

PKEM is poly(ethylene glycol) and X is a capping group such as alkoxy or a functional group as described above; and

R' is either H, an alkyl, alkoxy, aryl or aryloxy group or a substituted alkyl, alkoxyl, aryl or aryloxy group.

30 In the example above, the leaving group L should be sufficiently reactive to undergo SN2-type displacement when contacted with a sufficient concentration of the acetylene anion. The reaction conditions required to accomplish SN2 displacement of leaving groups by acetylene anions are known to those of ordinary skill in the art.

Purification of the crude product can usually be accomplished by methods known in the art including, but are not limited to, precipitation of the product followed by chromatography, if necessary.

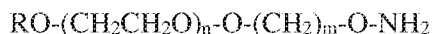
PKEM can be linked to the DDAH polypeptides described herein. The PKEM may be linked via a naturally encoded amino acid, a derivitized naturally encoded amino acid, or a non-naturally encoded amino acid incorporated in the DDAH polypeptide or any functional group or substituent of a non-naturally encoded or naturally encoded amino acid, or any functional group or substituent added to a non-naturally encoded or naturally encoded amino acid. Alternatively, the PKEM are linked to a DDAH polypeptide incorporating a non-naturally encoded amino acid via a naturally-occurring amino acid (including but not limited to, cysteine, lysine or the amine group of the N-terminal residue). In some cases, the DDAH polypeptides comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 non-natural amino acids, wherein one or more non-naturally-encoded amino acid(s) are linked to a pharmacokinetic enhancing moiety or moieties. In some cases, the DDAH polypeptides further comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more naturally-encoded amino acid(s) linked to a pharmacokinetic enhancing moiety or moieties. In some cases, the DDAH polypeptides described herein can comprise one or more non-naturally encoded amino acid(s) linked to PKEM and one or more naturally-occurring amino acids linked to PKEM. In some embodiments, the PKEM can enhance the serum half-life of the DDAH polypeptide relative to the unconjugated form.

The number of PKEM linked to a DDAH polypeptide described herein can be adjusted to provide an altered (including but not limited to, increased or decreased) pharmacologic, pharmacokinetic or pharmacodynamic characteristic such as in vivo half-life. In some embodiments, the half-life of DDAH is increased at least about 10, 20, 30, 40, 50, 60, 70, 80, 90 percent, 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 50-fold, or at least about 100-fold over an unmodified polypeptide.

PKEM derivatives containing a strong nucleophilic group (i.e., hydrazide, hydrazine, hydroxylamine or semicarbazide)

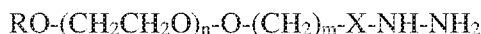
In one embodiment, a DDAH polypeptide comprising a carbonyl-containing non-naturally encoded amino acid is modified with a PKEM derivative that contains a terminal hydrazine, hydroxylamine, hydrazide or semicarbazide moiety that is linked directly to the PKEM backbone.

In some embodiments, the hydroxylamine-terminal PKEM derivative will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e.,
5 average molecular weight is between 5-40 kDa).

In some embodiments, the hydrazine- or hydrazide-containing PKEM derivative will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 and X is
10 optionally a carbonyl group (C=O) that can be present or absent.

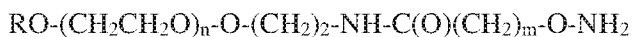
In some embodiments, the semicarbazide-containing PKEM derivative will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000.

15 In another embodiment, a DDAH polypeptide comprising a carbonyl-containing amino acid is modified with a PKEM derivative that contains a terminal hydroxylamine, hydrazide, hydrazine, or semicarbazide moiety that is linked to the PKEM backbone by means of an amide linkage.

20 In some embodiments, the hydroxylamine-terminal PKEM derivatives have the structure:



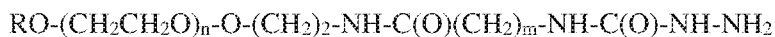
where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e.,
average molecular weight is between 5-40 kDa).

25 In some embodiments, the hydrazine- or hydrazide-containing PKEM derivatives have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, n is 100-1,000 and X is
optionally a carbonyl group (C=O) that can be present or absent.

30 In some embodiments, the semicarbazide-containing PKEM derivatives have the structure:

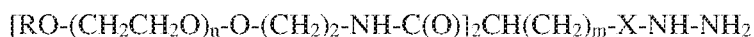


where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000.

In another embodiment, a DDAH polypeptide comprising a carbonyl-containing amino acid is modified with a branched PKEM derivative that contains a terminal hydrazine,

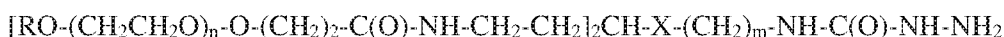
hydroxylamine, hydrazide or semicarbazide moiety, with each chain of the branched PKEM having a MW ranging from 10-40 kDa and, may be from 5-20 kDa.

In another embodiment, a DDAH polypeptide comprising a non-naturally encoded amino acid is modified with a PKEM derivative having a branched structure. For instance, in some embodiments, the hydrazine- or hydrazide-terminal PKEM derivative will have the following structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000, and X is optionally a carbonyl group (C=O) that can be present or absent.

In some embodiments, the PKEM derivatives containing a semicarbazide group will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), X is optionally NH, O, S, C(O) or not present, m is 2-10 and n is 100-1,000.

In some embodiments, the PKEM derivatives containing a hydroxylamine group will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), X is optionally NH, O, S, C(O) or not present, m is 2-10 and n is 100-1,000.

Methods and chemistry for activation of polymers as well as for conjugation of peptides are described in the literature and are known in the art. Commonly used methods for activation of polymers include, but are not limited to, activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxydes, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, etc. (see, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTAL AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson et al., (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., et al., Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Several reviews and monographs on the functionalization and conjugation of PKEM are available. See, for example, Harris, Macromol. Chem. Phys. C25: 325-373 (1985); Scouten, Methods in Enzymology 135: 30-65 (1987); Wong et al., Enzyme Microb. Technol.

14: 866-874 (1992); Delgado et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 9: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* 6: 150-165 (1995).

Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and WO 93/15189, and for conjugation between activated polymers and enzymes including but not limited to Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese et al., *App. Biochem. Biotech.* 11: 141-52 (1985)). All references and patents cited are incorporated by reference herein.

PKEMylation (i.e., addition of any water-soluble polymer) of DDAH polypeptides containing a non-naturally encoded amino acid, such as p-azido-L-phenylalanine, is carried out by any convenient method. For example, DDAH polypeptide is PKEMylated with an alkyne-terminated PKEM derivative. Briefly, an excess of solid PKEM(5000)-O-CH₂-C≡CH is added, with stirring, to an aqueous solution of p-azido-L-Phe-containing DDAH polypeptide at room temperature. Typically, the aqueous solution is buffered with a buffer having a pKa near the pH at which the reaction is to be carried out (generally about pH 4-10). Examples of suitable buffers for PKEMylation at pH 7.5, for instance, include, but are not limited to, HEPES, phosphate, borate, TRIS-HCl, EPPS, and TES. The pH is continuously monitored and adjusted if necessary. The reaction is typically allowed to continue for between about 1-48 hours.

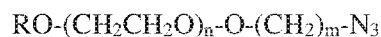
The reaction products are subsequently subjected to hydrophobic interaction chromatography to separate the PKEMylated DDAH polypeptide variants from free PKEM(5000)-O-CH₂-C≡CH and any high-molecular weight complexes of the pegylated DDAH polypeptide which may form when unblocked PKEM is activated at both ends of the molecule, thereby crosslinking DDAH polypeptide variant molecules. The conditions during hydrophobic interaction chromatography are such that free PKEM(5000)-O-CH₂-C≡CH flows through the column, while any crosslinked PKEMylated DDAH polypeptide variant complexes elute after the desired forms, which contain one DDAH polypeptide variant molecule conjugated to one or more PKEM groups. Suitable conditions vary depending on the relative sizes of the cross-linked complexes versus the desired conjugates and are readily determined by those of ordinary skill in the art. The eluent containing the desired conjugates is concentrated by ultrafiltration and desalted by diafiltration.

A pharmacokinetic enhancing moiety linked to an amino acid of a DDAH polypeptide can be further derivatized or substituted without limitation.

Azide-containing PKEM derivatives

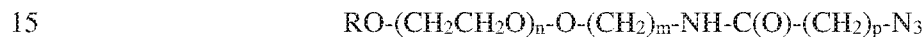
In another embodiment, a DDAH polypeptide is modified with a PKEM derivative that contains an azide moiety that will react with an alkyne moiety present on the side chain of the non-naturally encoded amino acid. In general, the PKEM derivatives will have an average molecular weight ranging from 1-100 kDa and, in some embodiments, from 10-40 kDa.

In some embodiments, the azide-terminal PKEM derivative will have the structure:



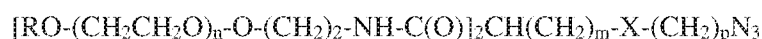
where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

In another embodiment, the azide-terminal PKEM derivative will have the structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

In another embodiment, a DDAH polypeptide comprising a alkyne-containing amino acid is modified with a branched PKEM derivative that contains a terminal azide moiety, with each chain of the branched PKEM having a MW ranging from 10-40 kDa and may be from 5-20 kDa. For instance, in some embodiments, the azide-terminal PKEM derivative will have the following structure:

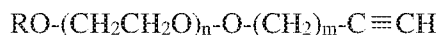


where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10, and n is 100-1,000, and X is optionally an O, N, S or carbonyl group (C=O), in each case that can be present or absent.

Alkyne-containing PKEM derivatives

In another embodiment, a DDAH polypeptide is modified with a PKEM derivative that contains an alkyne moiety that will react with an azide moiety present on the side chain of the non-naturally encoded amino acid.

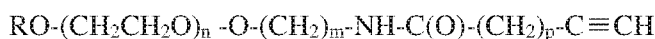
In some embodiments, the alkyne-terminal PKEM derivative will have the following structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10 and n is 100-1,000 (i.e., average molecular weight is between 5-40 kDa).

In another embodiment, a DDAH polypeptide comprising an alkyne-containing non-naturally encoded amino acid is modified with a PKEM derivative that contains a terminal azide or terminal alkyne moiety that is linked to the PKEM backbone by means of an amide linkage.

In some embodiments, the alkyne-terminal PKEM derivative will have the following structure:



where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10 and n is 100-1,000.

In another embodiment, a DDAH polypeptide comprising an azide-containing amino acid is modified with a branched PKEM derivative that contains a terminal alkyne moiety, with each chain of the branched PKEM having a MW ranging from 10-40 kDa and may be from 5-20 kDa. For instance, in some embodiments, the alkyne-terminal PKEM derivative will have the following structure:

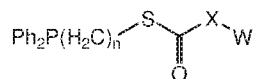


where R is a simple alkyl (methyl, ethyl, propyl, etc.), m is 2-10, p is 2-10, and n is 100-1,000, and X is optionally an O, N, S or carbonyl group (C=O), or not present.

Phosphine-containing PKEM derivatives

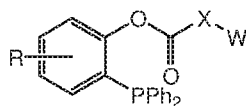
In another embodiment, a DDAH polypeptide is modified with a PKEM derivative that contains an activated functional group (including but not limited to, ester, carbonate) further comprising an aryl phosphine group that will react with an azide moiety present on the side chain of the non-naturally encoded amino acid. In general, the PKEM derivatives will have an average molecular weight ranging from 1-100 kDa and, in some embodiments, from 10-40 kDa.

In some embodiments, the PKEM derivative will have the structure:



wherein n is 1-10; X can be O, N, S or not present, Ph is phenyl, and W is a pharmacokinetic enhancing moiety.

