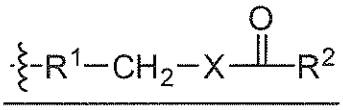
administration. When glucagon is used in the acute medical treatment of hypoglycemia, a crystalline form of glucagon is solubilized with a dilute acid buffer and the solution is injected intramuscularly. While this treatment is effective, the methodology is cumbersome and dangerous for someone that is semi-conscious. Accordingly, there is a need for a glucagon analog that maintains the biological performance of the parent molecule but is sufficiently soluble and stable, under relevant physiological conditions, that it can be pre-formulated as a solution, ready for injection.

Additionally, diabetics are encouraged to maintain near normal blood glucose levels to delay or prevent microvascular complications. Achievement of this goal usually requires intensive insulin therapy. In striving to achieve this goal, physicians have encountered a substantial increase in the frequency and severity of hypoglycemia in their diabetic patients. Accordingly, improved pharmaceuticals and methodologies are needed for treating diabetes that are less likely to induce hypoglycemia than current insulin therapies.

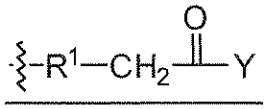
As described herein, high potency glucagon agonists are provided that exhibit enhanced biophysical stability and aqueous solubility at physiological pH in pharmaceutical compositions suitable for commercial use. Native glucagon is neither soluble, nor stable in the physiological pH range and thus must be manufactured as a dry product that requires reconstitution and immediate use. The glucagon analogs described herein have enhanced physical properties that render them superior for use in current medicinal settings where the native hormone is currently employed. These compounds can be used in accordance with one embodiment to prepare pre-formulated solutions ready for injection to treat hypoglycemia. Alternatively, the glucagon agonists can be co-administered with insulin to buffer the effects of insulin to allow for a more stable maintenance of blood glucose levels. In addition, other beneficial uses of compositions comprising the modified glucagon peptides disclosed herein are described in detail below.

SUMMARY

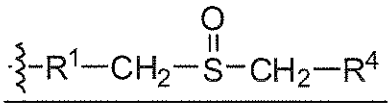
In one aspect, the present invention refers to a glucagon peptide with glucagon agonist activity, comprising an amino acid sequence with up to 10 amino acid modifications, relative to native glucagon (SEQ ID NO: 1), wherein the glutamine at position 3 is substituted with a different amino acid comprising a side chain of Structure I, II, or III:



Structure I



Structure II



Structure III

wherein R^1 is C_{0-3} alkylene or C_{0-3} heteroalkylene; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4, SR^3, or OR^3,

and wherein the glucagon peptide comprises (i) an intramolecular bridge which connects the side chains of an amino acid at position i and an amino acid at position i+4, wherein i is 12, 16, 20, or 24, (ii) an α,α-disubstituted amino acid at one, two, three, or all of positions 16, 20, 21, and 24, or (iii) both (i) and (ii).

One ~~embodiment~~example of the ~~invention~~ disclosure provides glucagon peptides that retain glucagon receptor activity and exhibit improved solubility relative to the native glucagon peptide (SEQ ID NO: 1). -Native glucagon exhibits poor solubility in

aqueous solution, particularly at physiological pH, with a tendency to aggregate and precipitate over time. In contrast, the glucagon peptides of one ~~embodiment~~example of the ~~invention~~ disclosure exhibit at least 2-fold, 5-fold, or even higher solubility compared to native glucagon at a pH between 6 and 8, or between 6 and 9, for example, at pH 7 after 24 hours at 25°C.

In one ~~embodiment~~example the glucagon peptides retain at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75% activity, 80% activity, 85% activity, or 90% of the activity of native glucagon (calculated as the inverse ratio of EC50s for the glucagon peptide vs. glucagon, e.g., as measured by cAMP production using the assay generally described in Example 13). In one ~~embodiment~~example, the glucagon peptides of the present ~~invention~~ disclosure have the same or greater activity (used synonymously with the term "potency" herein) than glucagon. In some ~~embodiments~~examples, the glucagon peptides retain up to about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon.

Glucagon normally has about 1% of the activity of native GLP-1 at the GLP-1 receptor. GLP-1(7-36) amide (SEQ ID NO: 57) or GLP-1(7-37)acid (SEQ ID NO: 58) are biologically potent forms of GLP-1, that demonstrate essentially equivalent activity at the GLP-1 receptor. Glucagon is also 10- to 20- fold more selective for the glucagon receptor compared to GLP-1 receptor (selectivity calculated as the inverse ratio of EC50 of glucagon for the glucagon receptor vs. for the GLP-1 receptor). For example, for an assay in which glucagon's EC50 at the glucagon receptor is 0.22 nM and glucagon's EC50 at the GLP-1 receptor is 3.85 nM, the calculated selectivity is 17.5-fold. Activity can be measured, e.g., by cAMP production using the assay generally described in Example 13. In some ~~embodiments~~examples, the glucagon peptides ~~of the present invention~~ as disclosed herein exhibit less than about 5%, 4%, 3%, 2% or 1% of the activity of native GLP-1 at the GLP-1 receptor and/or a greater than about 5-fold, 10-fold, or 15-fold selectivity for glucagon receptor compared to GLP-1 receptor. For example, ~~in some embodiments,~~ the glucagon peptides ~~of the present invention~~as disclosed herein exhibit less than 5% of the activity of native GLP-1 at the GLP-1 receptor and exhibit a greater than 5-fold selectivity for glucagon receptor compared to GLP-1 receptor.

