GLUCAGON ANALOGS EXHIBITING ENHANCED SOLUBILITY AND STABILITY IN PHYSIOLOGICAL PH BUFFERS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to the following: U.S. Provisional Patent Application No. 61/073,193 filed on June 17, 2008, U.S. Provisional Patent Application No. 61/078,165 filed July 3, 2008, and U.S. Provisional Patent Application No. 61/090,415 filed on August 20, 2008. The disclosure of each application is hereby expressly incorporated by reference in its entirety.

BACKGROUND

Pre-proglucagon is a 158 amino acid precursor polypeptide that is processed in different tissues to form a number of different proglucagon-derived peptides, including glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and oxyntomodulin (OXM), that are involved in a wide variety of physiological functions, including glucose homeostasis, insulin secretion, gastric emptying, and intestinal growth, as well as the regulation of food intake. Glucagon is a 29-amino acid peptide that corresponds to amino acids 33 through 61 of pre-proglucagon, while GLP-1 is produced as a 37-amino acid peptide that corresponds to amino acids 72 through 108 of pre-proglucagon.

Hypoglycemia occurs when blood glucose levels drops too low to provide enough energy for the body's activities. In adults or children older than 10 years, hypoglycemia is uncommon except as a side effect of diabetes treatment, but it can result from other medications or diseases, hormone or enzyme deficiencies, or tumors. When blood glucose begins to fall, glucagon, a hormone produced by the pancreas, signals the liver to break down glycogen and release glucose, causing blood glucose levels to rise toward a normal level. Thus, glucagon's most recognized role in glucose regulation is to counteract the action of insulin and maintain blood glucose levels. However for diabetics, this glucagon response to hypoglycemia may be impaired, making it harder for glucose levels to return to the normal range.

Hypoglycemia is a life threatening event that requires immediate medical attention. The administration of glucagon is an established medication for treating acute hypoglycemia and it can restore normal levels of glucose within minutes of

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

One embodiment of the invention 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 of the invention 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 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, the glucagon peptides of the present invention have the same or greater activity (used synonymously with the term "potency" herein) than glucagon. In some embodiments, 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, the glucagon peptides of the present invention 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 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 may additionally exhibit improved stability and/or reduced degradation, for example, retaining at least 95% of the original peptide after 24 hours at 25 ℃. In some embodiments, the glucagon

peptides of the invention 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 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 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 C-terminal to position 27. In accordance with one embodiment 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, 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 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 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 include glucagon peptides of SEQ ID NOS: 24, 25, 26, 33, 35, 36 and 37.

In accordance with another embodiment, 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, 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 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, 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, 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 has a molecular weight selected from the range of about 500 to about 40,000 Daltons. In one embodiment the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons. In another embodiment the polyethylene glycol chain has a molecular weight of about 10,000 to about 20,000 Daltons. In yet other exemplary embodiments the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

In accordance with some embodiments, 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, 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, an enhancement in activity at the GLP-1 and glucagon receptors is observed upon acylation of the peptide with a spacer. In selected aspects, 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, 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, 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, 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, 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, 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, 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, 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 further encompasses pharmaceutically acceptable salts of said glucagon agonists.

In other exemplary embodiments, 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 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, 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, 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, "i" and "i+4" are 12 and 16, 16 and 20, or 20 and 24, or 24 and 28. In some embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In exemplary embodiments, the bridge or linker is about 8 (or about 7-9) atoms in length. In other embodiments, 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, the bridge or linker is about 6 (or about 5-7) atoms in length. In yet other embodiments, 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, 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, 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 of the invention, 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, 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,

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, one, two, three or more of positions 16, 20, 21 or 24 are substituted with AIB.

Thus, in some embodiments the invention 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, the intramolecular bridge is a lactam bridge. In some embodiments, 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, 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, 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, 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, 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, the invention 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

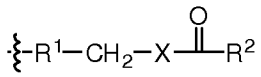
or addition of an amino acid covalently attached to a hydrophilic moiety; or a combination thereof.

In yet further exemplary embodiments, 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, Asp at position 15 is substituted with a Glu, homo-Glu, cysteic acid, or homo-cysteic acid.

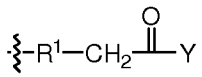
Alternatively, any of the glucagon peptides described herein can be further modified to improve stability by modifying the amino acid at position 16 of SEQ ID NO: 1. In exemplary embodiments, Ser at position 16 is substituted with Thr or AIB, or any of the amino acids substitutions described above which 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 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, 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.