In some embodiments, the PKEM derivative will have the structure:



wherein X can be O, N, S or not present, Ph is phenyl, W is a pharmacokinetic enhancing moiety and R can be H, alkyl, aryl, substituted alkyl and substituted aryl groups. Exemplary R groups include but are not limited to -CH₂, -C(CH₃)₃, -OR', -NR'R'', -SR', -halogen, -

5 C(O)R', -CONR'R'', -S(O)₂R', -S(O)₂NR'R'', -CN and -NO₂. R', R'', R''' and R'''' each independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, including but not limited to, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound includes more than one R group, for example, each of the R groups is independently selected

10 as are each R', R'', R''' and R'''' groups when more than one of these groups is present. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R'' is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents,

one of skill in the art will understand that the term "alkyl" is meant to include groups

15 including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (including but not limited to, -CF₃ and -CH₂CF₃) and acyl (including but not limited to, -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

Additional polymer and PEG derivatives including but not limited to, hydroxylamine (aminooxy) PEG derivatives, are described in the following patent applications which are all

20 incorporated by reference in their entirety herein: U.S. Patent Publication No. 2006/0194256, U.S. Patent Publication No. 2006/0217532, U.S. Patent Publication No. 2006/0217289, U.S. Provisional Patent No. 60/755,338; U.S. Provisional Patent No. 60/755,711; U.S. Provisional Patent No. 60/755,018; International Patent Application No. PCT/US06/49397; WO 2006/069246; U.S. Provisional Patent No. 60/743,041; U.S. Provisional Patent No.

25 60/743,040; International Patent Application No. PCT/US06/47822; U.S. Provisional Patent No. 60/882,819; U.S. Provisional Patent No. 60/882,500; and U.S. Provisional Patent No. 60/870,594.

Heterologous Fc Fusion Proteins

30 The DDAH compounds described above may be fused directly or via a peptide linker to the Fc portion of an immunoglobulin. Immunoglobulins are molecules containing polypeptide chains held together by disulfide bonds, typically having two light chains and

two heavy chains. In each chain, one domain (V) has a variable amino acid sequence depending on the antibody specificity of the molecule. The other domains (C) have a rather constant sequence common to molecules of the same class.

Depending on the desired in vivo effect, the heterologous fusion proteins may contain
5 any of the isotypes described above or may contain mutated Fc regions wherein the complement and/or Fc receptor binding functions have been altered. Thus, the heterologous fusion proteins may contain the entire Fc portion of an immunoglobulin, fragments of the Fc portion of an immunoglobulin, or analogs thereof fused to an interferon beta compound.

The fusion proteins described here can include single chain proteins or as multi-chain
10 polypeptides. Two or more Fc fusion proteins can be produced such that they interact through disulfide bonds. These multimers can be homogeneous with respect to the interferon beta compound or they may contain different interferon beta compounds fused at the N-terminus of the Fc portion of the fusion protein.

Regardless of the final structure of the fusion protein, the Fc or Fc-like region may
15 serve to prolong the in vivo plasma half-life of the interferon beta compound fused at the N-terminus.

Heterologous Albumin Fusion Proteins

DDAH described herein may be fused to albumin, such as directly or via a peptide
20 linker, water soluble polymer, or prodrug linker to albumin or an analog, fragment, or derivative thereof. Generally, the albumin proteins that are part of the fusion proteins may be derived from albumin cloned from any species, including human. A variety of polymorphic variants as well as analogs and fragments of albumin have been described. [See Weitkamp, et al., (1973) Ann. Hum. Genet. 37:219]. For example, in EP 322,094, various shorter forms of
25 HSA. Some of these fragments of HSA are disclosed, including HSA(1-373), HSA(1-388), HSA(1-389), HSA(1-369), and HSA(1-419) and fragments between 1-369 and 1-419. EP 399,666 discloses albumin fragments that include HSA(1-177) and HSA(1-200) and fragments between HSA(1-177) and HSA(1-200).

It is understood that the heterologous fusion proteins include DDAH compounds that
30 are coupled to any albumin protein including fragments, analogs, and derivatives wherein such fusion protein is biologically active and has a longer plasma half-life than the DDAH compound alone. Thus, the albumin portion of the fusion protein need not necessarily have a plasma half-life equal to that of native human albumin. Fragments, analogs, and derivatives

are known or can be generated that have longer half-lives or have half-lives intermediate to that of native human albumin and the DDAH compound of interest.

The heterologous fusion proteins encompass proteins having conservative amino acid substitutions in the DDAH compound and/or the Fc or albumin portion of the fusion protein.

5 A "conservative substitution" is the replacement of an amino acid with another amino acid that has the same net electronic charge and approximately the same size and shape. Amino acids with aliphatic or substituted aliphatic amino acid side chains have approximately the same size when the total number carbon and heteroatoms in their side chains differs by no more than about four. They have approximately the same shape when the number of branches
10 in their side chains differs by no more than one. Amino acids with phenyl or substituted phenyl groups in their side chains are considered to have about the same size and shape. Except as otherwise specifically provided herein, conservative substitutions are preferably made with naturally occurring amino acids.

Numerous methods exist to characterize the fusion proteins. Some of these methods
15 include, but are not limited to, SDS-PAGE coupled with protein staining methods or immunoblotting using anti-IgG or anti-HSA antibodies. Other methods include matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS), liquid chromatography/mass spectrometry, isoelectric focusing, analytical anion exchange, chromatofocusing, and circular dichroism.

20

Enhancing affinity for serum albumin

Various molecules can also be fused to the DDAH polypeptides to modulate the half-life of DDAH polypeptides in serum. In some embodiments, molecules are linked or fused to DDAH polypeptides to enhance affinity for endogenous serum albumin in an animal.

25 For example, in some cases, a recombinant fusion of a DDAH polypeptide and an albumin binding sequence is made. Exemplary albumin binding sequences include, but are not limited to, the albumin binding domain from streptococcal protein G (see, e.g., Makrides et al., J. Pharmacol. Exp. Ther. 277:534-542 (1996) and Sjolander et al., J. Immunol. Methods 201:115-123 (1997)), or albumin-binding peptides such as those described in, e.g.,
30 Dennis, et al., J. Biol. Chem. 277:35035-35043 (2002).

In other embodiments, the DDAH polypeptides can be acylated with fatty acids. In some cases, the fatty acids promote binding to serum albumin.

In other embodiments, the DDAH polypeptides are fused directly with serum albumin (including but not limited to, human serum albumin). Those of skill in the art will recognize

that a wide variety of other molecules can also be linked to DDAH polypeptides to modulate binding to serum albumin or other serum components.

Glycosylation of DDAH Polypeptides

5 Also described are DDAH polypeptides incorporating one or more non-naturally encoded amino acids bearing saccharide residues. The saccharide residues may be either natural (including but not limited to, N-acetylglucosamine) or non-natural (including but not limited to, 3-fluorogalactose). The saccharides may be linked to the non-naturally encoded amino acids either by an N- or O-linked glycosidic linkage (including but not limited to, N-
10 acetylglactose-L-serine) or a non-natural linkage (including but not limited to, an oxime or the corresponding C- or S-linked glycoside).

The saccharide (including but not limited to, glycosyl) moieties can be added to DDAH polypeptides either in vivo or in vitro. In some embodiments, a DDAH polypeptide comprising a carbonyl-containing non-naturally encoded amino acid is modified with a
15 saccharide derivatized with an aminooxy group to generate the corresponding glycosylated polypeptide linked via an oxime linkage. Once attached to the non-naturally encoded amino acid, the saccharide may be further elaborated by treatment with glycosyltransferases and other enzymes to generate an oligosaccharide bound to the DDAH polypeptide.

In some embodiments, a DDAH polypeptide comprising a carbonyl-containing non-
20 naturally encoded amino acid is modified directly with a glycan with defined structure prepared as an aminooxy derivative. One of ordinary skill in the art will recognize that other functionalities, including azide, alkyne, hydrazide, hydrazine, and semicarbazide, can be used to link the saccharide to the non-naturally encoded amino acid.

In some embodiments, a DDAH polypeptide comprising an azide or alkynyl-
25 containing non-naturally encoded amino acid can then be modified by, including but not limited to, a Huisgen [3+2] cycloaddition reaction with, including but not limited to, alkynyl or azide derivatives, respectively. This method allows for proteins to be modified with extremely high selectivity.

DDAH Dimers and Multimers

30 Also provided are DDAH and DDAH analog combinations such as dimers, homodimers, heterodimers, multimers, homomultimers, or heteromultimers (i.e., trimers, tetramers, etc.) where a DDAH or DDAH variant polypeptide is bound to another DDAH or DDAH variant thereof or any other polypeptide that is not DDAH or DDAH variant thereof,

either directly to the polypeptide N-terminus, C-terminus, or peptide backbone or via a linker or directly through the functional groups or modified functional groups of an amino acid in the DDAH polypeptide. Due to its increased molecular weight compared to monomers, the DDAH dimer or multimer conjugates may exhibit new or desirable properties, including but not limited to different pharmacological, pharmacokinetic, pharmacodynamic, modulated therapeutic half-life, or modulated plasma half-life relative to the monomeric DDAH. In some embodiments, DDAH dimers or multimers will modulate enzymatic activity of the DDAH.

In some embodiments, one or more of the DDAH molecules present in a DDAH containing dimer or multimer is linked to a pharmacokinetic enhancing moiety.

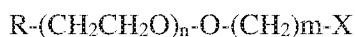
In some embodiments, the DDAH polypeptides are linked directly, including but not limited to, at their N-termini, via a Gly residue at the N-terminus through the enzyme sortase, via an Asn-Lys amide linkage or Cys-Cys disulfide linkage. In some embodiments, the DDAH polypeptides, and/or the linked non-DDAH molecule, will comprise different amino acids to facilitate dimerization, including but not limited to, an alkyne in one non-naturally encoded amino acid of a first DDAH polypeptide and an azide in a second amino acid of a second molecule will be conjugated via a Huisgen [3+2] cycloaddition. Alternatively, DDAH, and/or the linked non-DDAH molecule comprising a ketone-containing amino acid can be conjugated to a second polypeptide comprising a hydroxylamine-containing amino acid and the polypeptides are reacted via formation of the corresponding oxime.

Alternatively, the two DDAH polypeptides, and/or the linked non-DDAH molecule, are linked via a linker. Any hetero- or homo-bifunctional linker can be used to link the two molecules, and/or the linked non-DDAH molecules, which can have the same or different primary sequence. In some cases, the linker used to tether the DDAH, and/or the linked non-DDAH molecules together can be a bifunctional pharmacokinetic enhancing moiety. The linker may have a wide range of molecular weight or molecular length. Larger or smaller molecular weight linkers may be used to provide a desired spatial relationship or conformation between DDAH and the linked entity or between the linked entity and its binding partner, if any. Linkers having longer or shorter molecular length may also be used to provide a desired space or flexibility between DDAH and the linked entity, or between the linked entity and its binding partner, if any.

Also provided are water-soluble bifunctional linkers that have a dumbbell structure that include: a) an azide, an alkyne, a hydrazine, a hydrazide, a hydroxylamine, or a carbonyl-containing moiety on at least a first end of a polymer backbone; and b) at least a second

functional group on a second end of the polymer backbone. The second functional group can be the same or different as the first functional group. The second functional group, in some embodiments, is not reactive with the first functional group. Also provided, in some embodiments, are water-soluble compounds that comprise at least one arm of a branched molecular structure. For example, the branched molecular structure can be dendritic.

In some embodiments, also provided are multimers comprising one or more DDAH polypeptide, formed by reactions with water soluble activated polymers that have the structure:



wherein n is from about 5 to 3,000, m is 2-10, X can be an azide, an alkyne, a hydrazine, a hydrazide, an aminooxy group, a hydroxylamine, an acetyl, or carbonyl-containing moiety, and R is a capping group, a functional group, or a leaving group that can be the same or different as X. R can be, for example, a functional group selected from the group consisting of hydroxyl, protected hydroxyl, alkoxyl, N-hydroxysuccinimidyl ester, 1-benzotriazolyl ester, N-hydroxysuccinimidyl carbonate, 1-benzotriazolyl carbonate, acetal, aldehyde, aldehyde hydrates, alkenyl, acrylate, methacrylate, acrylamide, active sulfone, amine, aminooxy, protected amine, hydrazide, protected hydrazide, protected thiol, carboxylic acid, protected carboxylic acid, isocyanate, isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxals, diones, mesylates, tosylates, and tresylate, alkene, and ketone.

First, PKEMylation of DDAH is performed to generate a longer half life DDAH1 adduct. PEGylation of proteins is widely used in order to prolong their in vivo half life. Several PEGylation sites are identified to be present in DDAH1. PEGylation conditions will be determined to generate an active enzyme with extended half-life, such as a circulating half life of 6-24hours. This will enable 1-2 injections daily to reduce ADMA levels for a desired treatment duration depending upon the condition of the patient.