Any of the glucagon peptides ~~of the invention~~as disclosed herein may additionally exhibit improved stability and/or reduced degradation, for example, retaining at least 95% of the original peptide after 24 hours at 25 °C. In some ~~embodiments~~examples, the glucagon

peptides of the ~~invention~~ disclosure exhibit improved stability, such that at least 75% (e.g., at least 80%, at least 85%, at least 90%, at least 95%, more than 95%, up to 100%) of a concentration of the peptide or less than about 25% (e.g., less than 20%, less than 15%, less than 10%, less than 5%, 4%, 3%, 2%, 1%, down to 0%) of degraded peptide is detectable at 280 nm by an ultraviolet (UV) detector after 1 or more weeks (e.g., 2 weeks, 4 weeks, 1 month, two months, four months, six months, eight months, ten months, twelve months) in solution at a temperature of at least 20 °C (e.g., 21 °C, 22 °C, 23 °C, 24 °C, 25 °C, 26 °C, at least 27.5 °C, at least 30 °C, at least 35 °C, at least 40 °C, at least 50 °C) and less than 100 °C, less than 85 °C, less than 75 °C, or less than 70 °C.

In accordance with one ~~embodiment~~example a glucagon peptide is provided with improved solubility, wherein the peptide is modified by amino acid substitutions and/or additions that introduce a charged amino acid into the C-terminal portion of the peptide, and in one ~~embodiment~~example at a position C-terminal to position 27 of SEQ ID NO: 1. Optionally, one, two or three charged amino acids may be introduced within the C-terminal portion, and in one ~~embodiment~~example C-terminal to position 27. In accordance with one ~~embodiment~~example the native amino acid(s) at positions 28 and/or 29 are substituted with a charged amino acid, and/or one to three charged amino acids are added to the C-terminus of the peptide, after position 29. In exemplary ~~embodiments~~examples, one, two or all of the charged amino acids are negatively charged. Additional modifications, e.g. conservative substitutions, may be made to the glucagon peptide that still allow it to retain glucagon activity.

In accordance with one exemplary ~~embodiment~~example the glucagon peptide comprises an amino acid sequence of SEQ ID NO: 11, or an analog thereof that contains 1 to 3 further amino acid modifications relative to native glucagon, or a glucagon agonist analog thereof. SEQ ID NO: 11 represents a modified glucagon peptide wherein the asparagine residue at position 28 of the native protein has been substituted with an aspartic acid. In another exemplary ~~embodiment~~example the glucagon peptide comprises an amino acid sequence of SEQ ID NO: 38, wherein the asparagine residue at position 28 of the native protein has been substituted with glutamic acid. Other exemplary ~~embodiments~~examples include glucagon peptides of SEQ ID NOS: 24, 25, 26, 33, 35, 36 and 37.

In accordance with another ~~embodiment~~example, glucagon peptides are provided that have enhanced potency at the glucagon receptor, wherein the peptides comprise an amino acid modification at position 16 of native glucagon (SEQ ID NO: 1). By way of nonlimiting example, such enhanced potency can be provided by substituting the naturally occurring serine at position 16 with glutamic acid or with another negatively charged amino acid having a side chain with a length of 4 atoms, or alternatively with any one of glutamine, homoglutamic acid, or homocysteic acid, or a charged amino acid having a side chain containing at least one heteroatom, (e.g. N, O, S, P) and with a side chain length of about 4 (or 3-5) atoms. Substitution of serine at position 16 with glutamic acid enhances glucagon activity at least 2-fold, 4-fold, 5-fold and up to 10-fold greater at the glucagon receptor. In some ~~embodiments~~examples, glucagon peptide retains selectivity for the glucagon receptor relative to the GLP-1 receptors, e.g., at least 5-fold, 10-fold, or 15-fold selectivity.

The solubility of any of the foregoing compounds can be further improved by attaching a hydrophilic moiety to the peptide. Introduction of such groups also increases duration of action, e.g. as measured by a prolonged half-life in circulation. In one ~~embodiment~~example the hydrophilic moiety is a polyethylene glycol (PEG) chain or other water soluble polymer that is covalently linked to the side chain of an amino acid residue at one or more of positions 16, 17, 21, 24, 29, 40 of said glucagon peptide, within a C-terminal extension, or at the C-terminal amino acid. In some ~~embodiments~~examples, the native amino acid at that position is substituted with an amino acid having a side chain suitable for crosslinking with hydrophilic moieties, to facilitate linkage of the hydrophilic moiety to the peptide. Exemplary amino acids include Cys, Lys, Orn, homo-Cys, or acetyl phenylalanine (Ac-Phe). In other ~~embodiments~~examples, an amino acid modified to comprise a hydrophilic group is added to the peptide at the C-terminus. The polyethylene glycol chain in accordance with one ~~embodiment~~example has a molecular weight selected from the range of about 500 to about 40,000 Daltons. In one ~~embodiment~~example the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons. In another ~~embodiment~~example the polyethylene glycol chain has a molecular weight of about 10,000 to about 20,000 Daltons. In yet other exemplary ~~embodiments~~examples the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

In accordance with some ~~embodiments~~examples, the glucagon peptides disclosed herein are modified to comprise an acyl group or alkyl group, e.g., an acyl or alkyl group which is non-native to a naturally-occurring amino acid. Acylation or alkylation can increase the half-life of the glucagon peptides in circulation, can advantageously delay the onset of action and/or extend the duration of action at the glucagon and/or GLP-1 receptors and/or improve resistance to proteases such as DPP-IV. Acylation or alkylation may also enhance solubility of the peptide at neutral pH. As shown herein, the activity at the glucagon receptor and GLP-1 receptor of the glucagon peptide is maintained if not enhanced after acylation.