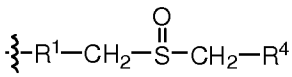
In some embodiments, the glutamine analog is a naturally occurring or a non-naturally occurring amino acid comprising a side chain of Structure I, II or III:



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, X is NH or Y is NHR^4. In some embodiments, R^1 is C_{0-2} alkyl or C_1 heteroalkyl. In some embodiments, R^2 is NHR^4 or C_1 alkyl. In some embodiments, R^4 is H or C^1 alkyl. In exemplary embodiments, 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, 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 , 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, 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, the acylated or alkylated glucagon peptides lack an

intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam). In certain aspects, 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, 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, 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, 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 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, 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, Lys at position 12 is substituted with Arg. In other exemplary embodiments 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, 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, 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, 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 is Thr or Gly. In other specific embodiments, 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, 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, the linker is selected from the group consisting of a bifunctional thiol crosslinker and a bi-functional amine crosslinker. In certain embodiments, the linker is PEG, e.g., a 5 kDa PEG, 20 kDa PEG. In some embodiments, 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. In some aspects of the invention, 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, the monomers are not connected via an N-terminal amino acid. In some aspects, 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.

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, 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, 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 accordance with one embodiment 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, 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, 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 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 to prepare pre-formulated solutions ready for injection. In other embodiments the pharmaceutical compositions comprise a lyophilized powder. 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 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 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, the aqueous composition is pre-packaged in a device that is used to administer the composition to the patient. In another embodiment 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 yet another embodiment 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. 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 further embodiments, methods of treating hyperglycemia or diabetes involving co-administering insulin and a glucagon peptide of the invention 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 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 accordance with one embodiment 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 the glucagon peptide and the insulin are co-administered as a single composition.

All therapeutic methods, pharmaceutical compositions, kits and other similar embodiments 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 the glucagon peptide is pegylated. In one embodiment 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 contemplates that glucagon analogs described herein may optionally be joined to this 8 amino acid carboxy terminal extension (SEQ ID NO: 21), the invention in some embodiments 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, and additional embodiments 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 includes any one or all embodiments of the invention that are narrower in scope in any way than the variations defined by specific paragraphs herein. For example, where certain aspects of the invention are described as a genus, it should be understood that every member of a genus is, individually, an embodiment of the invention, and that combinations of two or more members of the genus are embodiments 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 ■.

Fig. 10 represents data showing the change in serum glucose concentrations in beagle dogs after intramuscular administration of glucagon and glucagon analogs. The animals were administered a 0.005 mg/kg dose of either glucagon, a glucagon analog comprising glucagon with the sequence of SEQ ID NO: 31 linked to the carboxy terminus of glucagon (glucagon-CEX) or a glucagon analog comprising an aspartic acid substitution at amino acid 28 (glucagon-Asp28) SEQ ID NO: 11.

Figs. 11A and 11B respectively represent data showing glucagon receptor mediated cAMP induction, and GLP-1 receptor mediated cAMP induction, by a glucagon analog having multiple substitutions: T16,A20,E21,A24,Nle27,D28,E29.

Fig. 12 is a graph of the area under the curve of the UV absorbtion at 280 nm of the formulation comprising the peptide of SEQ ID NO: 71 as a function of time (months).

Fig. 13 is a graph of the area under the curve of the UV absorbtion at 280 nm of the formulation comprising the peptide of SEQ ID NO: 76 as a function of time (months).

Fig. 14 is a graph of the area under the curve of the UV absorbtion at 280 nm of the formulation comprising the peptide of SEQ ID NO: 78 as a function of time (months).

Figure 15 represents a graph of the total change in body weight (%) of mice injected with vehicle control, Liraglutide, (C16) Glucagon Amide, γE-γE-C16 Glucagon Amide, AA-C16 Glucagon Amide, or βAβA-C16 Glucagon Amide at the indicated dose.

Figure 16 represents a graph of the fat mass (g) as measured on Day 7 of the study of mice injected with vehicle control, Liraglutide, (C16) Glucagon Amide, γE-γE-C16 Glucagon Amide, AA-C16 Glucagon Amide, or βAβA-C16 Glucagon Amide at the indicated dose.

Figure 17 represents a graph of the change in blood glucose (mg/dL) of mice injected with vehicle control, Liraglutide, (C16) Glucagon Amide, γE-γE -C16 Glucagon Amide, AA-C16 Glucagon Amide, or βAβA-C16 Glucagon Amide at the indicated dose.

DETAILED DESCRIPTION

DEFINITIONS

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

As used herein the term "pharmaceutically acceptable salt" refers to salts of compounds that retain the biological activity of the parent compound, and which are not biologically or otherwise undesirable. Many of the compounds disclosed herein are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. Salts derived from inorganic bases, include by way of example only, sodium, potassium, lithium, ammonium, calcium and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary and tertiary amines.

Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like. Salts derived from organic acids include acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluene-sulfonic acid, salicylic acid, and the like.

As used herein, the term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. For example, as used herein the term "treating diabetes" will refer in general to altering glucose blood levels in the direction of normal levels and may include increasing or decreasing blood glucose levels depending on a given situation.

As used herein an "effective" amount or a "therapeutically effective amount" of a glucagon peptide refers to a nontoxic but sufficient amount of the peptide to provide the desired effect. For example one desired effect would be the prevention or treatment of hypoglycemia, as measured, for example, by an increase in blood glucose level. The amount that is "effective" will vary from subject to subject, depending on the age and general condition of the individual, mode of administration, and the like. Thus, it is not always possible to specify an exact "effective amount." However, an appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

The term, "parenteral" means not through the alimentary canal but by some other route such as subcutaneous, intramuscular, intraspinal, or intravenous.

As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free of contaminants normally associated with the molecule or compound in a native or natural environment. As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. The term "purified polypeptide" is used herein to describe a polypeptide which has been separated from other compounds including, but not limited to nucleic acid molecules, lipids and carbohydrates.

The term "isolated" requires that the referenced material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide present in a living animal is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term “native glucagon” refers to a peptide consisting of the sequence of SEQ ID NO: 1, and the term “native GLP-1” is a generic term that designates GLP-1(7-36)amide (consisting of the sequence of SEQ ID NO: 57), GLP-1(7-37)acid (consisting of the sequence of SEQ ID NO: 58) or a mixture of those two compounds. As used herein, a general reference to “glucagon” or “GLP-1” in the absence of any further designation is intended to mean native glucagon or native GLP-1, respectively.

A "glucagon peptide" as used herein includes any peptide comprising, either the amino acid sequence of SEQ ID NO: 1, or any analog of the amino acid sequence of SEQ ID NO: 1, including amino acid substitutions, additions, or deletions, or post

translational modifications (e.g. methylation, acylation, ubiquitination and the like) of the peptide, wherein the analog stimulates glucagon or GLP-1 receptor activity, e.g., as measured by cAMP production using the assay described in Example 13.

The term "glucagon agonist" refers to a complex comprising a glucagon peptide that stimulates glucagon receptor activity, e.g., as measured by cAMP production using the assay described in Example 13.

As used herein a "glucagon agonist analog" is a glucagon peptide comprising a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13 or analog of such a sequence that has been modified to include one or more conservative amino acid substitutions at positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29.

As used herein an amino acid “modification” refers to a substitution, addition or deletion of an amino acid, and includes substitution with or addition of any of the 20 amino acids commonly found in human proteins, as well as atypical or non-naturally occurring amino acids. Throughout the application, all references to a particular amino acid position by number (e.g. position 28) refer to the amino acid at that position in native glucagon (SEQ ID NO:1) or the corresponding amino acid position in any analogs thereof. For example, a reference herein to “position 28” would mean the corresponding position 27 for a glucagon analog in which the first amino acid of SEQ ID NO: 1 has been deleted. Similarly, a reference herein to “position 28” would mean the corresponding position 29 for a glucagon analog in which one amino acid has been added before the N-terminus of SEQ ID NO: 1.

As used herein an amino acid "substitution" refers to the replacement of one amino acid residue by a different amino acid residue.

As used herein, the term "conservative amino acid substitution" is defined herein as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:
Ala, Ser, Thr, Pro, Gly;

II. Polar, negatively charged residues and their amides:
Asp, Asn, Glu, Gln, cysteic acid and homocysteic acid;

III. Polar, positively charged residues:

His, Arg, Lys; Ornithine (Orn)

IV. Large, aliphatic, nonpolar residues:

Met, Leu, Ile, Val, Cys, Norleucine (Nle), homocysteine

V. Large, aromatic residues:

Phe, Tyr, Trp, acetyl phenylalanine

As used herein the general term "polyethylene glycol" or "PEG", refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH_2CH_2)_nOH$, wherein n is at least 9. Absent any further characterization, the term is intended to include polymers of ethylene glycol with an average total molecular weight selected from the range of 500 to 40,000 Daltons. "polyethylene glycol" or "PEG" is used in combination with a numeric suffix to indicate the approximate average molecular weight thereof. For example, PEG-5,000 refers to polyethylene glycol having a total molecular weight average of about 5,000.

As used herein the term "pegylated" and like terms refers to a compound that has been modified from its native state by linking a polyethylene glycol polymer to the compound. A "pegylated glucagon peptide" is a glucagon peptide that has a PEG chain covalently bound to the glucagon peptide.