With respect to diseases such as heart failure and fibrotic disease, activity of a DDAH or modified DDAH may also be determined using one or more in vivo assays. Said assays typically involve administration of a DDAH in an animal model of said disease and determining the effect on disease progression, severity, or other indicia of efficacious treatment. Such assays may be used to determine efficacious dosages and treatment regimens in the model system, and based thereon predict a dosing regimen for clinical use, e.g., for human patients. Exemplary HF assays that may be utilized include: 1) the mouse left anterior descending (LAD) coronary ligation model which mimics the cardiac changes of patients

suffering a myocardial infarction and progression to HF (Samuel et al., Lab. Investigation 91:675-690, 2011); 2) limited AngII-infusion model in which minimal concentrations AngII are utilized to induce cardiac fibrosis (Xu et al., J. Cardiovasc. Pharmacol. 51:62-70, 2008); 3) Dahl-Salt sensitive rat model which is characterized by hypertension, renal impairment, and blood volume overload (Sakata et al., Circulation 109:2143-2149, 2004); 4) Aged-Spontaneously Hypertensive Rat (SHR) model of cardiac and renal fibrosis which was previously utilized to demonstrate WT-RLX efficacy (Lekgabe et al., Hypertension 46:412-418, 2005); and 5) rat thoracic aortic constriction model of pressure overload (Kuster et al., Circulation 111:420-427, 2005). In addition to these models, activity of modified DDAHs may also be determined in a dog model, such as in normal and tachypacing-induced HF dogs. Additionally, these assays may be performed to determine whether a DDAH or modified DDAH is efficacious when given not only in preventative mode, but also in therapeutic mode. Further, modified DDAHs may be tested in models of fibrosis including renal (Yoshida et al., Nephrol. Dialysis Transplant 27: 2190-2197, 2012), lung (Huang et al., Am. J. Pathol. 179:2751-2765, 2011), and liver fibrosis (Williams et al., Gut 49:577-583, 2001). For example, efficacy of a DDAH or modified DDAH may be compared to efficacy of wild-type DDAH (e.g., wild-type human DDAH).

The exact amount of DDAH, DDAH polypeptides, and/or DDAH analogues described herein is a matter of preference subject to such factors as the exact type and/or severity of the condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. The compositions and methods described herein also provide for administration of a therapeutically effective amount of another active agent. The amount to be given may be readily determined by one of ordinary skill in the art based upon therapy with DDAH, available DDAH therapies, and/or other DDAH analogues.

The invention will now be described in more detail with respect to the following, specific, non-limiting examples.

EXAMPLES

Example 1

This example details cloning and expression of a DDAH polypeptide in *E. coli*.

Methods for cloning DDAH are known to those of ordinary skill in the art. Polypeptide and polynucleotide sequences for DDAH and cloning of DDAH into host cells are detailed in U.S. Patent No. 4,758,516; U.S. Patent No. 5,166,191; U.S. Patent No. 5,179,195, 5,945,402; and 5,759,807; all of which patents are herein incorporated by reference.

cDNA encoding DDAH 1 and DDAH 2 are shown as SEQ ID NO: 3 and SEQ ID NO:4 and the DDAH 1 and DDAH 2 polypeptide amino acid sequences are shown as SEQ ID NO: 1 and SEQ ID NO:2. Polypeptide sequence of bacterial DDAH amino acid sequence *Pseudomonas aeruginosa* shown in SEQ ID NO 13.

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TABLE 2: Human DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
1	Human DDAH1 amino acid sequence	MAGLGHPAAF GRATHAVVRA LPESLGQHAL RSAKGEEVDV ARAERQHQLY VGVLGSKLGL QVVELPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK EVDMMKEALE KLQLNIVEMK DENATLDGGD VLFTGREFFV GLSKRTNQRG AEILADTFKD YAVSTVPVAD GLHLKSFCMS AGPNLIAIGS SESAQKALKI MQQMSDHRYD KLTVPDDIAA NCIYLNIPNK GHVLLHRTPE EYPESAKVYE KLKDHMLIPV SMSELEKVDGLLTCCSVLIN KKVDS
2	Human DDAH 2 amino acid sequence	MGTPGEGGLGR CSHALIRGVP ESLASGEGAG AGLPALDLAK AQREHGVGG KLRQRLGLQL LELPPEESLP LGPLLGD TAV IQGDTALITR PWSPARRPEV DGVRKALQDL GLRIVEIGDE NATLDGTDVL FTGREFFVGL SKWTNHRGAE IVADTFR DFA VSTVPVSGPS HLRGLCGMGG PRTVVAGSSD AAQKAVRAMA VLTDHPYASL TLPDDAAADC LFLRPGLPGV PPFLLRHGGG DLPNSQEALQ KLSVDTLVPV SCSELEKAGA GLSSLCLVLS TRPHS
3	Human DDAH1 mRNA nucleotide sequence	aacttaattg ttttgattg gactttgagt taagattatt ttttaaatcc tgaaggactagcattaattga cagctgaccc aggtgctaca cagaagtga ttcagtgaat ctaggaagacagcagcagac aggattccag gaaccagtgt ttgatgaagc taggactgag gagcaagcgagcaagcagca gttcgtggaa tctgtctgc tctgtcttc ctggtttagg agccgacggcgctcgcagg ctcagcgcgc gctgcccgcg gcaggaccgc gccgcctccg ccgcgcgcgc cgccttaag cctcccgaag ccatggccgg gctcgccac cccgcgcct tcggccgggccaccacgc gtggtgcggg cgtaccga gtcgtcggc cagcagcgc tgaagaagcgaagggcgag gaggtggacg tgcgcgcgc ggaacggcag caccagctct acgtggcgctgctgggcagc aagctggggc tgcaggtggt ggagctgcc gccgacgaga gcctccggactgcgtcttc gtggaggacg tggcgtggt gtgcgaggag acggccctca tcaccgacc cggggcgccg agccggagga aggaggtga catgatgaaa gaagcattag aaaaactcagctcaatata gtagagatga aagatgaaaa tgcaacttta gatggcgag atgtttattcacaggcaga gaatttttg tgggcttgc caaaaggaca aatcaacgag gtgctgaaatcttgctgat actttaagg actatgcagt ctccacagt ccagtggcag atgggttcatttgaagagt ttctgcagca tggctgggac taacctgac gcaattgggt ctagtgaatc tgcacagaag gcccttaaga tcatgcaaca gatgagtac caccgctac aaaaactcactgctgat gacatagcag caaactgtat atatctaat atcccaaca aagggcacgtctgtgcac cgaaccccg aagagtatcc agaaagtga aaggtttatg agaaactgaaggaccatag ctgatcccg tgagcatgct gaactggaa aaggtgtagt ggtgctcactgctgctca gttttaatta acaagaaagt agactctga gctgcagagt ccccccggt agccggcaag accgcacagg caaggccgat gactctgtgc ccactctgt tgtttcttgacaatctac tgtgccactg tctactaac tctgtttac aaaatttgat

		tctaagttgaattgcttcat tcaacacccc caccctccct ccccregagg tggtagctaa gctgtggatttgctaaatga attaagcaac ctagaagata cagagctaat gaattatcaa aatgtgattaatcccagtaa ggaacacactc atttagtgct tgtatttttg gtgnaaaat tatttagttg ccagtataft ctgaagaatg tcttcttgat cagtcagata agcttgcttt tttttttttttcatgaa tcatgtttgg ttcctgtgaa agtccttggt ccagggatcc tectctttctcttttaact ctg
4	Human DDAH2 mRNA nucleotide sequence	ccgcttagac aatgccccgg agccgccaga ccgtcgcgcc cctgccccat cgtagtatatgagctcgctt acacaaggac ccccgctaaa agccagagct ccagtccec gaggctgaagacggggact cctttctcca ccaactctgt cctcggggggg tggggcccca gccgagatcacagcgcgaca ggagtggggg tggccgctgg agacaggtga agaacaaga aaactaagaaatccgagcgg ttggaggggg agtctgtgtg gatgggatgg ggaagccggg ggaggggctg ggccgctgct cccatgccct gatccgggga gtcccagaga gcctggcgtc gggggaaggtgcgggggctg gccttccgc tctggatctg gccaaagtc aaaggagca cgggggtgctgggaggtaaac tgaggcaacg actggggcta cagetctag aactgccacc tgaggagtcattgcgctgg gaccgctgct tggcgacacg gccgtgatcc aaggggacac ggccctaatacagcggccct ggagccccgc tctaggccca gaggtcgatg gattccgcaa agccctgcaa gacctggggc tccgaattgt ggaaatagga gacgagaacg cgacgctgga tggcactgacgttcttca ccggccggga gttttcgtg ggccctctcca aatggacca tcaccgaggagctgagatcg tggcggacac gtccggggac ttcccgctct ccaactgtgc agtctcgggtccctcccacc tgcgcggtct ctgcggcatg gggggacctc gcaactgtgt ggcaggcagcagcagctg cccaaaaggc tgccgggga atggcagtc tgacagatca cccataatgccc tccctgaccc tcccagatga cgcagctgct gactgcctct ttcttgctc tgggttgctggtgtgcccc ctctctctct gcaccgtgga ggtggggatc tgcccaacag ccaggaggcactgcagaagc tctctgatgt caccctggta cctgtgtct gctcagaact ggagaaggctggcgcgggc ttagctccct ctgcttggtg ctcagcacac gcccacacag ctgagggcctggccttgggg tactgctggc caggggtagg atagtatagg aagtagaagg ggaaggaggg ttagatagag aatgctgaat aggcagtagt tgggagagag cctcaatatt gggggaggggagagtgtagg gaaaaggatc cactgggtga atcctccctc tcagaaccaa taaaatagaattgacctttt aaaaaaaaaa aaaaaaaaaa a
5	Human DDAH1 isoform 2 amino acid sequence (missing residues 1-103 of DDAH1)	MMKEALEKLQ LNIVEMKDEN ATLDGGDVLV TGREFFVGLS KRTNQRGAEI LADTFKDYAV STVPVADGLH LKSFCMAGP NLIAIGSSES AQKALKIMQQ MSDHRYDKLT VPDDIAANCI YLNIPNKGHV LLHRTPEEYP ESAKVYEKLK DHMLIPVSMS ELEKVDGLLT CCSVLINKKV DS

TABLE 3: Bovine DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
6	Bovine DDAH1 amino acid sequence	MASLGHPATF GRATHVVVRA LPESLAQQAL RRTKGDEVDF ARAERQHQLY VGVLGSKLGLQVVQLPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK EADMMKEALE KLQLNIVEMK DENATLDGGD VLFTGREFFV GLSKRTNQRG AEILADTFKD YAVSTVPVVD ALHLKSFCM AGPNLIAIGS SESAQKALKI MQQMSDHRYD KLTVPDDTAA NCIYLNIPSK GHVLLHRTPE EYPESAKVYE KLKDHMLIPV SNSELEKVDG LLTCSSVLIN KKVDS

7	Bovine DDAH 2 amino acid sequence	MGTPGEGGLGR CSHALIRGVP ESLASGEGAA AGLPALDLAK AQREHGVVLGG KLRQRLGLQL VELPPEESLP LGPLLGD TAV IQGDTALITR PWSPARRPEV DGVRKALQDL GLRIVEMGDE NATLDGTDVL FTGREFFVGL SKWTNHRGAE IVADTFRDFA VSTVPVTSTS HLRGLCGMGG PRTVVAGSSE AAQKAVRAMA VLTDHPYASL TLPDDAAADC LFLRPGQPGL PPFLLRGGG DLPNSQEALQ KLSDVTLVPV SCSELEKAGA GLSSLCLVLS TRPHN
8	Bovine DDAH1 nucleotide sequence	atggcttctc teggccaacc agccaccttt ggccggggcca cccatgtcgt ggtacgggcgctgcccagat cctcggccca acaggcgctg aggcgcacca agggcgacga ggtggaattcgcggcgctg agcggcagca ccagctctac gtggcgctgc tgggcagtaa actggggctgcagggtgtgc agctgcccgc cgacgagage ctcccagact gcgtgttcgt ggaggacgtggccgtggtgt gcgaggagac ggccctgac acccgccccg gggcgccgag ccggaggaag gagcgctgaca tgatgaaaga agcactagaa aaactcagc tcaacatagt agagatgaaagatgaaaatg caactttaga tggaggagat gtcttattca caggcagaga atTTTTgtgggctttcca aaaggacaaa tcaacgaggt gcggaaatct tggctgatac ttttaaggactatgcggtct ccacgggtccc tgtggtggat gctttgact tgaagagttt ctgcagcatggctgggecta acctaatcgc tattggatcc agtgaatctg cacagaagggc cctcaagatc atgcaacaga tgagtatca tgcctacgac aaactcacag tgcctgatga cacggccgcaactgcatat acctgaatat cccagcaaa ggccacgtct tctgcaccg aaccacagaagagtaccag agagtgcataa ggtttatgaa aagctgaagg accatatgct gatccccgtgagcaattctg aactggaaaa ggtggacggg ctgctcact gcagctcggt tttaattaacaagaaagtag actcctga