In some ~~aspects~~examples, the glucagon peptide is covalently attached to an acyl or alkyl group via a spacer, e.g., an amino acid, dipeptide, tripeptide, hydrophilic bifunctional spacer, or hydrophobic bifunctional spacer. In certain ~~aspects~~examples, an enhancement in activity at the GLP-1 and glucagon receptors is observed upon acylation of the peptide with a spacer. In selected ~~aspects~~examples, such as, for example, when the peptide lacks an intramolecular bridge (e.g., a covalent intramolecular bridge), a further enhancement in activity at the GLP-1 and glucagon receptors is observed upon acylation of the peptide with a spacer, as compared to an acylated peptide, wherein the acyl group is attached to the peptide without a spacer. In accordance with some ~~embodiments~~examples, the spacer is an amino acid or dipeptide having an amino acid or peptide backbone structure that is 3 to 10 atoms (e.g., 6 to 10 atoms) in length. In accordance with certain specific ~~embodiments~~examples, the total length of the spacer and acyl or alkyl group is 14 to 28 atoms, e.g., 17 to 28, 19 to 26 atoms, 19 to 21 atoms. Suitable spacers for purposes of enhancing glucagon activity are further described herein. In some ~~embodiments~~examples, the acylated or alkylated peptides described herein further comprise a modification which selectively decreases activity at the GLP-1 receptor, e.g., a modification of the Thr at position 7, such as a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., aminobutyric acid (Abu) or Ile; deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28 (e.g., deleting one or both of the amino acids at positions 28 and 29), yielding a peptide 27 or 28 amino acids in length).

Glucagon peptides may be acylated or alkylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position.

In some ~~embodiments~~examples, the invention provides a glucagon peptide modified to

comprise an acyl group or alkyl group covalently linked to the amino acid at position 10 of the glucagon peptide. The glucagon peptide may further comprise a spacer between the amino acid at position 10 of the glucagon peptide and the acyl group or alkyl group. In some ~~embodiments~~examples, the acyl group is a fatty acid or bile acid, or salt thereof, e.g. a C4 to C30 fatty acid, a C8 to C24 fatty acid, cholic acid, a C4 to C30 alkyl, a C8 to C24 alkyl, or an alkyl comprising a steroid moiety of a bile acid. The spacer is any moiety with suitable reactive groups for attaching acyl or alkyl groups. In exemplary ~~embodiments~~examples, the spacer comprises an amino acid, a dipeptide, a tripeptide, a hydrophilic bifunctional, e.g., an amino poly(alkyloxy)carboxylate, or a hydrophobic bifunctional spacer. In some ~~embodiments~~examples, the spacer is selected from the group consisting of: Trp, Glu, Asp, Cys and a spacer comprising $NH_2(CH_2CH_2O)n(CH_2)_mCOOH$, wherein m is any integer from 1 to 6 and n is any integer from 2 to 12. Such acylated or alkylated glucagon peptides may also further comprise a hydrophilic moiety, optionally a polyethylene glycol. Any of the foregoing glucagon peptides may comprise two acyl groups or two alkyl groups, or a combination thereof.

The present ~~invention~~disclosure further encompasses pharmaceutically acceptable salts of said glucagon agonists.

In other exemplary ~~embodiments~~examples, any of the foregoing compounds can be further modified to alter its pharmaceutical properties by the addition of a second peptide to the carboxy terminus of the glucagon peptide. In one ~~embodiment~~example a glucagon peptide is covalently bound through a peptide bond to a second peptide, wherein the second peptide comprises a sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO: 21 and SEQ ID NO: 22.

In some ~~embodiments~~examples, modifications at position 1 or 2 can increase the peptide's resistance to dipeptidyl peptidase IV (DPP IV) cleavage. For example, the amino acid at position 2 may be substituted with D-serine, D-alanine, valine, glycine, N-methyl serine, N-methyl alanine, or amino isobutyric acid. Alternatively, or in addition, the amino acid at position 1 may be substituted with D-histidine (D-His), desaminohistidine, hydroxyl-histidine, acetyl-histidine, homo-histidine, N-methyl histidine, alpha-methyl histidine, imidazole acetic acid, or alpha, alpha-dimethyl imidiazole acetic acid (DMIA).

It was observed that modifications at position 2 (e.g. AIB at position 2) and in some cases modifications at position 1 (e.g. DMIA at position 1) may reduce glucagon activity, sometimes significantly; surprisingly, this reduction in glucagon activity can be restored by stabilization of the alpha-helix structure in the C-terminal portion of glucagon (around amino acids 12-29). In some ~~embodiments~~examples, stabilization is via a covalent bond between amino acids at positions "i" and "i+4", wherein i is any integer from 12 to 25. In some specific ~~embodiments~~examples, "i" and "i+4" are 12 and 16, 16 and 20, or 20 and 24, or 24 and 28. In some ~~embodiments~~examples, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In exemplary ~~embodiments~~examples, the bridge or linker is about 8 (or about 7-9) atoms in length. In other ~~embodiments~~examples, stabilization is via a covalent bond between amino acids at positions "j" and "j+3," wherein j is any integer between 12 and 27. In exemplary ~~embodiments~~examples, the bridge or linker is about 6 (or about 5-7) atoms in length. In yet other ~~embodiments~~examples, stabilization is via a covalent bond between amino acids at positions "k" and "k+7," wherein k is any integer between 12 and 22. In some ~~embodiments~~examples, this covalent bond is an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods (i.e., means of forming a covalent intramolecular bridge) include any one or more of olefin metathesis, lanthionine-based cyclization, disulfide bridge or modified sulfur-containing bridge formation, the use of α, ω-diaminoalkane tethers, the formation of metal-atom bridges, and other means of peptide cyclization.

