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FGF21 mutants and uses thereof

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(56) Related Art
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Abstract

The invention provides nucleic acid molecules encoding FGF21 mutant polypeptides, FGF21 mutant polypeptides, pharmaceutical compositions comprising FGF21 mutant polypeptides, and methods for treating metabolic disorders using such nucleic acids, polypeptides, or pharmaceutical compositions.

FGF21 MUTANTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/195,761 filed October 10, 2008.

BACKGROUND OF THE INVENTION

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

1. Field of the Invention

The invention relates to nucleic acid molecules encoding FGF21 mutant polypeptides, FGF21 mutant polypeptides, pharmaceutical compositions comprising FGF21 mutant polypeptides, and methods for treating metabolic disorders using such nucleic acids, polypeptides, or pharmaceutical compositions.

2. Background of the Invention

FGF21 is a secreted polypeptide that belongs to a subfamily of fibroblast growth factors (FGFs) that includes FGF19, FGF21, and FGF23 (Itoh et al., 2004, Trend Genet. 20: 563-69). FGF21 is an atypical FGF in that it is heparin independent and functions as a hormone in the regulation of glucose, lipid, and energy metabolism.

FGF21 was isolated from a liver cDNA library as a hepatic secreted factor. It is highly expressed in liver and pancreas and is the only member of the FGF family to be primarily expressed in liver. Transgenic mice overexpressing FGF21 exhibit metabolic phenotypes of slow growth rate, low plasma glucose and triglyceride levels, and an absence of age-associated type 2 diabetes, islet hyperplasia, and obesity. Pharmacological administration of recombinant FGF21 protein in diabetic rodent models results in normalized levels of plasma glucose, reduced triglyceride and cholesterol levels, and improved glucose tolerance and insulin sensitivity. In addition, FGF21 reduces body weight and body fat by increasing energy expenditure, physical activity, and metabolic rate. Experimental research provides support for the pharmacological administration of FGF21 for the treatment of type 2 diabetes, obesity, dyslipidemia, and other metabolic conditions or disorders in humans.

Human FGF21 has a short half-life in vivo. In mice and cynomolgus monkey, the effective half-life of human FGF21 is 1 to 2 hours. In developing an FGF21 protein for use as a therapeutic in the treatment of type 2 diabetes, an increase in half-life would be desirable. FGF21 proteins having an enhanced half-life would allow for less frequent dosing of patients being administered the protein.

SUMMARY OF THE INVENTION

According to one aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) an arginine residue at one or more of positions 56, 59, 69, and 122;
(b) a glycine residue at position 171; or
(c) combinations of (a) – (b).

According to another aspect, the present invention provides an isolated nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d),

and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (d) is not further modified.

According to another aspect, the present invention provides a process of producing a polypeptide encoded by a vector comprising an isolated nucleic acid according to any aspect, embodiment or example herein, said process comprising culturing a host cell comprising said vector under suitable conditions to express the polypeptide, and optionally isolating the polypeptide.

According to another aspect, the present invention provides a polypeptide produced by the process of the previous aspect.

According to another aspect, the present invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d).

According to another aspect, the present invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d),

and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (d) is not further modified.

According to another aspect, the present invention provides a composition comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;

(d) a glycine residue at position 171; or
(e) combinations of (a) – (d),

joined by a linker to a second polypeptide comprising a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d).

According to another aspect, the present invention provides a pharmaceutical composition comprising the isolated polypeptide, or the composition according to any aspect, embodiment or example herein, and a pharmaceutically acceptable formulation agent.

According to another aspect, the present invention provides a method for treating a metabolic disorder comprising administering to a human patient in need thereof the pharmaceutical composition according to the previous aspect.

According to another aspect, the present invention provides use of the isolated polypeptide, or the composition according to any aspect, embodiment or example herein, in the treatment of a metabolic disorder in a human patient, or in the manufacture of a medicament for the treatment of a metabolic disorder in a human patient.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

In one embodiment, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide of SEQ ID NO: 4 having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e).

In another embodiment, the present invention provides an isolated nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e), and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (e) is not further modified.

The present invention also provides vectors and host cells comprising the nucleic acid molecules of the present invention.

In a further embodiment, the present invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e), In yet another embodiment, the present invention provides an isolated nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 91, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e), and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (e) is not further modified.

In still another embodiment, the present invention provides an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 91, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e), and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (e) is not further modified.

Additionally, the present invention provides a composition comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO: 4 optionally having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 91, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e), joined by a linker to a second polypeptide comprising a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 optionally having at least one amino acid substitution that is: (a) a lysine residue at one or more of positions 36, 72, 77, 126 and 175; (b) a cysteine residue at one or more of positions 37, 38, 46, 91, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179; (c) an arginine residue at one or more of positions 56, 59, 69, and 122; (d) a glycine residue at position 170; (e) a glycine residue at position 171; and combinations of (a) – (e).

The present invention also provides chemically modified forms of the polypeptides of the present invention. The chemically modified forms of the polypeptides comprise a polymer attached to the N-terminus and/or a naturally or non-naturally occurring polymer attachment site. The present invention further provides pharmaceutical compositions and methods of treating metabolic disorders such as obesity and diabetes comprising administering the pharmaceutical compositions of the present invention to a patient in need thereof.

Specific embodiments of the present invention will become evident from the following more detailed description of certain embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a cartoon depicting a FGF21 molecule having two polymers (*e.g.*, PEG molecules) attached to the sequence.

Figure 2 comprises four SDS-PAGE gels, showing the degree of PEGylation of nine FGF21 mutants having a single engineered polymer attachment site that have been chemically modified with PEG, namely E37C, R77C and H125C (upper left), D38C, D46C and D79C (upper right), H87C, E91C, G113C (lower left) and G120C, R126C, N121C (lower right).

Figure 3 comprises an SDS-PAGE gel, showing the degree of PEGylation of three FGF21 mutants having a single engineered polymer attachment site that have been chemically modified with a 20kDa methoxy PEG maleimide molecule, namely K69C, R175C and Y179C.

Figure 4 comprises two plots depicting the results of an ELK-luciferase assay performed on FGF21 mutant polypeptides having a single engineered polymer attachment sites that have been chemically modified by the attachment of a 20kDa methoxy PEG maleimide molecule, namely E37C, R77C, E91C, wild-type FGF21 and N-terminally PEGylated FGF21 (upper plot) and G113C, N121C, D46C, wild-type FGF21 and N-terminally PEGylated FGF21 (lower plot).

Figure 5 comprises two plots depicting the results of an ELK-luciferase assay performed on FGF21 mutant polypeptides having a single engineered polymer attachment site, that have been chemically modified by the attachment of a 20kDa methoxy-PEG maleimide, namely H125C, G120C, R126C, wild-type FGF21 and N-terminally PEGylated FGF21 (upper plot) and D79C, D38C, wild-type FGF21 and N-terminally PEGylated FGF21 (lower plot).

Figure 6 comprises two plots depicting results of an ELK-luciferase assay performed on wild-type FGF21 and FGF21 mutant polypeptides having a single engineered polymer attachment site that has been chemically modified by the attachment of a 20kDa methoxy PEG maleimide molecule, namely K69C, D79C, wild-type FGF21 and N-terminally PEGylated FGF21 (upper plot), and R175C, Y179C, wild-type FGF21 and N-terminally PEGylated FGF21 (lower plot).

Figure 7 is a cartoon depicting a Tethered Molecule of the present invention.

Figure 8 is a plot depicting the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (PBS), wild-type FGF21 or N-terminally PEGylated wild-type FGF21.

Figure 9 is a plot depicting the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (PBS), or wild-type FGF21 that was N-terminally PEGylated with 20, 30 or 40 kDa methoxy PEG maleimide molecules.

Figure 10 comprises two plots depicting the percent change in blood glucose levels in mice over a nine day period from time 0 after a single injection of PBS or N-terminally PEGylated FGF21 mutant polypeptides comprising the mutations R77C or R126K, which were further PEGylated at these introduced polymer attachment sites with 20kDa methoxy PEG maleimide molecules and a fusion comprising an Fc molecule and a G170E FGF21 mutant polypeptide(upper plot); or an N-terminally PEGylated FGF21 mutant polypeptide comprising the mutations R77C, which was further PEGylated at this introduced polymer attachment site with 20kDa methoxy PEG maleimide molecule, and P171G (lower plot).

Figure 11 is a plot showing the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8) or FGF21 mutant polypeptides which were dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E91C/H125C, E91C/R175C, E37C/G120C, E37C/H125C, and E37C/R175C; a fusion comprising an Fc molecule and a P171G FGF21 mutant polypeptide was also studied.

Figure 12 is a plot showing the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8) or FGF21 mutant polypeptides which were dually PEGylated with

20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E91C/H121C, G120C/H125C, or E37C/R77C; a fusion comprising an Fc molecule and a G170E FGF21 mutant polypeptide was also studied.

Figure 13 is a plot showing the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8) or FGF21 mutant polypeptides which were dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C, E91C/R175C, E37C/H125C, E37C/R77C/P171G, E91C/R77C/P171G and E37C/R125C/P171G.

Figure 14 is a plot showing the percent change in blood glucose levels in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8), FGF21 mutant polypeptides which were dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C/P171G and E91C/R125C/P171G, or Tethered Molecules comprising two identical FGF21 mutant polypeptides having the same introduced mutations, namely R77C/P171G (2x) and R78C/P172G (2x), which were joined together via a 20kDa methoxy PEG maleimide molecules.

Figure 15 is a plot showing the percent change in blood glucose levels in mice as a function of dose from time 0 after a single injection of vehicle (10mM Tris HCl, 150mM NaCl, pH 8.5), or an FGF21 mutant polypeptide which was dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C/P171G, and administered at doses of 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg or 1 mg/kg.

Figure 16 is a plot showing body weight change in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8) or FGF21 mutant polypeptides which were dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C, E91C/R175C, E37/H125C, E37C/R77C/P171G, E91C/R77C/P171G and E37C/R125C/P171G.

Figure 17 is a plot showing body weight change in mice from time 0 after a single injection of vehicle (10 mM potassium phosphate, 5% sorbitol, pH 8), FGF21 mutant polypeptides which were dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C/P171G and E91C/R125C/P171G, or Tethered Molecules comprising two

FGF21 mutant polypeptides having the same introduced mutations, namely R37C/P171G (2x) and R77C/P171G (2x), which were joined together via a 20kDa methoxy PEG maleimide molecule.

Figure 18 is a plot showing body weight change in mice as a function of dose from time 0 after a single injection of vehicle (10mM Tris HCl, 150mM NaCl, pH 8.5), or an FGF21 mutant polypeptide which was dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C/P171G, and administered at five different doses.

Figure 19A-19F is a series of six plots showing the change in body weight of mice during an eight week kidney vacuole study using once weekly dosing of vehicle (squares), 5 mg/kg (triangles) and 25 mg/kg (open circles) PEGylated FGF21 molecules. Mice dosed with dual cysteine targeted PEG-FGF21 showed a sustained weight loss, while those dosed with Tethered Molecules showed primarily transient weight loss.

Figure 20 comprises two bar graphs depicting the results of an eight week kidney vacuole study in mice injected with vehicle or an FGF21 mutant polypeptide which was dually PEGylated with 20kDa methoxy PEG maleimide molecules at introduced polymer attachment sites, namely E37C/R77C/P171G; E37/H125C/P171G; E91C/H125C/P171G; E37C/P171G; R77C/P171G; and R77C/P171G; two different doses were tested.

DETAILED DESCRIPTION OF THE INVENTION

A human FGF21 protein having enhanced properties such as an increased half-life can be prepared using the methods disclosed herein and standard molecular biology methods. It is known that by binding one or more water soluble polymers, such as PEG molecules, to a protein the half life of the protein can be extended. Thus, in various embodiments, the half life of native FGF21 can be extended by introducing amino acid substitutions into the protein to form points at which a polymer can be attached to the FGF21 protein. Such modified proteins are referred to herein as FGF21 mutants and form embodiments of the present invention. Polymers can also be introduced at the N-terminus of the FGF21 molecule in conjunction with the introduction of a non-naturally occurring polymer attachment site.

Recombinant nucleic acid methods used herein, including in the Examples, are generally those set forth in Sambrook *et al., Molecular Cloning: A Laboratory*

Manual (Cold Spring Harbor Laboratory Press, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., Green Publishers Inc. and Wiley and Sons 1994), both of which are incorporated herein by reference for any purpose.

1. General Definitions

As used herein, the term "a" means one or more unless specifically indicated otherwise.

The term "isolated nucleic acid molecule" refers to a nucleic acid molecule of the invention that (1) has been separated from at least about 50 percent of proteins, lipids, carbohydrates, or other materials with which it is naturally found when total nucleic acid is isolated from the source cells, (2) is not linked to all or a portion of a polynucleotide to which the "isolated nucleic acid molecule" is linked in nature, (3) is operably linked to a polynucleotide which it is not linked to in nature, or (4) does not occur in nature as part of a larger polynucleotide sequence. Preferably, the isolated nucleic acid molecule of the present invention is substantially free from any other contaminating nucleic acid molecules or other contaminants that are found in its natural environment that would interfere with its use in polypeptide production or its therapeutic, diagnostic, prophylactic or research use.

The term "isolated polypeptide" refers to a polypeptide of the present invention that (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is naturally found when isolated from the source cell, (2) is not linked (by covalent or noncovalent interaction) to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature, (3) is operably linked (by covalent or noncovalent interaction) to a polypeptide with which it is not linked in nature, or (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic or research use.

The term "vector" is used to refer to any molecule (*e.g.*, nucleic acid, plasmid, or virus) used to transfer coding information to a host cell.

The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control the expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

The term "host cell" is used to refer to a cell which has been transformed, or is capable of being transformed with a nucleic acid sequence and then of expressing a selected gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present.

The term "naturally occurring" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature and are not manipulated by man. Similarly, "non-naturally occurring" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by man. When used in connection with nucleotides, the term "naturally occurring" refers to the bases adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U). When used in connection with amino acids, the term "naturally occurring" refers to the 20 amino acids alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), valine (V), tryptophan (W), and tyrosine (Y).

The term "FGF21 polypeptide" refers to any naturally occurring wild-type polypeptide expressed in humans. For purposes of this application, the term "FGF21 polypeptide" can be used interchangeably to refer to the full-length FGF21 polypeptide, which consists of 209 amino acid residues (SEQ ID NO: 2) and which is encoded by the nucleotide sequence of SEQ ID NO: 1; and the mature form of the polypeptide, which consists of 181 amino acid residues (SEQ ID NO: 4), which is encoded by the nucleotide sequence of SEQ ID NO: 3, and in which the 28 amino acid residues at the amino-terminal end of the full-length FGF21 polypeptide (*i.e.*, which constitute the signal peptide) have been removed. An FGF21 polypeptide can be expressed with or without an N-terminal Methionine residue; as noted herein, an N-terminal Methionine residue can be added by design or as a function of a bacterial expression system.

The term "biologically active," as applied to an FGF21 polypeptide, including FGF21 mutant polypeptides described herein, refers to a naturally occurring activity of a wild-type FGF21 polypeptide, such as the ability to lower blood glucose, insulin, triglyceride, or cholesterol; reduce body weight; and improve glucose tolerance, energy expenditure, or insulin sensitivity. As applied to a FGF21 mutant polypeptide, the term is not dependent on the type or number of modifications that have been introduced into the FGF21 mutant polypeptide. For example, some FGF21 mutant polypeptides possess a somewhat decreased level of FGF21 activity relative to the wild-type FGF21 polypeptide but are nonetheless be considered to be biologically active FGF21 mutant polypeptides. Differences in the activity of a particular FGF21 mutant polypeptide may be observed between *in vivo* and *in vitro* assays; any such differences are related to the particular assays used. Such an observation, however, does not affect the meaning of the term "biologically active," and FGF21 mutant polypeptides showing a naturally occurring activity of a wild-type FGF21 polypeptide, such as the ability to lower blood glucose, insulin, triglyceride, or cholesterol; reduce body weight; and improve glucose tolerance, energy expenditure, or insulin sensitivity, in any *in vivo* or *in vitro* assay are "biologically active."

The terms "effective amount" and "therapeutically effective amount" are used interchangeably and refer to the amount of an FGF21 mutant polypeptide used to support an observable level of one or more biological activities of the wild-type FGF21 polypeptide, such as the ability to lower blood glucose, insulin, triglyceride, or cholesterol levels; reduce body weight; or improve glucose tolerance, energy expenditure, or insulin sensitivity.

The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of an FGF21 mutant polypeptide. Examples of such materials can be found in *Remington, supra*, incorporated herein by reference.

The term "Tethered Molecule" refers to a construct comprising two or more FGF21 molecules tethered together by a linker molecule. A Tethered Molecule comprises at least two FGF21 polypeptides, at least one of which is an FGF21 mutant polypeptide as described herein, but can comprise three, four or more FGF21 or FGF21 mutant polypeptides joined together by linkers. Thus, the term Tethered Molecule is not restricted to a molecule comprising combinations of only one or two FGF21 or FGF21 mutant polypeptides.

The term "polymer attachment site" refers to a region of the primary amino acid sequence of a polypeptide (*e.g.*, an FGF21 polypeptide) that is chemically adaptable to covalent association with a polymer (*e.g.*, PEG molecules of all molecular weights, polymeric mannose, glycans, etc). A polymer attachment site can mean a single amino acid (*e.g.*, cysteine, lysine, arginine or a suitable non-naturally occurring amino acid) or the term can refer to two or more amino acids that are adjacent to each other either in sequence or in space.

The term "chemically modified," when used in relation to a FGF21 wild-type or FGF21 mutant polypeptide as disclosed herein, refers to a FGF21 polypeptide that has been modified from its naturally occurring state by the covalent attachment of one or more heterologous molecules. Examples of heterologous molecules include polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, hydroxyl ethyl starch (HES), and polyvinyl alcohol. Examples of chemically modified FGF21 polypeptides include PEGylated wild-type FGF21 and FGF21 mutant polypeptides.

2. FGF21 Mutant Polypeptides

In various aspects, the present invention discloses a series of methods for the site-directed PEGylation of FGF21 and FGF21 mutant polypeptides, which can enhance the pharmacokinetic properties of the FGF21 molecule while minimizing the impact on the *in vitro* activity. The enhanced pharmacokinetic profile of these PEGylated FGF21 molecules has an impact on the *in vivo* efficacy of the molecule by increasing exposure to the therapeutic agent. In addition, the strategies described herein are compatible with creating multiple PEGylation sites, which may both further enhance the pharmacokinetic properties of the molecule, and lower their vacuole-forming potential. Two principle strategies were employed to accomplish this, as described herein.

In one aspect, the present invention relates to FGF21 sequences into which one or more modifications have been introduced. Thus, the terms "FGF21 mutant polypeptide" and "FGF21 mutant," which can be used interchangeably, refer to an FGF21 polypeptide in which a wild-type FGF21 amino acid sequence (*e.g.*, SEQ ID NOs 2 or 4) has been modified. Such modifications include, but are not limited to, one or more amino acid substitutions, including substitutions with non-naturally occurring amino acid analogs, insertions and truncations. Thus, FGF21 polypeptide mutants include, but are not limited to, site-directed FGF21 mutants, such as those introducing a non-naturally occurring polymer attachment site, or which impart a degree of resistance to proteolysis, as described herein. For the purpose of identifying the specific amino acid substitutions of the FGF21 mutants of the present invention, the numbering of the amino acid residues truncated or mutated corresponds to that of the mature 181-residue FGF21 polypeptide (*i.e.*, the N terminus of the sequence begins HPIPD, and these residues are designated as residues 1, 2, 3, 4 and 5, respectively). An N-terminal methionine residue can but does not need to be present; this N-terminal methionine residue is not included in the numbering scheme of the protein.

As stated, FGF21 mutants, including truncated forms of FGF21 comprising one or more substitutions or insertions, which comprise non-naturally occurring amino acids form an embodiment of the present invention. Such insertions or substitutions can impart various properties, including acting as sites for polymer attachment. In such cases, non-naturally occurring amino acids can be incorporated into an FGF21 sequence in addition to the various mutations described herein. Accordingly, an FGF21 mutant can comprise one or more of the mutations described herein and can further comprise one or more non-naturally occurring amino acids. A non-limiting lists of examples of non-naturally occurring amino acids that can be inserted into an FGF21 sequence or substituted for a wild-type residue in an FGF21 sequence include β-amino acids, homoamino acids, cyclic amino acids and amino acids with derivatized side chains. Examples include (in the L-form or D-form; abbreviated as in parentheses): para-acetyl-phenylalanine, para-azido-phenylalanine, para-bromo-phenylalanine, para-iodo-phenylalanine and para-ethynyl-phenylalanine, citrulline (Cit), homocitrulline (hCit), Nα-methylcitrulline (NMeCit), Nα-methylhomocitrulline (Nα-MeHoCit), ornithine (Orn), Nα-Methylornithine (Nα-MeOrn or NMeOrn), sarcosine (Sar), homolysine (hLys or hK), homoarginine (hArg or hR), homoglutamine (hQ), Nα-methylarginine (NMeR), Nα-methylleucine (Nα-MeL or NMeL), N-methylhomolysine (NMeHoK), Nα-methylglutamine (NMeQ), norleucine (Nle), norvaline (Nva), 1,2,3,4-tetrahydroisoquinoline (Tic), Octahydroindole-2-carboxylic acid (Oic), 3-(1-naphthyl)alanine (1-Nal), 3-(2-naphthyl)alanine (2-Nal), 1,2,3,4-tetrahydroisoquinoline (Tic), 2-indanylglycine (IgI), para-iodophenylalanine (pI-Phe), para-aminophenylalanine (4AmP or 4-Amino-Phe), 4-guanidino phenylalanine (Guf), glycyllysine (abbreviated "K(Nε-glycyl)" or "K(glycyl)" or "K(gly)"), nitrophenylalanine (nitrophe), aminophenylalanine (aminophe or Amino-Phe), benzylphenylalanine (benzylphe), γ-carboxyglutamic acid (γ-carboxyglu), hydroxyproline (hydroxypro), p-carboxyl-phenylalanine (Cpa), α-aminoadipic acid (Aad), Nα-methyl valine (NMeVal), N-α-methyl leucine (NMeLeu), Nα-methylnorleucine (NMeNle), cyclopentylglycine (Cpg), cyclohexylglycine (Chg), acetylarginine (acetylarg), α, β-diaminopropionoic acid (Dpr), α, γ-diaminobutyric acid (Dab), diaminopropionic acid (Dap), cyclohexylalanine (Cha), 4-methyl-phenylalanine (MePhe), β, β-diphenyl-alanine (BiPhA), aminobutyric acid (Abu), 4-phenyl-phenylalanine (or biphenylalanine; 4Bip), α-amino-isobutyric acid (Aib), beta-alanine, beta-aminopropionic acid, piperidinic acid, aminocaprioic acid, aminoheptanoic acid, aminopimelic acid, desmosine, diaminopimelic acid, N-ethylglycine, N-ethylaspargine, hydroxylysine, allo-hydroxylysine, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, 4-hydroxyproline (Hyp), γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-methylarginine, 4-Amino-O-Phthalic Acid (4APA), and other similar amino acids, and derivatized forms of any of those specifically listed.

In other embodiments of the present invention, an FGF21 mutant polypeptide comprises an amino acid sequence that is at least about 85 percent identical to a wild-type FGF21 amino acid sequence (*e.g.*, SEQ ID NOs: 2 or 4), but wherein the specific residues introducing non-naturally occurring polymer attachment sites in the FGF21 mutant polypeptide have not been further modified. In other words, with the exception of residues in the FGF21 mutant sequence that have been modified in order to introduce a non-naturally occurring polymer attachment site or a mutation to increase resistance to proteolysis, about 15 percent of all other amino acid residues in the FGF21 mutant sequence may be modified. For example, in the FGF21 mutant polypeptide G170C, up to 15 percent of all amino acid residues other than the glycine residue at position 170 could be modified. In still other embodiments, an FGF21 polypeptide mutant comprises an amino acid sequence that is at least about 90 percent, or about 95, 96, 97, 98, or 99 percent identical to a wild-type FGF21 amino acid sequence (*e.g.*, SEQ ID NO: 2, 4, 6 or 8), but wherein the specific residues that have been modified to introduce a non-naturally occurring polymer attachment site or enhance proteolysis resistance have not been further modified. Such FGF21 mutant polypeptides possess at least one activity of the wild-type FGF21 polypeptide.

FGF21 mutant polypeptides can be generated by introducing amino acid substitutions, either conservative or non-conservative in nature and using naturally or non-naturally occurring amino acids, at particular positions of the FGF21 polypeptide. Such substitutions can be made in addition to substitutions designed or observed to impart a desirable property to the FGF21 polypeptide. By way of example, a FGF21 mutant polypeptide can comprise a substitution designed to achieve a desirable property, such as introducing a non-naturally occurring polymer attachment site or enhancing resistance to proteolysis, and can further comprise one or more conservative or non-conservative substitutions which may, but need not, maintain the biological activity of the wild-type FGF21 polypeptide.

FGF21 mutations can be conservative or non-conservative. A "conservative amino acid substitution" can involve a substitution of a native amino acid residue (*i.e.*, a residue found in a given position of the wild-type FGF21 polypeptide sequence) with a nonnative residue (*i.e.*, a residue that is not found in a given position of the wild-type FGF21 polypeptide sequence) such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues that are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties.

Naturally occurring residues can be divided into classes based on common side chain properties:

(1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
(2) neutral hydrophilic: Cys, Ser, Thr;
(3) acidic: Asp, Glu;
(4) basic: Asn, Gln, His, Lys, Arg;
(5) residues that influence chain orientation: Gly, Pro; and
(6) aromatic: Trp, Tyr, Phe.

Conservative substitutions can involve the exchange of a member of one of these classes for another member of the same class. Non-conservative substitutions can involve the exchange of a member of one of these classes for a member from another class.

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. An exemplary (but not limiting) list of amino acid substitutions is set forth in Table 1.

Table 1

Amino Acid Substitutions

Original Residue	Exemplary Substitutions
Ala	Val, Leu, Ile
Arg	Lys, Gln, Asn
Asn	Gln
Asp	Glu
Cys	Ser, Ala
Gln	Asn
Glu	Asp
Gly	Pro, Ala
His	Asn, Gln, Lys, Arg
Ile	Leu, Val, Met, Ala, Phe
Leu	Ile, Val, Met, Ala, Phe
Lys	Arg, Gln, Asn
Met	Leu, Phe, Ile
Phe	Leu, Val, Ile, Ala, Tyr
Pro	Ala
Ser	Thr, Ala, Cys
Thr	Ser
Trp	Tyr, Phe
Tyr	Trp, Phe, Thr, Ser
Val	Ile, Met, Leu, Phe, Ala

2.A. FGF21 Mutant Polypeptides Comprising a Proteolysis-resistant Mutation

It has been determined that the mature form of FGF21 (*i.e.*, the 181 residue form) undergoes *in vivo* degradation, which was ultimately determined to arise from proteolytic attack. The *in vivo* degradation of mature FGF21 has been found to lead to a shorter effective half-life, which can adversely affect the therapeutic potential of the molecule. Accordingly, a directed study was performed to identify FGF21 mutants that exhibit a resistance to proteolysis. As a result of this investigation, the sites in the mature FGF21 polypeptide that were determined to be particularly susceptible to proteolysis include the peptide bond between the amino acid residues at positions 4-5, 20-21, 151-152, and 171-172.

A non-limiting list of exemplary substitutions that eliminate the proteolytic effect observed in mature FGF21 while not affecting the biological activity of the protein to an unacceptable degree that can be employed in the present invention is presented in Table 2. Table 2 is demonstrative only and other proteolysis resistant substitutions can be identified and employed in the present invention. These proteolysis-resistant substitutions can be made in addition to substitutions that introduce one or more non-naturally occurring polymer attachment sites, thus generating a FGF21 mutant polypeptide exhibiting the desirable characteristics imparted by each type of mutation.

Table 2

Representative Substitutions that Provide Proteolysis Resistance

Amino Acid Position	Native Residue	Mutations
19	Arg	Gln, Ile, Lys
20	Tyr	His, Leu, Phe
21	Leu	Ile, Phe, Tyr, Val
22	Tyr	Ile, Phe, Val
150	Pro	Ala, Arg
151	Gly	Ala, Val
152	Ile	His, Leu, Phe, Val
170	Gly	Ala, Asn, Asp, Cys, Gln, Glu, Pro, Ser
171	Pro	Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Lys, Ser, Thr, Trp, Tyr
172	Ser	Leu, Thr
173	Gln	Arg, Glu

Preferably, but not necessarily, FGF21 mutant polypeptides comprising a proteolysis-resistant mutation have biological activity essentially the same as, or greater than, the activity of wild-type FGF21. Therefore, another embodiment of the present invention is directed to FGF21 mutant polypeptides that comprise one or more non-naturally occurring polymer attachment sites and are resistant to proteolysis, yet still retain biological activity that is the same as, or greater than, wild-type FGF21. Although less desirable in some cases, FGF21 mutants that comprise one or more non-naturally occurring polymer attachment sites and are resistant to proteolysis but exhibit somewhat decreased biological activity form another embodiment of the present invention. In some cases it can be desirable to maintain a degree of proteolysis, and consequently, FGF21 mutants comprising one or more non-naturally occurring polymer attachment sites and which are resistant to proteolysis and yet still allow some degree of proteolysis to occur also form another embodiment of the present invention.