TABLE 4: Murine DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
9	Murine DDAH1 amino acid sequence	MAGLGHPSAF GRATHAVVRA PPESLCRHAL RRSQGEEVDF ARAERQHELY VGVLGSKLGL QVVQLPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK EVDMMKEALE KLQNLIVEMK DENATLDGGD VLFTGREFFV GLSKRTNQRG AEILADTFKD YAVSTVPVAD SLHLKSFCM AGPNLIAIGS SESAQKALKI MQQMSDHRD KLTVPDDMAA NCIYLNIPSK GHVLLHRTPE EYPESAKVYE KLKDHLLIPV SENSEMEKVDG LLTCCSVFIN KKIDS
10	Murine DDAH 2 amino acid sequence	MGTPGEGGLGR CSHALIRGVP ESLASGEGAG AGLPALDLAK AQREHGVVLGG KLRQRLGLQL LELPPEESLP LGPLLGD TAV IQGDTALITR PWSPARRPEV DGVRKALQDL GLRIVEMGDE NATLDGTDVL FTGREFFVGL SKWTNHRGAE IVADTFRDFA VSTVPVSGSS HLRGLCGMGG PRTVVAGSSE AAQKAVRAMA ALTDHPYASL TLPDDAASDC LFLRPGLPGA TPFLLRGGG DLPNSQEALQ KLSDVTLVPV SCSELEKAGA GLSSLCLVLS TRPHC

TABLE 5: Rat DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
11	Rat DDAH1 amino acid sequence	MAGLSHPSVF GRATHAVVRA PPESLCRHAL RRSQGEEVDF ARAERQHQLY VGVLGSKLGLQVVQLPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK EVDMMKEALE KLQLNIVEMKDENATLDGGD VLFTGREFFV GLSKRTNQRG AEILADTFKD YAVSTVPVAD SLHLKSFCM AGPNLIAIGS SESAQKALKI MQQMSDHRYD KLTVPDDMAA NCIYLNIPSK GHVLLHRTPE EYPESAKVYE KLKDHLLIPV SNSEMEKVDG LLTCCSVFIN KKTDS
12	Rat DDAH 2 amino acid sequence	MGTPGEGLGR CSHALIRGVP ESLASGEGAG AGLPALDLAK AQREHGVGG KLRQRLGLQLELPPEESLP LGPLLGD TAV IQGDTALITR PWSPARRPEV DGVRKALQDL GLRIVEMGDENATLDGTDVL FTGREFFVGL SKWTNHRGAE IVADTFRDFA VSTVPVSGAS HLRGLCGMGPRTVVAGSSE AAQKAVRAMA ALTDHPYASL TLPDDAASDC LFLRPGLPGT TPFLLHRGGGDL PNSQEALQ KLSDVTLVPV SCSELEKVGA GLSSLCLVLS TRPHC

TABLE 6: Bacterial DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
13	Bacterial DDAH amino acid sequence Pseudomonas aruginosa	MFKHIIARTP ARSLVDGLTS SHLGKPDYAK ALEQHNAYIR ALQTCDDVIT LLPDERFPDSVFVEDPVLC TSRCAITRP GAESRRGETE IIEETVQRFY PGKVERIEAP GTVEAGDIMMVGDFHYIGES ARTNAEGARQ MIAILEKHGL SGSVVRLEKV LHLKTGLAYL EHNNLLAAGEFVSKPEFQDF NIIIEPEEES YAANCIWVNE RVIMPAGYPR TREKIARLGY RVIEVDTSEYRKIDGGVSCM SLRF

TABLE 7: Non-Human Primate DDAH Sequences

SEQ ID NO:	Sequence Name	Sequence
14	Rhesus Monkey DDAH1 amino acid sequence	MAGLGHPAAF GRATHAVVRA LPESLGQHAL RSAKGEEVDV ARAERQHQLY VGVLGSKLGLQVVQLPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK EVDMMKEALE KLQLNIVEMK DENATLDGGD VLFTGREFFV GLSKRTNQRG AEILADTFKD YAVSTVPVAD GLHLKSFCMAGPNLIAIGS SESAQKALKI MQQMSDHRYD KLTVPDDIAA NCIYLNIPNK GHVLLHRTPE EYPESAKVYE KLKDHMLIPV SMSELEKVDG LLTCCSVLIN KKVDS

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The transformation of *E. coli* with plasmids containing the DDAH or modified DDAH or DDAH analog gene allows for biosynthesis of the DDAH polypeptide.

Wild type mature DDAH is amplified by PCR from a cDNA synthesis reaction using standard protocols and cloned into pET30 (NcoI-BamHI). Alternatively, the DNA sequence was synthesized. Prior to or alternatively following sequence confirmation, DDAH encoding nucleic acid sequences are subcloned into an expression vector under constitutive or
 5 inducible control of a synthetic promoter derived from *E. coli* or other suitable source. Expression of DDAH is under control of the T7 promoter. Any desired mutations are introduced using standard quick change mutation protocols (Stratagene; La Jolla, California). Constructs are sequence verified.

Expression plasmids (e.g. pET and pBAD) are used to transform into the Escherichia coli strain W3110B57 to produce strains of *E. coli* in which expression of the T7 polymerase
 10 is under control of an arabinose-inducible promoter. Overnight bacterial cultures are diluted 1:100 into shake flasks containing 2X YT culture media and grown at 37°C to an OD₆₀₀ of ~0.8. Protein expression is induced by the addition of arabinose (0.2% final). Cultures are incubated at 37 °C for 5 hours or overnight. Cells are pelleted and resuspended in B-PER
 15 lysis buffer (Pierce) 100ul/OD/ml + 10ug/ml DNase and incubated at 37°C for 30 min. Cellular material is removed by centrifugation and the supernatant removed. The pellet is re-suspended in an equal amount of SDS-PAGE protein loading buffer. All samples are loaded on a 4-12% PAGE gel with MES and DTT. Methods for purification of DDAH are known to those of ordinary skill in the art and are confirmed by SDS-PAGE, Western Blot analyses, or
 20 electrospray-ionization ion trap mass spectrometry and the like.

His-tagged mutant DDAH proteins can be purified using methods known to those of ordinary skill in the art. The ProBond Nickel-Chelating Resin (Life Technologies, Carlsbad, CA) may be used via the standard His-tagged protein purification procedures provided by the manufacturer.

25 Figure 1 shows a DDAH plasmid construct used for expression.

This example also details expression of DDAH polypeptides by *E. coli*. This example describes the scale up of DDAH polypeptide production using a five (5) liter fermentor. These methods and scale up may also be used for 10L, 30L, 150L and 1000L batches. In some embodiments, at least 2 g (e.g., at least 4 g, at least 6 g, at least 8 g, at least 10 g, at least
 30 15 g, or at least 20g) of DDAH protein is produced for each liter of cell culture.

DDAH Cloning

Human and PA-DDAH or various mutants were cloned by adding 2 ul of PCR linearized pE-SUMO vector, 2 ul of geneBlock IDT (PA-DDAH_pSUMO or HS-DDAH_pSUMO), 6ul of H2O to In Fusion HD EcoDry Mix. The mixture was

incubated at 37 C for 15 min and then at 50 C for 15 min. To 100 ul tubes of Stellar competent bacterial cells 2.5 ul of the above reactions mix was added and then incubated on ice for 30 min. The cells were heat shocked at 42 C for 1 min. Sterile LB (900 ul) was added and placed on shaker at 225 rpm for 1 hr. The cells were centrifuged and 200ul added to
 5 plate on LB agar KAN plates and incubated overnight at 37 C. Colonies were picked and grown in 5 ml of LB + KAN for 12-16 hrs with shaking at 250 rpm at 37 C. DNA was extracted using QIAprep Spin Minikit, purified and plasmid DNA sequence was confirmed.

DDAH Expression

The plasmid was transformed by adding 0.5 ul of DNA to 50 ul of BL21(DE3) cells.
 10 Cells were incubated on ice for 30min and then heat shocked at 42 C for 1 min. LB, without antibiotics (450 ul) was added to the cells and placed on a shaker at 225 rpm at 37 C for 60 min. 5-10 ul of the transformed cells were plated on LB-agar KAN plate and grown overnight at 37C. Colonies were used for inoculation of 5 ml of LB-KAN in culture tubes and placed on shaker at 250 rpm at 37C and grown until OD600 reached to ~0.8-0.9. 750 ul was removed
 15 for making glycerol stock by adding 50 ul of sterile glycerol. 50 ml of LB-KAN with glycerol stock was inoculated with BL21(DE) cells and grown overnight. 25 ml was then added to 500 ml of LB-KAN and grown at 37 C until the OD600 reached to ~0.7. The temperature was then lowered to 25 C. Induction was carried out by adding with 0.2 mM IPTG for ~20 hrs. Cells were then centrifuged for 15 min at 5,000 rpm. Cell extract was prepared by
 20 resuspending the pellet in 20 mM Tris, 300 mM NaCl and 1 mM beta mercaptoethanol (BME), pH 8.0 (~20 ml), sonication on ice for 3 min and centrifugation at 30,000 rpm for 30 min at 4 C. Supernatant was used for DDAH purification.

Example 2 – DDAH Purification

The supernatant from the cell extract containing DDAH was applied to a 5 ml NiNTA
 25 column equilibrated with 20 mM Tris, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM BME at pH 8.0. The column was washed with 25 ml buffer and then eluted using 20 mM Tris, 300 mM NaCl, 10% glycerol, 500 mM imidazole, 1mM BME, pH 8.0. Eluted fractions were assayed for DDAH activity and protein concentration. Active fractions were pooled. Sumo and His-tag from DDAH was cleaved by adding 37.5 ul of ULP-1 to pool
 30 DDAH (~4.92 mg/ml). Cleaved preparation was dialyzed against 2 L of 20 mM Tris, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1mM BME, pH 8.0. Dialyzed fraction was added to a new 5 ml NiNTA column, equilibrated with 20 mM Tris, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1mM BME, pH 8.0. Flow through fractions containing DDAH

were collected for activity and SDS-PAGE. Active fractions were pooled. Further purification was achieved by Sepharose Q chromatography.

DDAH enzyme assay: DDAH activity is determined by modification of method published in the art (Markus Knipp and Milan Vas'ak Analytical Biochemistry 286, 257–264 (2000)). The enzyme activity in cell extracts generated by homogenization in 0.1 M sodium phosphate buffer pH 6.9 and purified preparations will be determined by L-citrulline generation from ADMA. A 100 µl of sample will be transferred to a tube and 400 µl of 1mM ADMA in sodium phosphate buffer will be added and incubated at 37 °C for 45 min. The reaction will be terminated by addition of 500 µl of 4% Sulfosalicyclic acid. The mixture will be centrifuged at 3000 g for 10 minutes. A 60 µl of supernatant will be transferred to NUNC 96 well plate in triplicates. A 200 µl of COLDER (color development reagent) will be added. COLDER is prepared by mixing 1 volume of solution A [80 mM DAMO (diacetyl monoxime) and 2.0 mM TSC (thiosemicarbazide)] and 3 volume of solution B [3 M H3PO4, 6 M H2SO4, and 2 mM NH4Fe(SO4)2]. The plates will be sealed and heated at 95 °C for 20 minutes. After cooling, they will be read at 530 nM. DDAH activity will be expressed as µM citrulline produced per gram protein per minute at 37 °C.