In yet other ~~embodiments~~examples, the helix is stabilized by non-covalent bonds (i.e., non-covalent intramolecular bridges), including but not limited to hydrogen-bonding, ionic interactions, and salt bridges.

In other ~~embodiments~~examples of the ~~invention~~disclosure, stabilization of the alpha-helix structure in the C-terminal portion of the glucagon peptide (around amino acids 12-29) is achieved through purposeful introduction of one or more α, α-disubstituted amino acids at positions that retain the desired activity. In some ~~embodiments~~examples, one, two, three, four or more of positions 16, 17, 18, 19, 20, 21, 24 or 29 of a glucagon peptide is substituted with an α, α-disubstituted amino acid. For example, substitution of position 16 of a glucagon peptide with amino iso-butyric acid (AIB) provides a stabilized alpha helix in the absence of a salt bridge or lactam. Such peptides are considered herein as a peptide lacking an intramolecular bridge. In specific ~~aspects~~examples,

stabilization of the alpha-helix is accomplished by introducing one or more α, α-disubstituted amino acids without introduction of a covalent intramolecular bridge, e.g., a lactam bridge, a disulfide bridge. Such peptides are considered herein as a peptide lacking a covalent intramolecular bridge. In some ~~embodiments~~examples, one, two, three or more of positions 16, 20, 21 or 24 are substituted with AIB.

Thus, in some ~~embodiments~~examples the ~~invention~~ disclosure provides a glucagon peptide with glucagon agonist activity, comprising the amino acid sequence:

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 39) with 1 to 3 amino acid modifications thereto,

wherein X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

wherein Z is selected from the group consisting of –COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids, and

wherein an intramolecular bridge, preferably a covalent bond, connects the side chains of an amino acid at position i and an amino acid at position i+4, wherein i is 12, 16, 20 or 24.

In some ~~embodiments~~examples, the intramolecular bridge is a lactam bridge. In some ~~embodiments~~examples, the amino acids at positions i and i+4 of SEQ ID NO: 39 are Lys and Glu, e.g., Glu16 and Lys20. In some ~~embodiments~~examples, X1 is selected from the group consisting of: D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, and alpha, alpha-dimethyl imidiazole acetic acid (DMIA). In other ~~embodiments~~examples, X2 is selected from the group consisting of: D-Ser, D-Ala, Gly, N-methyl-Ser, Val, and alpha, amino isobutyric acid (AIB). In some ~~embodiments~~examples, the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, 29, 40, within a C-terminal extension, or at the C-terminal amino acid. In exemplary ~~embodiments~~examples, this hydrophilic moiety is covalently linked to a Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine residue at any of these positions. Exemplary hydrophilic moieties include polyethylene glycol (PEG), for example, of a molecular weight of about 1,000 Daltons to about 40,000 Daltons, or about 20,000 Daltons to about 40,000 Daltons.

In other ~~embodiments~~examples, the ~~invention~~ disclosure provides a glucagon peptide with glucagon agonist activity, comprising the amino acid sequence:

X1-X2-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Z (SEQ ID NO: 39),

wherein X1 and/or X2 is a non-native amino acid that reduces susceptibility of (or increases resistance of) the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

wherein one, two, three, four or more of positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α, α-disubstituted amino acid, and

wherein Z is selected from the group consisting of –COOH (the naturally occurring C-terminal carboxylate), -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids.

Exemplary further amino acid modifications to the foregoing glucagon peptides or analogs include substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile, optionally, in combination with substitution or addition of an amino acid comprising a side chain covalently attached (optionally, through a spacer) to an acyl or alkyl group, which acyl or alkyl group is non-native to a naturally-occurring amino acid, substitution of Lys at position 12 with Arg; substitution of Asp at position 15 with Glu; substitution of Ser at position 16 with Thr or AIB; substitution of Gln at position 20 with Ser, Thr, Ala or AIB; substitution of Asp at position 21 with Glu; substitution of Gln at position 24 with Ser, Thr, Ala or AIB; substitution of Met at position 27 with Leu or Nle; substitution of Asn at position 28 with a charged amino acid; substitution of Asn at position 28 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 28 with Asn, Asp, or Glu; substitution at position 28 with Asp; substitution at position 28 with Glu; substitution of Thr at position 29 with a charged amino acid; substitution of Thr at position 29 with a charged amino acid selected from the group consisting of Lys, Arg, His, Asp, Glu, cysteic acid, and homocysteic acid; substitution at position 29 with Asp, Glu, or Lys; substitution at position 29 with Glu; insertion of 1-3 charged amino acids after position 29; insertion at position 30 (i.e., after position 29) of Glu or Lys; optionally with insertion at position 31 of Lys; addition of SEQ ID NO: 20 to the C-terminus, optionally, wherein the amino acid at position 29 is Thr or Gly; substitution

or addition of an amino acid covalently attached to a hydrophilic moiety; or a combination thereof.