As with all FGF21 mutant polypeptides of the present invention, proteolysis-resistant FGF21 mutant polypeptides comprising one or more non-naturally occurring polymer attachment sites can be prepared as described herein. Those of ordinary skill in the art, for example, those familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the proteolysis-resistant FGF21 mutants comprising one or more non-naturally occurring polymer attachment sites of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

Proteolysis-resistant FGF21 mutants comprising one or more non-naturally occurring polymer attachment sites which are resistant to proteolysis can be chemically modified using methodology known in the art and described herein. Chemically modifying (*e.g.*, PEGylating) a proteolysis-resistant FGF21 mutant polypeptide comprising one or more non-naturally occurring polymer attachment sites can generate molecules that exhibit both proteolysis resistance and desirable pharmacokinetic and pharmacodynamic properties.

2.B. FGF21 Mutant Polypeptides Comprising a Non-naturally Occurring Polymer Attachment Site

In various aspects of the present invention, FGF21 mutant polypeptides are disclosed. In another aspect, the FGF21 mutant polypeptides of the present invention include FGF21 polypeptides into which a non-naturally occurring polymer (*e.g.*, PEGylation) attachment site(s) has been introduced. In yet another aspect of the present invention, truncated forms of FGF21 mutant polypeptides into which a non-naturally occurring polymer (*e.g.*, PEG) attachment site(s) has been introduced are disclosed. FGF21 mutant polypeptides comprising a non-naturally occurring polymer attachment site and one or more conservative or non-conservative substitutions, which may but need not maintain the biological activity of the wild-type FGF21, form another aspect of the invention. The various FGF21 polypeptide mutants of the present invention can be prepared as described herein and in references provided herein.

In one embodiment, FGF21 polypeptide mutants of the present invention are modified by introducing a non-naturally occurring polymer attachment site. Indeed, in one aspect this is a goal of the FGF21 mutants of the present invention, namely the introduction of one or more non-naturally occurring polymer attachment sites such that half life-extending polymers can be attached to the FGF21 polypeptide mutant at desired locations. The polymer selected is typically, but not necessarily, water-soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Included within the scope of suitable polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable, such as PEG of a suitable molecular weight. Non-water soluble polymers, such as PEG fatty acid blockcopolymers can also be conjugated to FGF21 polypeptide mutants of the present invention and forms an aspect of the invention.

The activity of the FGF21 mutant polypeptides of the present invention can be assayed in a variety of ways, for example, using an *in vitro* ELK-luciferase assay as described herein in Example 10.

The activity of the FGF21 mutant polypeptides of the present invention can also be assessed in an *in vivo* assay, such as with ob/ob mice as shown in Example 12. Generally, to assess the *in vivo* activity of one or more of these polypeptides, the polypeptide can be administered to a test animal intraperitoneally. After one or more desired time periods, a blood sample can be drawn, and blood glucose levels can be measured.

As with all FGF21 mutant polypeptides of the present invention, these polypeptides can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

The FGF21 mutant polypeptides of the present invention can be prepared as described in Example 7. Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the FGF21 mutant polypeptides of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See*, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

Following the preparation of a FGF21 mutant polypeptide, the polypeptide can be chemically modified by the attachment of a polymer, as described herein in Example 9.

3. Truncated FGF21 Mutant Polypeptides Comprising a Non-naturally Occurring Polymer Attachment Site

One embodiment of the present invention is directed to truncated forms of a mutant FGF21 polypeptide comprising one or more non-naturally occurring polymer attachment sites. Such truncated mutant polypeptides can, but need not, be chemically modified.

As used herein, the term "truncated FGF21 mutant polypeptide" refers to an FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide in which one or more amino acid residues have been removed from the amino-terminal (or N-terminal) end of the FGF21 polypeptide, one or more amino acid residues have been removed from the carboxyl-terminal (or C-terminal) end of the FGF21 mutant polypeptide or chemically modified FGF21 polypeptide, or one or more amino acid residues have been removed from both the N-terminal and C-terminal ends of the

FGF21 mutant polypeptide or chemically modified FGF21 polypeptide.

The activity of N-terminally truncated mutant FGF21, C-terminally truncated mutant FGF21 and mutant FGF21 molecules truncated at both the N- and C-terminal ends of the molecule, as well as chemically modified forms of these mutants, can be assayed in a variety of ways, for example, using an *in vitro* ELK-luciferase assay as described herein in Example 10.

The activity of the truncated mutant FGF21 polypeptides and chemically modified truncated mutant FGF21 polypeptides of the present invention can also be assessed in an *in vivo* assay, such as ob/ob mice as shown in Example 12. Generally, to assess the *in vivo* activity of one or more of these polypeptides, the polypeptide can be administered to a test animal intraperitoneally. After one or more desired time periods, a blood sample can be drawn, and blood glucose levels can be measured.

As with all FGF21 mutants of the present invention, truncated mutant FGF21 and chemically modified truncated mutant FGF21 polypeptides can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

The truncated FGF21 mutant polypeptides of the present invention can be prepared as described in Examples 7. Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the truncated mutant FGF21 polypeptides of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

Following the preparation of a truncated mutant FGF21 polypeptide, the polypeptide can be chemically modified by the attachment of a polymer, as described in Example 9.

4. Capped and C-terminal FGF21 Mutant Polypeptides

In another embodiment, the present invention is directed to mutant FGF21 polypeptides comprising one or more non-naturally occurring polymer attachment sites which have been capped by the addition of another one or more residues to the C-terminus of the polypeptide, extending the amino acid sequence beyond that of the wild –type protein. In yet another embodiment, the present invention is directed to FGF21 mutant polypeptides comprising one or more non-naturally occurring polymer attachments sites that further comprise one or more C-terminal mutations. Such capped and C-terminally mutated FGF21 mutant polypeptides can, but need not, be chemically modified.

As used herein, the term "capped FGF21 mutant polypeptide" refers to an FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide in which one or more amino acid residues have been added to the C terminus of the FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide. Any naturally or non-naturally occurring amino acid can be used to cap an FGF21 mutant polypeptide, including one or more proline residues and one or more glycine residues. Although the wild-type FGF21 sequence is only 181 residues long, a capped FGF21 mutant polypeptide extends the length of the polypeptide one residue for each added capping residue; consistent with the numbering scheme of the present disclosure, cap residues are numbered beginning with 182. Thus, a single proline capping residue is indicated as P182. Longer caps are possible and are numbered accordingly (e.g., X182, Y183, Z184, where X, Y and Z are any naturally or non-naturally occurring amino acid). Capping residues can be added to a mutant FGF21 polypeptide using any convenient method, such as chemically, in which an amino acid is covalently attached to the C-terminus of the polypeptide by a chemical reaction. Alternatively, a codon encoding a capping residue can be added to the FGF21 mutant polypeptide coding sequence using standard molecular biology techniques. Any of the mutant FGF21 polypeptides described herein can be capped with one or more residues, as desired.

C-terminal mutations form another aspect of the present invention. As used herein, the term "C-terminal mutation" referes to one or more changes in the region of residues 91-181 (or longer if the polypeptide is capped) of a mutant FGF21 polypeptide. A C-terminal mutation introduced into a FGF21 mutant polypeptide sequence will be in addition to one or more mutations which introduce a non-naturally occurring polymer attachment site. Although C-terminal mutations can be introduced at any point in the region of 91-181 of the FGF21 mutant polypeptide sequence, exemplary positions for C-terminal mutations include positions 171, 172, 173, 174, 175, 176, 177, 178, 179, 180 and 181. C-terminal mutations can be introduced using standard molecular biological techniques, such as those described herein. Any of the mutant FGF21 polypeptides described herein can comprise a C-terminal mutation.

Examples of positions and identities for capped and/or C-terminally mutations are shown in Table 3:

Table 3

Examples of Capping Positions and/or C-terminally Mutations

E37C, R77C, P171G, P182
P171G, S181P, P182
P171G, S181P
P171G, S181T
P171G, S181G
P171G, S181A
P171G, S181L
P171G, A180P
P171G, A180G
P171G, A180S
P171G, Y179P
P171G, Y179G
P171G, Y179S
P171G, Y179A
P171G, L182
P171G, G182
P171G, P182
P171G, G182, G183
P171G, G182, G183, G184, G185, G186

The activity of capped and/or C-terminally mutated FGF21 mutant polypeptides, as well as chemically modified forms of these mutants, can be assayed in a variety of ways, for example, using an *in vitro* ELK-luciferase assay as described herein in Example 10.

The activity of the capped and/or C-terminally mutated FGF21 mutant polypeptides, and chemically modified capped and/or C-terminally mutated FGF21 mutant polypeptides, of the present invention can also be assessed in an *in vivo* assay, such as ob/ob mice as shown in Example 12. Generally, to assess the *in vivo* activity of one or more of these polypeptides, the polypeptide can be administered to a test animal intraperitoneally. After one or more desired time periods, a blood sample can be drawn, and blood glucose levels can be measured.

As with all FGF21 mutants of the present invention, capped and/or C-terminally mutated FGF21 mutant polypeptides, and chemically modified capped and/or C terminally mutated FGF21 mutant polypeptides, can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

The capped and/or C-terminally mutated FGF21 mutant polypeptides of the present invention can be prepared as described in Example 7. Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the capped and/or C-terminally mutated FGF21 mutant polypeptides of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

Following the preparation of a capped and/or C terminally mutated FGF21 mutant polypeptide, the polypeptide can be chemically modified by the attachment of a polymer, as described in Example 9.

5. FGF21 Mutant Polypeptides Containing No Naturally Occurring Cysteine Residues

In a further aspect of the present invention, FGF21 mutant polypeptides can be prepared in which both cysteine residues in the wild-type FGF21 polypeptide sequence are replaced with residues that do not form disulfide bonds and do not serve as polymer attachment sites, such as alanine or serine. Subsequently, substitutions can be made in the FGF21 mutant polypeptide sequence that introduce non-naturally occurring polymer attachment sites, in the form of thiol-containing residues (*e.g.*, cysteine residues or non-naturally occurring amino acids having thiol groups) or free amino groups (*e.g.*, lysine or arginine residues or non-naturally occurring amino acids having free amino groups). Polymers that rely on thiol or free amino groups for attachment, such as PEG, can then be targeted to cysteine, lysine or arginine residues that have been introduced into the FGF21 mutant polypeptide sequence at known positions. This strategy can facilitate more efficient and controlled polymer placement.

In one approach, the two naturally occurring cysteine residues in the wild-type FGF21 polypeptide, which are located at positions 75 and 93, can be substituted with non-thiol containing residues. Subsequently, a cysteine residue can be introduced at a known location. The FGF21 mutant polypeptide can also comprise other mutations, which can introduce still more polymer attachments sites (*e.g.*, cysteine residues) or can be designed to achieve some other desired property. Examples of such FGF21 mutant polypeptides include C75A/E91C/C93A/H125C/P171G and C75S/E91C/C93S/H125C/P171G. In these examples, the naturally occurring cysteines at positions 75 and 93 have been mutated to alanine or serine residues, polymer attachment sites have been introduced at positions 91 and 125 (in this case for a thiol-reactive polymer such as PEG) and an additional mutation has been made at position 171, namely the substitution of proline 171 with a glycine residue.

Like all of the FGF21 mutant polypeptides disclosed herein, the activity of FGF21 mutant polypeptides which contain neither of the cysteines found in the wild-type FGF21 polypeptide sequence but instead comprise an introduced polymer attachment site and optionally one or more additional mutations, as well as chemically modified forms of these mutants, can be assayed in a variety of ways, for example, using an *in vitro* ELK-luciferase assay as described herein in Example 10. The in vivo activity of these polypeptides can be assessed in an *in vivo* assay, such as with ob/ob mice as shown in Example 12 and as described herein

As with all FGF21 mutants of the present invention, the activity of FGF21 mutant polypeptides which contain neither of the cysteines found in the wild-type FGF21 polypeptide sequence but instead comprise an introduced polymer attachment site and optionally one or more additional mutations and chemically modified forms of these FGF21 mutant polypeptides can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

FGF21 mutant polypeptides which contain neither of the cysteines found in the wild-type FGF21 polypeptide sequence but instead comprise an introduced polymer attachment site and optionally one or more additional mutations can be prepared as described herein, for example in Example 7. Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use these FGF21 mutant polypeptides. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

Following the preparation of FGF21 mutant polypeptides which contain neither of the cysteines found in the wild-type FGF21 polypeptide sequence but instead comprise an introduced polymer attachment site and optionally one or more additional mutations, the polypeptide can be chemically modified by the attachment of a polymer, as described in Example 9.

6. "Tethered Molecules"

In still another aspect of the present invention, a "Tethered Molecule" can be prepared as described herein. A "Tethered Molecule" is a molecule comprising two FGF21 polypeptides tethered together by a linker molecule. By joining two FGF21 polypeptides together, the effective half-life and potency of a Tethered Molecule can be extended beyond the half-life and potency of a single FGF21 polypeptide.

A Tethered Molecule of the present invention comprises a linker and two FGF21 polypeptides, which can be two naturally occurring FGF21 polypeptides into which no mutations have been introduced, two FGF21 mutant polypeptides having a linker attachment site introduced into the FGF21 polypeptides or a combination of one naturally occurring FGF21 polypeptide and one FGF21 mutant polypeptide. Tethered Molecules comprising at least one FGF21 polypeptide having a non-naturally occurring linker attachment site and one or more additional mutations are also contemplated and form another aspect of the invention. Such Tethered Molecules can thus comprise a mutation that forms a site for the attachment of a linker molecule as well as another mutation to impart another desirable property to the Tethered Molecule.

As used herein, the term "linker attachment site" means a naturally or non-naturally occurring amino acid having a functional group with which a linker can be associated. In one example, a linker attachment site is a residue containing a thiol group, which can be associated with a PEG molecule.

6.A. <u>FGF21 Polypeptides in a Tethered Molecule</u>

When a Tethered Molecule comprises two FGF21 mutant polypeptides, the FGF21 mutant polypeptides can comprise one or more mutations introduced into the sequence, but the mutations need not be at the same amino acid position in each of the FGF21 mutant polypeptides. By way of example, if a Tethered Molecule comprises two FGF21 mutant polypeptides, one FGF21 mutant polypeptide may contain an H125C mutation, which may form an attachment point for a linker molecule. In contrast, the other FGF21 mutant polypeptide can contain a mutation at a position other than H125 which can serve as an attachment point for the linker tethering the two FGF21 mutant polypeptides together. Even if one or two FGF21 mutant polypeptides are employed, the linker can be attached at the N terminal end of the FGF21 mutant polypeptide; introduced attachment points need not necessarily be used.

When a Tethered Molecule comprises one or two naturally-occurring FGF21 polypeptides the linker can be attached at a point in the FGF21 polypeptide that is amenable to the attachment chemistry. For example, naturally occurring disulfide bonds can be reduced and the cysteine residues can serve as attachment points for a linker, such as PEG. In another embodiment, a linker can be attached to a FGF21 polypeptide at the N-terminus or on lysine sidechains.

One or both of the FGF21 mutant polypeptides of a Tethered Molecule can comprise a truncated FGF21 mutant polypeptide. As described herein, a truncated FGF21 mutant polypeptide can be prepared by removing any number of residues on either the N-terminus, the C-terminus or both the N- and C-termini.

Tethered Molecules can also comprise one or both FGF21 polypeptides which comprise a mutation in the polypeptide sequence that may not be preferred as a linker attachment site, but instead may impart some other desirable property to the Tethered Molecule. Thus, Tethered Molecules comprising one or more FGF21 mutant polypeptides into which a mutation imparting a desirable property to the Tethered Molecule form a further aspect of the present invention.

The activity of Tethered Molecules can be assayed in a variety of ways, for example, using an *in vitro* ELK-luciferase assay as described herein in Example 10.

The activity of the Tethered Molecules of the present invention can also be assessed in an *in vivo* assay, such as with ob/ob mice as shown in Example 12. Generally, to assess the *in vivo* activity of one or more of these polypeptides, the polypeptide can be administered to a test animal intraperitoneally. After one or more desired time periods, a blood sample can be drawn, and blood glucose levels can be measured.

As with all FGF21 mutants of the present invention, the FGF21 polypeptides that comprise a Tethered Molecule, which can be FGF21 mutant polypeptides, wild-type FGF21 polypeptides or a combination of both, can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the Tethered Molecules of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (*e.g.*, electroporation, lipofection). *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Processes for associating linkers with FGF21 polypeptides will depend on the nature of the linker, but are known to those of skill in the art. Examples of linker attachment chemistries are described herein. Guidance on how a Tethered Molecule of the present invention can be formed is provided herein, for example in Example 9.

Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

6.B. Linkers in Tethered Molecules

Any linker can be employed in a Tethered Molecule to tether the two FGF21 mutant polypeptides together. Linker molecules can be branched or unbranched and can be attached to a FGF21 mutant polypeptide using various known chemistries, such as those described herein. The chemical structure of a linker is not critical, since it serves primarily as a spacer. The linker can be independently the same or different from any other linker, or linkers, that may be present in a Tethered Molecule (*e.g.*, a Tethered Molecule comprising three or more FGF21 mutant or wild-type polypeptides). In one embodiment, a linker can be made up of amino acids linked together by peptide bonds. Some of these amino acids can be glycosylated, as is well understood by those in the art. For example, a useful linker sequence constituting a sialylation site is $X_1X_2NX_4X_5G$ (SEQ ID NO: 5), wherein X_1, X_2, X_4 and X_5 are each independently any amino acid residue. In another embodiment a linker molecule can be a PEG molecule of any size, such as 20kDa, 30 kDa or 40 kDa.

In embodiments in which a peptidyl linker is present (*i.e.*, made up of amino acids linked together by peptide bonds) that is made in length, preferably, of from 1 up to about 40 amino acid residues, more preferably, of from 1 up to about 20 amino acid residues, and most preferably of from 1 to about 10 amino acid residues. In one embodiment, the amino acid residues in the linker are selected from any the twenty canonical amino acids. In another embodiment the amino acid residues in the linker are selected from cysteine, glycine, alanine, proline, asparagine, glutamine, and/or serine. In yet another embodiment, a peptidyl linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, serine, and alanine linked by a peptide bond. It is often desirable that, if present, a peptidyl linker be selected that avoids rapid proteolytic turnover in circulation *in vivo*. Thus, preferred peptidyl linkers include polyglycines, particularly $(Gly)_4$ (SEQ ID NO: 6); $(Gly)_5$ (SEQ ID NO: 7); poly(Gly-Ala); and polyalanines. Other preferred peptidyl linkers include GGGGS (SEQ ID NO: 8); GGGGSGGGGS (SEQ ID NO: 9); GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 10) and any linkers used in the Examples provided herein. The linkers described herein, however, are exemplary; linkers within the scope of this invention can be much longer and can include other residues.

In embodiments of a Tethered Molecule that comprise a peptide linker moiety, acidic residues, for example, glutamate or aspartate residues, are placed in the amino acid sequence of the linker moiety. Examples include the following peptide linker sequences:

GGEGGG (SEQ ID NO: 11);
GGEEEGGG (SEQ ID NO: 12);
GEEEG (SEQ ID NO: 13);
GEEE (SEQ ID NO: 14);
GGDGGG (SEQ ID NO: 15);
GGDDDGG (SEQ ID NO: 16);
GDDDG (SEQ ID NO: 17);
GDDD (SEQ ID NO: 18);
GGGGSDDSDEGSDGEDGGGGS (SEQ ID NO: 19);
WEWEW (SEQ ID NO: 20);
FEFEF (SEQ ID NO: 21);
EEEWWW (SEQ ID NO: 22);
EEEFFF (SEQ ID NO: 23);
WWEEEWW (SEQ ID NO: 24); or
FFEEEFF (SEQ ID NO: 25).

In other embodiments, a peptidyl linker constitutes a phosphorylation site, e.g., $X_1X_2YX_3X_4G$ (SEQ ID NO: 26), wherein X_1, X_2,X_3 and X_4 are each independently any amino acid residue; $X_1X_2SX_3X_4G$ (SEQ ID NO: 27), wherein X_1, X_2,X_3 and X_4 are each independently any amino acid residue; or $X_1X_2TX_3X_4G$ (SEQ ID NO: 28), wherein X_1, X_2,X_3 and X_4 are each independently any amino acid residue.

Non-peptide linkers can also be used in a Tethered Molecule. For example, alkyl linkers such as $-NH-(CH_2)_s-C(O)-$, wherein $s = 2$ to 20 could be used. These alkyl linkers can further be substituted by any non-sterically hindering group such as lower alkyl (*e.g.*, C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc.

Any sutiable linker can be employed in the present invention to from Tethered Molecules. In one example, the linker used to produce Tethered Molecules described herein were homobifunctional bis-maleimide PEG molecules having the general structure:

$$X-(CH_2CH_2O)_nCH_2CH_2-X$$

where X is a maleimide group. In other embodiments, X can be an orthopyridyl-disulphide, an iodoacetamide, a vinylsulfone or any other reactive moiety known to the art to be specific for thiol groups. In yet another embodiment X can be an amino-specific reactive moiety used to tether two mutant polypeptides through either the N-terminus or an engineered lysyl group. (*See, e.g.*, Pasut and Veronese, 2006, "PEGylation of Proteinsas Tailored Chemistry for Optimized Bioconjugates," *Adv. Polym. Sci.* 192:95-134).

In still another embodiment, a linker can have the general structure:

$$X-(CH_2CH_2O)_nCH_2CH_2-Y$$

where X and Y are different reactive moieties selected from the groups above. Such a linker would allow conjugation of different mutant polypeptides to generate Tethered heterodimers or hetero-oligomers.

In a further embodiment, a linker can be a PEG molecule, which can have a molecular weight of 1 to 100 kDa, preferably 10 to 50 kDa (e.g., 10, 20, 30 or 40 kDa) and more preferably 20 kDa. The peptide linkers can be altered to form derivatives in the same manner as described above.

Other examples of useful linkers include aminoethyloxyethyloxy-acetyl linkers as disclosed in International Publication No. WO 2006/042151, incorporated herein by reference in its entirety.

When forming a Tethered Molecule of the present invention, standard chemistries can be employed to associate a linker with a wild-type or mutant FGF21 molecule. The precise method of association will depend on the attachment site (*e.g.*, which amino acid side chains) and the nature of the linker. When a linker is a PEG molecule, attachment can be achieved by employing standard chemistry and a free sulfhydryl or amine group, such as those found on cysteine residues (which can be introduced into the FGF21 polypeptide sequence by mutation or can be naturally occurring) or on lysine (which can be introduced into the FGF21 polypeptide sequence by mutation or can be naturally occurring) or N-terminal amino groups.

7. Chemical Modification of FGF21 Mutants

In an aspect of the present invention, FGF21 mutant polypeptides are chemically modified. The term "chemically modified" refers to a polypeptide (e.g., an FGF21 mutant polypeptide) that has been modified by the addition of a polymer at one or more sites on the polypeptide. Examples of chemically modified forms of a FGF21 mutant polypeptide include PEGylated and glycosylated forms of an FGF21 mutant polypeptide.

Chemically modified FGF21 mutant polypeptides of the present invention can comprise any type of polymer, including water soluble polymers, such as PEG. Exemplary polymers each can be of any molecular weight and can be branched or unbranched. The polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations of a water-soluble polymer, some molecules will weigh more and some less than the stated molecular weight). The average molecular weight of each polymer is preferably between about 5 kDa and about 50 kDa, more preferably between about 10 kDa and about 40 kDa, and most preferably between about 10 kDa and about 20 kDa.

Suitable water-soluble polymers or mixtures thereof include, but are not limited to, carbohydrates, polyethylene glycol (PEG) (including the forms of PEG that have been used to derivatize proteins, including mono-(C_1-C_{10}), alkoxy-, or aryloxy-polyethylene glycol), monomethoxy-polyethylene glycol, dextran (such as low molecular weight dextran of, for example, about 6 kD), cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (*e.g.*, glycerol), and polyvinyl alcohol. Also encompassed by the present invention are bifunctional crosslinking molecules that can be used to prepare covalently attached FGF21 polypeptide mutant multimers. Also encompassed by the present invention are FGF21 mutants covalently attached to polysialic acid.

In some embodiments of the present invention, an FGF21 mutant polypeptide is covalently, modified to include one or more water-soluble polymers, including, but not limited to, polyethylene glycol (PEG), polyoxyethylene glycol, or polypropylene glycol. *See, e.g.*, U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; and 4,179,337. In some embodiments of the present invention, an FGF21 mutant comprises one or more polymers, including, but not limited to, monomethoxy-polyethylene glycol, dextran, cellulose, another carbohydrate-based polymer, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, or mixtures of such polymers.

In yet other embodiments of the present invention, a peptide or a protein can be conjugated to FGF21 in a site directed manner though the aforementioned engineered residues in order impart favorable properties to FGF21 (e.g. potency, stability, selectivity). Thus the present invention encompasses FGF21 mutant polypeptides conjugated to a heteroprotein or heteropeptide at an introduced polymer attachment site. Examples of suitable proteins include HSA and antibodies that do not bind to FGF21.

7.A. PEGylated FGF21 Mutant Polypeptides

In some embodiments of the present invention, an FGF21 mutant polypeptide is covalently-modified with PEG subunits. In some embodiments, one or more water-soluble polymers are bonded at one or more specific positions (for example, at the N-terminus) of the FGF21 mutant. In some embodiments, one or more water-soluble polymers are attached to one or more side chains of an FGF21 mutant; these side chains can be naturally occurring or can form a component of an engineered polymer attachment site. In some embodiments, PEG is used to improve the therapeutic capacity of an FGF21 mutant polypeptide. Certain such methods are discussed, for example, in U.S. Patent No. 6,133,426, which is hereby incorporated by reference for any purpose.

In embodiments of the present invention wherein the polymer is PEG, the PEG group can be of any convenient molecular weight, and can be linear or branched. The average molecular weight of the PEG group will preferably range from about 2 kD to about 100 kDa, and more preferably from about 5 kDa to about 50 kDa, *e.g.*, 10, 20, 30, 40, or 50 kDa. The PEG groups will generally be attached to the FGF21 mutant via acylation or reductive alkylation through a reactive group on the PEG moiety (*e.g.*, an aldehyde, NHS, or maleimide, vinylsulfone, alkylhalide) to a reactive group on the FGF21 mutant (*e.g.*, an amino or thiol group).

When the polymer(s) attached to a FGF21 mutant polypeptide is PEG, the PEGylation of a FGF21 mutant polypeptide of the present invention, can be specifically carried out using any of the PEGylation reactions known in the art. Such reactions are described, for example, in the following references: Zalipsky, 1995, *Functionalized Poly(ethylene glycol) for Preparation of Biologically Relevant Conjugates,* Bioconjugate Chemistry 6:150-165; Francis *et al.*, 1992, *Focus on Growth Factors* 3: 4-10; European Patent Nos. 0 154 316 and 0 401 384; and U.S. Patent No. 4,179,337. For example, when the target residue is a lysine residue (*i.e.*, a residue with a reactive amine group) PEGylation can be carried out via an acylation reaction or an alkylation reaction with an amino-reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described herein. For the acylation reactions, a selected polymer can have a single reactive ester group. For reductive alkylation, a selected polymer can have a single reactive aldehyde group. A reactive aldehyde is, for example, polyethylene glycol propionaldehyde, which is water stable, or mono C_1-C_{10} alkoxy or aryloxy derivatives thereof (*see* U.S. Patent No. 5,252,714). Various reactive PEG polymers activated with different amino-specific moieties will also known to those of ordinary skill in the art and can also be employed as circumstances dictate.

In another example, when the target residue is a cysteine residue (i.e., a residue with a reactive sulfhydryl group) PEGylation can be carried out via standard maleimide chemistry. For this reaction, the selected polymer can contain one or more reactive maleimide groups or other thiol reactive moiety such as vinylsulfone, orthopyridyl-disulphide or iodoacetamide. *See, e.g.*, Pasut & Veronese, 2006, "PEGylation of Proteins as Tailored Chemistry for Optimized Bioconjugates," *Adv. Polym. Sci.* 192:95-134; Zalipsky, 1995, "Functionalized Poly(ethylene glycol) for Preparation of Biologically Relevant Conjugates," *Bioconjugate Chemistry* 6:150-165, and Hermanson, *Bioconjugate Techniques, 2nd Ed., Academic Press, 2008,* each of which is incorporated herein by reference.

In some embodiments of the present invention, a useful strategy for the attachment of the PEG group to a FGF21 mutant involves combining through the formation of a conjugate linkage in solution, a FGF21 mutant and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The FGF21 mutant is "preactivated" with an appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the FGF21 mutant with PEG usually takes place in aqueous phase and can be easily monitored by SDS-PAGE or reverse phase analytical HPLC. Detailed analysis can be done by LC-MS based peptide mapping. Detailed analysis can be done by LC-MS based peptide mapping.

7.B. Polysaccharide FGF21 Mutant Polypeptides

Polysaccharide polymers are another type of water-soluble polymer that can be used for protein modification. Therefore, the FGF21 mutant polypeptides of the present invention can be attached to a polysaccharide polymer to form embodiments of the present invention. Thus, an engineered non-naturally occurring polymer attachment site in an FGF21 mutant polypeptide can comprise a residue to which a polysaccharide is attached. Dextrans are polysaccharide polymers comprised of individual subunits of glucose predominantly linked by alpha 1-6 linkages. The dextran itself is available in many molecular weight ranges, and is readily available in molecular weights from about 1 kD to about 70 kD. Dextran is a suitable water-soluble polymer for use as a vehicle by itself or in combination with another vehicle (*e.g.*, Fc). *See, e.g.*, International Publication No. WO 96/11953. The use of dextran conjugated to therapeutic or diagnostic immunoglobulins has been reported. *See, e.g.*, European Patent Publication No. 0 315 456, which is hereby incorporated by reference. The present invention also encompasses the use of dextran of about 1 kDa to about 20 kDa.