As used herein a general reference to a peptide is intended to encompass peptides that have modified amino and carboxy termini. For example, an amino acid chain comprising an amide group in place of the terminal carboxylic acid is intended to be encompassed by an amino acid sequence designating the standard amino acids.

As used herein a "linker" is a bond, molecule or group of molecules that binds two separate entities to one another. Linkers may provide for optimal spacing of the two entities or may further supply a labile linkage that allows the two entities to be separated from each other. Labile linkages include photocleavable groups, acid-labile moieties, base-labile moieties and enzyme-cleavable groups.

As used herein a "dimer" is a complex comprising two subunits covalently bound to one another via a linker. The term dimer, when used absent any qualifying language, encompasses both homodimers and heterodimers. A homodimer comprises

two identical subunits, whereas a heterodimer comprises two subunits that differ, although the two subunits are substantially similar to one another.

As used herein the term "pH stabilized glucagon peptide" refers to a glucagon agonist analog that exhibits superior stability and solubility, relative to native glucagon, in aqueous buffers in the broadest pH range used for pharmacological purposes.

As used herein the term "charged amino acid" refers to an amino acid that comprises a side chain that is negatively charged (i.e., de-protonated) or positively charged (i.e., protonated) in aqueous solution at physiological pH. For example negatively charged amino acids include aspartic acid, glutamic acid, cysteic acid, homocysteic acid, and homoglutamic acid, whereas positively charged amino acids include arginine, lysine and histidine. Charged amino acids include the charged amino acids among the 20 amino acids commonly found in human proteins, as well as atypical or non-naturally occurring amino acids.

Non-naturally occurring amino acids refer to amino acids that do not naturally occur in vivo but which, nevertheless, can be incorporated into the peptide structures described herein. Commercial sources of atypical amino acids include Sigma-Aldrich (Milwaukee, WI), ChemPep Inc. (Miami, FL), and Genzyme Pharmaceuticals (Cambridge, MA). Atypical amino acids may be purchased from commercial suppliers, synthesized de novo, or chemically modified or derivatized from other amino acids.

As used herein the term "acidic amino acid" refers to an amino acid that comprises a second acidic moiety, including for example, a carboxylic acid or sulfonic acid group.

The term “alkyl” refers to a linear or branched hydrocarbon containing the indicated number of carbon atoms. Exemplary alkyls include methyl, ethyl, and linear propyl groups.

The term “heteroalkyl” refers to a linear or branched hydrocarbon containing the indicated number of carbon atoms and at least one heteroatom in the backbone of the structure. Suitable heteroatoms for purposes herein include but are not limited to N, S, and O.

EMBODIMENTS

Enhanced solubility

Applicants have discovered that native glucagon can be modified by introducing charge at its carboxy terminus to enhance the solubility of the peptide while retaining the agonist properties of the peptide. The enhanced solubility allows for the preparation and storage of glucagon solutions at near neutral pH. Formulating glucagon solutions at relatively neutral pHs (e.g. pH of about 6.0 to about 8.0) improves the long term stability of the glucagon peptides.

Accordingly, one embodiment of the present invention is directed to a glucagon agonist that has been modified relative to the wild type peptide of His-Ser-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-Asn-Thr (SEQ ID NO: 1) to improve the peptide's solubility in aqueous solutions, particularly at a pH ranging from about 5.5 to about 8.0, while retaining the native peptide's biological activity. In one embodiment charge is added to the peptide by the substitution of native non-charged amino acids with charged amino acids selected from the group consisting of lysine, arginine, histidine, aspartic acid and glutamic acid, or by the addition of charged amino acids to the amino or carboxy terminus of the peptide. Surprisingly, applicants have discovered that substituting the normally occurring amino acid at position 28 and/or 29 with charged amino acids, and/or the addition of one to two charged amino acids at the carboxy terminus of the glucagon peptide, enhances the solubility and stability of the glucagon peptides in aqueous solutions at physiologically relevant pHs (i.e., a pH of about 6.5 to about 7.5) by at least 5-fold and by as much as 30-fold.