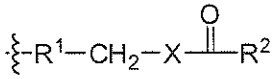
In yet further exemplary ~~embodiments~~examples, any of the foregoing peptides can be further modified to improve stability by modifying the amino acid at position 15 of SEQ ID NO: 1 to reduce degradation of the peptide over time, especially in acidic or alkaline buffers. In exemplary ~~embodiments~~examples, Asp at position 15 is substituted with a Glu, homo-Glu, cysteic acid, or homo-cysteic acid.

Alternatively, any of the glucagon peptides described herein can be further modified to improve stability by modifying the amino acid at position 16 of SEQ ID NO: 1. In exemplary ~~embodiments~~examples, Ser at position 16 is substituted with Thr or AIB, or any of the amino acids substitutions described above which enhance potency at the glucagon receptor. Such modifications reduce cleavage of the peptide bond between Asp15-Ser16.

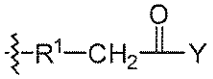
Maintained or enhanced activity at the glucagon receptor may be achieved by modifying the Gln at position 3 with a glutamine analog. For example, a glucagon peptide comprising a glutamine analog at position 3 may exhibit about 5%, about 10%, about 20%, about 50%, or about 85% or greater the activity of native glucagon (e.g. SEQ ID NO: 1) at the glucagon receptor. In some ~~embodiments~~examples a glucagon peptide comprising a glutamine analog at position 3 may exhibit about 20%, about 50%, about 75%, about 100%, about 200% or about 500% or greater the activity of a corresponding glucagon peptide having the same amino acid sequence as the peptide comprising the glutamine analog, except for the modified amino acid at position 3 (e.g. SEQ ID NO: 69 or SEQ ID NO: 70) at the glucagon receptor. In some ~~embodiments~~examples, a glucagon peptide comprising a glutamine analog at position 3 exhibits enhanced activity at the glucagon receptor, but the enhanced activity is no more than 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon or of a corresponding glucagon peptide having the same amino acid sequence as the peptide comprising the glutamine analog, except for the modified amino acid at position 3.

In another aspect, the present invention refers to a peptide comprising SEQ ID NO: 71, or a pharmaceutically acceptable salt thereof.

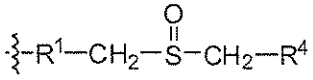
In some ~~embodiments~~examples, the glutamine analog is a naturally occurring or a non-naturally occurring amino acid comprising a side chain of Structure I, II or III:



Structure I



Structure II



Structure III

wherein R^1 is C_{0-3} alkyl or C_{0-3} heteroalkyl; R^2 is NHR^4 or C_{1-3} alkyl; R^3 is C_{1-3} alkyl; R^4 is H or C_{1-3} alkyl; X is NH, O, or S; and Y is NHR^4, SR^3, or OR^3. In some ~~embodiments~~examples, X is NH or Y is NHR^4. In some ~~embodiments~~examples, R^1 is C_{0-2} alkyl or C_1 heteroalkyl. In some ~~embodiments~~examples, R^2 is NHR^4 or C_1 alkyl. In some ~~embodiments~~examples, R^4 is H or C^1 alkyl. In exemplary ~~embodiments~~examples, an amino acid comprising a side chain of Structure I is provided where, R^1 is CH_2-S, X is NH, and R^2 is CH_3 (acetamidomethyl-cysteine, C(Acm)); R^1 is CH_2, X is NH, and R^2 is CH_3 (acetyldiaminobutanoic acid, Dab(Ac)); R^1 is C_0 alkyl, X is NH, R^2 is NHR^4, and R^4 is H (carbamoyldiaminopropanoic acid, Dap(urea)); or R^1 is CH_2-CH_2, X is NH, and R^2 is CH_3 (acetylornithine, Orn(Ac)). In exemplary ~~embodiments~~examples, an amino acid comprising a side chain of Structure II is provided where, R^1 is CH_2, Y is NHR^4, and R^4 is CH_3 (methylglutamine, Q(Me)); In exemplary ~~embodiments~~examples , an amino acid comprising a side chainof Structure III is provided where, R^1 is CH_2 and R^4 is H (methionine-sulfoxide, M(O)); In specific ~~embodiments~~examples, the amino acid at position 3 is substituted with Dab(Ac). For example, glucagon agonists can comprise the amino acid sequence of SEQ ID NO: 63, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, and SEQ ID NO: 74.

Enhanced activity at the glucagon receptor of the glucagon peptide also may be achieved by covalently attaching an acyl or alkyl group, e.g., an acyl or alkyl group which is non-native to a naturally occurring amino acid (e.g., a C4 to C30 fatty acyl group, a C4 to C30 alkyl group), to the side chain of an amino acid of the glucagon peptide. In some ~~embodiments~~examples, the acylated or alkylated glucagon peptides lack an

intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam). In certain ~~aspects~~examples, the acyl or alkyl group is attached to the side chain of the amino acid of the glucagon peptide through a spacer, e.g., a spacer which is 3 to 10 atoms in length. In some ~~embodiments~~examples, the acyl or alkyl group is attached to the side chain of the amino acid at position 10 of the glucagon peptide through a spacer. In specific ~~embodiments~~examples, the acylated or alkylated glucagon peptides further comprise a modification which selectively decreases the activity of the peptide at the GLP-1 receptor. For example, the acylated or alkylated glucagon peptide may comprise a C-terminal alpha carboxylate, a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile, a deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28, yielding a 27- or 28-amino acid peptide, or a combination thereof.