7.C. Methods of Chemically Modifying a FGF21 Mutant Polypeptide

In general, chemical modification (*e.g.*, PEGylation or glycosylation) can be performed under any suitable condition used to react a protein with an activated polymer molecule. Methods for preparing chemically modified FGF21 mutant polypeptides will generally comprise the steps of: (a) reacting the polypeptide with the activated polymer molecule (such as a reactive ester, maleimide or aldehyde derivative of the polymer molecule) under conditions whereby an FGF21 mutant polypeptide becomes attached to one or more polymer molecules, and (b) obtaining the reaction products. The optimal reaction conditions will be determined based on known parameters and the desired result. For example, the larger the ratio of polymer molecules to protein, the greater the percentage of attached polymer molecule. In one embodiment of the present invention, chemically modified FGF21 mutant polypeptides can have a single polymer molecule at the amino-terminus, while in other embodiments an FGF21 mutant polypeptide can have two or more polymers associated with the primary sequence, for example one polymer at the N-terminus of the polypeptide and a second at another residue in the polypeptide. Alternatively, a FGF21 mutant can have two or more polymers associated at two different residues in the primary sequence but not at the N-terminus.

Generally, conditions that can be alleviated or modulated by the administration of the present chemically modified FGF21 mutant polypeptides include those described herein for the native FGF21 polypeptide. However, the chemically modified FGF21 mutant polypeptides disclosed herein can have additional activities, such as increased half-life, as compared to wild-type FGF21 and FGF21 mutants.

8. Therapeutic Compositions of FGF21 Mutants and Administration Thereof

Therapeutic compositions comprising FGF21 mutant polypeptides are within the scope of the present invention, and are specifically contemplated in light of the identification of Tethered Molecules, FGF21 mutant polypeptides and chemically modified FGF21 mutant polypeptides that exhibit enhanced properties. Such Tethered Molecule, FGF21 mutant polypeptide, and chemically modified FGF21 mutant polypeptide therapeutic compositions, can comprise a therapeutically effective amount of a Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide, which can be chemically modified, in a mixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration.

Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The pharmaceutical composition can contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrogen-sulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrins), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or polyethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), tonicity enhancing agents (such as alkali metal halides – preferably sodium or potassium chloride – or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants (*see, e.g., Remington's Pharmaceutical Sciences* (18th Ed., A.R. Gennaro, ed., Mack Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The optimal pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage (*see, e.g., Remington's Pharmaceutical Science*). Such compositions can influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the Tethered Molecule, FGF21 mutant polypeptide (which can be truncated, capped or C-terminally mutated) or chemically modified FGF21 mutant polypeptide.

The primary vehicle or carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier for injection can be water, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute. In one embodiment of the present invention, Tethered Molecule, FGF21 mutant polypeptide and chemically modified FGF21 mutant polypeptide compositions can be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (*Remington's Pharmaceutical Sciences, supra*) in the form of a lyophilized cake or an aqueous solution. Further, the Tethered Molecule, FGF21 mutant polypeptide and chemically modified FGF21 mutant polypeptide product can be formulated as a lyophilizate using appropriate excipients such as sucrose.

The pharmaceutical compositions of the present invention can be selected for parenteral delivery. Alternatively, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

When parenteral administration is contemplated, the therapeutic compositions for use in this invention can be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which a Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which can then be delivered via a depot injection. Hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a pharmaceutical composition can be formulated for inhalation. For example, Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide can be formulated as a dry powder for inhalation. Such inhalation solutions can also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions can be nebulized. Pulmonary administration is further described in International Publication No. WO 94/20069, which describes the pulmonary delivery of chemically modified proteins.

It is also contemplated that certain formulations can be administered orally. In one embodiment of the present invention, a Tethered Molecule, FGF21 mutant polypeptide (which can be truncated, capped or C-terminally mutated) or chemically modified FGF21 mutant polypeptide that is administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the Tethered Molecule, FGF21 mutant polypeptide (which can be truncated, capped or C-terminally mutated) or chemically modified FGF21 mutant polypeptide. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Another pharmaceutical composition can involve a therapeutically effective quantity of a Tethered Molecule, FGF21 mutant polypeptide (which can be truncated, capped or C-terminally mutated) or chemically modified FGF21 mutant polypeptide in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide pharmaceutical compositions will be evident to those skilled in the art, including formulations involving Tethered Molecule, FGF21 mutant polypeptides or chemically modified FGF21 mutant polypeptides in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art (*see*, *e.g.*, International Publication No. WO 93/15722, which describes the controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions).

Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.*, films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides (U.S. Patent No. 3,773,919 and European Patent No. 0 058 481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, 1983, *Biopolymers* 22: 547-56), poly(2-hydroxyethyl-methacrylate) (Langer *et al.*, 1981, *J. Biomed. Mater. Res.* 15: 167-277 and Langer, 1982, *Chem. Tech.* 12: 98-105), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D(-)-3-hydroxybutyric acid (European Patent No. 0 133 988). Sustained-release compositions can also include liposomes, which can be prepared by any of several methods known in the art. *See, e.g.*, Epstein *et al.*, 1985, *Proc. Natl. Acad. Sci. U.S.A.* 82: 3688-92; and European Patent Nos. 0 036 676, 0 088 046, and 0 143 949.

A Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide pharmaceutical composition to be used for *in vivo* administration typically must be sterile. This can be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method can be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration can be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations can be stored either in a ready-to-use form or in a form (*e.g.*, lyophilized) requiring reconstitution prior to administration.

In a specific embodiment, the present invention is directed to kits for producing a single-dose administration unit. The kits can each contain both a first container having a dried protein and a second container having an aqueous formulation. Also included within the scope of this invention are kits containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes).

A therapeutically effective amount of a Tethered Molecule, FGF21 mutant polypeptide (which can be truncated, capped or C-terminally mutated) or chemically modified FGF21 mutant polypeptide pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide is being used, the route of administration, and the size (body weight, body surface, or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage can range from about 0.1 μg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In other embodiments, the dosage can range from 0.01 mg/kg up to about 10 mg/kg, for example 0.05 mg/kg, 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1.0 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg or 10 mg/kg.

The frequency of dosing will depend upon the pharmacokinetic parameters of the Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide in the formulation being used. Typically, a clinician will administer the composition until a dosage is reached that achieves the desired effect. The composition can therefore be administered as a single dose, as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages can be ascertained through use of appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, *e.g.*, orally; through injection by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, intraportal, or intralesional routes; by sustained release systems; or by implantation devices. Where desired, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device.

Alternatively or additionally, the composition can be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration.

10. Therapeutic Uses of FGF21 Polypeptide Mutants

The Tethered Molecules, FGF21 mutant polypeptides (which can be truncated, capped or C-terminally mutated) and chemically modified FGF21 mutant polypeptides of the present invention can be used to treat, diagnose, ameliorate, or prevent a number of diseases, disorders, or conditions, including, but not limited to metabolic disorders. In one embodiment, the metabolic disorder to be treated is diabetes. In another embodiment, the metabolic disorder is obesity. Other embodiments include metabolic conditions or disorders such as dyslipidimia; hypertension; hepatosteaotosis, such as non-alcoholic steatohepatitis (NASH); cardiovascular disease, such as atherosclerosis; and aging.

In application, a disorder or condition such as diabetes or obesity can be treated by administering a Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide as described herein to a patient in need thereof in the amount of a therapeutically effective dose. The administration can be performed as described herein, such as by IV injection, intraperitoneal injection, intramuscular injection, or orally in the form of a tablet or liquid formation. In most situations, a desired dosage can be determined by a clinician, as described herein, and can represent a therapeutically effective dose of the Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide. It will be apparent to those of skill in the art that a therapeutically effective dose of Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide will depend, *inter alia,* upon the administration schedule, the unit dose of antigen administered, whether the nucleic acid molecule or polypeptide is administered in combination with other therapeutic agents, the immune status and the health of the recipient. The term "therapeutically effective dose," as used herein, means that amount of Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide that elicits the biological or medicinal response in a tissue system, animal, or human being sought by a researcher, medical doctor, or other clinician, which includes alleviation of the symptoms of a disease or disorder being treated.

11. Antibodies

Antibodies and antibody fragments that specifically bind to the Tethered Molecules, FGF21 mutant polypeptides and chemically modified FGF21 mutant polypeptides of the present invention but do not specifically bind to wild-type FGF21 polypeptides are contemplated and are within the scope of the present invention. The antibodies can be polyclonal, including monospecific polyclonal; monoclonal (MAbs); recombinant; chimeric; humanized, such as complementarity-determining region (CDR)-grafted; human; single chain; and/or bispecific; as well as fragments; variants; or chemically modified molecules thereof. Antibody fragments include those portions of the antibody that specifically bind to an epitope on an FGF21 mutant polypeptide. Examples of such fragments include Fab and F(ab') fragments generated by enzymatic cleavage of full-length antibodies. Other binding fragments include those generated by recombinant DNA techniques, such as the expression of recombinant plasmids containing nucleic acid sequences encoding antibody variable regions.

Polyclonal antibodies directed toward a Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide generally are produced in animals (*e.g.*, rabbits or mice) by means of multiple subcutaneous or intraperitoneal injections of the FGF21 mutant polypeptide and an adjuvant. It can be useful to conjugate an FGF21 mutant polypeptide to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet hemocyanin, serum, albumin, bovine thyroglobulin, or soybean trypsin inhibitor. Also, aggregating agents such as alum are used to enhance the immune response. After immunization, the animals are bled and the serum is assayed for anti-Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide antibody titer.

Monoclonal antibodies directed toward Tethered Molecules, FGF21 mutant polypeptides or chemically modified FGF21 mutant polypeptides can be produced using any method that provides for the production of antibody molecules by continuous cell lines in culture. Examples of suitable methods for preparing monoclonal antibodies include the hybridoma methods of Kohler *et al.*, 1975, *Nature* 256: 495-97 and the human B-cell hybridoma method (Kozbor, 1984, *J. Immunol.* 133: 3001; Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications* 51-63 (Marcel Dekker, Inc., 1987). Also provided by the invention are hybridoma cell lines that produce monoclonal antibodies reactive with Tethered Molecules, FGF21 mutant polypeptides or chemically modified FGF21 mutant polypeptides.

The anti-FGF21 mutant antibodies of the invention can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays (*see, e.g.*, Sola, *Monoclonal Antibodies: A Manual of Techniques* 147-158 (CRC Press, Inc., 1987), incorporated herein by reference in its entirety) for the detection and quantitation of FGF21 mutant polypeptides. The antibodies will bind FGF21 mutant polypeptides with an affinity that is appropriate for the assay method being employed.

For diagnostic applications, in certain embodiments, anti-Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide antibodies can be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, ^{99}Tc, ^{111}In, or ^{67}Ga; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, β-galactosidase, or horseradish peroxidase (Bayer *et al.*, 1990, *Meth. Enz.* 184: 138-63).

Competitive binding assays rely on the ability of a labeled standard (*e.g.*, an FGF21 mutant polypeptide, or an immunologically reactive portion thereof) to compete with the test sample analyte (*e.g.*, a Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide) for binding with a limited amount of anti-Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide antibody, depending on the analyte. The amount of Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies typically are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies can conveniently be separated from the standard and analyte that remain unbound.

Sandwich assays typically involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected and/or quantitated. In a sandwich assay, the test sample analyte is typically bound by a first antibody that is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. *See, e.g.*, U.S. Patent No. 4,376,110. The second antibody can itself be labeled with a detectable moiety (direct sandwich assays) or can be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assays). For example, one type of sandwich assay is an enzyme-linked immunosorbent assay (ELISA), in which case the detectable moiety is an enzyme.

The anti-Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide antibodies of the present invention are also useful for *in vivo* imaging. An antibody labeled with a detectable moiety can be administered to an animal, preferably into the bloodstream, and the presence and location of the labeled antibody in the host assayed. The antibody can be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

The invention also relates to a kit comprising Tethered Molecules, FGF21 mutant polypeptides or chemically modified FGF21 mutant polypeptide antibodies and other reagents useful for detecting Tethered Molecule, FGF21 mutant polypeptide or chemically modified FGF21 mutant polypeptide levels in biological samples. Such reagents can include a detectable label, blocking serum, positive and negative control samples, and detection reagents.

EXAMPLES

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and should not be construed as limiting the scope of the invention in any way.

EXAMPLE 1

Preparation of FGF21 Polypeptide Expression Constructs

A nucleic acid sequence encoding the mature FGF21 polypeptide was obtained by polymerase chain reaction (PCR) amplification using primers having nucleotide sequences corresponding to the 5' and 3' ends of the mature FGF21 sequence. Table 4 lists the primers that were used to amplify the mature FGF21 sequence.

Table 4

PCR Primers for Preparing FGF21 Construct

Primer	Sequence	SEQ ID NO:
Sense	5'-AGGAGGAATAACATATGCATCCAATTCCAGATTCTTCTCC-3'	33
Antisense	5'-TAGTGAGCTCGAATTCTTAGGAAGCGTAGCTGG-3'	34

The primers used to prepare the FGF21 expression construct incorporated restriction endonuclease sites for directional cloning of the sequence into a suitable expression vector (*e.g.*, pET30 (Novagen/EMD Biosciences; San Diego, CA) or pAMG33 (Amgen; Thousand Oaks, CA)). The expression vector pAMG33 contains a low-copy number R-100 origin of replication, a modified *lac* promoter, and a kanamycin-resistance gene. The expression vector pET30 contains a pBR322-derived origin of replication, an inducible T7 promoter, and a kanamycin-resistance gene. While expression from pAMG33 was found to be higher, pET30 was found to be a more reliable cloning vector. Thus, the majority of the constructs described in the instant application were first generated in pET30 and then screened for efficacy. Selected sequences were then transferred to pAMG33 for further amplification.

The FGF21 sequence was amplified in a reaction mixture containing 40.65 µL dH_2O, 5µL PfuUltra II Reaction Buffer (10x), 1.25 µL dNTP Mix (40 mM – 4 x 10mM), 0.1 µL Template (100 ng/mL), 1 µL Primer1 (10 µM), 1 µL Primer2 (10 µM), and 1 µL PfuUltra II fusion HS DNA Polymerase (Stratagene; La Jolla, CA). Amplification reactions were performed by heating for two minutes at 95°C; followed by ten cycles at 95°C for 20 seconds, 60°C for 20 seconds (with an additional 1°C subtracted per cycle), and 72°C for 15 seconds/kilobase of desired product; followed by 20 cycles at 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 15 seconds/kilobase of desired product; followed by 72°C for three minutes. Amplification products were digested with the restriction endonucleases NdeI and

EcoRI; ligated into a suitable vector; and then transformed into competent cells.

As a result of the bacterial expression system employed, the expressed mature FGF21 polypeptide included an N-terminal methionine residue or a variant methionine residue such as fMet or gluconylated Met.

EXAMPLE 2

Purification of Wild-type FGF21 Polypeptides from Bacteria

In the Examples that follow, wild-type FGF21 polypeptides were expressed in a bacterial expression system. After expression, which is described below, the wild-type FGF21 polypeptides were purified as described in this Example, unless otherwise indicated.

To purify the wild-type FGF21 polypeptide from bacterial inclusion bodies, double-washed inclusion bodies (DWIBs) were solubilized in a solubilization buffer containing guanidine hydrochloride and DTT in Tris buffer at pH 8.5 and then mixed for one hour at room temperature, and the solubilization mixture was added to a refold buffer containing urea, arginine, cysteine, and cystamine hydrochloride at pH 9.5 and then mixed for 24 hours at 5°C (*see, e.g.*, Clarke, 1998, *Curr. Opin. Biotechnol.* 9: 157-63; Mannall *et al.*, 2007, *Biotechnol. Bioeng.* 97: 1523-34; Rudolph *et al.*, 1997, "Folding proteins," in *Protein Function: A Practical Approach* (Creighton, ed., New York, IRL Press), pp 57-99; and Ishibashi *et al.*, 2005, *Protein Expr. Purif.* 42: 1-6).

Following solubilization and refolding, the mixture was filtered through a 0.45 micron filter. The refold pool was then concentrated approximately 10-fold with a 10 kD molecular weight cut-off Pall Omega cassette at a transmembrane pressure (TMP) of 20 psi, and dialfiltered with 3 column volumes of 20 mM Tris, pH 8.0 at a TMP of 20 psi.

The clarified sample was then subjected to anion exchange (AEX) chromatography using a Q Sepharose HP resin. A linear salt gradient of 0 to 250 mM NaCl in 20 mM Tris was run at pH 8.0 at 5°C. Peak fractions were analyzed by SDS-PAGE and pooled.

The AEX eluate pool was then subjected to hydrophobic interaction chromatography (HIC) using a Phenyl Sepharose HP resin. Protein was eluted using a decreasing linear gradient of 0.7 M to 0 M ammonium sulfate at pH 8.0 and ambient temperature. Peak fractions were analyzed by SDS-PAGE (Laemmli, 1970, *Nature* 227: 680-85) and pooled.

The HIC pool was concentrated with a 10 kD molecular weight cut-off Pall Omega 0.2 m^2 cassette to 7 mg/mL at a TMP of 20 psi. The concentrate was dialfiltered with 5 volumes of 10 mM KPO_4, 5% sorbitol, pH 8.0 at a TMP of 20 psi, and the recovered concentrate was diluted to 5 mg/mL. Finally, the solution was filtered through a Pall mini-Kleenpac 0.2 μM Posidyne membrane.

EXAMPLE 3

Identification of FGF21 Mutants

Wild-type FGF21 has a relatively short half life, and in some cases this can be undesirable for the use of wild-type FGF21 as a therapeutic. Additionally, traditional methods of extending half life, such as PEGylation of the polypeptide are limited by the number and location of suitable PEGylation sites in the FGF21 sequence. In the wild-type FGF21 polypeptide sequence there are seven naturally occurring PEGylation sites, namely the alpha amino group, four lysine residues, and two cysteine residues. These sites are not ideal for PEGylation because the PEG molecule can adversely affect the ability of FGF21 to obtain its native structure, or it can adversely affect the interaction between FGF21 and its receptor or beta-klotho. In addition, with the exception of the alpha amino group, these reactive residues do not allow for the site specific PEGylation at a single targeted location. Accordingly, a directed and focused study was undertaken to identify residues within the wild-type FGF21 polypeptide that could be mutated to a residue suitable for chemical modification. Considerations in the study included the location of the residue in the FGF21 sequence, as well as its position on the protein surface.

Two different strategies were employed in an effort to identify individual residues in the wild-type FGF21 polypeptide that would be suitable for mutation to a residue useful for chemical modification, described herein.

EXAMPLE 3.A

Mutation Candidates Identified Using a Homology Model

In the first strategy, a homology model was employed in a systematic rational protein engineering approach to identify residues with a high probability of having surface exposed sidechains that were likely to tolerate PEGylation without interfering with FGF21 activity. Since there are no published X-ray or NMR structures of FGF21 that could be used to identify such residues, a high resolution (1.3 Å) X-ray crystal structure of FGF19 (1PWA) obtained from the Protein Databank (PDB) was used to create a 3D homology model of FGF21 using MOE (Molecular Operating Environment; Chemical Computing Group; Montreal, Quebec, Canada) modeling software. FGF19 was chosen as a template, since among the proteins deposited in the PDB, FGF19 is the most closely related protein to FGF21 in terms of the amino acid sequence homology.

The FGF21 homology model was then extended to represent FGF-21 bound to an FGF receptor. This model was used to identify residues that would likely be exposed on the surface of the FGF21 molecule and available for reaction with an activated polymer (*e.g.*, PEG) moiety, while avoiding those residues that might interfere with FGF21 interaction with its receptor. Considerations were also made for the sequence conservation of the residues between species as well as the biochemical properties of the native side chain. Residues identified as good candidates by this screen were ranked according to the estimated probability of successful PEGylation of this site with minimal disruption of activity. Residues in Group A reflect the best candidates, and residues in Group D reflect viable but less preferred candidates. Group A includes N121, H125, H112, R77, H87, E37 and K69. Group B includes R126, G113, D79, E91, D38, D46 and G120. Group C includes S71, D89, L86, T70, G39, T40, R36, P49, S48, S123, K122, A81, A111, E110 and R96. Finally, Group D includes Q18, R19, A26, Q28, E34, A44, A45, E50, L52, Q54, L55, K59, G61, L66, V68, R72, P78, G80, Y83, S85, F88, P90, A92, S94, L98, E101, D102, Q108, L114, H117, P119, P124, D127, P128, A129, P130, R131, and P140.

In addition, potential PEGylation sites within the amino and carboxy-terminal segments of the molecule were identified based on sequence alignments and the biochemical properties of the sidechains, since no three dimensional structural data is available for these portions of the molecule. Residues that were believed to be most suitable for mutation were identified and subsequently grouped according to how well the residues fit a set of selection criteria. With respect to the N-terminus of the FGF21 polypeptide sequence, residues 1-13 were evaluated, and D5 was determined to be the best candidate for mutation, with residues H1, P2, I3, P4 and S6 also determined to be viable. With respect to the C-terminus of the FGF21 polypeptide sequence, residues 141-181 were evaluated, and residues Y179 and R175 were determined to be the best candidates for mutation, with residues P143, P171, S172, Q173, G174, S176, P177, S178, A180, and S181 forming another group of candidates, and residues P144, A145, P147, E148, P149, P150, I152 A154, Q156 and G170 forming a third group of candidates for mutation.

EXAMPLE 3.B

FGF21 Mutants Generated by Removing Undesired Naturally Occurring Polymer Attachment Sites

The second strategy employed an amino-specific conjugation chemistry for coupling PEG to FGF21. Site-selective dual-PEGylation of potentially vacuologenic conjugates may significantly reduce their vacuologenic potential (*see, e.g.*, U.S. Patent No. 6,420,339). Accordingly, in one aspect of the present invention, site-selective dual-PEGylation of FGF21 is accomplished by mutating the protein so that only two primary amino groups remain for PEGylation.

The FGF21 protein contains four lysine and ten arginine residues as highlighted by bold (R and K residues) and underlining (K residues) in the wild-type FGF21 sequence (SEQ ID NO:4). The two naturally occurring cysteine residues are highlighted in bold and italic:

HPIPDSSPLLQFGGQV**R**Q**R**YLYTDDAQQTEAHLEI**R**EDGTVGGAADQS
PESLLQL**K**AL**K**PGVIQILGV**K**TS**R**FL***C***Q**R**PDGALYGSLHFDPEA***C***SF**R**E
LLLEDGYNVYQSEAHGLPLHLPGN**K**SPH**R**DPAP**R**GPA**R**FLPLPGLPPA
PPEPPGILAPQPPDVGSSDPLSMVGPSQG**R**SPSYAS SEQ ID NO:4

The four naturally occurring lysines were first mutated to arginines to create a parent molecule containing only one primary amine group as an α-amino group at the N-terminus. This was tested for *in vitro* activity and found to be fully active. Next, selected arginines were step-wise replaced with lysine to create up to ten analogs containing a second primary amino group for PEGylation at various positions on the FGF21 molecule. An examination of the homology model of FGF21 bound to its receptor (as described in Example 3A) allowed ranking of the proposed conjugation sites as a function of their proximity to the putative receptor interface. Sites that appeared buried were not addressed.

The various positions were ranked and characterized as follows: (a) Sites that are solvent exposed and distal to the receptor interface: R36, R77, K122 & R126; (b) Sites that are solvent exposed and proximal to the receptor interface: K56, K59, K69, R72 & R175; and (c) Sites that are buried: R17, R19, R131 & R135.

The final constructs, each containing a single α-amino and ε-amino group were purified, and tested for activity both before and after conjugation with PEG as described in Examples 9 (conjugation) and 10 (*in vitro* activity assay).

EXAMPLE 4

Identification of FGF21 Mutants Comprising a Single Mutation

A summary of FGF21 mutants comprising a single mutation that were generated through the rational protein engineering approach described above in Example 3.A and 3.B is provided in Table 3. These single mutants provide and incorporate a non-naturally occurring polymer attachment site, notably a cysteine or lysine residue. The side chains of these residues are particularly suited to chemical modification by the attachment of a polymer, such as a PEG molecule. It was recognized that in addition to the introduced non-naturally occurring polymer attachment site, a polymer could optionally be attached to the N-terminus of the protein, as desired.

Since the introduced residues were designed to maintain the wild-type levels of FGF21 biological activity, these FGF21 mutant polypeptides are expected to maintain FGF21 biological activity and yet still provide one or more non-naturally occurring polymer attachment sites. After chemical modification (*e.g.*, PEGylation) these mutants are expected to have a longer *in vivo* half life than wild-type FGF21, while maintaining significant *in vivo* wild-type levels of biological activity.

The numbers of the positions targeted for mutagenesis are given in Table 5 and correspond to the residue position in the mature FGF21 protein, which consists of 181 amino acid residues. Nucleic acid sequences encoding the FGF21 mutant polypeptides listed in Table 5 below were prepared using the techniques described below.

Table 5

FGF21 Mutant Polypeptides Comprising a Single Mutation

Residue Number	WT	Mutation	
36	R	K	R36K
37	E	C	E37C
38	D	C	D38C
46	D	C	D46C
56	K	R	K56R
60	K	R	K60R
91	E	C	E91C
69	K	C	K69C
69	K	R	K69R
72	R	K	R72K
77	R	C	R77C
77	R	K	R77K
79	D	C	D79C
86	H	C	H86C
91	E	C	E91C
112	H	C	H112C
113	G	C	G113C
120	G	C	G120C
121	N	C	N121C
122	K	R	K122R
125	H	C	H125C
126	R	C	R126C
126	R	K	R126K
171	P	G	P171G
175	R	C	R175C
175	R	K	R175K
170	G	C	G170C
179	Y	C	Y179C

EXAMPLE 5

Identification of FGF21 Mutants Comprising Two Mutations

Further analysis of the homology model and data obtained from the single cysteine mutants was performed in order to identify combinations of mutants that would provide two non-naturally occurring polymer attachment sites. Following chemical modification, these FGF21 mutants have two polymers (*e.g.*, PEG molecules) attached to the polypeptide at desired locations. As with all the FGF21 mutant polypeptides of the present invention, a polymer can also be attached to the N-terminus of the polypeptide, depending on the nature of the polymer itself. A cartoon depicting a dually PEGylated FGF21 mutant is shown graphically in Figure 1.

A summary of FGF21 mutants comprising two mutations that were generated through this rational protein engineering approach is provided in Table 6. These double mutants incorporate a non-naturally occurring polymer attachment site, notably a cysteine. The side chains of these residues are particularly suited to chemical modification by the attachment of a polymer, such as a PEG molecule. It was recognized that in addition to the introduced non-naturally occurring polymer attachment site, a polymer could optionally be attached to the N terminal of the protein, as desired.

Since the introduced residues were designed to maintain FGF21 biological activity, these FGF21 mutants are expected to preserve FGF21 biological activity and yet still provide one or more non-naturally occurring polymer attachment sites. After chemical modification (*e.g.*, PEGylation) these mutants are expected to have a longer half life than wild-type FGF21, while maintaining substantial *in vivo* potency.

The numbers of the positions given in Table 6 correspond to the residue position in the mature FGF21 protein, which consists of 181 amino acid residues. Nucleic acid sequences encoding the FGF21 mutant polypeptides listed in Table 6 below were prepared using the techniques described below.

Table 6

FGF21 Mutant Polypeptides Comprising Two Mutations

Residue 1	WT	Mutation	Residue 2	WT	Mutation	
37	E	C	77	R	C	E37C,R77C
120	G	C	125	H	C	G120C, H125C
77	R	C	91	E	C	R77C, E91C
77	R	C	125	H	C	R77C, H125C
91	E	C	125	H	C	E91C, H125C
77	R	C	120	G	C	R77C, G120C
37	E	C	91	E	C	E37C, E91C
91	E	C	175	R	C	E91C, R175C
37	E	C	175	R	C	E37C, R175C
91	E	C	120	G	C	E91C, G120C

37	E	C	120	G	C	E37C, G120C
77	R	C	175	R	C	R77C, R175C
37	E	C	125	H	C	E37C, H125C
37	E	C	69	K	C	E37C, K69C
69	K	C	91	E	C	K69C, E91C
120	G	C	175	R	C	G120C, R175C
69	K	C	120	G	C	K69C, G120C
69	K	C	125	H	C	K69C, H125C
69	K	C	77	R	C	K69C, R77C
125	H	C	175	R	C	H125C, R175C
69	K	C	175	R	C	K69C, R175C
37	E	C	170	G	C	E37C, G170C

EXAMPLE 6

Identification of FGF21 Mutants Comprising Three Mutations

As described above, non-naturally occurring polymer attachment sites can be introduced into the wild-type FGF21 polypeptide sequence. This affords the opportunity for site-selective chemical modification at desired locations in the polypeptide. In addition, it has previously been determined that residue P171 in the wild-type FGF21 sequence is susceptible to proteolytic degradation. Accordingly, by introducing combinations of the above modifications, plus a mutation at the P171 position, FGF21 molecules having both enhanced proteolytic stability and site-specific polymer attachment sites can be generated.

Further analysis of the homology model was performed in order to identify combinations of mutants that would provide two non-naturally occurring polymer attachment sites. Following chemical modification, these mutants would have two polymers (*e.g.*, PEG molecules) attached to the polypeptide at desired locations. As with all the FGF21 mutant polypeptides of the present invention, a polymer can also be attached to the N-terminus of the polypeptide, depending on the nature of the polymer itself. The analysis included the evaluation of a mutation at the P171 position in addition to the two introduced polymer attachment sites, which was designed to enhance the proteolytic stability of the FGF21 mutant polypeptide, and provide selected polymer attachment sites and enhanced proteolytic stability, while at the same time maintaining or enhancing the *in vivo* biological activity of wild-type FGF21.

In a parallel study, a select subset of single mutations from Table 5, presented in Example 4, were combined with the stability enhancing P171 mutation for the purpose of PEGylating site selectively at both the α-amino N-terminus and the introduced cysteine mutation using site-selective mixed chemistries as previously described (*see* U.S. Patent No. 6,420,339, incorporated herein by reference). These double mutants were designated R77C/P171G and H125C/P171G and are exemplary of any other combinations that might be contemplated using the mutations disclosed in Table 5 of Example 4.