Accordingly, glucagon peptides of one embodiment of the invention retain glucagon activity and exhibit at least 2-fold, 5-fold, 10-fold, 15-fold, 25-fold, 30-fold or greater solubility relative to native glucagon at a given pH between about 5.5 and 8, e.g., pH 7, when measured after 24 hours at 25 °C. Any of the glucagon peptides disclosed herein may additionally exhibit improved stability at a pH within the range of 5.5 to 8, for example, retaining at least 75%, 80%, 90%, 95%, 96%, 97%, 98% or 99% of the original peptide after 24 hours at 25 °C. In some embodiments, the glucagon peptides of the invention 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 about 1 or more weeks (e.g., about 2 weeks, about 4 weeks, about 1 month, about two months, about four months, about six months, about eight months, about ten months, about 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. The glucagon peptides may include additional modifications that alter its pharmaceutical properties, e.g. increased potency, prolonged half-life in circulation, increased shelf-life, reduced precipitation or aggregation, and/or reduced degradation, e.g., reduced occurrence of cleavage or chemical modification after storage.

In one embodiment a glucagon peptide with improved solubility may be prepared, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, and in one embodiment at a position C-terminal to position 27. Such a charged amino acid can be introduced, for example by substituting a native amino acid with a charged amino acid, e.g. at positions 28 or 29, or alternatively by adding a charged amino acid, e.g. after position 27, 28 or 29. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In other embodiments, one, two, three or all of the charged amino acids are positively charged. In specific exemplary embodiments, the glucagon peptide may comprise any one or two of the following modifications: substitution of N28 with E; substitution of N28 with D; substitution of T29 with D; substitution of T29 with E; insertion of E after position 27, 28 or 29; insertion of D after position 27, 28 or 29. For example, D28E29, E28E29, E29E30, E28E30, D28E30.

Further modifications and combinations

Additional modifications may be made to the glucagon peptide which may further increase solubility and/or stability and/or glucagon activity. The glucagon peptide may alternatively comprise other modifications that do not substantially affect solubility or stability, and that do not substantially decrease glucagon activity. In exemplary embodiments, the glucagon peptide may comprise a total of 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, or up to 10, or up to 11, or up to 12, or up to 13, or up to 14 amino acid modifications relative to the native glucagon sequence. In some embodiments, such glucagon analogs retain at least 22, 23, 24, 25,

26, 27 or 28 of the naturally occurring amino acids at the corresponding positions in native glucagon (e.g. have 1-7, 1-5 or 1-3 modifications relative to naturally occurring glucagon).

In some embodiments 1, 2, 3, 4 or 5 non-conservative substitutions are carried out at any of positions 2, 5, 7, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 27, 28 or 29 and up to 5 further conservative substitutions are carried out at any of these positions. In some embodiments 1, 2, or 3 amino acid modifications are carried out within amino acids at positions 1-16, and 1, 2 or 3 amino acid modifications are carried out within amino acids at positions 17-26.

Exemplary modifications include but are not limited to:

(a) non-conservative substitutions, conservative substitutions, additions or deletions while retaining at least partial glucagon agonist activity, for example, conservative substitutions at one or more of positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29, substitution of Tyr at position 10 with Val or Phe, substitution of Lys at position 12 with Arg, substitution of one or more of these positions with Ala;

(b) deletion of amino acids at positions 29 and/or 28, and optionally position 27, while retaining at least partial glucagon agonist activity;

(c) modification of the aspartic acid at position 15, for example, by substitution with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid, which may reduce degradation; or modification of the serine at position 16, for example, by substitution of threonine, AIB, 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, which likewise may reduce degradation due to cleavage of the Asp15-Ser16 bond;

(d) addition of a hydrophilic moiety such as the water soluble polymer polyethylene glycol, as described herein, e.g. at position 16, 17, 20, 21, 24, 29, 40 or at the C-terminal amino acid, which may increase solubility and/or half-life;

(e) modification of the methionine at position 27, for example, by substitution with leucine or norleucine, to reduce oxidative degradation;

(f) modification of the Gln at position 20 or 24, e.g. by substitution with Ser, Thr, Ala or AIB, to reduce degradation that occurs through deamidation of Gln

(g) modification of Asp at position 21, e.g. by substitution with Glu, to reduce degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate;

(h) modifications at position 1 or 2 as described herein that improve resistance to DPP-IV cleavage, optionally in combination with an intramolecular bridge such as a lactam bridge between positions "i" and "i+4", wherein i is an integer from 12 to 25, e.g., 12, 16, 20, 24;

(i) acylating or alkylating the glucagon peptide as described herein, which may increase the activity at the glucagon and GLP-1 receptors, increase half-life in circulation and/or extending the duration of action and/or delaying the onset of action, optionally combined with addition of a hydrophilic moiety, additionally or alternatively, optionally combined with a modification which selectively reduces activity at the GLP-1 peptide, 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., Abu or Ile; deleting amino acids C-terminal to the amino acid at position 27 (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); or a combination thereof.

(j) C-terminal extensions as described herein;

(k) homodimerization or heterodimerization as described herein; and

combinations of the above.