In some ~~embodiments~~examples, any of the glucagon peptides described herein can be further modified to reduce degradation at various amino acid positions by modifying any one, two, three, or all four of positions 20, 21, 24, or 27. Exemplary ~~embodiments~~examples include substitution of Gln at position 20 with Ala or AIB, substitution of Asp at position 21 with Glu, substitution of Gln at position 24 with Ala or AIB, substitution of Met at position 27 with Leu or Nle. Removal or substitution of methionine reduces degradation due to oxidation of the methionine. Removal or substitution of Gln or Asn reduces degradation due to deamidation of Gln or Asn. Removal or substitution of Asp reduces degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate.

In some ~~embodiments~~examples, any of the glucagon peptides described herein can be modified without adversely affecting activity at the glucagon receptor, while retaining at least partial glucagon receptor activity. For example, conservative or non-conservative substitutions, additions or deletions may be carried out at any of positions 2, 5, 7, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 27, 28 or 29. In exemplary ~~embodiments~~examples, Lys at position 12 is substituted with Arg. In other exemplary ~~embodiments~~examples amino acids at positions 29 and/or 28, and optionally at position 27, are deleted.

Any of the glucagon peptides described herein may exhibit an EC50 at the human glucagon receptor of about 100 nM, 75 nM, 50 nM, 40nM, 30 nM, 20 nM, 10

nM, 5 nM, 1 nM or less when tested for cAMP induction in HEK293 cells over-expressing glucagon receptor, e.g. using the assay of Example 13. Typically pegylated peptides will exhibit a higher EC50 compared to the unpegylated peptide. For example, the glucagon peptides described herein, when unpegylated, may exhibit activity at the glucagon receptor which is at least 20% (e.g., at least 30%, at least 40%, at least 50%, at least 60%, at least 75%, at least 80%, at least 90% at least 95%, at least 98%, at least 99%, 100%, 150%, 200%, 400%, 500% or more) of the activity of native glucagon (SEQ ID NO: 1) at the glucagon receptor. In certain ~~embodiments~~examples, the glucagon peptides described herein exhibit the indicated % activity of native glucagon at the glucagon receptor, when lacking a hydrophilic moiety, but exhibit a decreased % activity of native glucagon at the glucagon receptor, when comprising a hydrophilic moiety. For example, the glucagon peptides described herein, when pegylated, may exhibit activity at the glucagon receptor which is at least 2% (e.g. at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, or at least 10% of the activity of native glucagon. In some ~~embodiments~~examples, the glucagon peptides described herein may exhibit any of the above indicated activities but no more than 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon at the glucagon receptor.

In some specific ~~embodiments~~examples, the glucagon peptide comprises (a) an amino acid modification at position 1 and/or 2 that confers DPP-IV resistance, e.g., substitution with DMIA at position 1, or AIB at position 2, (b) an intramolecular bridge within positions 12-29, e.g. at positions 16 and 20, or one or more substitutions of the amino acids at positions 16, 20, 21, and 24 with an α,α disubstituted amino acid, optionally (c) linked to a hydrophilic moiety such as PEG, e.g., through Cys at position 24, 29 or at the C-terminal amino acid, optionally (d) an amino acid modification at position 27 that substitutes Met with, e.g., Nle, optionally (e) amino acid modifications at positions 20, 21 and 24 that reduce degradation, and optionally (f) linked to SEQ ID NO: 20. When the glucagon peptide is linked to SEQ ID NO: 20, the amino acid at position 29 in certain ~~embodiments~~examples is Thr or Gly. In other specific ~~embodiments~~examples, the glucagon peptide comprises (a) Asp28Glu29, or Glu28Glu29, or Glu29Glu30, or Glu28Glu30 or Asp28Glu30, and optionally (b) an amino acid modification at position 16 that substitutes Ser with, e.g. Thr or AIB, and optionally (c) an amino acid modification at position 27 that substitutes Met with,

e.g., Nle, and optionally (d) amino acid modifications at positions 20, 21 and 24 that reduce degradation. In a specific ~~embodiment~~example, the glucagon peptide is T16,A20,E21,A24,Nle27,D28,E29.

The glucagon peptide may be part of a dimer, trimer or higher order multimer comprising at least two, three, or more peptides bound via a linker, wherein at least one or both peptides is a glucagon peptide. The dimer may be a homodimer or heterodimer. In some ~~embodiments~~examples, the linker is selected from the group consisting of a bifunctional thiol crosslinker and a bi-functional amine crosslinker. In certain ~~embodiments~~examples, the linker is PEG, e.g., a 5 kDa PEG, 20 kDa PEG. In some ~~embodiments~~examples, the linker is a disulfide bond. For example, each monomer of the dimer may comprise a Cys residue (e.g., a terminal or internally positioned Cys) and the sulfur atom of each Cys residue participates in the formation of the disulfide bond. As described herein, ~~In some aspects of~~ the ~~invention~~disclosure, the monomers are connected via terminal amino acids (e.g., N-terminal or C-terminal), via internal amino acids, or via a terminal amino acid of at least one monomer and an internal amino acid of at least one other monomer. In specific ~~aspects~~examples, the monomers are not connected via an N-terminal amino acid. In some ~~aspects~~examples, the monomers of the multimer are attached together in a "tail-to-tail" orientation in which the C-terminal amino acids of each monomer are attached together.

In another aspect, the present invention refers to a conjugate, dimer, or fusion peptide comprising a glucagon peptide, or analog thereof.

A conjugate moiety may be covalently linked to any of the glucagon peptides described herein, including a dimer, trimer or higher order multimer. Fusion peptides comprising the amino acid sequence of any of SEQ ID NOs: 20 to 22 are also contemplated.