Determination of dimethylarginines: L-arginine, ADMA and SDMA will be quantified by LC-MS methods described in the art (Jens Martens-Lobenhoffer J. Mass Spectrom. 2004; 39: 1287–1294) or by high performance liquid chromatography (HPLC) after precolumn derivatization with o-phthaldialdehyde (OPA) ³⁹ L-homoarginine (10 µM) as an internal standard will be added to the tissue homogenate. Samples and standards will be extracted on solid phase extraction cartridges (CBA Bond Elut, Varian, Harbor City, California). The eluents will be dried over nitrogen and resuspended in bi-distilled water. Samples and standards will be incubated for 1 min with OPA reagent (5.4 mg/mL OPA in borate buffer, pH 8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivatives of L-arginine, ADMA and SDMA will be separated on a 250 x 4.5 mm I.D. 7 µm Nucleosil phenyl column (Supelco, Bellefonte, Pennsylvania) with the fluorescence detector set at $\lambda^{ex} = 340$ nm and $\lambda^{em} = 450$ nm. Samples will be eluted from the column with 0.96% citric acid/methanol 70:30, pH 6.8, at a flow rate of 1 mL/min. The variability of the method is 7%; the detection limit of the assay is 0.15 µmol/L.

Figure 2 shows the activity and purity of rDDAH.

Example 3 – Representative DDAH mutants and Activity

Table 8 shows the activity of DDAH mutants. Recombinant human DDAH and *Pseudomonas aruginosa* are noted as rhDDAH and rPa-DDAH respectively, with the change in amino acid residue is listed in the parenthesis.

5 **TABLE 8: Activity of DDAH mutants.**

DDAH mutants	Activity relative to rhDDAH
1. rhDDAH	
2. N-His-rhDDAH	
3. N-His-sumo-rhDDAH	1.0
4. rhDDAH (C74A)	1.0
5. rhDDAH (C84A)	1.0
6. rhDDAH (C178A)	1.2
7. rhDDAH(C275A)	2.0
8. rhDDAH(C274A)	1.5
9. rhDDAH (C74S)	1.0
10. rhDDAH (C84S)	0.0
11. rhDDAH (C178S)	1.5
12. rhDDAH (C275S)	2.2
13. rhDDAH (C274S)	1.5
14. rhDDAH (C222S)	1.0
15. rhDDAH (C222Q)	0.0
16. rhDDAH (C74A+C84A+C275A)	3.0
17. rPa-DDAH	1.0
18. N-His-rPa-DDAH	0.2
19. C-His-rPa-DDAH	20.0
20. N-His-sumo-rPa-DDAH	15.0
21. rPa-DDAH C74A+C84A+C275A+C178	50.0
22. rPa-DDAH (C74S+C84S+C275S+C178	15.0
23. C-Biotinylation site* rPa-DDAH	15.0
24. Sephadex conjugated ** rPa-DDAH	0.2
	20.0
* Biotinylation sequence add at C-terminal	20.0
** Conjugated to NHS Sephadex beads	
All sequence contain N-terminal methionine extra amino acid.	

Example 4

10 rDDAH lowers ADMA in blood and plasma: 1ml of heparinized whole blood was incubated with or without rDDAH for 15 minutes at room temperature. The reaction was terminated by addition of 10% trichloric acid. The mixture was centrifuged to remove precipitated proteins. The supernatant was used for determination of ADMA. In another study, human plasma containing 3uM added ADMA was incubated with different concentration

rDDAH for 30 min at room temperature. The reactions were terminated by addition of 10% trichloric acid. The mixture was centrifuged to remove precipitated proteins. The supernatant was used for determination of ADMA.

Figure 3 shows that rDDAH lowers ADMA in blood and plasma.

5 Example 5

Recombinant DDAH lowers ADMA in blood of animal model in vivo. rDDAH (10 mg/kg) was administered to mice or rats by single intravenous injection. Blood samples were withdrawn at various times

10 As shown in Table 9, rDDAH lowers ADMA in animal models, but has a relatively short circulating half life in plasma.

TABLE 9. ADMA levels and DDAH activity over time after intravenous administration of rDDAH to mice.

Animal Groups	Plasma ADMA (uM)	Plasma DDAH activity
1. 0 Min, no rDDAH	2.7+0.4	3.4+6.3
2. 15 min after rDDAH	1.4+0.2	129.8+27.2
3. 30 min after rDDAH	2.1+0.4	38.1+28.3
5. 60 min after rDDAH	2.5+0.2	7.3+3.1

Example 6

15 HELMylated recombinant human DDAH, PEGylated recombinant human or Pa-DDAH, or Acylated recombinant human DDAH pharmacokinetics. PEGylation of rDDAH was performed by addition of N-hydroxy succinamide PEG (NHS-PEG) (from Nanocs Inc.) with 10 KD PEG. PEG size from 2KD -40KD was tested. DDAH (1mg/ml) was incubated with 10 molar excess of NHS-PEG at 4C for 24 hours or 37C for 3 hours. PEGylation
 20 reaction was terminated by adding 1M Tris. PEGylated DDAH was separated from free PEG by filtering through a membrane of 50KD cutoff. PEGylated DDAH was also separated by Sepharose Q column chromatography. PEGylation was also performed using maleimide PEG with or without additional cysteine at the C or N terminal of DDAH. Site specific PEGylation is performed with 20 kDa mPEG-butyraldehyde. Activity of PEGylated DDAH (M-DDAH)
 25 is shown in Figures 4A-4C.

Example 7

In vivo studies are conducted in mice, rats, dogs and monkeys to characterize the pharmacokinetics (PK) of wild-type and modified DDAH polypeptides after intravenous (i.v.) and subcutaneous (s.c.) dosing. In some embodiments, recombinant human DDAH, HELMylated recombinant human DDAH, PEGylated recombinant human DDAH, or

5 Acylated recombinant human DDAH polypeptides of the invention are observed to exhibit increased *in vivo* half-life relative to wild-type. Sprague-Dawley rats are also dosed with wild-type and modified DDAH polypeptides (i.v., 1 mg/kg; s.c., 1mg/kg) and the PK profile determined. Three rats are bled at each time point and serum samples are analyzed for ADMA method or by DDAH activity method.

10 Full i.v. pharmacokinetics is also investigated in male Balb/c mice or Sprague Dawley rats for HELMylated DDAH, C12 or C14-DDAH or PEG-DDAH following a single tail vein bolus injection at a dose of 0.5 mg/kg (N=3 each time point, 5 mL/kg). HELMylated DDAH, C12 or C14-DDAH or PEG-DDAH is dosed as a solution in 0.2M Tris, 1M NaCl, 25% propylene glycol, pH 8.5 and the blood samples are collected through retro-orbital bleeding at
15 0.05, 0.25, 0.5, 1, 3, 5, 7, 9, 24 hours post i.v. dose (0.05, 1, 7 h from one group of 3 mice, 0.25, 3, 9 h from the second group of 3 mice and 0.5, 5, 24 h from the third group of 3 mice).

Subcutaneous (s.c.) pharmacokinetics is investigated in male Balb/c mice or Sprague Dowley rats for HELMylated DDAH, C12 or C14-DDAH or PEG-DDAH following a single s.c. injection at a dose of 1 mg/kg (N=3 each time point, 7 mL/kg). Each drug is dosed as a
20 solution in 0.2M Tris, 1M NaCl, 25% propylene glycol, pH 8.5 and the blood samples are collected through retro-orbital bleeding at 0.25, 0.5, 1, 3, 7, and 24 hours post dose (0.25, 1, 7 h from one group of 3 mice, and 0.5, 3, 24 h from the other group of 3 mice).

Following collection, blood samples are centrifuged at 10,000 rpm for 10 min at 4°C to obtain serum and serum samples are stored at -20°C until analysis. The serum
25 concentration of acylated DDAH is analyzed by enzyme-linked immunosorbent assay (ELISA) or DDAH activity method. Pharmacokinetic parameters are estimated using non-compartmental analysis by Kinetica software (Thermo Fisher Scientific Corporation, version 5.0). The peak concentration (C_{max}) and time for C_{max} (T_{max}) are recorded directly from experimental observations. The area under the curve from time zero to the last sampling time
30 [AUC_{last}] and the area under the curve from time zero to infinity [AUC_{total}] are calculated using a combination of linear and log trapezoidal summations. The total plasma clearance, steady-state volume of distribution (V_{ss}), apparent elimination half-life (t_{half}), and mean residence time (MRT) are estimated after i.v. administration. Estimations of AUC and t_{half} are made using a minimum of 3 time points with quantifiable concentrations. The absolute s.c.

bioavailability (F) is estimated as the ratio of dose-normalized AUC values following s.c. and i.v. doses. The PK parameters are calculated when applicable

Example 8

In vivo efficacy of M-DDAH. M-DDAH improved renal function in rat model of acute kidney injury. The efficacy of M-DDAH was assessed using a rat model of acute kidney injury (AKI). AKI was produced by 40 min of ligation of renal artery and then allowing reperfusion. In this model, ischemia- reperfusion leads to a major loss in DDAH activity and impairment of kidney function as measured by an increase in creatinine levels in the blood. M-DDAH (0.25mg/Kg) was administered intravenously 30 min prior to ischemia and a second dose after 3 hours. Fig. 3 shows that M-DDAH significantly attenuated the loss of renal function as indicated by the reduced serum creatinine at 24 hours of reperfusion relative to vehicle injected control rats ($P < 0.05$ by Student's t-test; $N = 7$ per group).

As shown in Figure 5, M-DDAH treatment improved kidney functions in acute kidney injury models as determined by lowering of creatinine levels.

Example 9

Modified human DDAH-2 polypeptides linked to a HELM including but not limited to C12 or C14 acyl group or a water-soluble polymer such as PEG, are synthesized for pharmacokinetic and pharmacodynamic testing. The DDAH polypeptide has the amino acid sequence of SEQ ID NO: 1 or 2, or comprising one or more naturally encoded or amino acid substituted at a single position. Different modified DDAH polypeptides are produced. The DDAH is then reacted with a linker comprising the HELM, C12 or C14 acyl group or a water-soluble polymer such as PEG. These modified DDAHs are then tested to determine activity and pharmacokinetic properties as described in the following examples.

Example 10 - Pharmacokinetic studies of C14-acylatedDDAHs

The C14-acylated DDAHs described in Example 5 and tested for activity and specificity *in vitro* as described in Example 6 are further tested to determine pharmacokinetic (PK) properties in the mouse and rat. As in the preceding examples, the modified DDAH polypeptides each consisted of the DDAH polypeptide of SEQ ID NO: 1 or 2 linked to a linker comprising the HELM, C12 or C14 acyl group, or a water-soluble polymer such as PEG, as shown in Example 7.

Example 11

In order to evaluate potential sites for chemical conjugation, a new method is developed and carried out in order to assess the potential exposure of fatty acids synthetically attached to the DDAH amino acid located at specified positions on the DDAH molecule. The

method is based upon a molecular dynamics protocol which analyzes molecular motions at the atomistic level and is therefore predictive of key biological phenomena. More specifically, a higher relative level of accessibility of the fatty acid is predicted to facilitate its interaction with human serum albumin and therefore should be indicative of increased in vivo half-life.

Accessibility of the fatty acid is assessed by determining the 2-shell water count in an MD simulation of the DDAH or modified DDAH polypeptides. The 2-shell water count is a count of the number of water molecules in direct contact with the pharmacokinetic enhancing moiety (constituting a first shell), as well as a second shell of water molecules in direct contact with the water molecules in the first shell. More specifically, the 2-shell water count includes all waters within 6 Å of the pharmacokinetic enhancing moiety and DDAH.

Accessibility of the fatty acid is also assessed by calculating the SASA (Solvent Accessible Surface Area) for the pharmacokinetic enhancing moiety and the position in the amino acid sequence of DDAH.

The simulation is carried out with a construct composed of a recombinant human DDAH polypeptide linked to a pharmacokinetic enhancing moiety.

All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document are individually indicated to be incorporated by reference for all purposes.