A summary of FGF21 mutants comprising two mutations, in addition to a mutation at the P171 site, that were generated through this rational protein engineering approach is provided in Table 7. These mutants incorporate two non-naturally occurring polymer attachment sites, notably cysteine. The side chains of these residues are particularly suited to chemical modification by the attachment of a polymer, such as a PEG molecule. It was recognized that in addition to the introduced non-naturally occurring polymer attachment site, a polymer could optionally be attached to the N-terminus of the protein, as desired. The triple FGF21 mutants described include not only the introduced polymer attachment sites described above, but also a proteolytic stability inducing mutation at position 171. Following chemical modification of the FGF21 triple mutant, this combination of mutations serves the function of enhancing FGF21 half life via the association of two polymers (*e.g.*, PEG molecules) with the polypeptide sequence as well as enhancing the half life via the elimination of a proteolytic cleavage site.

Since the introduced residues were designed to maintain the *in vivo* wild-type levels of FGF21 biological activity, these FGF21 mutants are expected to maintain FGF21 biological activity and yet still provide unique non-naturally occurring polymer attachment sites. After chemical modification (*e.g.*, PEGylation) these mutants are expected to have a longer half life than wild-type FGF21, while maintaining significant *in vivo* wild-type levels of biological activity.

The numbers of the positions given in Table 7 correspond to the residue position in the mature FGF21 protein, which consists of 181 amino acid residues. Nucleic acid sequences encoding the FGF21 mutant polypeptides listed in Table 7 were prepared using the techniques described herein.

Table 7

FGF21 Mutant Polypeptides Comprising Three Mutations

Residue 1	**WT**	**Mutation**	**Residue 2**	**WT**	**Mutation**	**Residue 3**	**WT**	**Mutation**	
37	E	C	77	R	C	171	P	G	E37C, R77C, P171G
91	E	C	125	H	C	171	P	G	E91C, H125C, P171G
77	R	C	120	G	C	171	P	G	R77C, G120C, P171G
37	E	C	91	E	C	171	P	G	E37C, E91C, P171G
91	E	C	175	R	C	171	P	G	E91C, R175C, P171G
37	E	C	175	R	C	171	P	G	E37C, R175C, P171G
91	E	C	120	G	C	171	P	G	E91C, G120C, P171G
37	E	C	120	G	C	171	P	G	E37C, G120C, P171G
77	R	C	175	R	C	171	P	G	R77C,

									R175C, P171G
37	E	C	125	H	C	171	P	G	E37C, H125C, P171G

EXAMPLE 7

Preparation and Expression of FGF21 Mutant Polypeptides

Constructs encoding the FGF21 mutants listed in Tables 3 (single mutations), 4 (double mutations) and 5 (triple mutations, *i.e.*, double mutations + P171G), collectively "FGF21 mutants" in this Example, were prepared by PCR amplification of the wild-type FGF21 expression vector as described below (the construction of the wild-type FGF21 expression vector is described in Example 1). The goal of these experiments was to generate FGF21 mutants that comprise one or more non-naturally occurring polymer attachment sites, and in some cases are resistant to proteolysis. The chemically modified forms of these FGF21 mutants would be expected to exhibit longer half lives, and in some cases also resist proteolysis.

FGF21 mutant constructs were prepared using primers having sequences that are homologous to regions upstream and downstream of a codon (or codons) to be mutated. The primers used in such amplification reactions also provided approximately 15 nucleotides of overlapping sequence to allow for recircularization of the amplified product, namely the entire vector now having the desired mutant.

An exemplary FGF21 mutant construct, encoding an FGF21 mutant having a glutamic acid residue at position 170 instead of the native glycine residue (*i.e.*, a G170E FGF21 mutant polypeptide) was prepared using the primers shown in Table 8.

Table 8

PCR Primers for Preparing Exemplary FGF21 Mutant

Primer	**Sequence**	**SEQ ID NO:**
Sense	5'-ATGGTGGAACCTTCCCAGGGCCGAAGC-3'	29

	CTCCTCGGACCCTCTGAGCATGGTG**GGA**CCTTCCCAGGGCCGAAGCCCCA	30
	GAGGAGCCTGGGAGACTCGTACCAC**CCT**GGAAGGGTCCCGGCTTCGGGGT	31
Antisense	5'-GGAAGGTTCCACCATGCTCAGAGGGTCCGA-3'	32

The primers shown in Table 6 allow for the substitution of the glycine residue with a glutamic acid residue as shown below, wherein the upper sequence is the sense primer (SEQ ID NO: 29), the second and third sequences (SEQ ID NOs: 30 and 31) are portions of an FGF21 expression construct, and the fourth sequence is the antisense primer (SEQ ID NO: 32):

5'-ATGGTGG**A**ACCTTCCCAGGGCCGAAGC
CTCCTCGGACCCTCTGAGCATGGTG**GGA**CCTTCCCAGGGCCGAAGCCCCA
GAGGAGCCTGGGAGACTCGTACCAC**CCT**GGAAGGGTCCCGGCTTCGGGGT
AGCCTGGGAGACTCGTACCACC**T**TGGAAGG-5'

FGF21 mutant constructs were prepared using essentially the PCR conditions described in Example 1. Amplification products were digested with the restriction endonuclease DpnI, and then transformed into competent cells. The resulting clones were sequenced to confirm the absence of polymerase-generated errors.

FGF21 mutants were expressed by transforming competent BL21 (DE3) or BL21 Star (Invitrogen; Carlsbad, CA) cells with the construct encoding a particular mutant. Transformants were grown overnight with limited aeration in TB media supplemented with 40 μg/mL kanamycin, were aerated the next morning, and after a short recovery period, were induced in 0.4 mM IPTG. FGF21 mutant polypeptides were harvested by centrifugation 18-20 hours after induction.

As a result of the bacterial expression system employed, the FGF21 mutant polypeptides were expressed with an N-terminal methionine residue.

EXAMPLE 8

Purification of FGF21 and FGF21 Mutant Polypeptides from Bacteria

In the Examples that follow, FGF21 mutant polypeptides were expressed in a bacterial expression system. After expression, which is described in Example 7, FGF21 mutant polypeptides were purified as described in this Example, unless otherwise indicated.

To purify the FGF21 mutant polypeptide from bacterial inclusion bodies, double-washed inclusion bodies (DWIBs) were solubilized in a solubilization buffer containing guanidine hydrochloride and DTT in Tris buffer at pH 8.5 and then mixed for one hour at room temperature, and the solubilization mixture was added to a refold buffer containing urea, arginine, cysteine, and cystamine hydrochloride at pH 9.5 and then mixed for 24 hours at 5°C (*see, e.g.*, Clarke, 1998, *Curr. Opin. Biotechnol.* 9: 157-63; Mannall *et al.*, 2007, *Biotechnol. Bioeng.* 97: 1523-34; Rudolph *et al.*, 1997, "Folding Proteins," in *Protein Function: A Practical Approach* (Creighton, ed., New York, IRL Press), pp 57-99; and Ishibashi *et al.*, 2005, *Protein Expr. Purif.* 42: 1-6).

Following solubilization and refolding, the mixture was filtered through a 0.45 micron filter. The refold pool was then concentrated approximately 10-fold with a 5 kD molecular weight cut-off Pall Omega cassette at a transmembrane pressure (TMP) of 20 psi, and dialfiltered with 3 column volumes of 20 mM Tris, pH 8.0 at a TMP of 20 psi.

The clarified sample was then subjected to anion exchange (AEX) chromatography using a Q Sepharose HP resin. A linear salt gradient of 0 to 250 mM NaCl in 20 mM Tris was run at pH 8.0 at 5°C. Peak fractions were analyzed by SDS-PAGE and pooled.

The AEX eluate pool was then subjected to hydrophobic interaction chromatography (HIC) using a Phenyl Sepharose HP resin. Protein was eluted using a decreasing linear gradient of 0.6 M to 0 M ammonium sulfate with 20 mM TRIS at pH 8.0 and ambient temperature. Peak fractions were analyzed by SDS-PAGE (Laemmli, 1970, *Nature* 227: 680-85) and pooled.

The HIC pool was concentrated with a 5 kD molecular weight cut-off Pall Omega 0.2 m^2 cassette to 7 mg/mL at a TMP of 20 psi. The concentrate was diafiltered with 5 column volumes of 20 mM TRIS, pH 8.0 at a TMP of 20 psi, and the recovered concentrate was diluted to about 5 mg/mL. Finally, the solution was filtered through a 0.22 µm cellulose acetate filter.

EXAMPLE 9

Chemical Modification of FGF21 Mutants

Variants of FGF21 having cysteine substituted at the selected positions shown in Tables 3-5 were produced as described in Example 7, and the molecules were then subjected to a PEGylation reaction with a 20 kDa methoxy-PEG-maleimide. Unless indicated otherwise, all PEGylated FGF21 wild-type and mutant polypeptides disclosed herein comprise one or more 20kDa methoxy PEG maleimide polymers.

The partially purified FGF21 molecules were then reduced using 5 molar equivalents of TCEP for 30 minutes at 25 °C. The reduced FGF21 was then buffer exchanged in to 10 mM imidazole, pH 7.5 using a GE Healthcare Sephadex G25M column. The buffer exchanged FGF21 was then reacted with 5 molar equivalents of 20 kDa methoxy-PEG-maleimide for 30 minutes at 25 °C. The resulting reaction mixture was then subjected to ion-exchange chromatography to isolate the mono-PEGylated species from multi-PEGylated and un-PEGylated molecules. Most of the FGF21 mutant polypeptides reacted well with the methoxy-PEG-maleimide and produced principally mono-PEGylated products in high yield (Figures 2 and 3).

For production of the Tethered Molecules, partially purified FGF21 molecules were reduced using 5 molar equivalents of TCEP for 30 minutes at 25 °C. The reduced FGF21 was then buffer exchanged in to 10 mM imidazole, pH 7.5 using a GE Healthcare Sephadex G25M column. The buffer exchanged FGF21 was then reacted with 0.45 molar equivalents of 20 kDa PEG bis-maleimide for 60 minutes at 4 °C. The resulting reaction mixture was then subjected to ion-exchange chromatography to isolate the Tethered Molecule from other undesirable reaction products. Frequently an additional ion-exchange purification step was required, which was accomplished by diluting the first ion-exchange pool in about 4 volumes of water and reapplying to the ion-exchange column.

Alternatively amine specific coupling to the N-terminus was achieved by reductive alkylation using methoxy-PEG--propionaldehyde as previously described (*see* U.S. Patent No. 5,824,784). Briefly, mutant FGF21 at about 2 mg/ml was reacted overnight at 4 degrees C, with a 5-fold molar excess of 20kD mPEG-propionaldehyde in acetate buffer pH 5.5 and 10mM sodium cyanoborohydride. N-terminal PEGylation of any of the FGF21 wild-type and mutant molecules described herein can be achieved using this strategy.

When amine specific dual PEGylation was required at both the N-terminus and a mutant lysine, methoxyPEG-NHS (N-hydroxysuccinimidyl ester) was used. Briefly, mutant FGF21 at about 2 mg/ml was reacted for about 2 hours at 4 °C with a 5-fold molar excess (PEG:amino group) of 20kD mPEG-NHS in bicine buffer, pH 7.5.

When applying a mixed chemistry approach to dual PEGylate FGF21 mutants at both the N-terminus and a mutant Cys position, both methoxy-PEG–propionaldehyde and methoxy-PEG-maleimide were used sequentially as previously described (*see* U.S. Patent No. 6,420,339). Briefly, mutant FGF21 at about 2 mg/ml was reacted for about 2 hours with a 1.5-fold molar excess of 20kD mPEG-maleimide in phosphate buffer at pH 6.5 at 4 °C, then the pH was adjusted to about pH 5 and a 5-fold excess of 20kD mPEG-propionaldehyde added with 10mM sodium cyanoborohydride. The final reaction was allowed to continue at 4 °C overnight.

Purification of all the different reaction mixtures was by anion exchange chromatography in Tris buffer at pH 8 as previously described.

EXAMPLE 10

In vitro Activity of FGF21 Mutant Polypeptides and Chemically Modified FGF21 Mutant Polypeptides

PEGylated FGF21 mutant polypeptides were then subjected to an *in vitro* assay to assess their activity, as compared to the un-PEGylated form of the FGF21 mutant polypeptide and N-terminally PEGylated FGF21.

One goal of these experiments was to identify FGF21 mutant polypeptides and chemically-modified FGF21 mutant polypeptides that preserve FGF21 activity in an ELK-luciferase *in vitro* assay. ELK-luciferase assays were performed using a recombinant human 293T kidney cell system, in which the 293T cells overexpress beta-klotho and luciferase reporter constructs. Beta-klotho is a co-receptor that is required by FGF21 for activation of its FGF receptors. The FGF receptors used in this assay are endogenous levels of FGF receptors expressed in 293T kidney cell. The luciferase reporter constructs contain sequences encoding GAL4-ELK1 and a luciferase reporter driven by a promoter containing five tandem copies of the Gal4 binding site (5xUAS-Luc). Luciferase activity is regulated by the level of phosphorylated Erk/ELK1, and is used to indirectly monitor and quantify FGF21 activity.

ELK-luciferase assays were performed by culturing the 293T cells in the presence of different concentrations of wild-type FGF21 or FGF21 mutant polypeptide for 6 hours, and then assaying the cell lysates for luciferase activity. Figures 4-6 and Tables 3-5 summarize the *in vitro* results obtained for several exemplary FGF21 mutant polypeptides, including those having one, two or three mutations.

EXAMPLE 10.A

EC50 Values for PEGylated FGF21 Mutant Polypeptides Comprising One Mutation

Table 9 below summarizes the EC50 values of various FGF21 mutant polypeptides comprising two mutations, which introduce one non-naturally occurring polymer attachment sites at a specific, known location.

Various FGF21 mutant polypeptides were generated and the activity determined in the *in vitro* ELK-luciferase assay described herein. Table 9 summarizes the data obtained:

Table 9

Summary of EC50 for PEGylated FGF21 Mutant Polypeptides Comprising One Mutation

Mutation	**EC50 (nM)**	**WT EC50 (nM)**	**N-PEG20 EC50 (nM)**
H125C	4.5	2.8	36.7
R77C	4.9	3.9	43.3
K69C	5.3	2.8	36.7
G120C	6.0	2.8	36.7
E37C	6.9	3.9	43.3
R175C	7.9	0.9	21.5
E91C	8.3	3.9	43.3
N121C	9.2	3.9	43.3
R126C	10.0	2.8	36.7
G113C	12.0	3.9	43.3
D38C	13.1	2.8	36.7
D79C	20.4	3.4	40.0
D46C	27.8	3.9	43.3
Y179C	287.1	1.1	21.5

Table 9 summarizes the effect of PEGylation on the *in vitro* activity of some of the various FGF21 mutant polypeptides of the present invention.

Figures 4, 5 and 6 graphically depict the results and EC50 values for several PEGylated FGF21 mutants comprising a single point mutation. Figures 4 and 5 contain data on several of the FGF21 mutants for which data is presented in Table 9. More particularly, the upper plot of Figure 4 shows the results of the ERK-luciferase assay performed on PEGylated E37C, R77C, E91C mutants and N-terminally PEGylated wild-type FGF21, as well as un-PEGylated FGF21. In the lower plot in Figure 4, data is presented on PEGylated G113C, N121C, D46C mutants and N-terminally PEGylated wild-type FGF21, as well as un-PEGylated wild-type FGF21.

Turning to Figure 5, in the upper plot data is presented for H125C, G120C, R126C mutants and N-terminally PEGylated wild-type FGF21, as well as un-PEGylated wild-type FGF21. In the lower plot, data is presented for D79C, D38C mutants and N-terminally PEGylated wild-type FGF21, as well as un-PEGylated wild-type FGF21.

Continuing with the upper plot of Figure 6, graphical data is presented for PEGylated K69C and D79C mutants, as well as N-terminally PEGylated wild-type FGF21, and for un-PEGylated wild-type FGF21. In the lower plot of Figure 6, data is presented for PEGylated R175C and Y179C mutants and N-terminally PEGylated wild-type FGF21, as well as un-PEGylated wild-type FGF21. Surprisingly, many of these molecules have *in vitro* activity close to that of the unPEGylated molecule.

EXAMPLE 10.B

EC50 Values for Selected Arg/Lys FGF21 Mutants

As described in Example 3, a series of FGF21 mutant polypeptides was generated in which naturally-occurring polymer attachment sites (e.g., PEGylation sites) were removed by mutagenesis. In these FGF21 mutants reactive Lys groups were first mutated to arginine, leaving only the N-terminus α-amino group available for PEGylation. Next select arginine residues, either native or mutant, were converted one by one to lysine, thereby introducing a secondary PEGylation site at defined positions on the FGF21 surface. One goal of this strategy was to generate FGF21 mutant polypeptides in which a polymer (e.g., a PEG molecule) would be conjugated at one or more specific, known locations.

Various arginine/lysine FGF21 mutant polypeptides were generated and the activity determined in the *in vitro* ELK-luciferase assay described herein. Table 10 summarizes the data obtained:

Table 10

Summary of EC50 for PEGylated FGF21 Mutant Polypeptides Comprising Lysine/Arginine Mutations

Construct	**Type of PEG**	***In Vitro* Activity EC_{50} (nM)**
Native FGF21	No PEG	3
Native FGF21 (N-terminus)	1 x 20k	43.3
Native FGF21 (random)	2 x 20k	200
FGF21(all K to all R)	1 x 20k	nd
FGF21(all K to all R, R36K)	2 x 20k	73
FGF21(all K to all R, R77K)	2 x 20k	175
FGF21(K56/59/69R)	2 x 20k	500
FGF21(K all R, R126K)	2 x 20k	38
FGF21(K59R/K69R/K122R)	2 x 20k	499
FGF21(K56R/K69R/K122R)	2 x 20k	215
FGF21(K56R/K59R/K122R)	2 x 20k	531
FGF21(all K to all R, R72K)	2 x 20k	>1000
FGF21(all K to all R, R175K)	2 x 20k	>1000

EXAMPLE 10.C

EC50 Values for Selected PEGylated FGF21 Mutant Polypeptides Comprising Two Mutations

The activity of a number of FGF21 mutant polypeptides comprising two mutations was also examined in the ERK-luciferase assay described herein. These mutants were engineered to introduce two non-naturally occurring polymer attachment sites (in the form of a cysteine residue) and a mutation providing enhanced proteolytic stability (P171G) into the wild-type FGF21 sequence.

Various FGF21 mutant polypeptides were generated and the activity determined in the *in vitro* ELK-luciferase assay described herein. EC50 values from those experiments are shown below in Table 11.

Table 11

EC50 Values for Selected Chemically Modified FGF21 Mutant Polypeptides Comprising Two Mutations

Mutations	EC50 (nM)
Native FGF21	3
N-term PEG20K	37
E37C, R77C	21
G120C, H125C	23
R77C, E91C	26
R77C, H125C	30
E91, H125C	32
R77C, G120C	34
E37C, E91C	36
E91C, R175C	46
E37C, R175C	50
E91C, G120C	54
E37C, G120C	55
R77C, R175C	62
E37C, H125C	64
E37C, K69C	67
K69C, E91C	69
G120C, R175C	118

K69C, G120C	119
K69C, H125C	164
K69C, R77C	180
H125C, R175C	200
K69C, R175C	318
E37C, G170C	163

In parallel, dual-PEGylated FGF21 mutants were prepared using single cysteine mutants derived from Example 10A, but also carrying the stability enhancing P171G mutation. These mutants were site-selectively PEGylated at both the N-terminus and the engineered cysteine using the mixed PEGylation chemistry as previously described (*see* U.S. Patent No. 6,420,339). Because the mixed PEGylation chemistry allows the discrimination between the N-terminus and cysteine conjugation sites, it is also possible to site-selectively couple different polymers to different sites. In this case, a series of conjugates were prepared wherein combinations of 5kDa, 10kDa and 20kDa polymers were coupled to the N-terminus and a 20kDa polymer was consistently coupled to the cysteine mutant. This allowed assessment of the impact of N-terminal PEGylation on FGF21 *in vitro* activity. These conjugates were all tested in the *in vitro* ELK-luciferase assay described here. The results are presented in Table 12.

Table 12

EC50 Values for Selected Chemically Modified FGF21 Mutant Polypeptides Comprising a Cysteine Mutation and a P171G Mutation

Mutation	Type of PEG	EC50 (nM)
R77C, P171G	2 x 20k	64
H125C, P171G	2 x 20k	152
H125C, P171G	1 x 10k, 1 x 20k	65
H125C, P171G	1 x 5k, 1 x 20k	30

EXAMPLE 10.D

EC50 Values for Selected Chemically Modified FGF21 Mutant Polypeptides

Comprising Three Mutations

The activity of a number of FGF21 mutant polypeptides comprising three mutations was also examined in the ERK-luciferase assay described herein. These mutants were engineered to introduce two non-naturally occurring polymer attachment sites (in the form of a cysteine residue) and a mutation providing enhanced proteolytic stability (P171G) into the wild-type FGF21 sequence.

Various FGF21 mutant polypeptides were generated and the activity determined in the *in vitro* ELK-luciferase assay described herein. Table 13 summarizes the data obtained:

Table 13

EC50 Values for Selected Chemically Modified FGF21 Mutant Polypeptides Comprising Three Mutations

Mutations	**Type of PEG**	**EC50 (nM)**
E37C, R77C, P171G	2X 20 kDa	27
E91, H125C , P171G	2X 20 kDa	37
R77C, G120C , P171G	2X 20 kDa	35
E37C, E91C , P171G	2X 20 kDa	30
E91C, R175C , P171G	2X 20 kDa	155
E37C, R175C , P171G	2X 20 kDa	162
E91C, G120C , P171G	2X 20 kDa	34
E37C, G120C , P171G	2X 20 kDa	35
R77C, R175C , P171G	2X 20 kDa	ND
E37C, H125C , P171G	2X 20 kDa	37

Surprisingly, the data indicates that the majority of these dual-PEGylated FGF21 mutant polypeptides have activity surpassing the N-terminally mono-PEGylated molecule.

EXAMPLE 11

EC50 Values for Selected FGF21 Tethered Molecules

The Tethered Molecules of the present invention comprise two FGF21 polypeptide sequences tethered together by a linker molecule. Figure 7 graphically depicts an example of a Tethered Molecule. As described herein, it was predicted that these Tethered Molecules would provide longer half-lives, while still retaining a desirable level of biological activity.

Various Tethered Molecules were generated and the activity determined in the *in vitro* ELK-luciferase assay described here. The Tethered Molecules generated comprise FGF21 mutants in which the mutation introduces a linker attachment site. Several of the FGF21 mutants were double mutants and include the P171G mutation to enhance proteolytic stability. Conditions and procedures for this *in vitro* study were the same as those in Example 10. Table 14 summarizes the data obtained:

Table 14

EC50 Values for Selected FGF21 Tethered Molecules

Mutations	**Linker**	**EC50 (nM)**
H125C, P171G	20 kDa PEG	0.21
R77C, P171G	20 kDa PEG	0.25
G120C, P171G	20 kDa PEG	0.51
E37C, P171G	20 kDa PEG	0.36
R175C, P171G	20 kDa PEG	0.36
E91C, P171G	20 kDa PEG	0.20
G170C	20 kDa PEG	0.47
P171C	20 kDa PEG	0.21

It can be seem from the data in Table 14 that the Tethered Molecules possess a surprisingly high *in vitro* activity equal to or exceeding that of the unPEGylated molecule which, for reference, was determined to be 0.63.

EXAMPLE 12

In vivo Activity of Chemically-modified FGF21 Mutant Polypeptides

FGF21 possesses a number of biological activities, including the ability to lower blood glucose, insulin, triglyceride, or cholesterol levels; reduce body weight;

or improve glucose tolerance, energy expenditure, or insulin sensitivity. Following the initial *in vitro* evaluation described in Example 10, PEGylated FGF21 mutant polypeptides were further analyzed for *in vivo* FGF21 activity. PEGylated FGF21 polypeptides were introduced into insulin resistant ob/ob mice, and the ability of a particular PEGylated FGF21 polypeptide to lower blood glucose was measured. The procedure for the *in vivo* work was as follows.

The PEGylated FGF21 polypeptide to be tested was injected intraperitoneally into an 8 week old ob/ob mice (Jackson Laboratory), and blood samples were obtained at various time points following a single injection, *e.g.*, 0, 6, 24, 72, 120, and 168 hours after injection. Blood glucose levels were measured with a OneTouch Glucometer (LifeScan, Inc. Milpitas, CA), and the results expressed as a percent change of blood glucose relative to the baseline level of blood glucose (*i.e.*, at time 0).

The FGF21 mutants were generated as described herein and were chemically modified by the addition of two 20kDa methoxy PEG maleimide molecules, one at each of the introduced polymer attachment sites, which were typically cysteine residues. The mutations were selected so as to provide discrete, known attachment points for two PEG molecules. PEGylation of the FGF21 mutants was achieved using the methods described herein.

EXAMPLE 12.A

In vivo Activity of N-terminally PEGylated Wild-type FGF21

Wild-type FGF21 that was chemically modified by PEGylation at the N-terminus of the polypeptide was studied in an ob/ob mouse model. Un-PEGylated wild-type FGF21 was also studied in the same experiment and PBS was used as a control. A single 20kDa methoxy PEG maleimide molecule was used to N-terminally PEGylate wild-type FGF21. Figure 8 demonstrates the results of this experiment.

Both native and N-terminally PEGylated wild-type FGF21 reduced blood glucose levels by 30-40% after a single injection. However, the blood glucose levels returned to baseline 24 hours after the injection of native wild-type FGF21. In contrast, N-terminally PEGylated wild-type FGF21 has sustained blood glucose-lowering activity for at least 72 hours. The results of this study indicate PEGylation of wild-type FGF21 prolongs the pharmacodynamic effects of native molecule.

Turning to Figure 9, a dose response study was performed in an ob/ob mouse model using wild-type FGF21, which was PEGylated at the N-terminus with a 20, 30 or 40kDa PEG molecule. Figure 9 demonstrates that 30 and 40kDa PEG molecules have greater and longer glucose lowering efficacy compared with 20kDa PEG molecule, suggesting PEG size is positively correlated with *in vivo* pharmacodynamic effects.

EXAMPLE 12.A.1

***In Vivo* Activity of Selected Chemically Modified FGF21 Mutant Polypeptides Conjugated at Both the N-terminus and an Introduced Polymer Attachment Site**

Several chemically modified mutants of FGF21 that were site-selectively PEGylated at both the N-terminus and a second engineered site were studied in a mouse ob/ob model. These dual-PEGylated FGF21 constructs were all PEGylated at the N-terminus and a second site comprising either an engineered lysine as described in Example 4 or an engineered cysteine as described in Example 6.

A similar experiment was performed using a different group of PEGylated FGF21 mutant polypeptides. Figure 10 comprises two plots showing the percent change in blood glucose levels of mice injected with vehicle (PBS), or the PEGylated forms of FGF21 mutants comprising polymer attachment mutations, namely FGF21 R77C, which was also N-terminally PEGylated, and FGF21 R126K, which was also N-terminally PEGylated (upper plot), and N-terminally PEGylated F77/P171G (lower plot), which were also PEGylated at the introduced polymer attachment sites. 20kDa methoxy PEG maleimide molecules were used. The results of this experiment again confirm that the dually PEGylated FGF21 mutants demonstrate an enhanced pharmacodynamics relative to the wild type FGF21 protein with sustained glucose-lowering for at least 120 hours.

EXAMPLE 12.B

In vivo Activity of Selected Chemically Modified FGF21 Mutant Polypeptides Comprising Two or Three Mutations

A number of chemically modified FGF21 mutant polypeptides were studied in a mouse ob/ob model. These mutants were chemically modified by the addition of two 20kDa methoxy PEG maleimide molecules, one at each of the introduced cysteine residues, and in the case of FGF21 mutant polypeptides comprising a single mutation, at the N terminus of the polypeptide. The mutations were selected so as to provide discrete known attachment points for one or more PEG molecules. Some FGF21 mutants also included the P171G mutation to enhance proteolytic resistance. The PEGylated FGF21 mutant polypeptides were injected into the mice and the mice bled at intervals over a 9 day study.

EXAMPLE 12.B.1

Effect on Glucose Levels

An experiment was performed using yet another group of PEGylated FGF21 mutant polypeptides. Figure 11 is plot showing the percent change in blood glucose levels of mice injected with vehicle (PBS), or the dually PEGylated forms of FGF21 mutants comprising the mutations, namely E91/H125C, E91C/R175C, E37C/G120C, E37C/H125C, E37C/R175C; unPEGylated Fc-G170E FGF21 was also studied. 20kDa methoxy PEG maleimide molecules were used. The results of this experiment again confirm that the dually PEGylated FGF21 mutants demonstrate an enhanced pharmacodynamics relative to the wild type FGF21 protein.

A similar experiment was performed using yet another group of PEGylated FGF21 mutant polypeptides. Figure 12 is a plot showing the percent change in blood glucose levels of mice injected with vehicle (PBS), or the dually PEGylated forms of FGF21 mutants comprising the mutations, namely N-terminally PEGylated R77C which was also PEGylated at the introduced polymerization site, N-terminally PEGylated R126K which was also PEGylated at the introduced polymerization site, E91/G120C, G120C/H125C, and E37C/R77C; unPEGylated Fc-G170E FGF21 was also studied. 20kDa methoxy PEG maleimide molecules were used. The results of this experiment confirm that the dually PEGylated FGF21 mutants demonstrate enhanced pharmacodynamics relative to the wild-type FGF21 protein.