Exemplary modifications include at least one amino acid modification selected from Group A and one or more amino acid modifications selected from Group B and/or Group C,

wherein Group A is:

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 after position 29 of Glu or Lys;
insertion after position 29 of Gly-Lys or Lys-Lys;
or combinations thereof;

wherein Group B is:
substitution of Asp at position 15 with Glu;
substitution of Ser at position 16 with Thr or AIB;

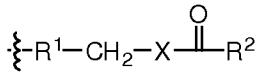
and wherein Group C is:
substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),
substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),
substitution of Lys at position 12 with Arg;
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;
deletion of amino acids at positions 27-29;
deletion of amino acids at positions 28-29;
deletion of the amino acid at positions 29;
or combinations thereof.

Modifications at position 3

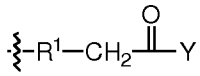
Glucagon receptor activity can be reduced by an amino acid modification at position 3, e.g. substitution of the naturally occurring glutamine at position 3, with an acidic, basic, or a hydrophobic amino acid. For example substitution at position 3 with glutamic acid, ornithine, or norleucine substantially reduces or destroys glucagon receptor activity.

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 (SEQ ID NO: 1) at the glucagon receptor. In some embodiments 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). In some embodiments, 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.

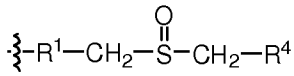
In some embodiments, the glutamine analog is a naturally occurring or a non-naturally occurring amino acid comprising a side chain of Structure I, II or III:



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, X is NH or Y is NHR^4. In some embodiments, R^1 is C_{0-2} alkyl or C_1 heteroalkyl. In some embodiments, R^2 is NHR^4 or C_1 alkyl. In some embodiments, R^4 is H or C^1 alkyl. In exemplary embodiments, 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, 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, an amino acid comprising a side chain of Structure III is provided where, R^1 is CH_2 and R^4 is H (methionine-sulfoxide, M(O)); In specific embodiments, 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 GLP-1 receptor is provided by replacing the carboxylic acid of the C-terminal amino acid with a charge-neutral group, such as an amide or ester. Conversely, retaining the native carboxylic acid at the C-terminus of the peptide maintains the relatively greater selectivity of the glucagon peptide for glucagon receptor vs. GLP-1 receptor (e.g., greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold).

DPP-IV Resistance

In some embodiments the glucagon peptides disclosed herein are further modified at position 1 or 2 to reduce susceptibility to cleavage by dipeptidyl peptidase IV. More particularly, in some embodiments, position 1 of the analog peptide is substituted with an amino acid selected from the group consisting of D-histidine, alpha, alpha-dimethyl imidiazole acetic acid (DMIA), N-methyl histidine, alpha-methyl histidine, imidazole acetic acid, desaminohistidine, hydroxyl-histidine, acetyl-histidine and homo-histidine.

More particularly, in some embodiments, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, D-alanine, valine, amino N-butyric acid, glycine, N-methyl serine and aminoisobutyric acid. In one embodiment position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, D-alanine, glycine, N-methyl serine and aminoisobutyric acid. In another embodiment position 2 of the analog

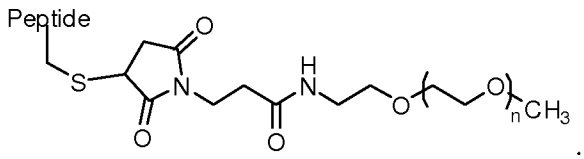
peptide is substituted with an amino acid selected from the group consisting of D-serine, glycine, and aminoisobutyric acid.

Where substitutions or modifications or derivatization at positions 1 or 2 reduce activity at the glucagon receptor, an intramolecular bridge in the C-terminal portion (amino acids 12-29) of the peptide (e.g., a lactam bridge between side chains of amino acids at positions "i" and "i+4", wherein i is an integer from 12 to 25), can improve glucagon activity at the glucagon receptor.

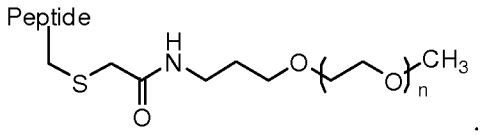
Addition of hydrophilic moieties

Hydrophilic moieties such as PEG groups can be attached to the glucagon peptides under any suitable conditions used to react a protein with an activated polymer molecule. Any means known in the art can be used, including via acylation, reductive alkylation, Michael addition, thiol alkylation or other chemoselective conjugation/ligation methods through a reactive group on the PEG moiety (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group) to a reactive group on the target compound (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group). Activating groups which can be used to link the water soluble polymer to one or more proteins include without limitation sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane, 5-pyridyl. and alpha-halogenated acyl group (e.g., alpha-iodo acetic acid, alpha-bromoacetic acid, alpha-chloroacetic acid). If attached to the peptide by reductive alkylation, the polymer selected should have a single reactive aldehyde so that the degree of polymerization is controlled. See, for example, Kinstler et al., *Adv. Drug. Delivery Rev.* 54: 477-485 (2002); Roberts et al., *Adv. Drug Delivery Rev.* 54: 459-476 (2002); and Zalipsky et al., *Adv. Drug Delivery Rev.* 16: 157-182 (1995).