CLAIMS

What is claimed is:

1. A DDAH or modified DDAH polypeptide comprising: a DDAH polypeptide having
5 at least 80% identity to SEQ ID NO: 1; SEQ ID NO:2; SEQ ID NO:5; SEQ ID NO 6;SEQ ID
NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ ID NO 13;or SEQ
ID NO 14, or fragment thereof, wherein said DDAH polypeptide or fragment thereof is
biologically active.
- 10 2. The DDAH or modified DDAH polypeptide of claim 1, and at least one
pharmacokinetic enhancing moiety (HELM).
3. The DDAH or modified DDAH polypeptide of claim 1, or 2 wherein said DDAH
comprises the amino acid sequence of SEQ ID NO: 1; SEQ ID NO:2; SEQ ID NO:5; SEQ
15 ID NO 6;SEQ ID NO 7;SEQ ID NO 9;SEQ ID NO 10;SEQ ID NO 11;SEQ ID NO 12;SEQ
ID NO 13;or SEQ ID NO 14 containing zero, one, two, three, four or more amino acid
substitutions, insertions, or deletions, wherein said substitutions are with natural or non-
naturally encoded amino acids.
- 20 4. The DDAH or modified DDAH polypeptide of any one of the foregoing claims,
wherein said HELM optionally comprises at least one acyl group, polyethylene glycol, lipid,
alkyl group, carbohydrate, polypeptide, polynucleotide, polysaccharide, antibody or antibody
fragment, sialic acid(s), a prodrug, serum albumin, XTEN molecule, Fc molecule, adnectin,
fibronectin, a biologically active molecule, water soluble polymer, one or more DDAH
25 polypeptides, or a combination thereof.
5. The DDAH polypeptide of claim 4, wherein the water-soluble polymer has a molecular
weight of between about 0.1 kDa and about 40 kDa.
- 30 6. The DDAH polypeptide of claim 4, wherein the water-soluble polymer has a molecular
weight of between about 0.1 kDa and about 20 kDa.
7. The DDAH or modified DDAH polypeptide of claim 2, wherein said HELM
comprises at least one acyl group.

8. The DDAH or modified DDAH polypeptide of claim 7, wherein said acyl group comprises a branched or unbranched C8-C30 acyl.
9. The DDAH or modified DDAH polypeptide of claim 7, wherein said acyl group
5 comprises a branched or unbranched C14 acyl, C16 acyl, C18 acyl, or C20 acyl.
10. The DDAH or modified DDAH polypeptide of claim 7, wherein said acyl group is of the formula: $\text{CH}_3(\text{CH}_2)_{12}\text{C}(=\text{O})-$ or $\text{CH}_3(\text{CH}_2)_{14}\text{C}(=\text{O})-$.
- 10 11. The DDAH or modified DDAH polypeptide of claim 7, wherein said acyl group is of the formula: $\text{CH}_3(\text{CH}_2)_{16}\text{C}(=\text{O})-$ or $\text{CH}_3(\text{CH}_2)_{18}\text{C}(=\text{O})-$.
12. The DDAH or modified DDAH polypeptide of claim 2, wherein said pharmacokinetic enhancing moiety comprises at least one alkyl group.
15
13. The DDAH or modified DDAH polypeptide of claim 12, wherein said alkyl group is branched or unbranched.
14. The DDAH or modified DDAH polypeptide of claim 12, wherein said alkyl group is a
20 C8-C30 alkyl group.
15. The DDAH or modified DDAH polypeptide of claim 12, wherein said alkyl group is a C14, C16, C18, or C20 alkyl group.
- 25 16. The DDAH or modified DDAH polypeptide of claim 2, wherein said pharmacokinetic enhancing moiety comprises at least one serum albumin molecule or Fc molecule.
17. The DDAH or modified DDAH polypeptide of claim 16, wherein said serum albumin comprises human serum albumin.
30
18. The DDAH or modified DDAH polypeptide of claim 16, which is linked to the Cys 34 residue of said human serum albumin.

19. The DDAH or modified DDAH polypeptide of claim 2, wherein said pharmacokinetic enhancing moiety comprises at least one XTEN molecule.
20. The DDAH or modified DDAH polypeptide of claim 19, wherein said XTEN
5 molecule is linked to a single modified DDAH polypeptide molecule.
21. The DDAH or modified DDAH polypeptide of claim 19, wherein the DDAH or modified DDAH polypeptide is linked to a site at or near the N-terminus of said XTEN molecule.
10
22. The DDAH or modified DDAH polypeptide of claim 19, wherein said XTEN molecule is linked to multiple modified DDAH polypeptide molecules.
23. The DDAH or modified DDAH polypeptide of claim 19, wherein each said XTEN
15 molecule is linked to one, two, three, four, or five modified DDAH polypeptide molecules.
24. The DDAH or modified DDAH polypeptide of claim 19, wherein each said XTEN molecule is linked to three modified DDAH polypeptide molecules.
- 20 25. The DDAH or modified DDAH polypeptide of claim 24, wherein said three modified DDAH polypeptide molecules are linked to the XTEN molecule at or near the N-terminus, C-terminus, and internal portion of the XTEN molecule, respectively.
- 25 26. The DDAH or modified DDAH polypeptide of any one of claims 19-25, wherein said XTEN molecule comprises an unstructured recombinant polymer (URP) comprising at least 40 contiguous amino acids, wherein: (a) the URP comprises at least three different types of amino acids selected from the group consisting of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P) residues, wherein the sum of said group of amino acids contained in the URP constitutes more than about 80% of the total
30 amino acids of the URP, and wherein said URP comprises more than one proline residue, and wherein said URP possesses reduced sensitivity to proteolytic degradation relative to a corresponding URP lacking said more than one proline residue; (b) at least 50% of the amino acids of said URP are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) the Tepitope score of said URP is less than -5.

27. The DDAH or modified DDAH polypeptide of any one of claims 19-25, wherein said XTEN molecule comprises an unstructured recombinant polymer (URP) comprising at least about 40 contiguous amino acids, and wherein (a) the sum of glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P) residues contained in the URP, constitutes at least 80% of the total amino acids of the URP, and the remainder, when present, consists of arginine or lysine, and the remainder does not contain methionine, cysteine, asparagine, and glutamine, wherein said URP comprises at least three different types of amino acids selected from glycine (G), aspartate (D), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P); (b) at least 50% of the at least 40 contiguous amino acids in said URP are devoid of secondary structure as determined by Chou-Fasman algorithm; and (c) wherein the URP has a Tepitope score less than -4.
28. The DDAH or modified DDAH polypeptide of any one of claims 19-25, wherein each modified DDAH polypeptide is linked to said XTEN molecule through a dibenzylcyclooctyne (DBCO).
29. The DDAH or modified DDAH polypeptide of any one of claims 19-25, wherein said XTEN molecule is further linked to a polyethylene glycol molecule.
30. The DDAH or modified DDAH polypeptide of claim 4, wherein said pharmacokinetic enhancing moiety comprises at least one adnectin.
31. The DDAH or modified DDAH polypeptide of claim 30, wherein said adnectin comprises one or more of a BC loop, a DE loop, and an FG loop.
32. The DDAH or modified DDAH polypeptide of claim 2, wherein said pharmacokinetic enhancing moiety comprises at least one additional DDAH or modified DDAH polypeptide.
33. The DDAH or modified DDAH polypeptide of claim 32, wherein said DDAH polypeptides are linked via their N-termini.

34. The DDAH or modified DDAH polypeptide of claim 32, wherein said DDAH polypeptides are linked to pharmacokinetics enhancing moiety via their C-termini.
35. The DDAH or modified DDAH polypeptide of claim 32, wherein said DDAH
5 polypeptides are linked to pharmacokinetic enhancing moiety via the lysine residues.
36. The DDAH or modified DDAH polypeptide of claim 32 wherein said DDAH polypeptides are linked to pharmacokinetic enhancing moiety via the cysteine residues
- 10 37. The DDAH or modified DDAH polypeptide of any of claims 1-36, which exhibits an *in vivo* half-life of at least 1, 2, 5, 10, 12, 15, 20, 25 or more hours.
38. The DDAH or modified DDAH polypeptide of claim 37, wherein said *in vivo* half-life is determined in human, mouse, rat, dog, cynomolgus monkey, rabbit, horse, cattle, cat,
15 hamster, or rhesus macaque.
39. The DDAH or modified DDAH polypeptide of claim 38, wherein said *in vivo* half-life is determined following subcutaneous or intravenous administration of said DDAH or modified DDAH polypeptide.
20
40. The DDAH or modified DDAH polypeptide of any foregoing claim, which is attached to another biologically active moiety.
41. The DDAH or modified DDAH polypeptide of any one of the foregoing claims,
25 which exhibits efficacy in a model of kidney disease.
42. The DDAH or modified DDAH polypeptide of any one of the foregoing claims, which includes at least one, at least two, or three additions, deletions, or substitutions of amino acids of the mature wild-type human or non-human DDAH.
30
43. A composition comprising a DDAH or modified DDAH polypeptide according to any foregoing claim and a pharmaceutically acceptable carrier.

44. The composition of claim 43, further comprising one or more other active compounds selected from: antidiabetics, hypotensive agents, perfusion-enhancing agents, lipid metabolism modulators, endothelin antagonist, PDE5 inhibitors, p38-kinase inhibitors, PAF-AH inhibitors, antiphlogistics, COX inhibitors, LTB₄-receptor antagonists, analgesics,
5 prostacyclin analogs, endothelin receptor antagonist, PDE5 inhibitor, ACE inhibitor, angiotensin receptor antagonist, diuretics and aspirin.
45. A method of treatment of disease, comprising administering a DDAH or modified DDAH polypeptide or a composition containing a DDAH or modified DDAH polypeptide
10 according to any one of claims 1-42 to a subject in need thereof.
46. The method of claim 45, wherein the disease comprises heart failure or kidney failure, hypertension, resistant hypertension, organ failure or sepsis, pulmonary hypertension, COPD.
- 15 47. The method of claim 45, wherein DDAH or modified DDAH polypeptide or composition containing a DDAH or modified DDAH improves endothelial function.
48. The method of claim 45, wherein said disease comprises one or more of acute decompensated heart failure, right heart failure, left heart failure, global failure, ischemic
20 cardiomyopathy, dilated cardiomyopathy, heart failure associated with congenital heart defects, heart failure associated with heart valve defects, mitral stenosis, mitral insufficiency, aortic stenosis, aortic insufficiency, tricuspid stenosis, tricuspid insufficiency, pulmonary stenosis, pulmonary valve insufficiency, heart failure associated with combined heart valve defects, myocardial inflammation (myocarditis), chronic myocarditis, acute myocarditis, viral
25 myocarditis, diabetic heart failure, alcoholic cardiomyopathy, heart failure associated with cardiac storage disorders, diastolic heart failure, and systolic heart failure.
49. The method of claim 45, wherein the disease comprises cardiovascular disease, lung disease, fibrotic disease or kidney disease.
30
50. The method of claim 45, wherein the disease comprises pancreatitis, inflammation, cancer, gastric cancer, scleroderma, pulmonary fibrosis, renal fibrosis, hepatic fibrosis, non-alcoholic hepatosteatosis.

51. The method of claim 45, wherein the disease comprises fibrosis of the lung, heart, kidney, bone marrow, liver, dermatological fibrosis, or a fibrotic eye disorder.

52. The method of claim 45, wherein the disease comprises of erythropoietin resistance.

53. The method of claim 45, wherein the disease comprises preeclampsia.

54. The method of claim 45, wherein the disease comprises one or more of ischemia, Alzheimer's disease, corneal injury, neurodegenerative disease, cardiovascular disease, fibrotic disease, inflammation, cancer, scleroderma, pulmonary fibrosis, renal fibrosis, hepatic fibrosis, thromboembolic disorders, reperfusion damage following ischemia, micro- and macrovascular lesions (vasculitis), arterial and venous thromboses, atrial fibrillation edemas, myocardial infarction, stroke, cardio protection in connection with coronary artery bypass operations, cardio protection in connection with primary percutaneous transluminal coronary angioplasties (PTCAs), PTCAs after thrombolysis, rescue PTCA, heart transplants and open-heart operations, organ protection in connection with transplants, bypass operations, respiratory disorders, chronic obstructive pulmonary disease, chronic bronchitis, asthma, pulmonary emphysema, bronchiectases, and pulmonary hypertension, in particular pulmonary arterial hypertension, kidney disease, acute and chronic kidney diseases and acute and chronic renal insufficiencies, as well as acute and chronic renal failure with or without the requirement of dialysis, underlying or related kidney diseases, renal hypoperfusion, dialysis induced hypotension, glomerulopathies, glomerular and tubular proteinuria, renal edema, hematuria, chronic glomerulonephritis (including primary, secondary, or acute), membranous and membranoproliferative glomerulonephritis, Alport-Syndrome, glomerulosclerosis, interstitial tubular diseases, nephropathic diseases, primary and inborn kidney diseases, renal inflammation, immunological renal diseases, renal transplant rejection, immune complex induced renal diseases, intoxication induced nephropathic diseases, diabetic and non-diabetic renal diseases, pyelonephritis, cystic kidneys, nephrosclerosis, hypertensive nephrosclerosis, nephrotic syndrome, diseases that are characterized and diagnostically associated with an abnormal reduction in creatinine clearance and/or water excretion, abnormal increased blood concentrations of urea, nitrogen, potassium and/or creatinine, alteration in the activity of renal enzymes including without limitation glutamylsynthetase, urine osmolarity and urine volume, increased microalbuminuria, macroalbuminuria, glomerular and arteriolar lesions, tubular dilation, hyperphosphatemia, disease requiring