In another experiment, Figure 13 shows the effect of the PEGylated FGF21 mutants on the blood glucose levels of the mice over the course of a 9 day study. This data demonstrates that the PEGylated molecules have enhanced *in vivo* potency compared to that of the native molecule. In Figure 13, the results were generated with PEGylated E37C/R77C, E91C/R175C, E37C/H125C, E37C/R77C/P171G, E91C/R175C/P171G, and E37C/H125C/P171G FGF21 mutants, which were dually PEGylated at the introduced polymer attachment sites. The FGF21 mutants were modified with two 20kDa methoxy PEG maleimide molecules in this study, and the vehicle used was 10 mM potassium phosphate, 5% sorbitol, pH 8.

As shown in Figure 13, the three double mutant dually PEGylated molecules E37C/R77C; E91C/R175C; E37C/H125C reduced blood glucose levels, and the effects were maintained for 72 hours after a single injection. Interestingly, introducing P171G mutation further prolonged the *in vivo* actions and the maximal glucose-lowering activities were maintained for additional 2 days with total duration of action of 120 hours.

Additionally, the glucose lowering ability of several FGF21 polypeptides comprising three mutations were studied alongside several Tethered Molecules. The triple mutants used were E37C/R77C/P171G and E91C/H125C/P171G; the Tethered Molecules comprised two identical FGF21 mutant polypeptides comprising two mutations, namely E37C/P171G and R77C/P171G. 20kDa methoxy PEG maleimide molecules were used for the dually PEGylated forms of FGF21, as well as for the linker molecule in the Tethered Molecule. The results of this experiment are shown in Figure 14. Compared with the Tethered FGF21 mutants, the dually PEGylated mutants have further enhanced pharmacodynamics. The glucose lowering activity of the dually PEGylated mutants sustained for at least 168 hours as compared to 120 hours with the Tethered FGF21 mutants.

Figure 15 is a plot showing the percent change in blood glucose as a function of dose in ob/ob mice injected with vehicle (10 mM TRIS, 150 mM NaCl, pH 8.5) or different doses of a dually PEGylated E37C/R77C/P171G FGF21 mutant polypeptide over a nine day period. The results of this experiment demonstrate that the dually PEGylated triple mutant E37C/R77C/P171G reduced blood glucose levels in ob/ob mice in a dose-dependent manner. The dose level of 0.3mg/kg nearly reached maximal glucose-lowering activity and the effects were maintained for at least 5 days. Further enhanced *in vivo* potency and duration of action were observed when the dose level increased to 1 mg/kg.

EXAMPLE 12.B.2

Effect on Body Weight

Type 2 diabetes is often accompanied with increased adiposity and body weight. A therapeutic molecule would therefore preferably have the desirable effect of reducing body weight as well as the desirable effect of lowering blood glucose levels. Accordingly, many of the PEGylated FGF21 mutants described herein were evaluated for their effect on mice body weight.

To study the effect of various FGF21 mutant polypeptides on body weight, PEGylated wild-type and FGF21 mutant polypeptides were injected intraperitoneally into 8 week old *ob/ob* mice (Jackson Laboratory), and body weight was monitored at various time points following a single injection, *e.g.*, 0, 24, 72, 120, and 168 hours after injection.

Figure 16 shows the effect of the PEGylated FGF21 mutants on the weight of the mice over the term of the study. The results were generated using dually PEGylated E37C/R77C, E91C/R175C, E37C/H125C, E37C/R77C/P171G, E91C/R175C/P171G, and E37C/H125C/P171G FGF21 mutant polypeptides. The FGF21 mutants were modified with 20kDa methoxy PEG maleimide molecules in this study. These FGF21 mutants were also studied with respect to changes in glucose levels and those results are shown in Figure 13. Taken together, this data demonstrates that the mice receiving the PEGylated FGF21 mutants gained less weight, relative to the vehicle control and that FGF21 mutant polypeptides comprising the proteolysis resistant mutation P171G are more efficacious than are their P171 wild-type counterparts.

Figure 17 is plot showing the change in body weight of mice injected with vehicle, or the dually PEGylated forms of a FGF21 mutant comprising the mutations, namely E37C/R77C/P171G; E91C/H125C/P171G; R77C/P171G; and Tethered Molecules comprising in one case two E37C/P171G FGF21 mutant polypeptides and in another case two R77C/P171G FGF21 mutant polypeptides. 20kDa methoxy PEG maleimide molecules were used in the PEGylated forms of FGF21, while 20 kDa PEG bis-maleimide was used for the Tethered Molecules. These FGF21 mutants were also studied with respect to changes in glucose levels and the results are shown in Figure 14. Taken together, this data demonstrates that the mice receiving the PEGylated FGF21 mutants gained less weight, relative to the vehicle control. It was also observed that the dually PEGylated FGF21 mutant polypeptides were more efficacious in reducing body weight than the Tethered Molecules studied.

The effect of a dually PEGylated FGF21 mutant polypeptide on body weight was also examined in a multi-dose study. Figure 18 is a plot showing the change in body weight as a function of dose in ob/ob mice injected with vehicle or different doses of dually PEGylated E37C/R77C/P171G over a nine day period. This FGF21 mutant was also studied with respect to changes in glucose levels and the results are shown in Figure 15. Taken together, this data demonstrates that a dose level of 0.3 mg/kg is sufficient to reduce body weight gain in ob/ob mice after a single injection.

EXAMPLE 13

Murine Kidney Vacuole Study

One consideration for employing PEG and other polymers to extend the half-life of a therapeutic protein is the possibility that the PEGylated molecule will form kidney vacuoles. This property of PEG is well-documented (*see, e.g.*, Bendele, et al 1998, "Short Communication: Renal Tubular Vacuolation in Animals Treated with Polyethylene-glycol-conjugated Proteins," *Toxicological Sciences*, 42:152-157 and Conover et al., 1996, "Transitional Vacuole Formation Following a Bolus Infusion of PEG-hemoglobin in the Rat," *Art. Cells, Blood Subs., and Immob. Biotech.* 24:599-611) and can be unpredictable. A PEGylated molecule that induces the formation of kidney vacuoles typically, but not necessarily, renders the molecule less desirable as a therapeutic protein. In some circumstances kidney vacuolization is of less concern and can be tolerated. Accordingly, a long term kidney vacuole study was performed in mice.

FGF21 mutant polypeptides were generated as described herein. One study was designed and carried out as follows. Multiple PEGylated FGF21 molecules were administered once daily for 7 consecutive days via subcutaneous injection to female C57BL/6 mice (3/group) at a dose of 10 mg/kg; a vehicle control (10mM $KHPO_4$, 150 mM NaCl, pH 8) and 2 positive controls were administered in the same manner. Detailed clinical observations were performed on all animals prior to each dose administration, and 1-2 hours post dose on days 1, 3, and 6; cage-side observations were collected 1-2 hours post dose on all other dosing days. Body weights were collected prior to each dose administration and at necropsy. The kidneys and liver were collected from all animals for histological evaluation. Table 15 summarizes the results of the study.

Table 15

Summary of Kidney Vacuole Study

Molecule	**Kidney**	**Liver**
Vehicle	0.0	0.0
Positive Control	3.7	0.0
FGF21-**1X**-NT(PEG20K)	2.0	0.0
H126C **1X**-PEG20K	2.3	0.0
R78C **1X**-PEG20K	2.3	0.0
K70C **1X**-PEG20K	2.0	0.0
G121C **1X**-PEG20K	2.0	0.0
E38C **1X**-PEG20K	2.0	0.0
R176C **1X**-PEG20K	3.0	0.0
E92C **1X**-PEG20K	2.7	0.0
N122C **1X**-PEG20K	2.0	0.0
R127C **1X**-PEG20K	3.0	0.0
G114C **1X**-PEG20K	2.7	0.0
D80C **1X**-PEG20K	2.0	0.0
D47C **1X**-PEG20K	3.0	0.0
H112C **1X**-PEG20K	2.6	0.0
K56R, K59R, K69R, K122R, R175K **2X**-PEG20K	0.0	0.0
K56R, K59R, K69R, K122R, R77K **2X**-PEG20K	0.0	0.0
K56R, K59R, K69R, K122R, R72K **2X**-PEG20K	0.0	0.0

Table 15 demonstrates the results of a 7-day murine vacuole study performed using vehicle (10 mM Tris, 150 mM NaCl, pH 8.5), N-terminally PEGylated form of FGF21, as well as PEGylated forms of FGF21 mutants comprising the mutations H125C, R77C, K69C, G120C, E37C, R175, E91C, N121C, R126C, G113C, D79C, D46C and H112C. Either one or two 20kDa methoxy PEG maleimide molecules was used, as indicated in the table. The vacuole indices are a range with 0 being no observed change in vacuoles relative to control and 4 being severe vacuole formation. As is evident from Table 15, all of the mono-PEGylated FGF21 mutants induced the formation of kidney vacuoles. However, none of the dual-PEGylated FGF21 mutants induced vacuole formation. It was also noted that no vacuolization was observed in the livers of the mice.

Following the one week study, an 8 week chronic study was undertaken. In this study, various PEGylated FGF21 molecules were administered once weekly via subcutaneous injection for 8 weeks to female C57BL/6 mice (5/group) at doses of either 5 or 25 mg/kg; a vehicle control (10 mM Tris, 150 mM NaCl, pH 8.5) was administered in the same manner. All animals were observed for clinical signs once daily prior to dosing, and 1-2 hours post dose on dosing days; once daily on non-dosing days. Body weights were collected prior to each dose administration starting on Day 1 and then on the third day after each dose, and at necropsy. The kidneys, liver, and spleen were collected from all animals (moribund sacrifice and final euthanasia) for histological evaluation.

Figures 19A-19F is a series of plots showing weight change results observed during the eight week kidney vacuole study employing a vehicle (squares) and two doses of dual PEGylated, FGF21 mutants, namely 5 mg/kg (triangles) and 25 mg/kg (open circles). The FGF21 mutants studied include R77C, P171G (Figure 19A); E37C, R77C, P171G (Figure 19B); E37C, H125C, P171G (Figure 19C); E91C, H125C, P171G (Figure 19D); E37C, P171G (Figure 19E) and R77C, P171G (Figure 19F). In Figure 19A, a mixed chemistry approach was employed, leading to N-terminal PEGylation of the FGF21 mutant as well as PEGylation at the introduced polymer attachment site, the non-naturally occurring cysteine at 77. In Figures 19B-19D, a single chemistry approach was employed, leading to PEGylation at the introduced polymer attachment sites, the non-naturally occurring cysteine residues (cysteines at positions 37 and 77 in Figure 19B, positions 37 and 125 in Figure 19C, and positions 91 and 125 in Figure 19D). Finally, in Figures 19D and 19E, a single bis maleimide PEG was used to join two FGF21 mutants together at the non-naturally occurring cysteine residues (cysteine at position 37 in Figure 21E and position 77 in Figure 19F).

Figure 20 comprises two bar graphs showing the average score of kidney vacuoles observed during the eight-week kidney vacuole study for two doses, 5 and 25 mg/kg, of six singly or dually PEGylated FGF21 mutants. A score of 0 indicates no more vacuoles were observed than found in the control animals, while a score of 4 indicates severe vacuole formation relative to the control. The FGF21 mutants were the same as those described in Figure 19A-19E. Thus, for one construct a mixed chemistry approach was employed, leading to N-terminal PEGylation of the FGF21 mutant as well as PEGylation at the introduced polymer attachment site, the non-naturally occurring cysteine at 77. A single chemistry approach was employed for three other constructs, leading to PEGylation at the introduced polymer attachment sites, the non-naturally occurring cysteine residues (cysteines at positions 37 and 77 in one construct, positions 37 and 125 in a second construct or positions 91 and 125 in a third construct). Finally, a single bis maleimide PEG was used to join two FGF21 mutants together at the non-naturally occurring cysteine residues (cysteine at position 37 or position 77). The P171G mutation was introduced into each of the six constructs.

While the present invention has been described in terms of various embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed. In addition, the section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

All references cited in this disclosure are expressly incorporated by reference herein.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

    (a) an arginine residue at one or more of positions 56, 59, 69, and 122;
    (b) a glycine residue at position 171; or
    (c) combinations of (a) – (b).

2. An isolated nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

    (a) a lysine residue at one or more of positions 72, 77, 126 and 175;
    (b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
    (c) an arginine residue at one or more of positions 56, 59, 69, and 122;
    (d) a glycine residue at position 171; or
    (e) combinations of (a) – (d),

    and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (d) is not further modified.

3. A vector comprising the nucleic acid molecule of claim 1 or 2.

4. A host cell comprising a vector of claim 3.

5. The host cell of claim 4 that is a eukaryotic cell.

6. The host cell of claim 4 that is a prokaryotic cell.

7. A process of producing a polypeptide encoded by a vector of claim 3 comprising culturing a host cell comprising a vector of claim 3 under suitable conditions to express the polypeptide, and optionally isolating the polypeptide.

8. A polypeptide produced by the process of claim 7.

9. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d).

10. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d),

and which comprises additions, deletions or further substitutions that make the polypeptide at least 85% identical to SEQ ID NO:4, provided that the at least one amino acid substitution of claim 1(a) – (d) is not further modified.

11. The polypeptide of claim 9 or claim 10, further comprising a proline or glycine residue added to the C-terminus of the polypeptide.

12. The isolated polypeptide of any one of claims 9 to 11, wherein the polypeptide is covalently linked to one or more polymers.

13. The isolated polypeptide of claim 12, wherein the polypeptide is covalently linked to one polymer.

14. The isolated polypeptide of claim 13, wherein the polymer is a water-soluble polymer.

15. The isolated polypeptide of claim 14, wherein the water-soluble polymer is polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol.

16. The isolated polypeptide of claim 15, wherein the water-soluble polymer is PEG.

17. The isolated polypeptide of claim 12, wherein the polymer is a branched polymer.

18. The isolated polypeptide of any one of claims 9 to 11, wherein the polypeptide has a PEG moiety covalently linked to its amino-terminus.

19. The isolated polypeptide of any one of claims 9 to 11, wherein the polypeptide is covalently linked to two polymers.

20. The isolated polypeptide of claim 19, wherein one of the two polymers is a water-soluble polymer.

21. The isolated polypeptide of claim 20, wherein the water-soluble polymer is polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol.

22. The isolated polypeptide of claim 21, wherein the water-soluble polymer is PEG.

23. The isolated polypeptide of claim 19, wherein one of the polymers is branched.

24. The isolated polypeptide of claim 19, wherein both of the polymers are branched.

25. The isolated polypeptide of claim 20, wherein the polypeptide has a PEG moiety covalently linked to its amino-terminus.

26. A composition comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d),

joined by a linker to a second polypeptide comprising a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 having a lysine residue at position 36 and at least one amino acid substitution that is:

(a) a lysine residue at one or more of positions 72, 77, 126 and 175;
(b) a cysteine residue at one or more of positions 37, 38, 46, 69, 77, 79, 87, 91, 112, 113, 120, 121, 125, 126, 175, 170, and 179;
(c) an arginine residue at one or more of positions 56, 59, 69, and 122;
(d) a glycine residue at position 171; or
(e) combinations of (a) – (d).

27. The composition of claim 26, wherein the first, second or both polypeptides further comprise a proline or glycine residue added to the C-terminus of the polypeptide.

28. The composition of claim 26 or 27, wherein the linker is a peptide.

29. The composition of claim 26 or 27, wherein the linker is a water soluble polymer.

30. The composition of claim 29, wherein the water-soluble polymer is polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol.

31. The composition of claim 30, wherein the water-soluble polymer is PEG.

32. The composition of claim 26 or 27, wherein the first, second or both polypeptides are further covalently linked to one polymer, in addition to the linker.

33. The composition of claim 32, wherein the polymer is a water-soluble polymer.

34. The composition of claim 33, wherein the water-soluble polymer is polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol.

35. The composition of claim 34, wherein the water-soluble polymer is PEG.

36. The composition of claim 29, wherein the polymer is branched.

37. The composition of claim 26 or 27, wherein the composition has a single PEG moiety covalently linked to its amino-terminus.

38. The composition of claim 26 or 27, wherein the composition is covalently linked to two polymers.

39. The composition of claim 38, wherein one of the two polymers is a water-soluble polymer.

40. The composition of claim 39, wherein the water-soluble polymer is polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol.

41. The composition of claim 40, wherein the water-soluble polymer is PEG.

42. The composition of claim 38, wherein both of the polymers are water soluble polymers.

43. The composition of claim 42, wherein the water-soluble polymers are independently polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran,

cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, or polyvinyl alcohol and combinations thereof.

44. The composition of claim 43, wherein both of the water soluble polymers are PEG.

45. The composition of claim 38, wherein one of the polymers is branched.

46. The composition of claim 38, wherein both of the polymers are branched.

47. A pharmaceutical composition comprising the isolated polypeptide of any one of claims 9 to 25 or the composition of any one of claims 26 to 46 and a pharmaceutically acceptable formulation agent.

48. The pharmaceutical composition of claim 47, wherein the pharmaceutically acceptable formulation agent is a carrier, adjuvant, solubilizer, stabilizer, or anti-oxidant.

49. A method for treating a metabolic disorder comprising administering to a human patient in need thereof the pharmaceutical composition of claim 48.

50. Use of the isolated polypeptide of any one of claims 9 to 25, or the composition of any one of claims 26 to 46 in the treatment of a metabolic disorder in a human patient, or in the manufacture of a medicament for the treatment of a metabolic disorder in a human patient.

51. The method of claim 49; or the use of claim 50, wherein the metabolic disorder is diabetes.

52. The method of claim 49; or the use of claim 50, wherein the metabolic disorder is obesity.

FIG. 1

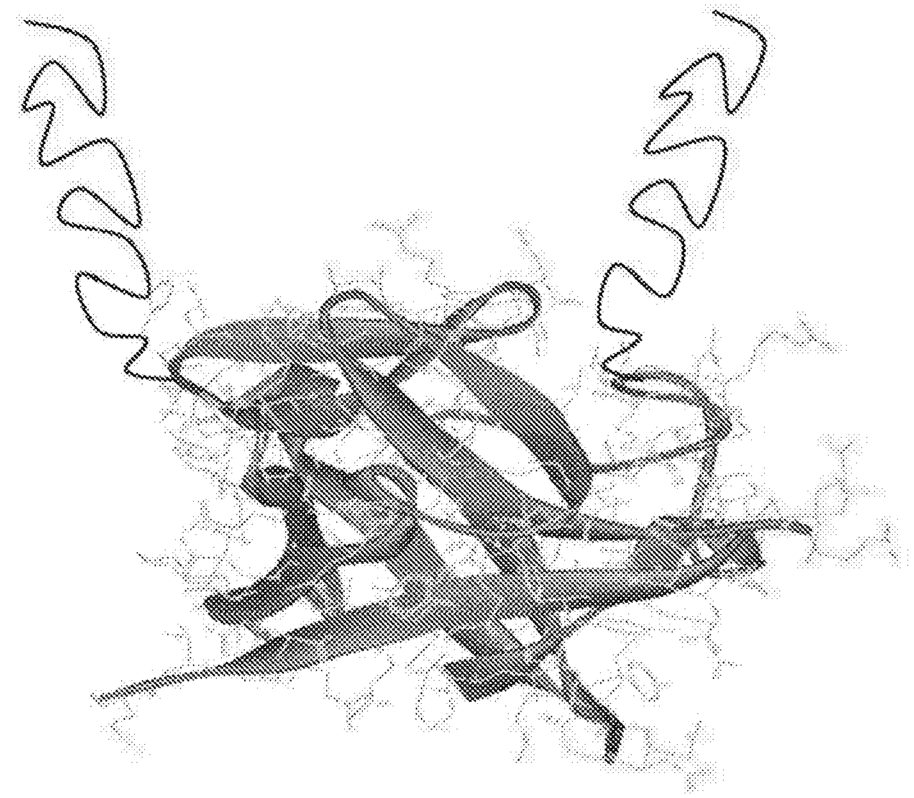


FIG. 2

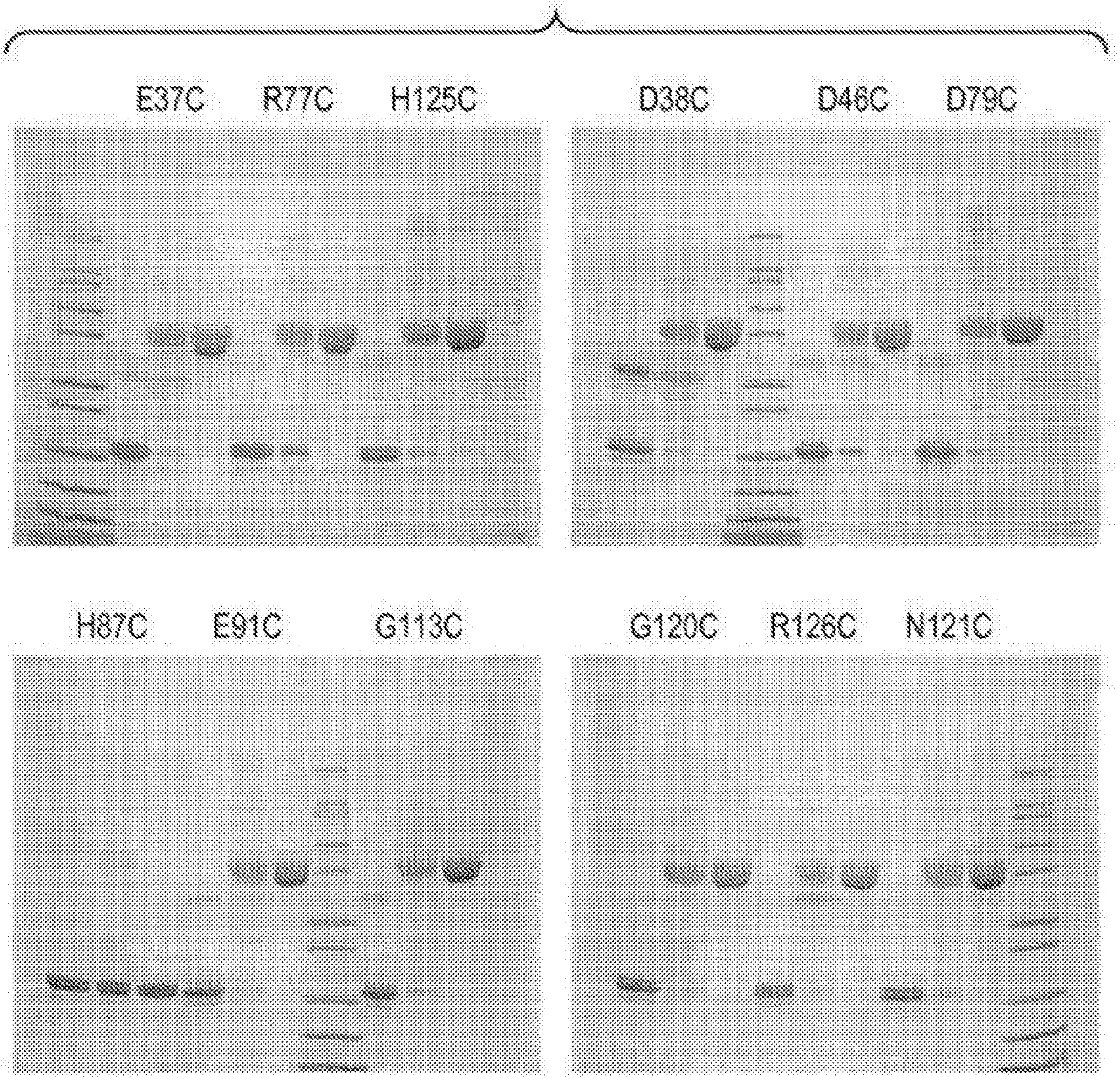

E37C
R77C
H125C
D38C
D46C
D79C
H87C
E91C
G113C
G120C
R126C
N121C

FIG. 3

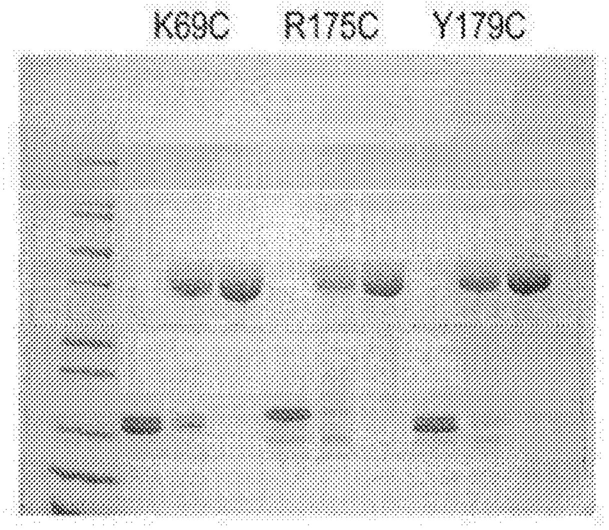


FIG. 4

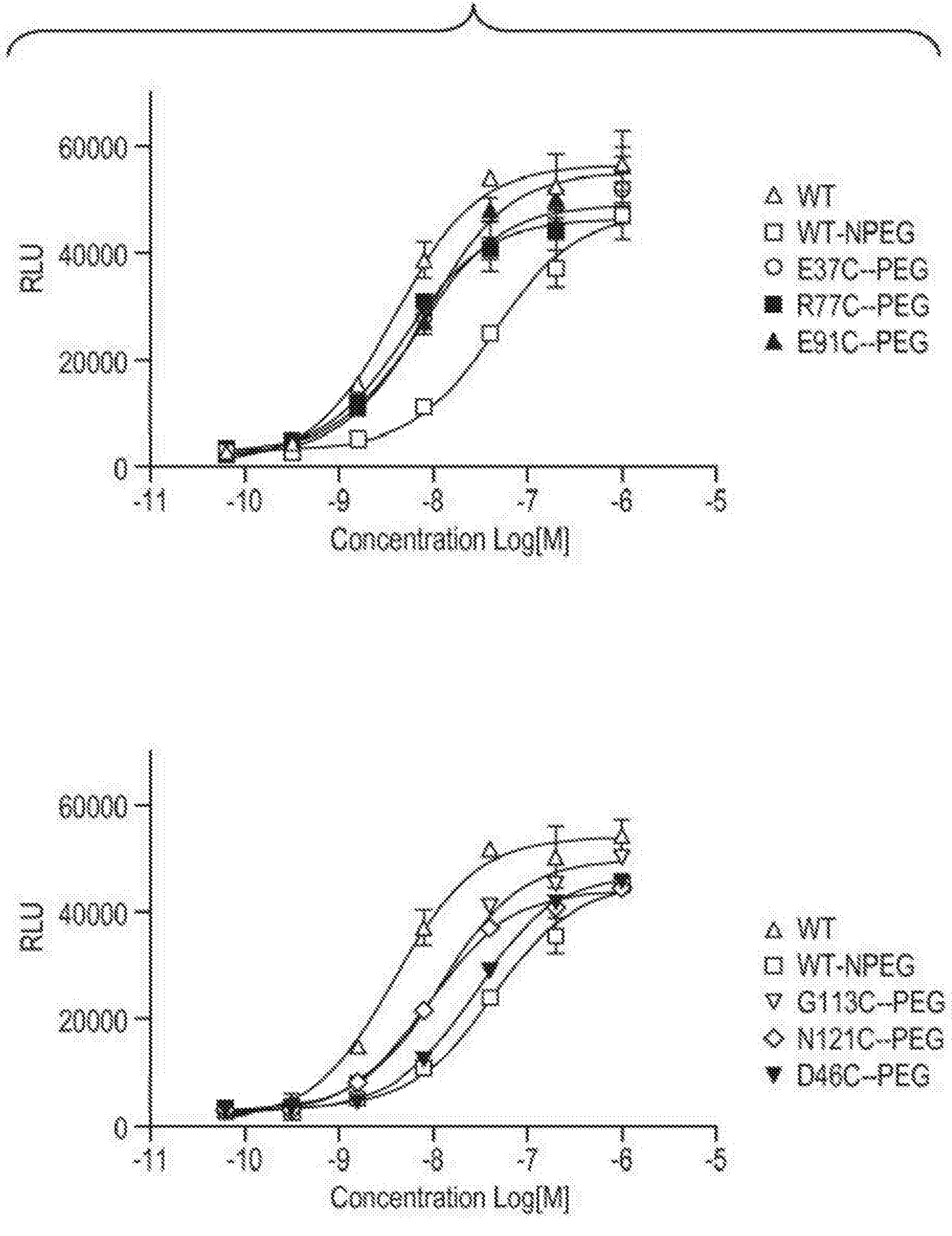

RLU
60000
40000
20000
0
-11
-10
-9
-8
-7
-6
-5
Concentration Log[M]
△ WT
□ WT-NPEG
○ E37C--PEG
■ R77C--PEG
▲ E91C--PEG
RLU
60000
40000
20000
0
-11
-10
-9
-8
-7
-6
-5
Concentration Log[M]
△ WT
□ WT-NPEG
▽ G113C--PEG
◇ N121C--PEG
▼ D46C--PEG