In a specific aspect of the invention, an amino acid residue on the glucagon peptide having a thiol is modified with a hydrophilic moiety such as PEG. In some embodiments, the thiol is modified with maleimide-activated PEG in a Michael addition reaction to result in a PEGylated peptide comprising the thioether linkage shown below:



In some embodiments, the thiol is modified with a haloacetyl-activated PEG in a nucleophilic substitution reaction to result in a PEGylated peptide comprising the thioether linkage shown below:



Suitable hydrophilic moieties include polyethylene glycol (PEG), polypropylene glycol, polyoxyethylated polyols (e.g., POG), polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), polyoxyalkylenes, polyethylene glycol propionaldehyde, copolymers of ethylene glycol/propylene glycol, monomethoxy-polyethylene glycol, mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol, carboxymethylcellulose, polyacetals, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, poly (β-amino acids) (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers (PPG) and other polyakylene oxides, polypropylene oxide/ethylene oxide copolymers, colonic acids or other polysaccharide polymers, Ficoll or dextran and mixtures thereof.

The polyethylene glycol chain in accordance with some embodiments has a molecular weight selected from the range of about 500 to about 40,000 Daltons. In one embodiment the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons, or about 1,000 to about 5,000 Daltons. In another embodiment the polyethylene glycol chain has a molecular weight of about 10,000 to about 20,000 Daltons. In yet other exemplary embodiments the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

Dextrans are polysaccharide polymers of glucose subunits, predominantly linked by α1-6 linkages. Dextran is available in many molecular weight ranges, e.g., about 1 kD to about 100 kD, or from about 5, 10, 15 or 20 kD to about 20, 30, 40, 50, 60, 70, 80 or 90 kD.

Linear or branched polymers are contemplated. Resulting preparations of conjugates may be essentially monodisperse or polydisperse, and may have about 0.5, 0.7, 1, 1.2, 1.5 or 2 polymer moieties per peptide.

Acylation

In accordance with one embodiment, the glucagon peptide is modified to comprise an acyl group, e.g., an acyl group which is not naturally-occurring on an amino acid (e.g., an acyl group which is non-native to a naturally-occurring amino acid). The addition of an acyl group causes the peptide to have one or more of a prolonged half-life in circulation, a delayed onset of action, an extended duration of action, an improved resistance to proteases, such as DPP-IV, and increased potency at the GLP-1 and glucagon receptors. As shown herein, acylation of the glucagon peptide does not lead to decreased activity at the glucagon and GLP-1 receptors. Rather, in some instances, acylation actually increases the activity at the GLP-1 and glucagon receptors. Accordingly, the potency of the acylated analogs is comparable to the unacylated versions of the glucagon co-agonist analogs, if not enhanced.

In accordance with one embodiment, the glucagon peptide is modified to comprise an acyl group which is attached to the glucagon peptide via an ester, thioester, or amide linkage for purposes of prolonging half-life in circulation and/or delaying the onset of and/or extending the duration of action and/or improving resistance to proteases such as DPP-IV.

Acylation can be carried out at any position within the glucagon peptide, including any of positions 1-29, a position within a C-terminal extension, or the C-terminal amino acid, provided that glucagon and/or GLP-1 activity is retained, if not enhanced. Nonlimiting examples include positions 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28, or 29. In specific embodiments, acylation occurs at position 10 of the glucagon peptide and the glucagon peptide lacks an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam bridge). Such acylated peptides lacking an intramolecular bridge demonstrate enhanced activity at the GLP-1 and glucagon receptors as compared to the corresponding non-acylated peptides lacking a covalent intramolecular bridge and in comparison to the corresponding peptides lacking an intramolecular bridge acylated at a position other than position 10. As shown herein, acylation at position 10 can even transform a glucagon analog having little activity at the glucagon receptor to a glucagon analog having activity at both the

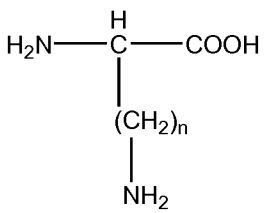
glucagon and GLP-1 receptors. Accordingly, the position at which acylation occurs can alter the overall activity profile of the glucagon analog.