dialysis for treatment, renal carcinomas, after incomplete resection of the kidney, dehydration after overuse of diuretics, uncontrolled blood pressure increase with malignant hypertension, urinary tract obstruction and infection, amyloidosis, systemic diseases associated with glomerular damage, Lupus erythematoses, rheumatic immunological systemic diseases, renal artery stenosis, renal artery thrombosis, renal vein thrombosis, analgetics induced nephropathy, renal tubular acidosis, contrast medium induced and drug induced acute and chronic interstitial kidney diseases, metabolic syndrome, dyslipemia, aftereffects associated with acute and/or chronic kidney diseases, pulmonary edema, heart failure, uremia, anemia, electrolyte disturbances, hyperkalemia, hyponatremia, bony and carbohydrate metabolism, lung diseases, asthmatic disorders, pulmonary arterial hypertension (PAH), pulmonary hypertension (PH), left-heart disease, HIV, sickle cell anemia, thromboembolisms (CTEPH), sarcoidosis, COPD, pulmonary fibrosis-associated pulmonary hypertension, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), acute lung injury (ALI), alpha-1-antitrypsin deficiency (AATD), pulmonary fibrosis, pulmonary emphysema (for example pulmonary emphysema induced by cigarette smoke), cystic fibrosis (CF), fibrotic disorders, fibrotic disorders of the internal organs, fibrotic disorders of the lung, fibrotic disorders of the heart, fibrotic disorders of the kidney, fibrotic disorders of the bone marrow fibrotic disorders of the liver, dermatological fibroses, fibrotic eye disorders, osteodegenerative joint dysfunction, angiotensin-II (AngII)-mediated vasoconstriction, endothelin-1 (ET-1)-mediated vasoconstriction, ischemic conditions, ischemia associated with myocardial infarct ischemia associated with wounds, renal pathologies, renal pathologies related to vasoconstriction, or hypertension.

55. The method according to any one of claims 45-54, further comprising administration of another therapeutic agent to the subject.

56. The method according to claim 55, wherein said other therapeutic agent includes an antidiabetics, hypotensive agents, perfusion-enhancing agents, lipid metabolism modulators, antithrombotic agents, antioxidants, chemokine receptor antagonists, p38-kinase inhibitors, NPY agonists, orexin agonists, anorectics, PAF-AH inhibitors, antiphlogistics, COX inhibitors, LTB₄-receptor antagonists, analgesics, prostacyclin analogs, endothelin receptor antagonist, PDE5 inhibitor, ACE inhibitor, angiotensin receptor antagonist, diuretics or aspirin.

57. A DDAH or modified DDAH polypeptide or composition containing a DDAH or modified DDAH polypeptide according to any one of claims 1-42 for use as a medicament.

58. Use of a DDAH or modified DDAH polypeptide or composition containing a DDAH or modified DDAH polypeptide according to any one of claims 1-42 for the manufacture of a medicament for treating heart failure, cardiovascular disease, lung disease, fibrotic disease, kidney disease, pancreatitis, inflammation, cancer, scleroderma, pulmonary fibrosis, renal fibrosis, hepatic fibrosis, coronary fibrosis, bone marrow fibrosis, dermatological fibrosis, a fibrotic eye disorder, ischemia, Alzheimer's diseases, or a corneal injury.

59. A DDAH or modified DDAH polypeptide or composition containing a DDAH or modified DDAH polypeptide according to any one of claims 1-42, wherein said DDAH or modified DDAH polypeptide is conjugated to at least one substance including but not limited to a label, a dye, a polymer, a water-soluble polymer, a derivative of polyethylene glycol, a photocrosslinker, a radionuclide, a cytotoxic compound, a drug, an affinity label, a photoaffinity label, a reactive compound, a resin, another polypeptide or protein, a polypeptide analog, an antibody, an antibody fragment, a metal chelator, a cofactor, a fatty acid, a carbohydrate, a polynucleotide, a DNA, a RNA, an antisense polynucleotide, a saccharide, a water-soluble dendrimer, a cyclodextrin, an inhibitory ribonucleic acid, a biomaterial, a nanoparticle, a spin label, a fluorophore, a metal-containing moiety, a radioactive moiety, a functional group, a group that covalently or noncovalently interacts with other molecules, a photocaged moiety, an actinic radiation excitable moiety, a photoisomerizable moiety, biotin, a derivative of biotin, a biotin analogue, a moiety incorporating a heavy atom, a chemically cleavable group, a photocleavable group, an elongated side chain, a carbon-linked sugar, a redox-active agent, an amino thioacid, a toxic moiety, an isotopically labeled moiety, a biophysical probe, a phosphorescent group, a chemiluminescent group, an electron dense group, a magnetic group, an intercalating group, a chromophore, an energy transfer agent, a biologically active agent, a detectable label, a small molecule, a quantum dot, a nanotransmitter, a radionucleotide, a radiotransmitter, a neutron-capture agent, or any combination of the above.

60. A composition comprising one or more polynucleotides which encode a DDAH or modified DDAH polypeptide having at least 80% identity to SEQ ID NO: 1; SEQ ID NO:2;

SEQ ID NO:5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; or SEQ ID NO 14.

61. An isolated cell, vector, plasmid, prokaryotic cell, eukaryotic cell, virus, mammalian
5 cell, yeast, bacterium, or cell-free translation system comprising the composition of claim 60.

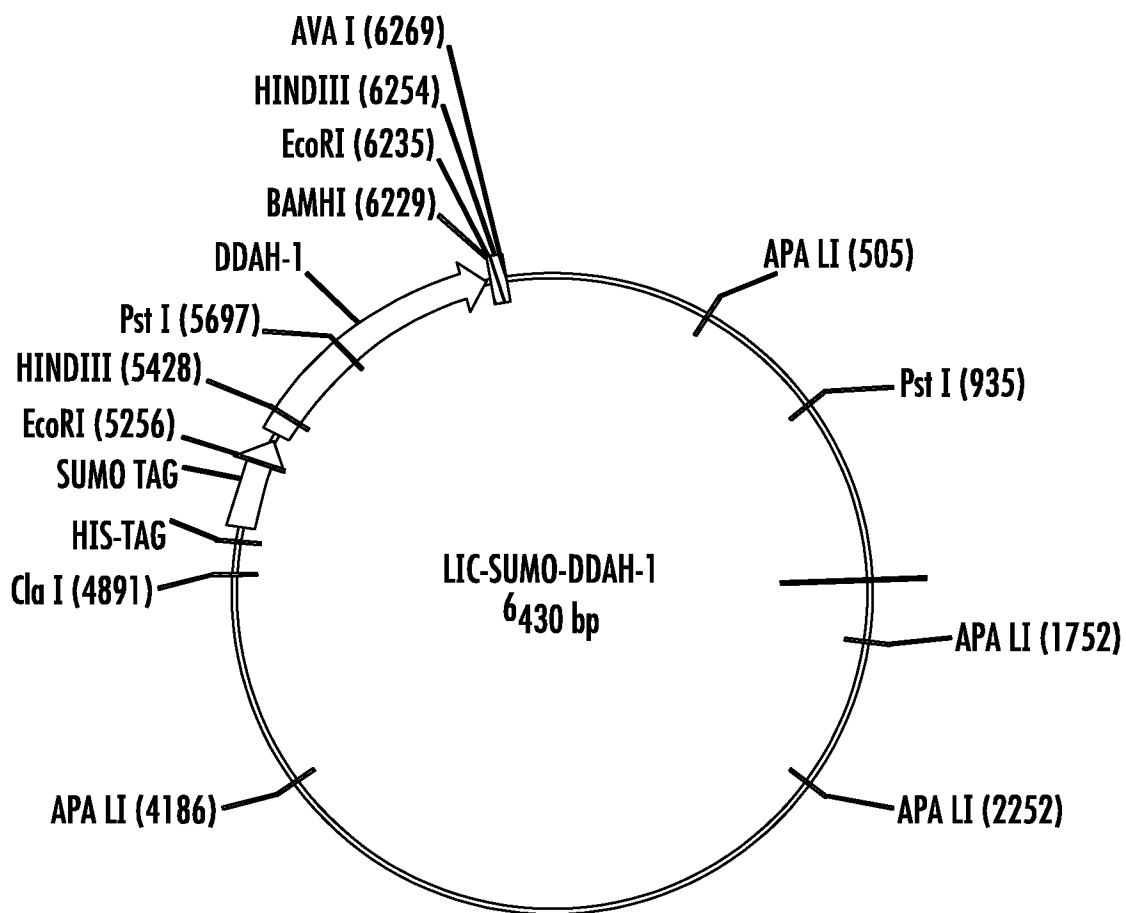
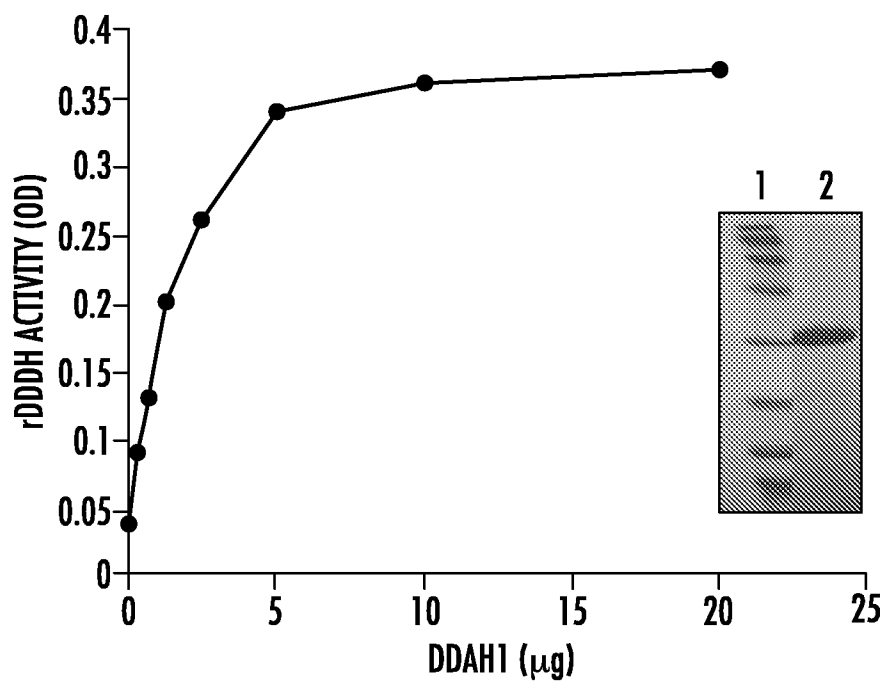
62. The DDAH or modified DDAH polypeptide of claim 3 wherein said one, two, three,
four or more amino acid substitutions comprises substitution of Ser or Ala for 1, 2, 3, 4, or 5
of the Cys residues except for Cys273 of DDAH1, or 1, 2, 3, or 4 of the Cys residues except
10 for Cys273 of DDAH2.

63. The DDAH or modified DDAH polypeptide of claim 3, wherein said DDAH
polypeptide is linked to one, two, three, four or more DDAH polypeptides to form
homodimers, homomultimers, heterodimers, or heteromultimers.