FIG. 5

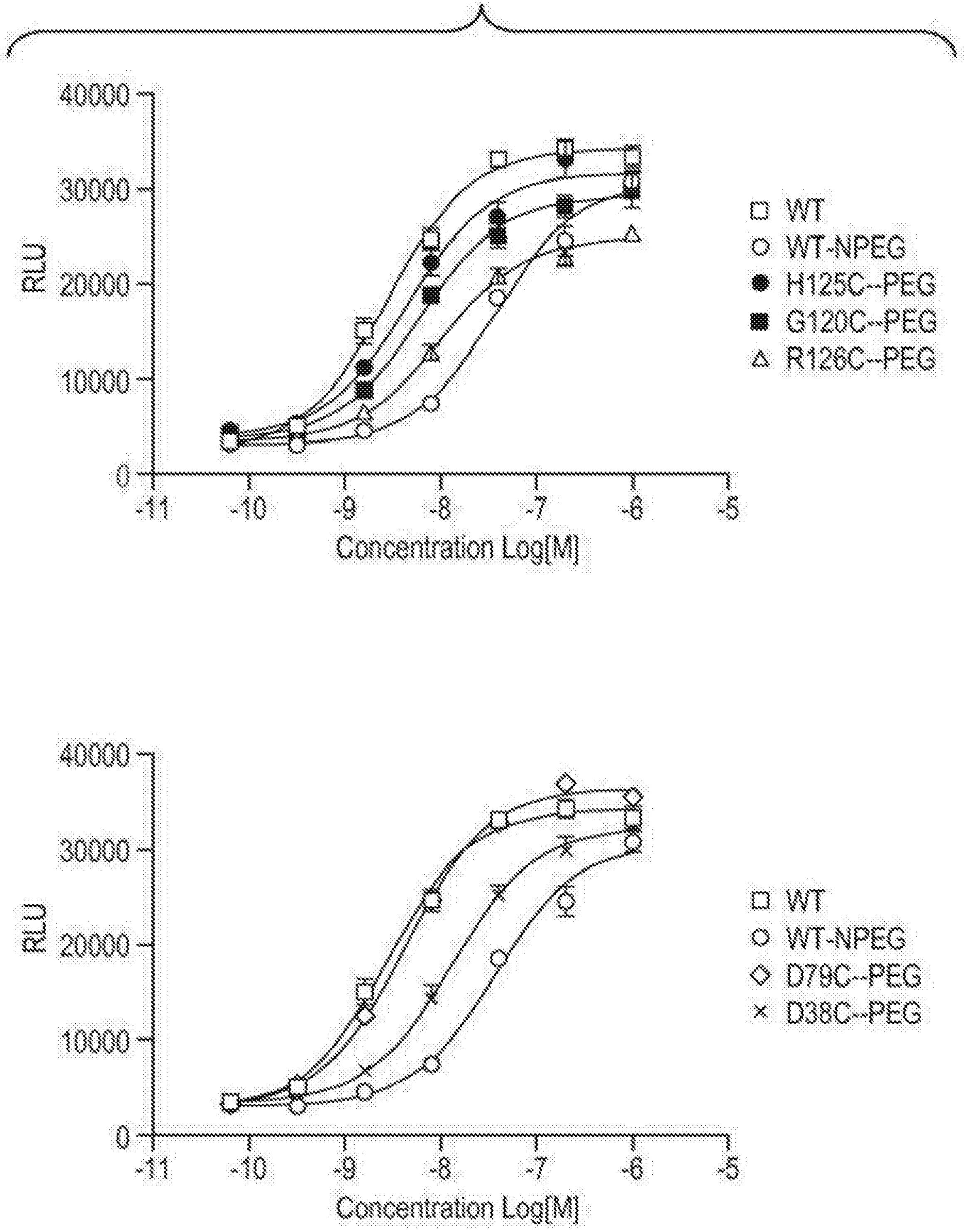

40000
30000
20000
10000
0
RLU
-11
-10
-9
-8
-7
-6
-5
Concentration Log[M]
□ WT
○ WT-NPEG
● H125C--PEG
■ G120C--PEG
△ R126C--PEG
40000
30000
20000
10000
0
RLU
-11
-10
-9
-8
-7
-6
-5
Concentration Log[M]
□ WT
○ WT-NPEG
◇ D79C--PEG
× D38C--PEG

FIG. 6

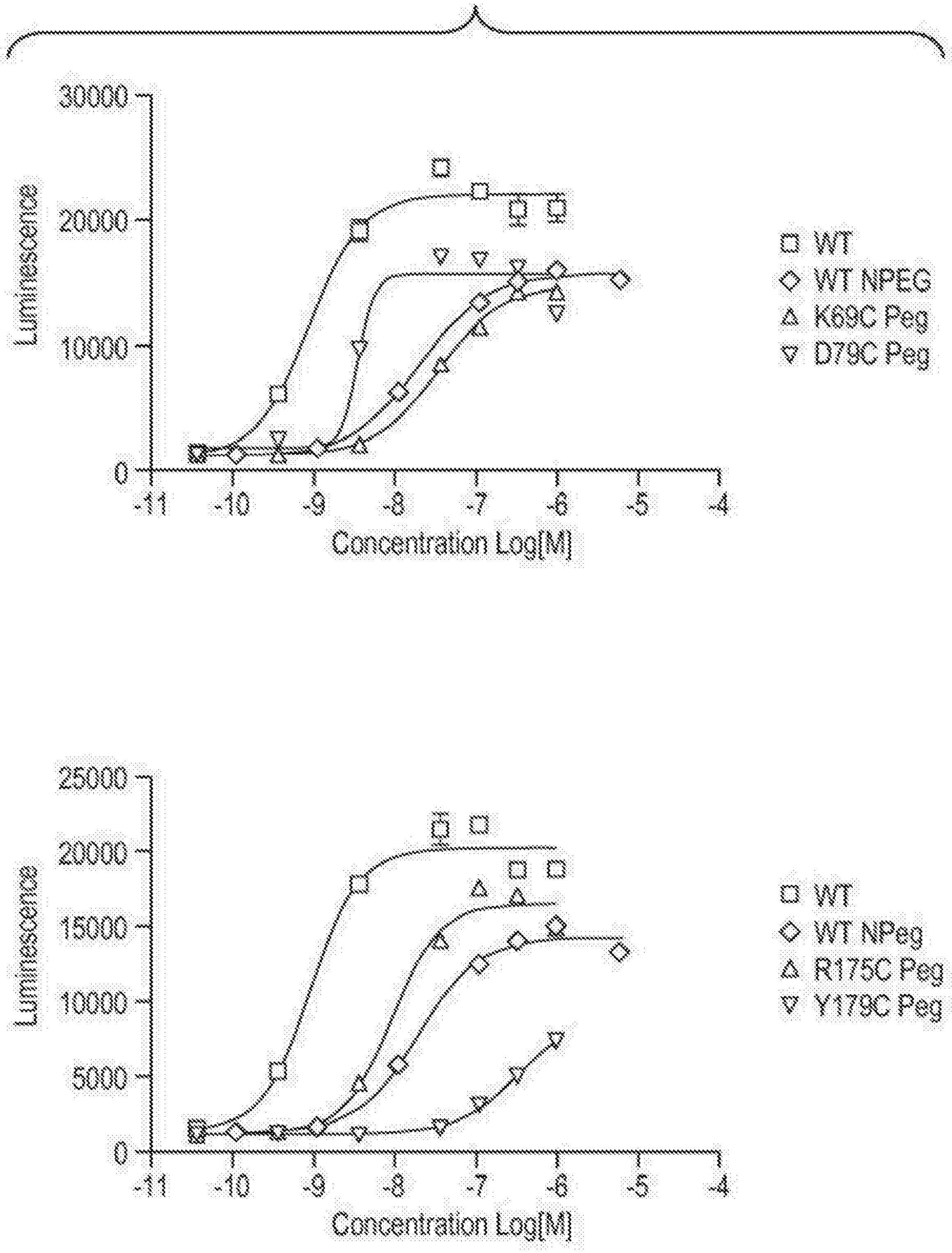

30000
20000
10000
0
Luminescence
-11
-10
-9
-8
-7
-6
-5
-4
Concentration Log[M]
□ WT
◇ WT NPEG
△ K69C Peg
▽ D79C Peg
25000
20000
15000
10000
5000
0
Luminescence
-11
-10
-9
-8
-7
-6
-5
-4
Concentration Log[M]
□ WT
◇ WT NPeg
△ R175C Peg
▽ Y179C Peg

FIG. 7

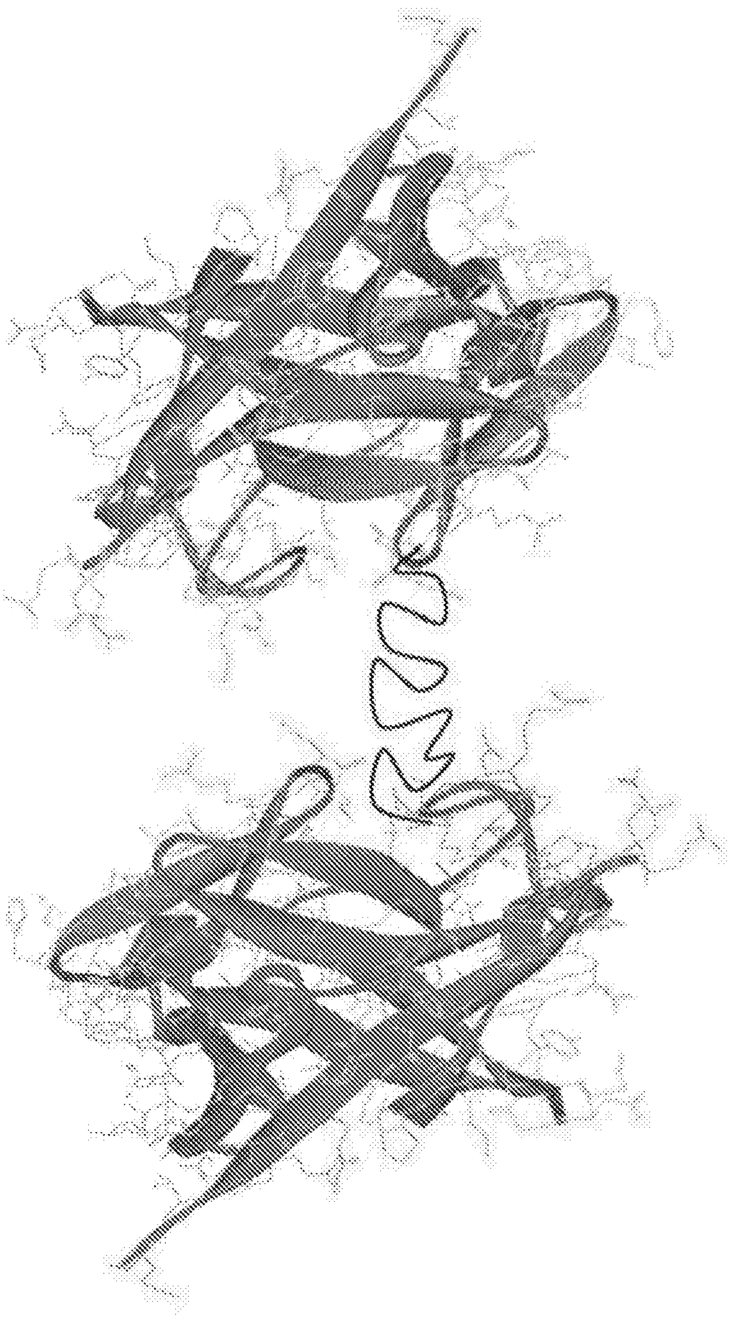


FIG. 8

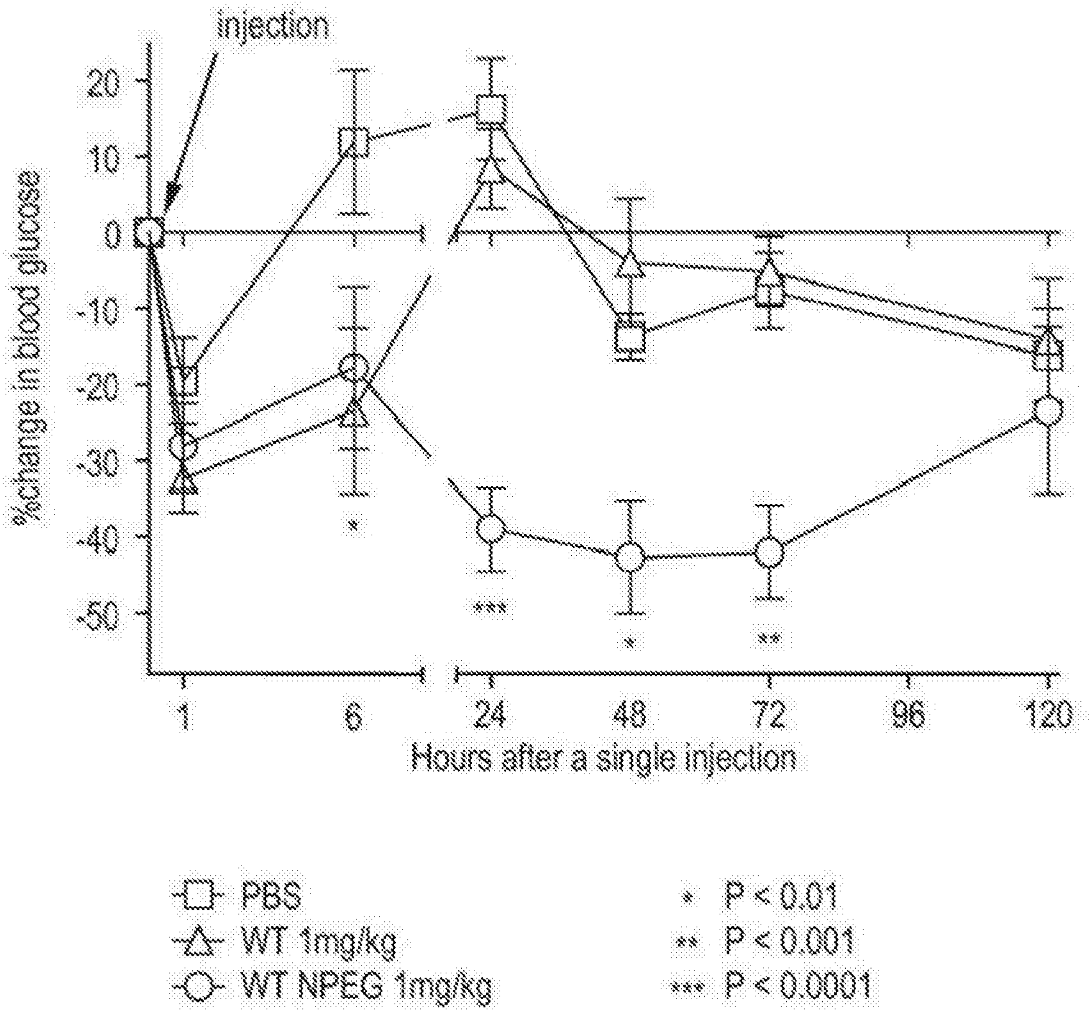

injection
20
10
0
-10
-20
-30
-40
-50
%change in blood glucose
1
6
24
48
72
96
120
Hours after a single injection
*
***
*
**
PBS
WT 1mg/kg
WT NPEG 1mg/kg
* P < 0.01
** P < 0.001
*** P < 0.0001

FIG. 9

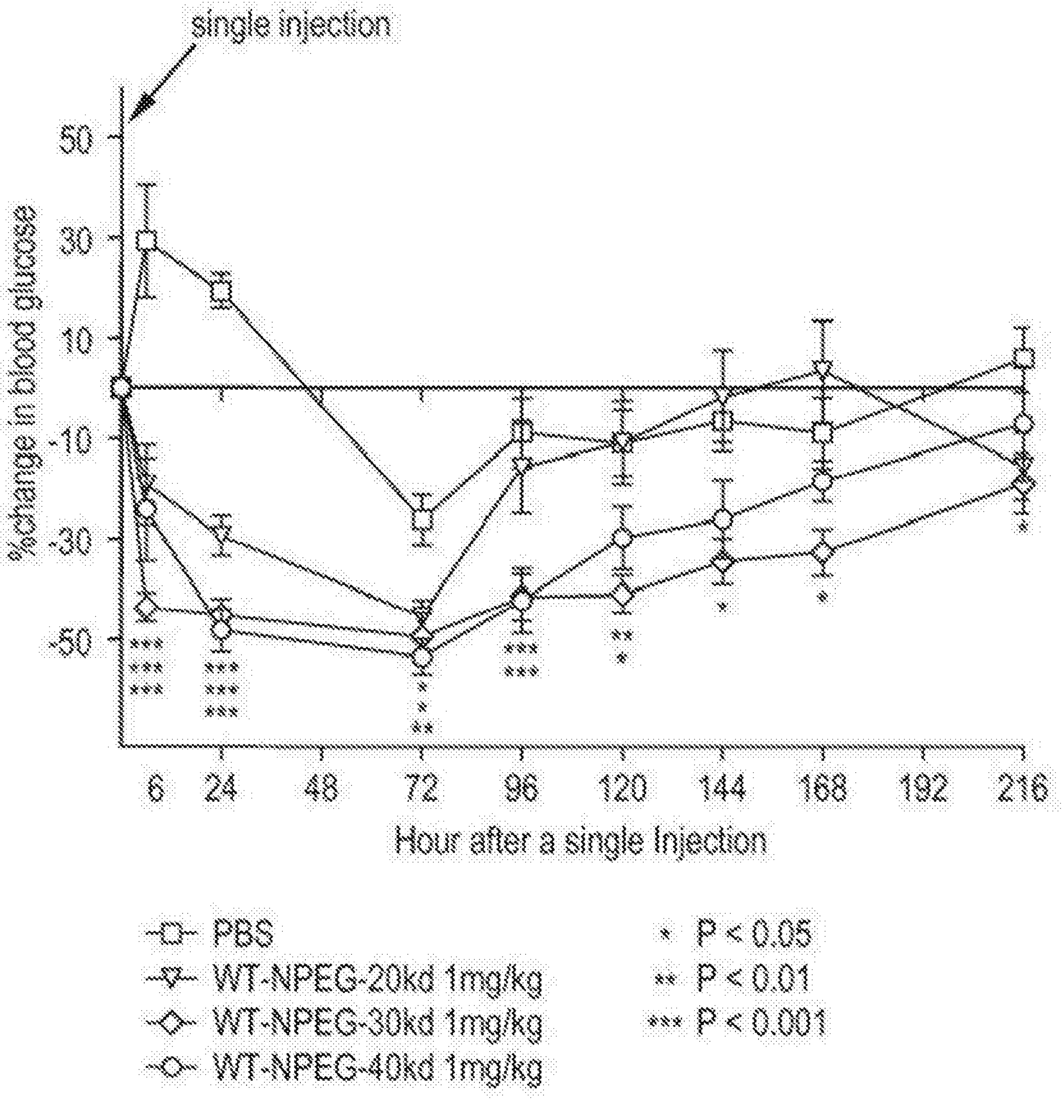

single injection
%change in blood glucose
50
30
10
-10
-30
-50
6
24
48
72
96
120
144
168
192
216
Hour after a single Injection
***
***
***
*
**
*
PBS
WT-NPEG-20kd 1mg/kg
WT-NPEG-30kd 1mg/kg
WT-NPEG-40kd 1mg/kg
* P < 0.05
** P < 0.01
*** P < 0.001

FIG. 10

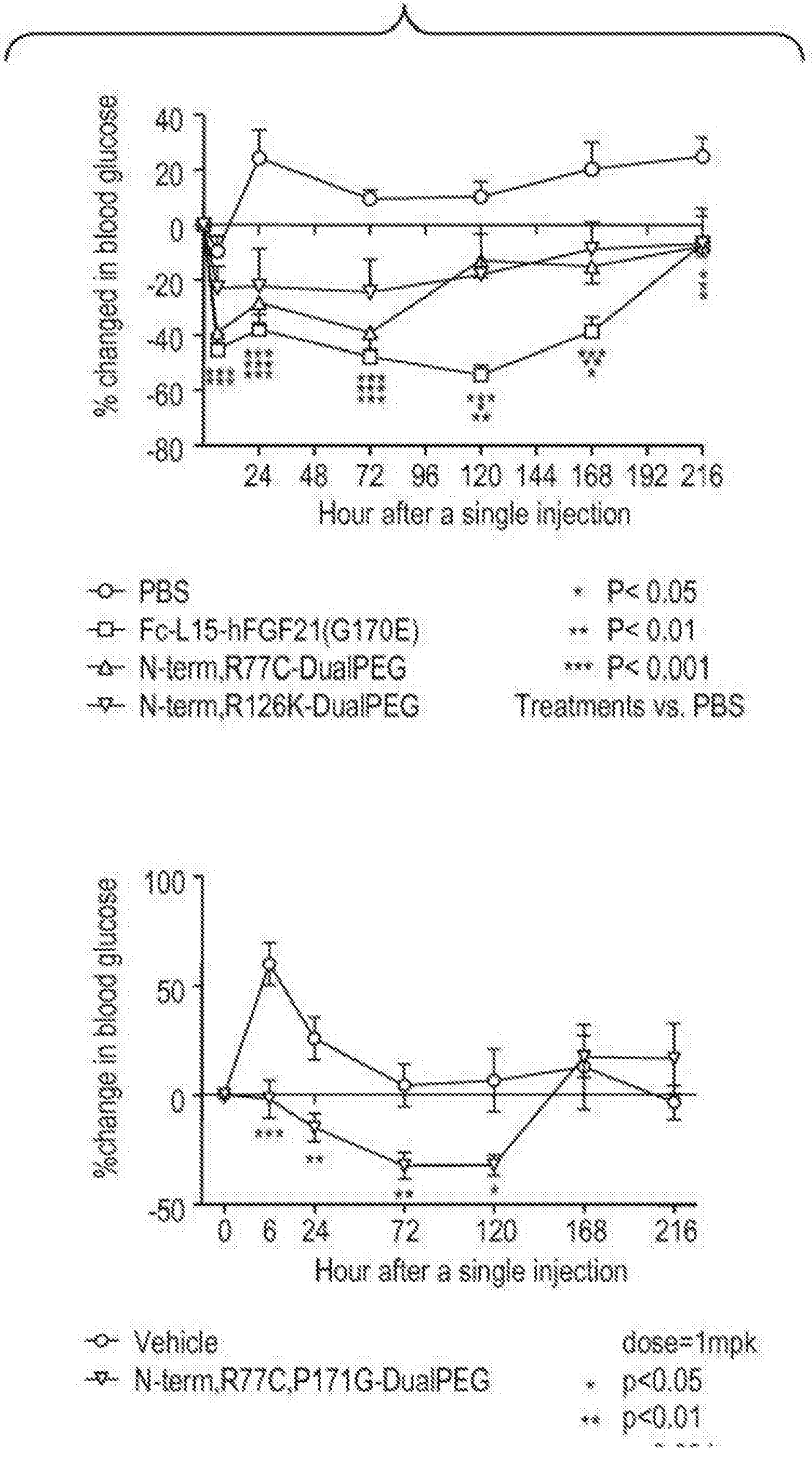

% changed in blood glucose
40
20
0
-20
-40
-60
-80
24
48
72
96
120
144
168
192
216
Hour after a single injection
PBS
Fc-L15-hFGF21(G170E)
N-term,R77C-DualPEG
N-term,R126K-DualPEG
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs. PBS
%change in blood glucose
100
50
0
-50
0
6
24
72
120
168
216
***
**
**
*
Hour after a single injection
Vehicle
N-term,R77C,P171G-DualPEG
dose=1mpk
* p<0.05
** p<0.01

FIG. 11

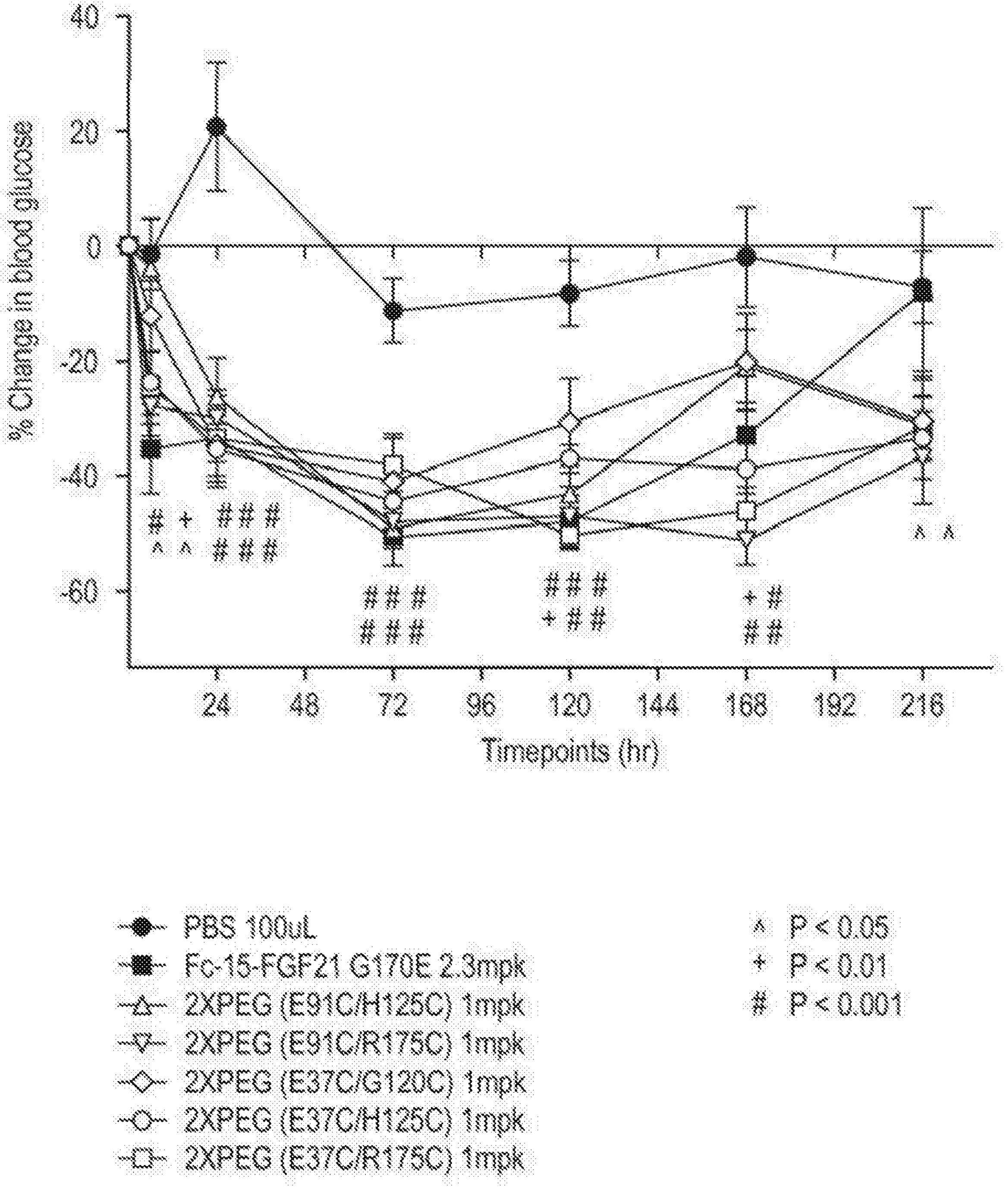

40
20
0
-20
-40
-60
% Change in blood glucose
24
48
72
96
120
144
168
192
216
Timepoints (hr)
+ # #
^ ^ # # #
# # #
# # #
# # #
+ # #
+ #
# #
^ ^
PBS 100uL
Fc-15-FGF21 G170E 2.3mpk
2XPEG (E91C/H125C) 1mpk
2XPEG (E91C/R175C) 1mpk
2XPEG (E37C/G120C) 1mpk
2XPEG (E37C/H125C) 1mpk
2XPEG (E37C/R175C) 1mpk
^ P < 0.05
+ P < 0.01
# P < 0.001

FIG. 12

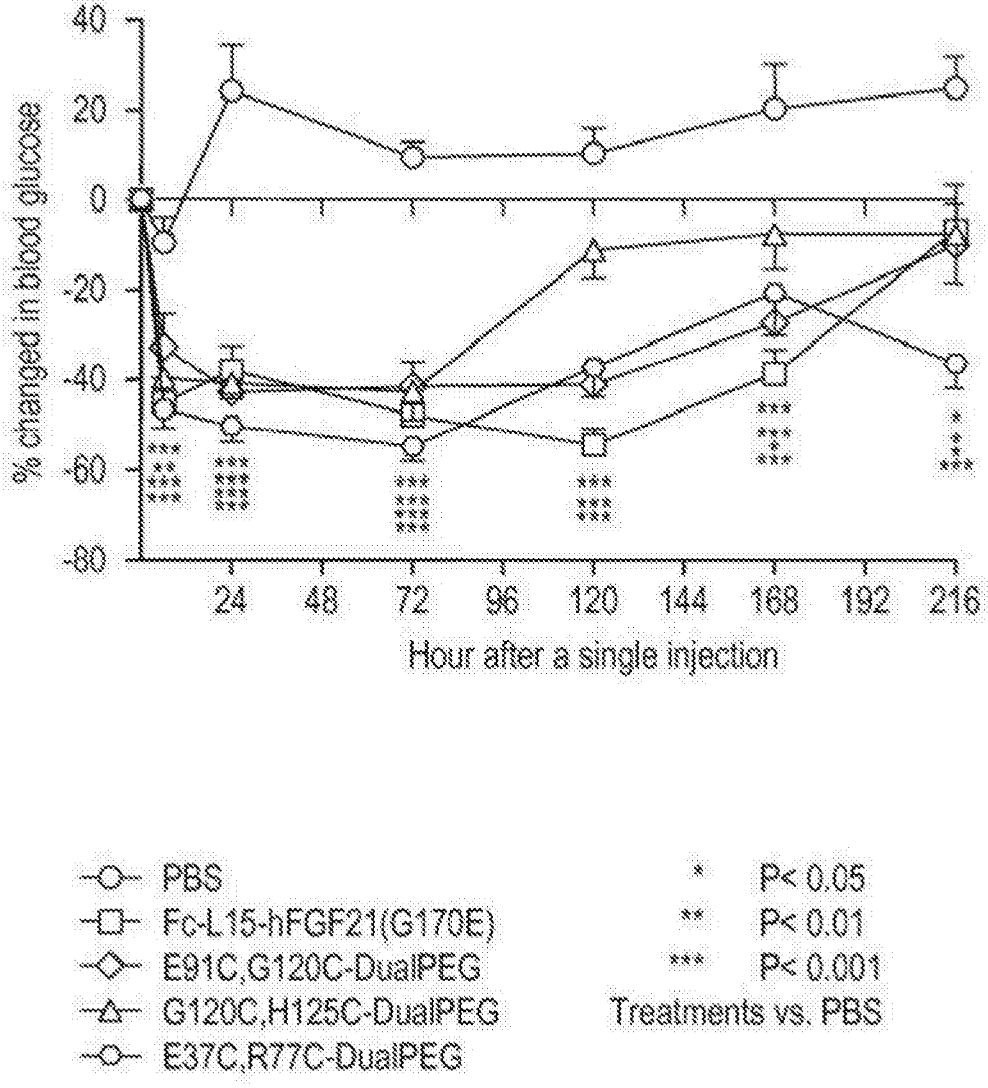

40
20
0
-20
-40
-60
-80
% changed in blood glucose
24
48
72
96
120
144
168
192
216
Hour after a single injection
PBS
Fc-L15-hFGF21(G170E)
E91C,G120C-DualPEG
G120C,H125C-DualPEG
E37C,R77C-DualPEG
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs. PBS