Any of the modifications described above which increase glucagon receptor activity, retain partial glucagon receptor activity, improve solubility, increase stability, or reduce degradation can be applied to glucagon peptides individually or in combination. Thus, glucagon peptides can be prepared that retain at least 20% of the activity of native glucagon at the glucagon receptor, and which are soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, (e.g. pH 7), and optionally retain at least 95% of the original peptide (e.g. 5% or less of the original peptide is degraded or cleaved) after 24 hours at 25◦C. Alternatively, high potency glucagon peptides can be prepared that exhibit at least about 100%, 125%, 150%, 175%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800%,

900% or 10-fold or more of the activity of native glucagon at the glucagon receptor, and optionally are soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, (e.g. pH 7), and optionally retains at least 95% of the original peptide (e.g. 5% or less of the original peptide is degraded or cleaved) after 24 hours at 25°C. In certain ~~embodiments~~examples, the glucagon peptides described herein exhibit the indicated % activity of native glucagon at the glucagon receptor, when lacking a hydrophilic moiety, but exhibit a decreased % activity of native glucagon at the glucagon receptor, when comprising a hydrophilic moiety. In some ~~embodiments~~examples, the glucagon peptides described herein may exhibit at least any of the above indicated relative levels of activity at the glucagon receptor but no more than 10,000%, 100,000% or 1,000,000% of the activity of native glucagon at the glucagon receptor.

In another aspect, the present invention refers to a pharmaceutical composition comprising a glucagon peptide, a dimer, a conjugate or a fusion peptide, or a combination thereof, and a pharmaceutically acceptable carrier.

In accordance with one ~~embodiment~~example a pharmaceutical composition is provided comprising any of the novel glucagon peptides disclosed herein, preferably sterile and preferably at a purity level of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, and a pharmaceutically acceptable diluent, carrier or excipient. Such compositions may contain a glucagon peptide at a concentration of at least A, wherein A is 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml, 10 mg/ml, 11 mg/ml, 12 mg/ml, 13 mg/ml, 14 mg/ml, 15 mg/ml, 16 mg/ml, 17 mg/ml, 18 mg/ml, 19 mg/ml, 20 mg/ml, 21 mg/ml, 22 mg/ml, 23 mg/ml, 24 mg/ml, 25 mg/ml or higher. In other ~~embodiments~~examples, such compositions may contain a glucagon peptide at a concentration of at most B, wherein B is 30 mg/ml, 25 mg/ml, 24 mg/ml, 23, mg/ml, 22 mg/ml, 21 mg/ml, 20 mg/ml, 19 mg/ml, 18 mg/ml, 17 mg/ml, 16 mg/ml, 15 mg/ml, 14 mg/ml, 13 mg/ml, 12 mg/ml, 11 mg/ml 10 mg/ml, 9 mg/ml, 8 mg/ml, 7 mg/ml, 6 mg/ml, 5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml, or 0.1 mg/ml. In some ~~embodiments~~examples, the compositions may contain a glucagon peptide at a concentration range of A to B mg/ml, for example, 0.001 to 30.0 mg/ml. In one ~~embodiment~~example the pharmaceutical compositions comprise aqueous solutions that are sterilized and optionally stored within various containers. Such solutions can be used in accordance with one

~~embodiment~~example to prepare pre-formulated solutions ready for injection. In other ~~embodiments~~examples the pharmaceutical compositions comprise a lyophilized powder. In another aspect, the present invention refers to a kit for administering a glucagon agonist to a patient in need thereof, said kit comprising a pharmaceutical composition and a device for administering said pharmaceutical composition to the patient. The pharmaceutical compositions can be further packaged as part of a kit that includes a disposable device for administering the composition to a patient. Devices may

include a syringe and needle, or a pre-filled syringe. The containers or kits may be labeled for storage at ambient room temperature or at refrigerated temperature.

In accordance with one ~~embodiment~~example a method of rapidly increasing glucose level, normalizing blood glucose level, stabilizing blood glucose level, or preventing or treating hypoglycemia using a pre-formulated aqueous composition of a glucagon peptide of the ~~invention~~ disclosure is provided. The method comprises the step of administering an effective amount of an aqueous solution comprising a novel modified glucagon peptide of the present disclosure. In some ~~embodiments~~examples, the aqueous composition is pre-packaged in a device that is used to administer the composition to the patient. In another aspect, it is referred to the use of the glucagon peptide of the present invention, a dimer, a conjugate or a fusion peptide, or a combination thereof, in the manufacture of a medicament for causing temporary paralysis of the intestinal tract in the patient. In another ~~embodiment~~example a method is provided for inducing the temporary paralysis of the intestinal tract. The method comprises the step of administering one or more of the glucagon peptides disclosed herein to a patient in need thereof.

In another aspect, it is referred to the use of the glucagon peptide of the present invention, a dimer, a conjugate or a fusion peptide, or a combination thereof, in the manufacture of a medicament for reducing weight gain or inducing weigh loss in the patient.

In yet another ~~embodiment~~example a method of reducing weight gain or inducing weight loss is provided, which involves administering an effective amount of an aqueous solution comprising a glucagon peptide of the ~~invention~~disclosure. Methods for reducing weight gain or inducing weight loss are expected to be useful to treat obesity of various causes, including drug-induced obesity, and reducing complications associated with obesity including vascular disease (coronary artery disease, stroke, peripheral vascular disease, ischemia reperfusion, etc.), hypertension, onset of diabetes type II, hyperlipidemia and musculoskeletal diseases.