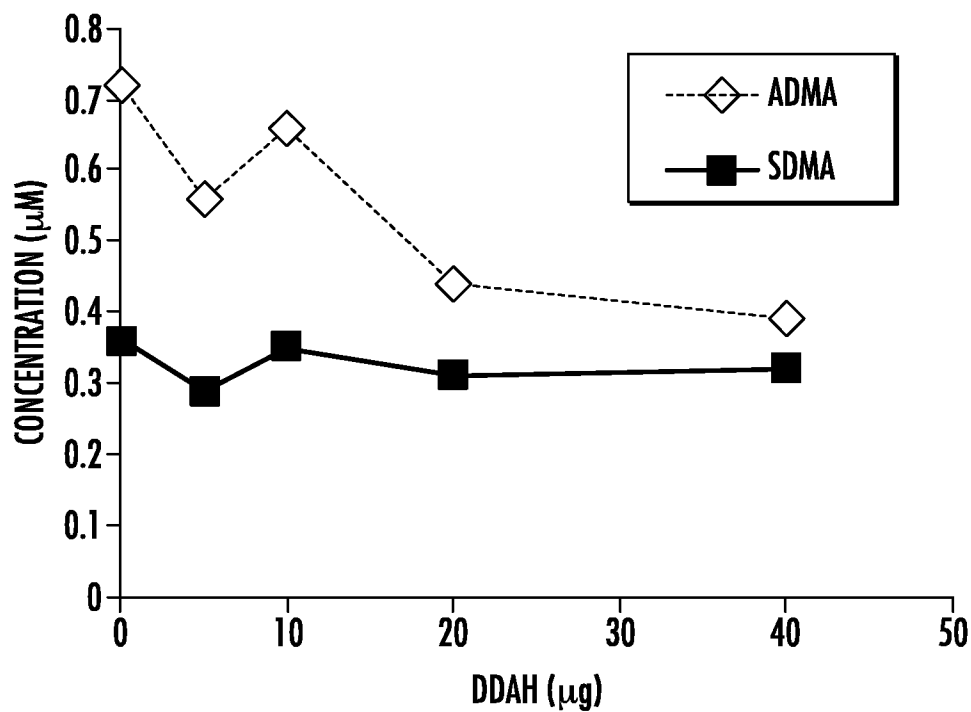
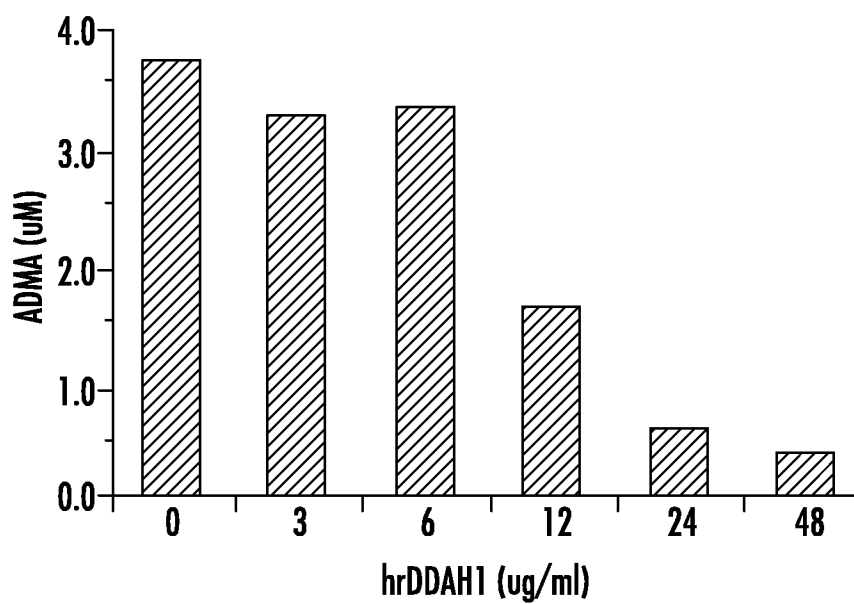
15

64. The DDAH or modified DDAH polypeptide of claim 60, wherein said DDAH
homodimers, homomultimers, heterodimers, or heteromultimers are linked via their N-
termini.

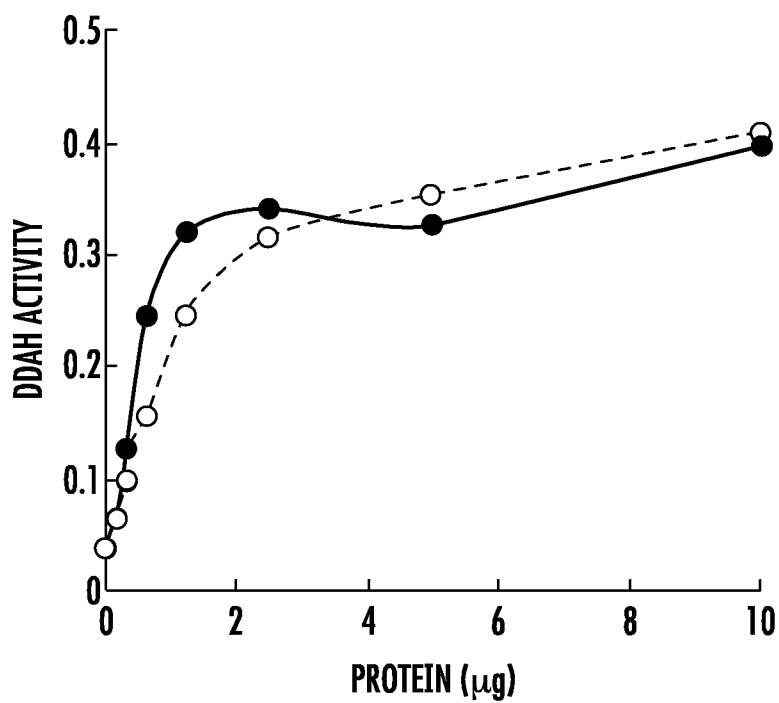
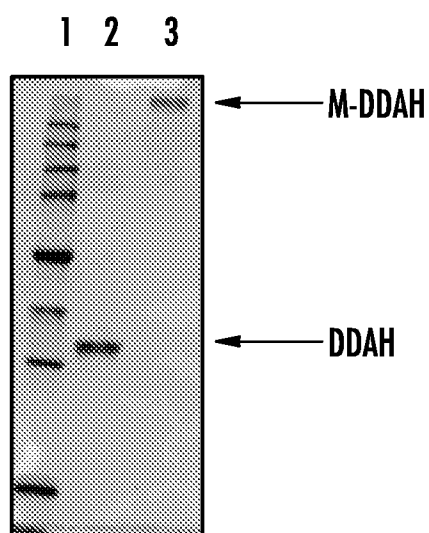
1/4

**FIG. 1****FIG. 2**

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**FIG. 3A****FIG. 3B**

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**FIG. 4A****FIG. 4B**

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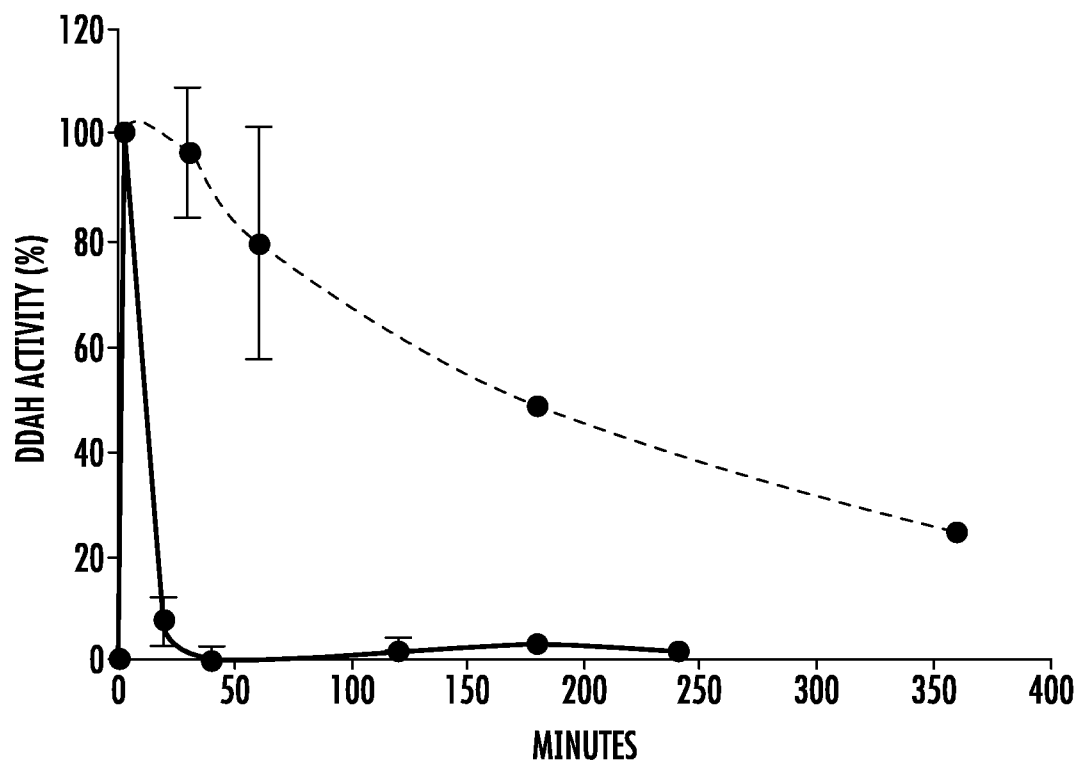


FIG. 4C

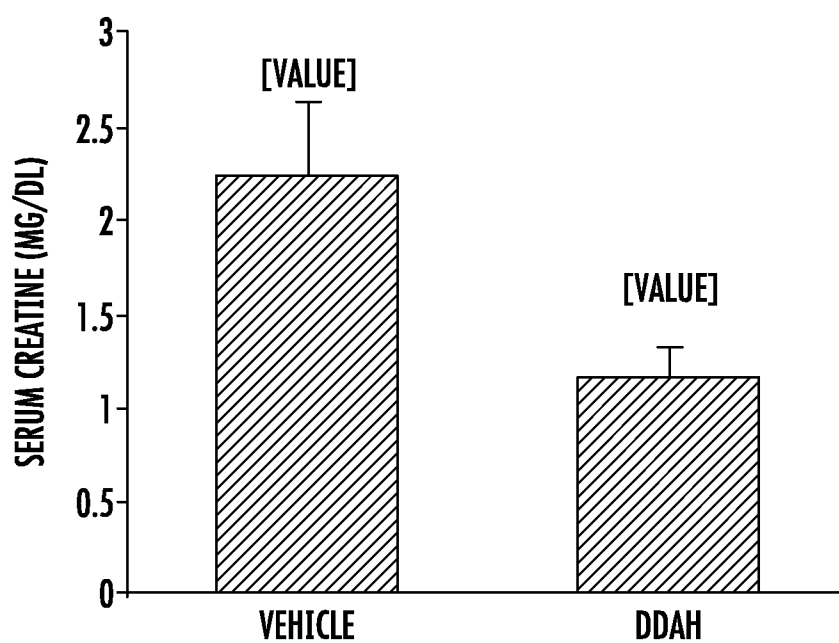


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/044627

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/50; C12N 9/78 (2018.01)

CPC - A61K 38/50; C07K 2319/35; C12N 9/78; C12Y 305/03018 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/94.6; 435/18 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/0153315 A1 (VALLANCE et al) 14 July 2005 (14.07.2005) entire document	1, 3, 60, 61
Y		2, 7-29, 32-36, 62-64
Y	US 2012/0220011 A1 (SCHELLENBERGER et al) 30 August 2012 (30.08.2012) entire document	2, 7-29, 32-36, 62
Y	US 2011/0065633 A1 (DIMARCHI et al) 17 March 2011 (17.03.2011) entire document	7-11
Y	SHARMA et al. "Tumor-targeted delivery of siRNA using fatty acyl-CGKRK peptide conjugates," Scientific Reports, 21 July 2017 (21.07.2017), Vol. 7, Pgs. 1-14. entire document	10, 11
Y	US 2008/0108557 A1 (BEHRENS et al) 08 May 2008 (08.05.2008) entire document	12-15
Y	US 2007/0207952 A1 (SILVA et al) 06 September 2007 (06.09.2007) entire document	16-18
Y	PANG et al. "A modular method for high yield synthesis of site-specific protein-polymer therapeutics," Angewandte Chemie International Edition English, 21 July 2016 (21.07.2016), Vol. 55, No. 35, Pgs. 10296-10300. entire document	28
Y	US 2005/0176060 A1 (MCDONALD et al) 11 August 2005 (11.08.2005) entire document	33, 63, 64
Y	FREY et al. "Structure of the Mammalian NOS Regulator Dimethylarginine Dimethylaminohydrolase: A Basis for the Design of Specific Inhibitors," Structure, 31 May 2006 (31.05.2006), Vol. 14, Iss. 5, Pgs. 901-911. entire document	62

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

07 November 2018

Date of mailing of the international search report

09 JAN 2019

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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/044627

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-14 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/044627

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-6, 30, 31, 37-59
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.