FIG. 13

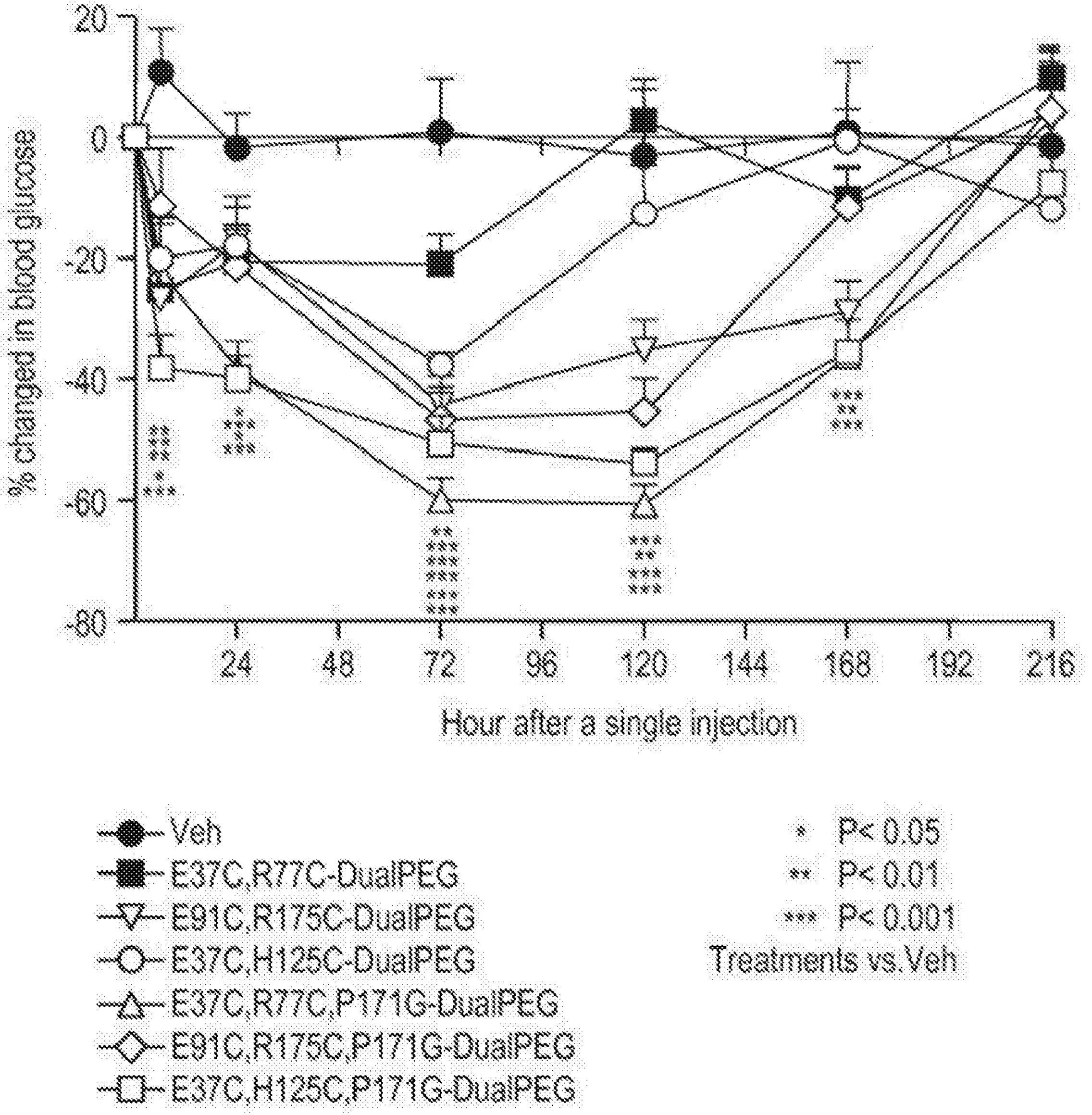

% changed in blood glucose
20
0
-20
-40
-60
-80
24
48
72
96
120
144
168
192
216
Hour after a single injection
Veh
E37C,R77C-DualPEG
E91C,R175C-DualPEG
E37C,H125C-DualPEG
E37C,R77C,P171G-DualPEG
E91C,R175C,P171G-DualPEG
E37C,H125C,P171G-DualPEG
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs.Veh

FIG. 14

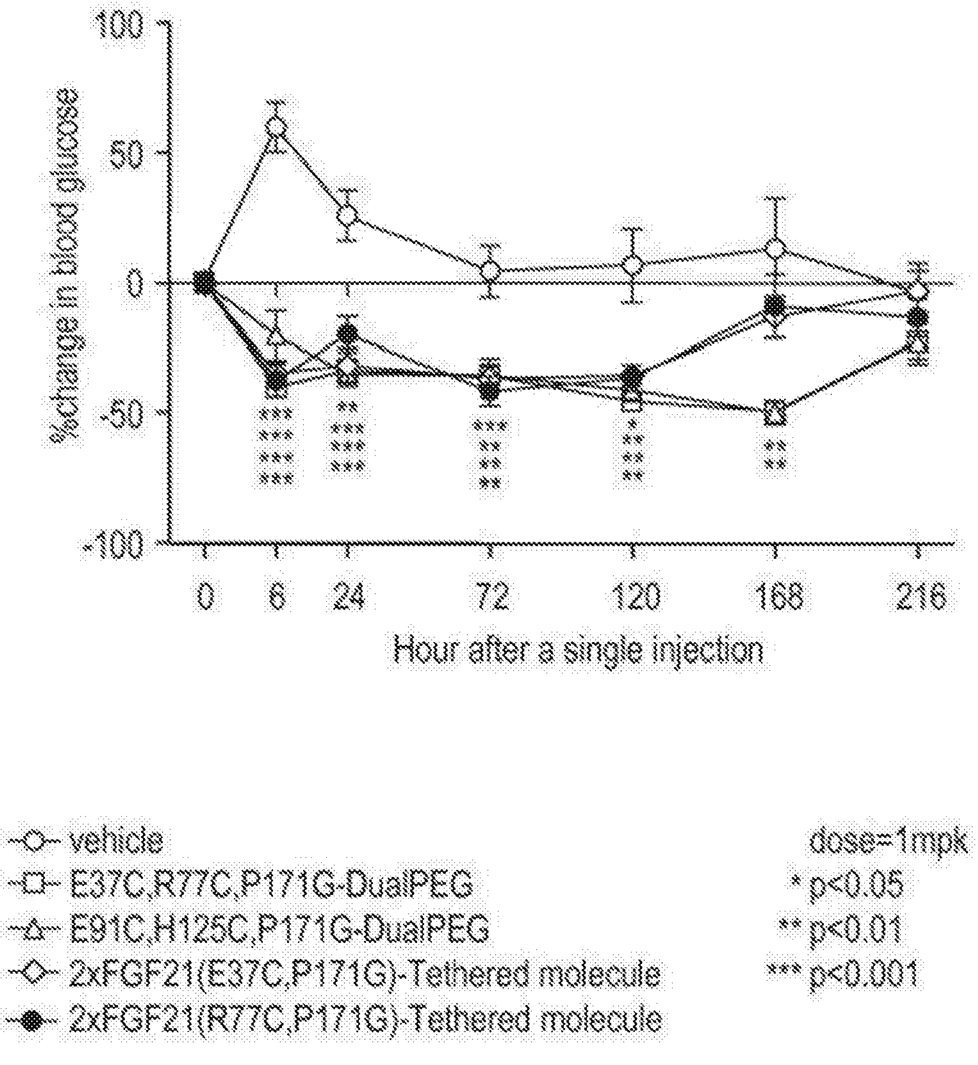

100
50
0
-50
-100
%change in blood glucose
0
6
24
72
120
168
216
Hour after a single injection
***
***
***
***
**
***
***
***
***
**
**
**
*
**
**
**
**
vehicle
E37C,R77C,P171G-DualPEG
E91C,H125C,P171G-DualPEG
2xFGF21(E37C,P171G)-Tethered molecule
2xFGF21(R77C,P171G)-Tethered molecule
dose=1mpk
* p<0.05
** p<0.01
*** p<0.001

FIG. 15

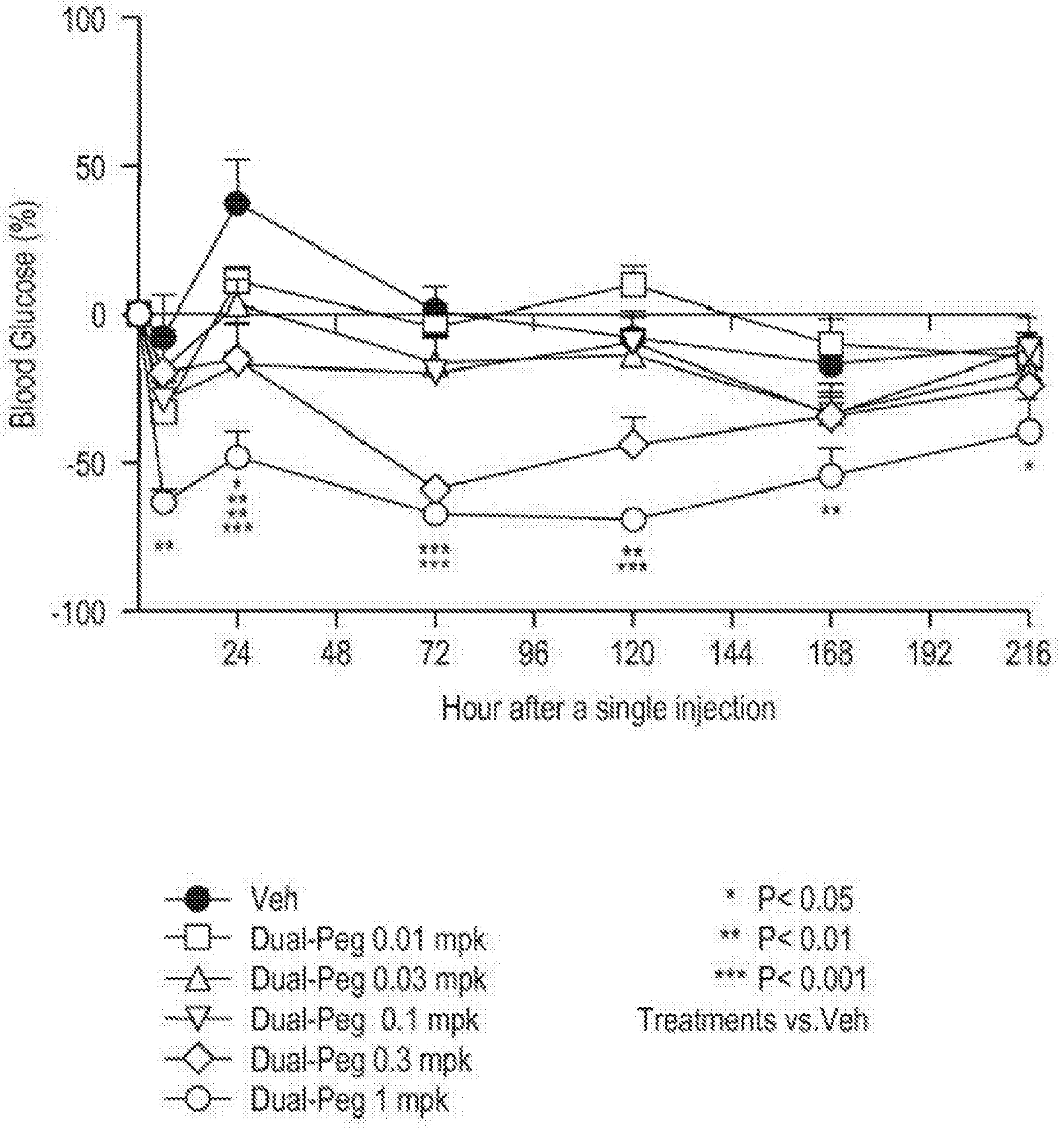

100
50
0
-50
-100
Blood Glucose (%)
24
48
72
96
120
144
168
192
216
Hour after a single injection
**
*
**
**
***
***
***
**
***
**
*
Veh
Dual-Peg 0.01 mpk
Dual-Peg 0.03 mpk
Dual-Peg 0.1 mpk
Dual-Peg 0.3 mpk
Dual-Peg 1 mpk
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs.Veh

FIG. 16

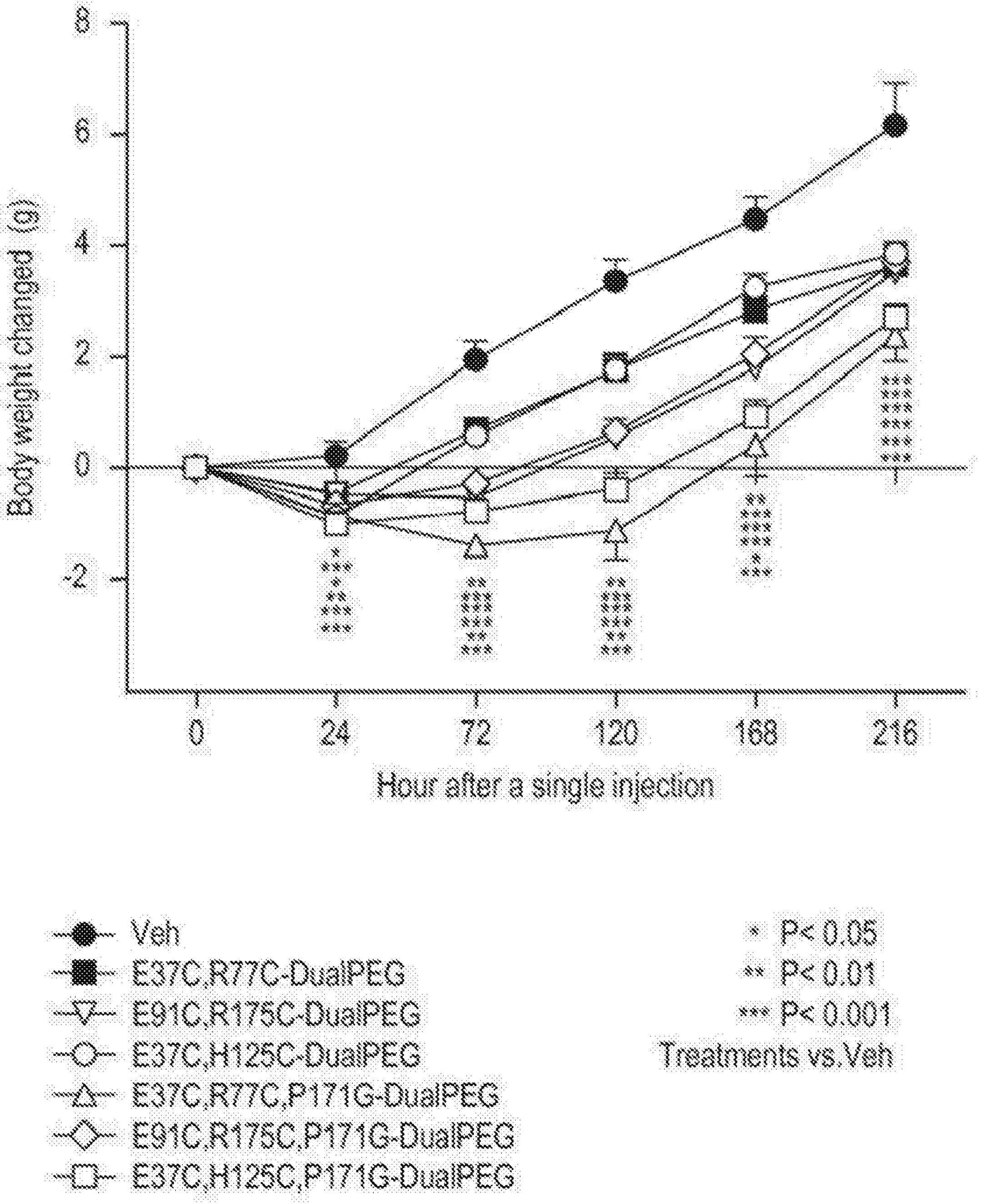

8
6
4
2
0
-2
Body weight changed (g)
0
24
72
120
168
216
Hour after a single injection
Veh
E37C,R77C-DualPEG
E91C,R175C-DualPEG
E37C,H125C-DualPEG
E37C,R77C,P171G-DualPEG
E91C,R175C,P171G-DualPEG
E37C,H125C,P171G-DualPEG
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs.Veh

FIG. 17

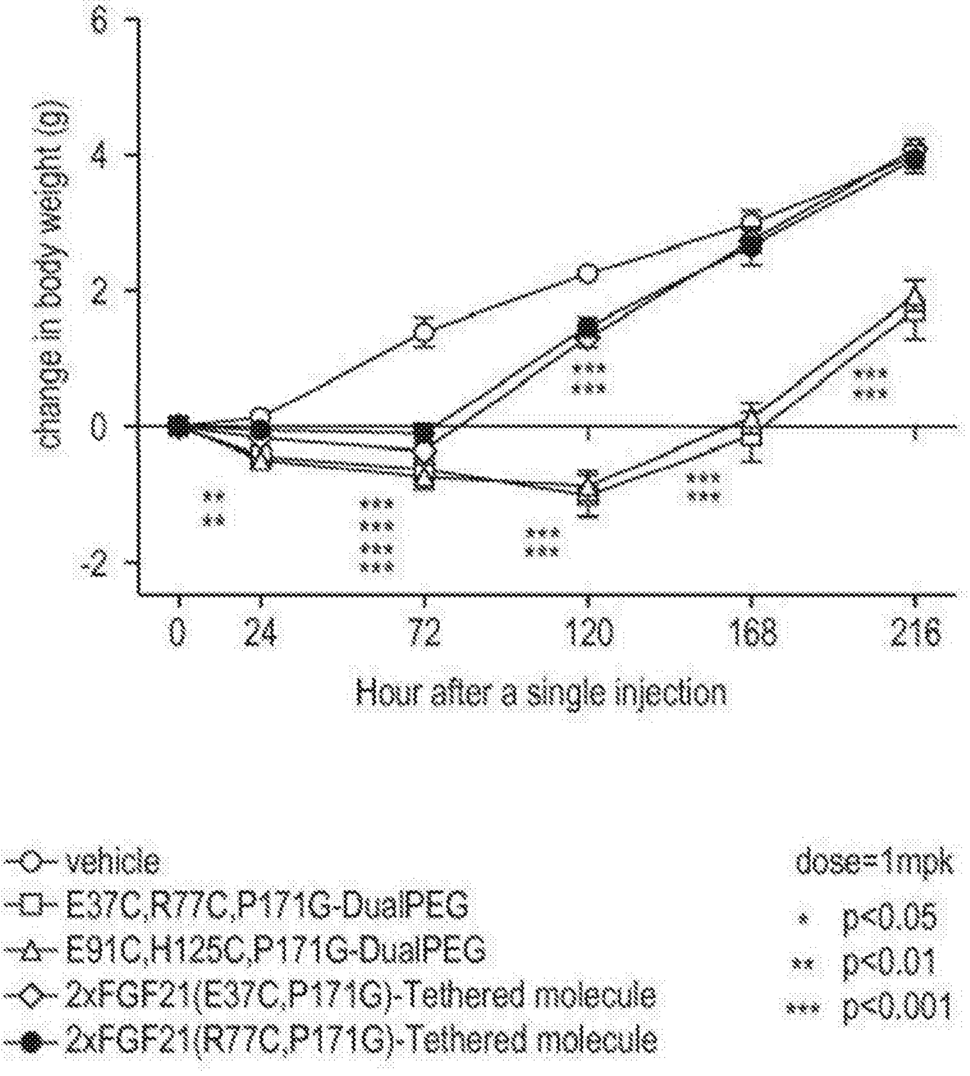

6
4
2
0
-2
change in body weight (g)
0
24
72
120
168
216
Hour after a single injection
**
**
***
***
***
***
***
***
***
***
***
***
***
***
vehicle
E37C,R77C,P171G-DualPEG
E91C,H125C,P171G-DualPEG
2xFGF21(E37C,P171G)-Tethered molecule
2xFGF21(R77C,P171G)-Tethered molecule
dose=1mpk
* p<0.05
** p<0.01
*** p<0.001

FIG. 18

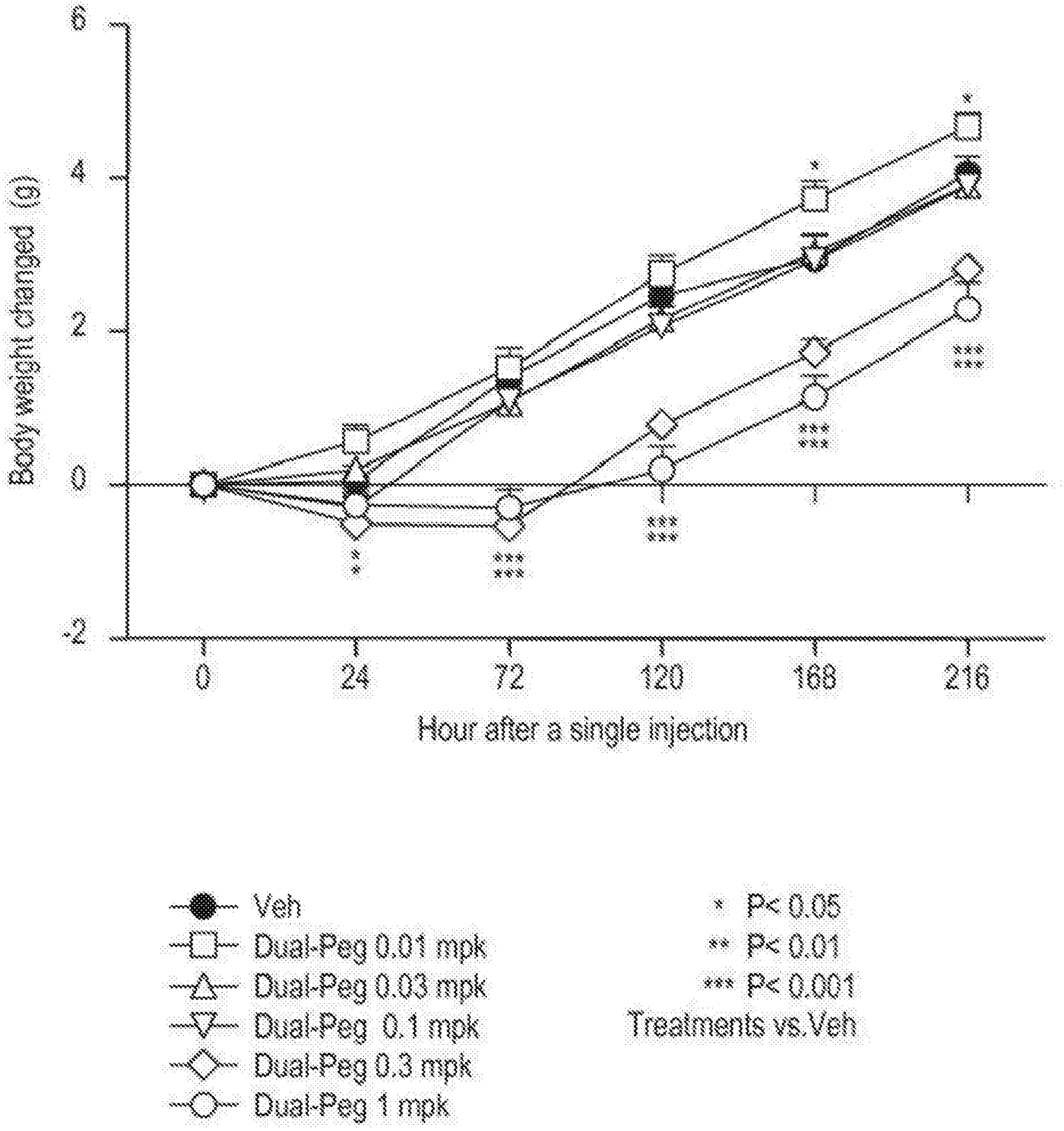

Body weight changed (g)
6
4
2
0
-2
0
24
72
120
168
216
Hour after a single injection
*
**
***
Veh
Dual-Peg 0.01 mpk
Dual-Peg 0.03 mpk
Dual-Peg 0.1 mpk
Dual-Peg 0.3 mpk
Dual-Peg 1 mpk
* P< 0.05
** P< 0.01
*** P< 0.001
Treatments vs.Veh

FIG. 19A

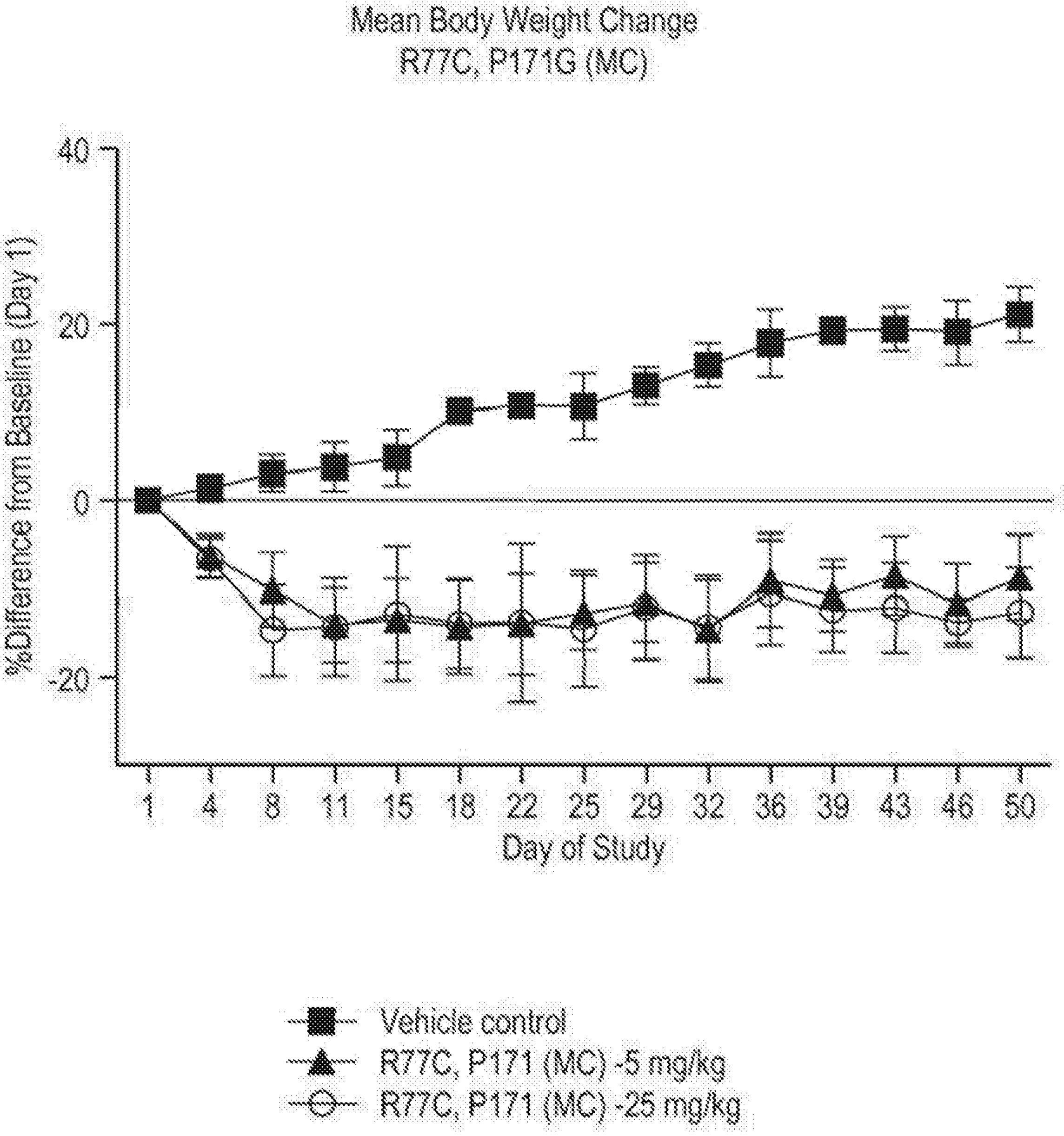

Mean Body Weight Change
R77C, P171G (MC)
%Difference from Baseline (Day 1)
40
20
0
-20
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
R77C, P171 (MC) -5 mg/kg
R77C, P171 (MC) -25 mg/kg