In another aspect, it is referred to the use of the glucagon peptide of the present invention, a dimer, a conjugate or a fusion peptide, or a combination thereof, in the manufacture of a medicament for treating or preventing hypoglycemia in the patient.

In further ~~embodiments~~examples, methods of treating hyperglycemia or diabetes involving co-administering insulin and a glucagon peptide of the ~~invention~~ disclosure are provided. Hyperglycemia includes diabetes, diabetes mellitus type I, diabetes mellitus type II, or gestational diabetes, either insulin-dependent or non-insulin-dependent, and reducing complications of diabetes including nephropathy, retinopathy and vascular disease. Co-administration of insulin and a glucagon peptide of the ~~invention~~ disclosure can reduce nocturnal hypoglycemia and/or buffer the hypoglycemic effects of insulin, allowing the same or higher doses of short-acting or long-acting insulin to be administered with fewer adverse hypoglycemic effects. Compositions comprising insulin together with a glucagon peptide of the invention are also provided.

In another aspect, the present invention refers to the use of the glucagon peptide of the present invention, a dimer, a conjugate or a fusion peptide, or a combination thereof, in the manufacture of a medicament for stabilizing the blood glucose level of the patient.

In accordance with one ~~embodiment~~example an improved method of regulating blood glucose levels in insulin dependent patients is provided. The method comprises the steps of administering insulin in an amount therapeutically effective for the control of

diabetes and administering a novel modified glucagon peptide of the present disclosure in an amount therapeutically effective for the prevention of hypoglycemia, wherein said administering steps are conducted within twelve hours of each other. In one ~~embodiment~~example the glucagon peptide and the insulin are co-administered as a single composition.

All therapeutic methods, pharmaceutical compositions, kits and other similar ~~embodiments~~examples described herein contemplate that the use of the term glucagon peptides, glucagon agonist analogs, glucagon agonists, or glucagon analogs includes all pharmaceutically acceptable salts or esters thereof.

Exemplary glucagon peptides are selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 33, wherein amino acid 29 of the glucagon peptide is bound to a second peptide through a peptide bond, and said second peptide comprises the sequence of SEQ ID NO: 20, SEQ ID NO: 21 or SEQ ID NO: 22. In one ~~embodiment~~example the glucagon peptide is pegylated. In one ~~embodiment~~example the method comprises the step of administering a peptide comprising the sequence of SEQ ID NO: 24, SEQ ID NO: 25, and SEQ ID NO: 26, wherein a polyethylene chain is covalently linked to amino acid position 21 or at position 24.

Oxyntomodulin is a 37 amino acid peptide that contains the 29 amino acid sequence of glucagon (i.e. SEQ ID NO: 1) followed by an 8 amino acid carboxy terminal extension of SEQ ID NO: 21 (KRNRNNIA). While the present ~~invention~~disclosure contemplates that glucagon analogs described herein may optionally be joined to this 8 amino acid carboxy terminal extension (SEQ ID NO: 21), the ~~invention~~disclosure in some ~~embodiments~~examples also specifically contemplates glucagon analogs and uses of glucagon analogs lacking the 8 contiguous carboxy amino acids of SEQ ID NO: 21.

The foregoing summary is not intended to define every aspect of the ~~invention~~disclosure, and additional ~~embodiments~~examples are described in other sections, such as the Detailed Description. The entire document is intended to be related as a unified disclosure, and it should be understood that all possible combinations of features described herein may be contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document.

Moreover, the ~~invention~~disclosure includes any one or all ~~embodiments~~examples of the invention that are narrower in scope in any way than the variations defined by specific paragraphs herein. For example, where certain ~~aspects~~examples of the ~~invention~~disclosure are described as a genus, it should be understood that every member of a genus is, individually, an ~~embodiment~~ example of the ~~invention~~disclosure, and that combinations of two or more members of the genus are ~~embodiments~~examples of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a bar graph representing the stability of Glucagon Cys^{21}maleimidoPEG$_{5K}$ at 37 °C incubated for 24, 48, 72, 96, 144 and 166 hours, respectively.

Fig. 2 represents data generated from HPLC analysis of Glucagon Cys^{21}maleimidoPEG$_{5K}$ at pH 5 incubated at 37 °C for 24, 72 or 144 hours, respectively.

Fig. 3 represents data showing the solubility of glucagon analogs (D28, E29, E30) relative to native glucagon after 60 hours at 25 °C at pH of 2, 4, 5.5, 7 and 8, respectively.

Fig. 4 represents data showing the solubility of glucagon analogs (E15D28, D28E29 and D28E30) relative to native glucagon after 24 hours at 25 °C and then 24 hours a 4 °C at pH of 2, 4, 5.5 and 7, respectively.

Fig. 5 represents the maximum solubility of glucagon analogs D28, D28E30 and E15,D28 after 24 hours, pH 7 at 4 °C.

Fig. 6 represents data showing a glucagon receptor mediated cAMP induction by glucagon analogs (K29 ▲, K30 ▼, and K29K30 ◆) relative to native glucagon ■.

Fig. 7 represents data showing a glucagon receptor mediated cAMP induction by glucagon analogs (D28 □, E29 △, E30 ▽, K30K31 ◇ and K30, ▼) relative to native glucagon ■.

Fig. 8 represents data showing a glucagon receptor mediated cAMP induction by glucagon analogs (D28 □, E28 ⬟ and K29, ▲) relative to native glucagon ■.

Fig. 9 represents data showing a glucagon receptor mediated cAMP induction by glucagon analogs (D28E29 +, D28E30 ×, E15D28 ✳ and E29 △) relative to native glucagon ■.