FIG. 19B

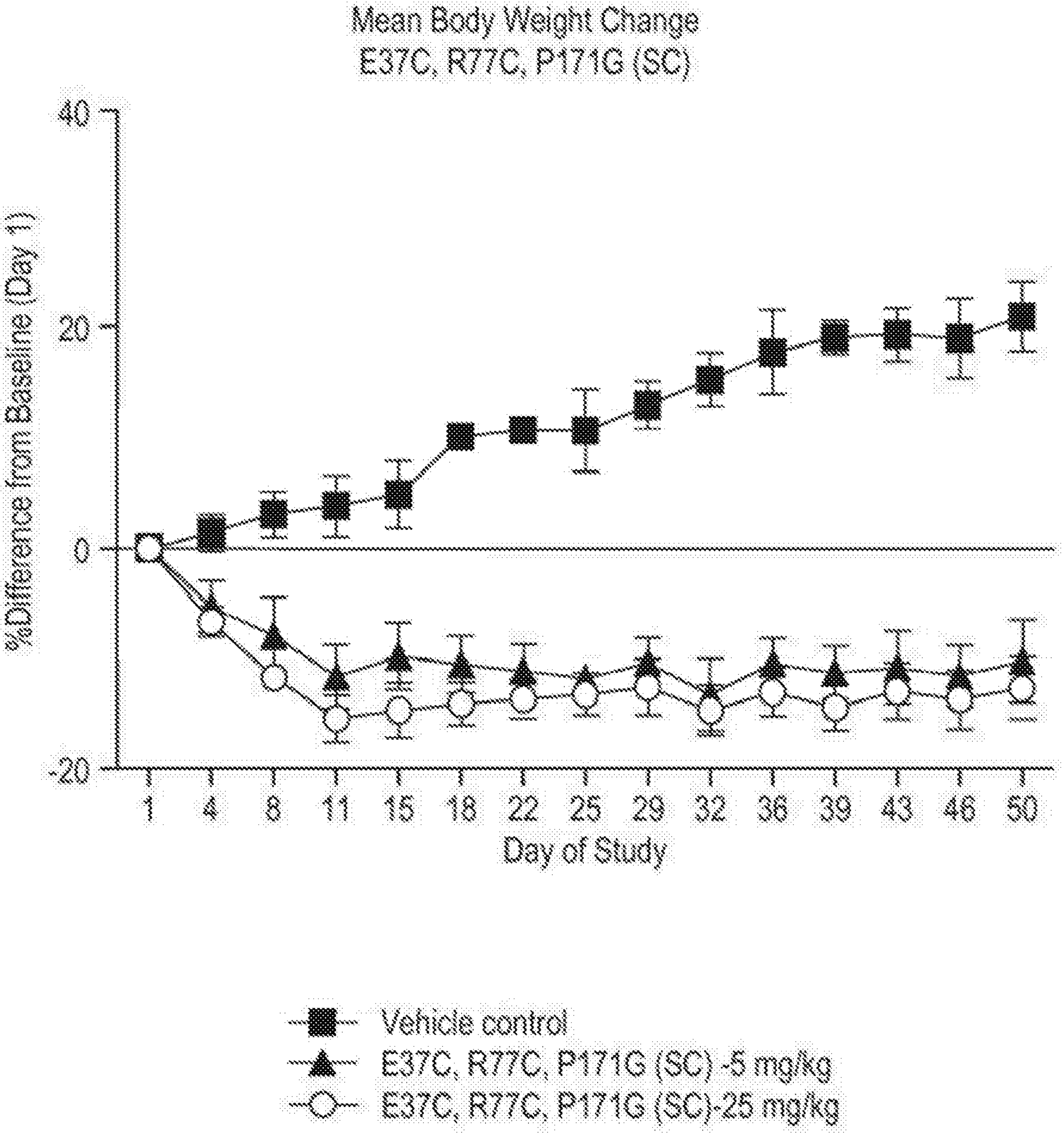

Mean Body Weight Change
E37C, R77C, P171G (SC)
%Difference from Baseline (Day 1)
40
20
0
-20
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
E37C, R77C, P171G (SC) -5 mg/kg
E37C, R77C, P171G (SC)-25 mg/kg

FIG. 19C

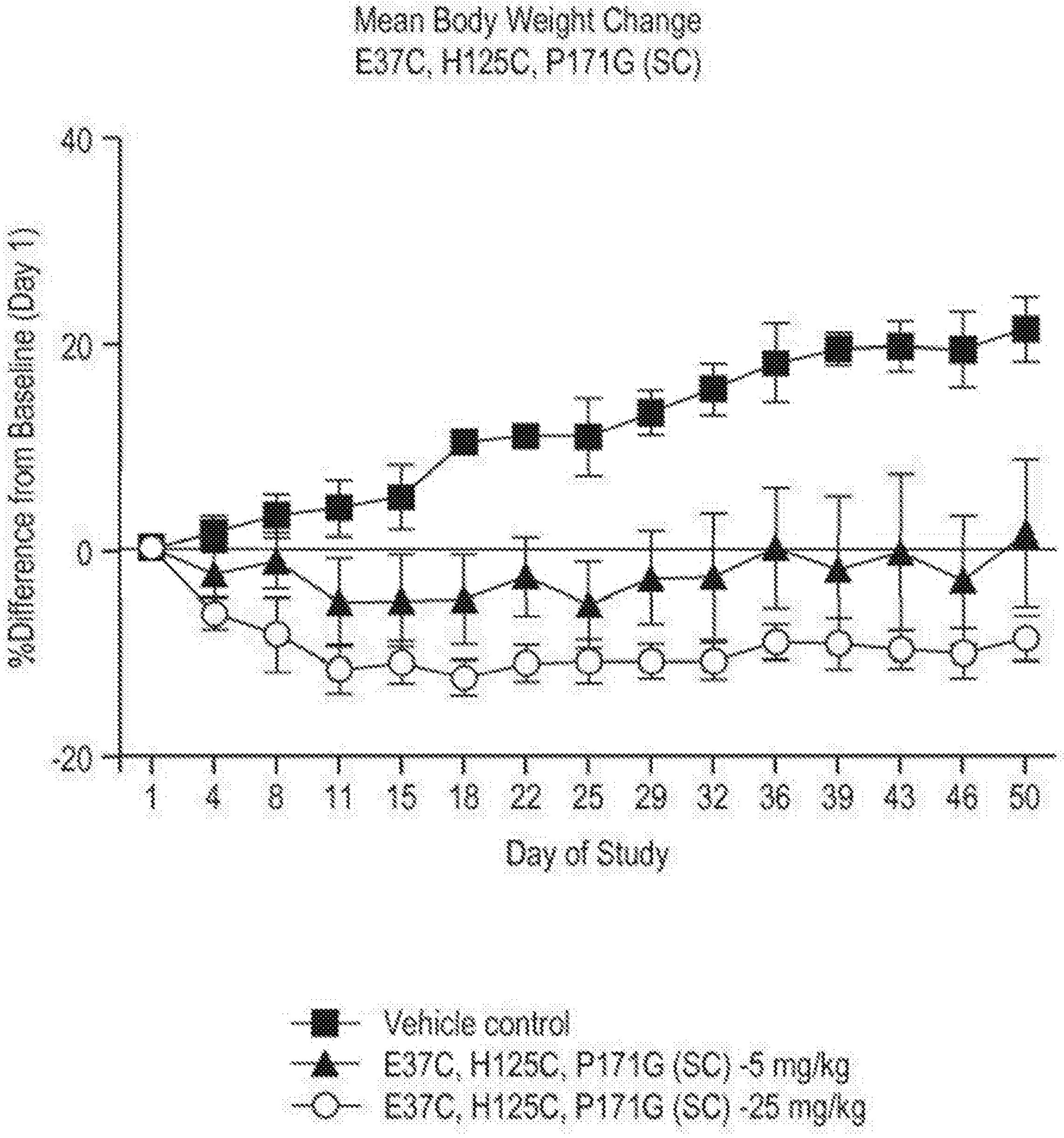

Mean Body Weight Change
E37C, H125C, P171G (SC)
40
20
0
-20
%Difference from Baseline (Day 1)
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
E37C, H125C, P171G (SC) -5 mg/kg
E37C, H125C, P171G (SC) -25 mg/kg

FIG. 19D

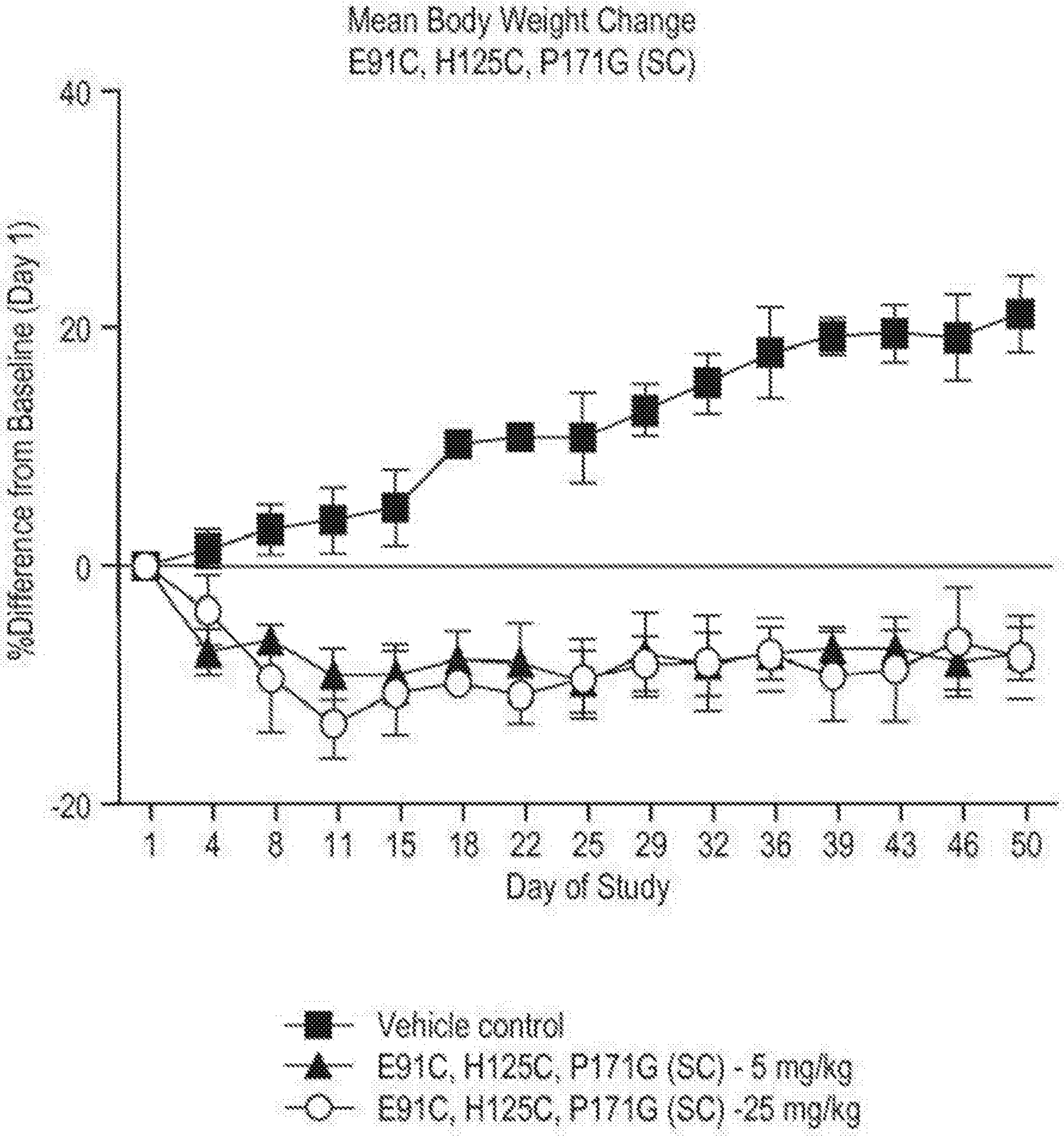

Mean Body Weight Change
E91C, H125C, P171G (SC)
%Difference from Baseline (Day 1)
40
20
0
-20
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
E91C, H125C, P171G (SC) - 5 mg/kg
E91C, H125C, P171G (SC) -25 mg/kg

FIG. 19E

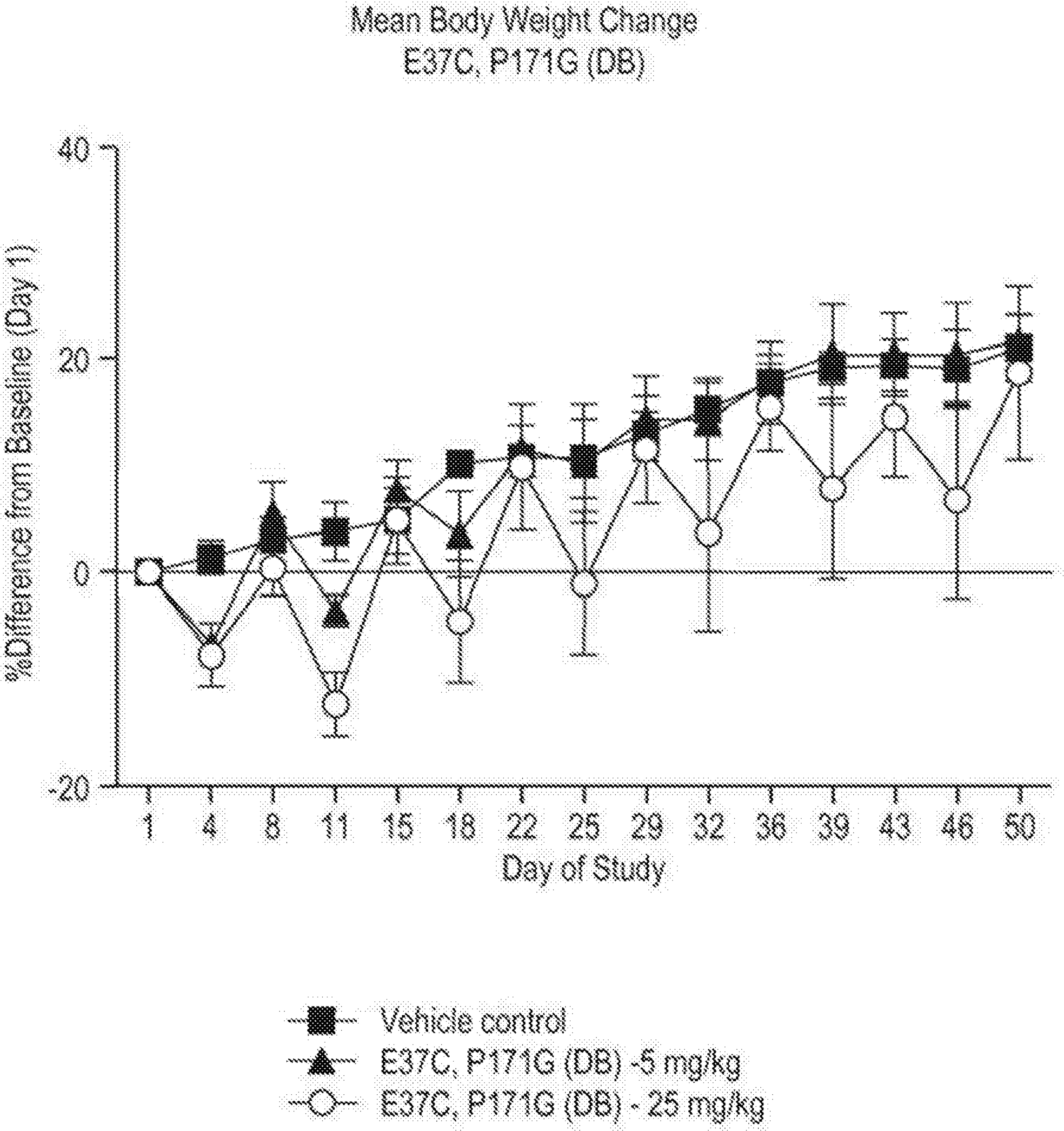

Mean Body Weight Change
E37C, P171G (DB)
%Difference from Baseline (Day 1)
40
20
0
-20
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
E37C, P171G (DB) -5 mg/kg
E37C, P171G (DB) - 25 mg/kg

FIG. 19F

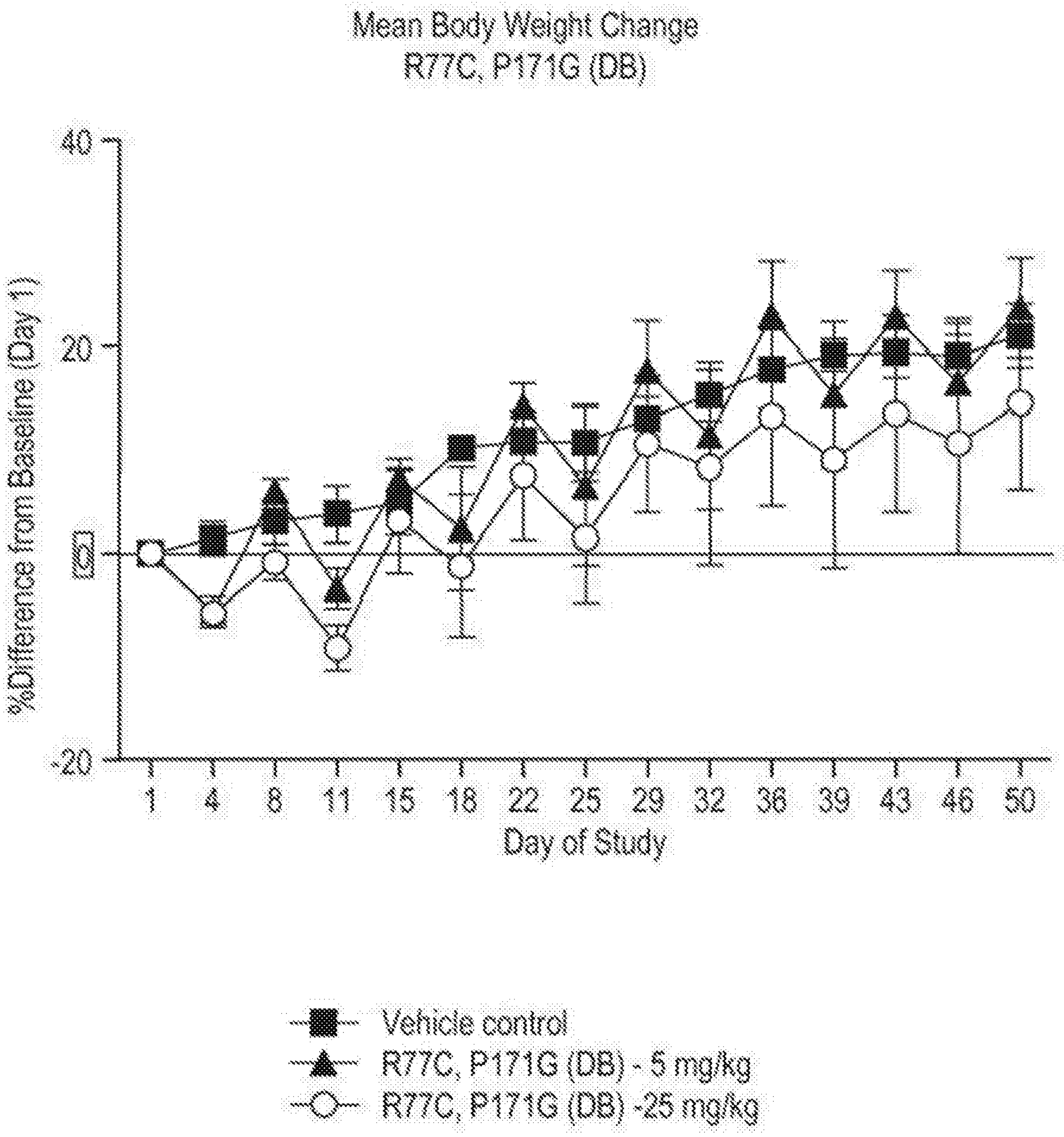
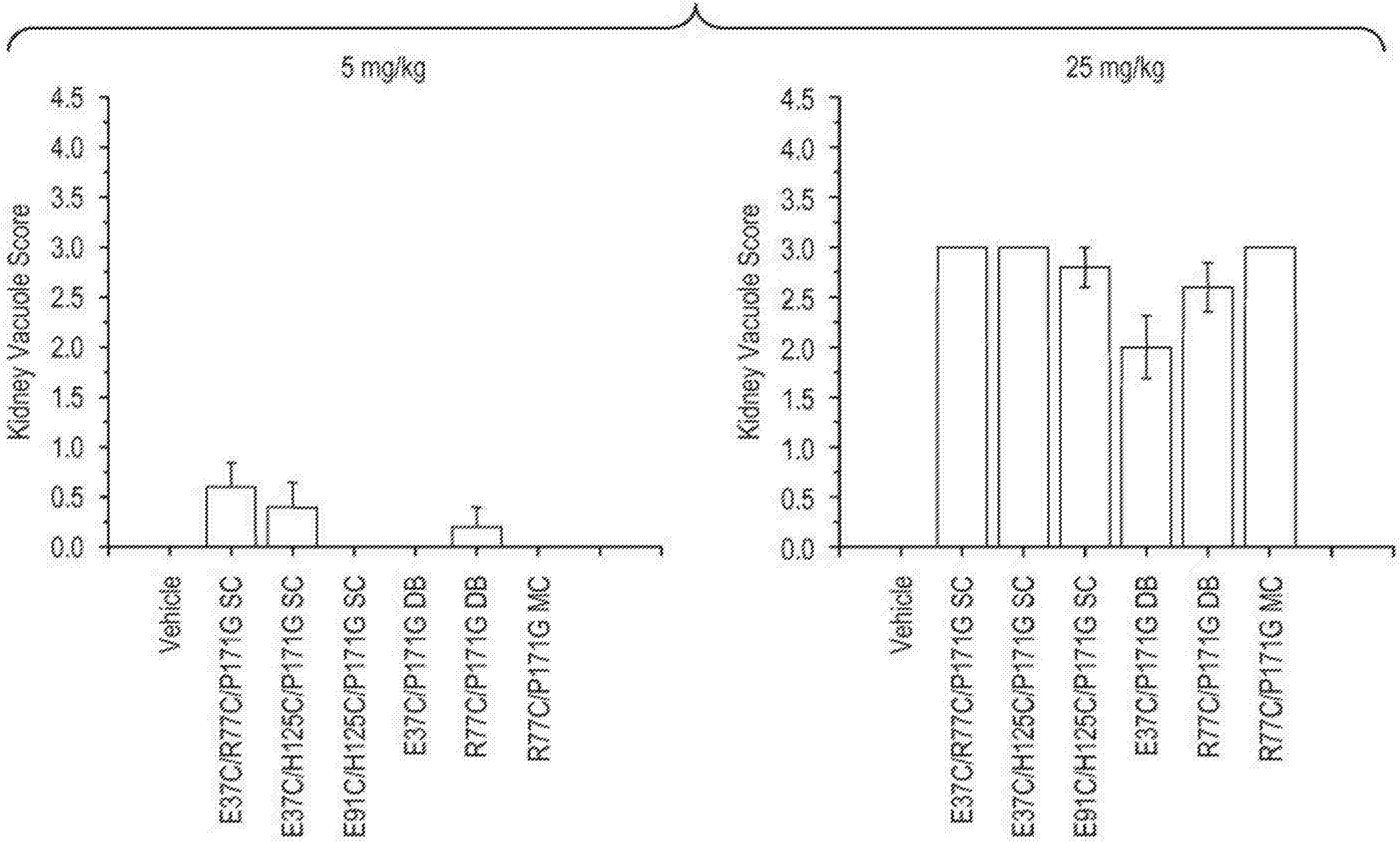

Mean Body Weight Change
R77C, P171G (DB)
%Difference from Baseline (Day 1)
40
20
0
-20
1
4
8
11
15
18
22
25
29
32
36
39
43
46
50
Day of Study
Vehicle control
R77C, P171G (DB) - 5 mg/kg
R77C, P171G (DB) -25 mg/kg

FIG. 20


5 mg/kg
25 mg/kg
Kidney Vacuole Score
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
E37C/R77C/P171G SC
E37C/H125C/P171G SC
E91C/H125C/P171G SC
E37C/P171G DB
R77C/P171G DB
R77C/P171G MC

SEQUENCE LISTING

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<110>  WALKER, Kenneth W.
       GEGG, Jr. , Colin V.
       HECHT, Randy I.
       BELOUSKI, Edward J.
       LI, Yue-Sheng
       MICHAELS, Mark L.
       XU, Jing
       ELLISON, Murielle M.

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gggggccaag tccggcagcg gtacctctac acagatgatg cccagcagac agaagcccac     180

ctggagatca gggaggatgg gacggtgggg ggcgctgctg accagagccc cgaaagtctc     240

ctgcagctga aagccttgaa gccgggagtt attcaaatct tgggagtcaa gacatccagg     300

ttcctgtgcc agcggccaga tggggccctg tatggatcgc tccactttga ccctgaggcc     360

tgcagcttcc gggagctgct tcttgaggac ggatacaatg tttaccagtc cgaagcccac     420

ggcctcccgc tgcacctgcc agggaacaag tccccacacc gggaccctgc accccgagga     480

ccagctcgct tcctgccact accaggcctg ccccccgcac ccccggagcc acccggaatc     540

ctggcccccc agccccccga tgtgggctcc tcggaccctc tgagcatggt gggaccttcc     600

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Met Asp Ser Asp Glu Thr Gly Phe Glu His Ser Gly Leu Trp Val Ser
1               5                   10                  15


Val Leu Ala Gly Leu Leu Leu Gly Ala Cys Gln Ala His Pro Ile Pro
            20                  25                  30


Asp Ser Ser Pro Leu Leu Gln Phe Gly Gly Gln Val Arg Gln Arg Tyr
        35                  40                  45
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Leu Tyr Thr Asp Asp Ala Gln Gln Thr Glu Ala His Leu Glu Ile Arg
    50                  55                  60

Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln Ser Pro Glu Ser Leu
65                  70                  75                  80

Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile Gln Ile Leu Gly Val
                85                  90                  95

Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp Gly Ala Leu Tyr Gly
            100                 105                 110

Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe Arg Glu Leu Leu Leu
        115                 120                 125

Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala His Gly Leu Pro Leu
    130                 135                 140

His Leu Pro Gly Asn Lys Ser Pro His Arg Asp Pro Ala Pro Arg Gly
145                 150                 155                 160

Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro Pro Ala Pro Pro Glu
                165                 170                 175

Pro Pro Gly Ile Leu Ala Pro Gln Pro Pro Asp Val Gly Ser Ser Asp
            180                 185                 190

Pro Leu Ser Met Val Gly Pro Ser Gln Gly Arg Ser Pro Ser Tyr Ala
        195                 200                 205

Ser


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gtgggggcg ctgctgacca gagccccgaa agtctcctgc agctgaaagc cttgaagccg     180

ggagttattc aaatcttggg agtcaagaca tccaggttcc tgtgccagcg gccagatggg     240

gccctgtatg gatcgctcca ctttgaccct gaggcctgca gcttccggga gctgcttctt     300

gaggacggat acaatgttta ccagtccgaa gcccacggcc tcccgctgca cctgccaggg     360

aacaagtccc cacaccggga ccctgcaccc cgaggaccag ctcgcttcct gccactacca     420

ggcctgcccc ccgcaccccc ggagccaccc ggaatcctgg ccccccagcc ccccgatgtg     480

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tcctga                                                                 546


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1               5                   10                  15


Arg Gln Arg Tyr Leu Tyr Thr Asp Asp Ala Gln Gln Thr Glu Ala His
            20                  25                  30


Leu Glu Ile Arg Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln Ser
        35                  40                  45


Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile Gln
    50                  55                  60


Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp Gly
65                  70                  75                  80


Ala Leu Tyr Gly Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe Arg
                85                  90                  95


Glu Leu Leu Leu Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala His
            100                 105                 110


Gly Leu Pro Leu His Leu Pro Gly Asn Lys Ser Pro His Arg Asp Pro
        115                 120                 125


Ala Pro Arg Gly Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro Pro
    130                 135                 140


Ala Pro Pro Glu Pro Pro Gly Ile Leu Ala Pro Gln Pro Pro Asp Val
145                 150                 155                 160


Gly Ser Ser Asp Pro Leu Ser Met Val Gly Pro Ser Gln Gly Arg Ser
                165                 170                 175


Pro Ser Tyr Ala Ser
            180


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<210>  8
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Gly Gly Gly Gly Ser
1               5


<210>  9
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<212>  PRT
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1               5                   10
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1               5                   10                  15


Gly Gly Gly Ser Gly Gly Gly Gly Ser
            20                  25


<210>  11
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<212>  PRT
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<220>
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<400>  11

Gly Gly Glu Gly Gly Gly
1               5


<210>  12
<211>  8
<212>  PRT
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<220>
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Gly Gly Glu Glu Glu Gly Gly Gly
1               5


<210>  13
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<400>  13

Gly Glu Glu Glu Gly
1               5


<210>  14
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Gly Glu Glu Glu
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<210>  15
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Gly Gly Asp Gly Gly Gly
1               5


<210>  16
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Gly Gly Asp Asp Asp Gly Gly
1               5


<210>  17
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Gly Asp Asp Asp Gly
1               5


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1


<210>  19
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1               5                   10                  15


Gly Gly Gly Gly Ser
            20


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Trp Glu Trp Glu Trp
1               5


<210>  21
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Phe Glu Phe Glu Phe
1               5


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Glu Glu Glu Trp Trp Trp
1               5


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Glu Glu Glu Phe Phe Phe
1               5


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Trp Trp Glu Glu Glu Trp Trp
1               5


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Phe Phe Glu Glu Glu Phe Phe
1               5


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1               5


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<400>  27

Xaa Xaa Ser Xaa Xaa Gly
1               5


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<400>  28

Xaa Xaa Thr Xaa Xaa Gly
1               5


<210>  29
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<400>  29
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<210>  30
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<210>  31
<211>  50
<212>  DNA
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<210>  32
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