Glucagon peptides may be acylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position. Nonlimiting examples include acylation at position 10 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28 or 29, within a C-terminal extension, or at the C-terminus (e.g., through adding a C-terminal Cys).

The acyl group can be covalently linked directly to an amino acid of the glucagon peptide, or indirectly to an amino acid of the glucagon peptide via a spacer, wherein the spacer is positioned between the amino acid of the glucagon peptide and the acyl group.

In a specific aspect of the invention, the glucagon peptide is modified to comprise an acyl group by direct acylation of an amine, hydroxyl, or thiol of a side chain of an amino acid of the glucagon peptide. In some embodiments, the glucagon peptide is directly acylated through the side chain amine, hydroxyl, or thiol of an amino acid. In some embodiments, acylation is at position 10, 20, 24, or 29. In this regard, the acylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO : 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or thiol. In some specific embodiments of the invention, the direct acylation of the glucagon peptide occurs through the side chain amine, hydroxyl, or thiol of the amino acid at position 10.

In some embodiments, the amino acid comprising a side chain amine is an amino acid of Formula I:

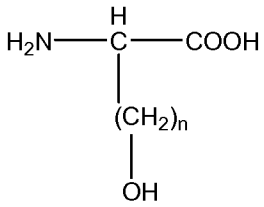


wherein n = 1 to 4

[Formula I]

In some exemplary embodiments, the amino acid of Formula I, is the amino acid wherein n is 4 (Lys) or n is 3 (Orn).

In other embodiments, the amino acid comprising a side chain hydroxyl is an amino acid of Formula II:

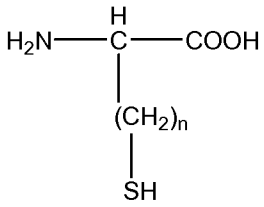


wherein n = 1 to 4

[Formula II]

In some exemplary embodiments, the amino acid of Formula II is the amino acid wherein n is 1 (Ser).

In yet other embodiments, the amino acid comprising a side chain thiol is an amino acid of Formula III:



wherein n = 1 to 4

[Formula III]

In some exemplary embodiments, the amino acid of Formula III is the amino acid wherein n is 1 (Cys).

In yet other embodiments, the amino acid comprising a side chain amine, hydroxyl, or thiol is a disubstituted amino acid comprising the same structure of Formula I, Formula II, or Formula III, except that the hydrogen bonded to the alpha carbon of the amino acid of Formula I, Formula II, or Formula III is replaced with a second side chain.

In one embodiment of the invention, the acylated glucagon peptide comprises a spacer between the peptide and the acyl group. In some embodiments, the glucagon peptide is covalently bound to the spacer, which is covalently bound to the acyl group.

The amino acid to which the spacer is attached can be any amino acid (e.g., a singly α-substituted amino acid or an α,α-disubstituted amino acid) comprising a

moiety which permits linkage to the spacer. For example, an amino acid comprising a side chain NH_2, –OH, or –COOH (e.g., Lys, Orn, Ser, Asp, or Glu) is suitable. In this respect, the acylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO: 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or carboxylate.

In some embodiments, the spacer is an amino acid comprising a side chain amine, hydroxyl, or thiol, or a dipeptide or tripeptide comprising an amino acid comprising a side chain amine, hydroxyl, or thiol.

When acylation occurs through an amine group of a spacer, the acylation can occur through the alpha amine of the amino acid or a side chain amine. In the instance in which the alpha amine is acylated, the amino acid of the spacer can be any amino acid. For example, the amino acid of the spacer can be a hydrophobic amino acid, e.g., Gly, Ala, Val, Leu, Ile, Trp, Met, Phe, Tyr, 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, 8-aminooctanoic acid. Alternatively, the amino acid of the spacer can be an acidic residue, e.g., Asp and Glu.

In the instance in which the side chain amine of the amino acid of the spacer is acylated, the amino acid of the spacer is an amino acid comprising a side chain amine, e.g., an amino acid of Formula I (e.g., Lys or Orn). In this instance, it is possible for both the alpha amine and the side chain amine of the amino acid of the spacer to be acylated, such that the glucagon peptide is diacylated. Embodiments of the invention include such diacylated molecules.

When acylation occurs through a hydroxyl group of a spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula II. In a specific exemplary embodiment, the amino acid is Ser.

When acylation occurs through a thiol group of a spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula III. In a specific exemplary embodiment, the amino acid is Cys.

In some embodiments, the spacer is a hydrophilic bifunctional spacer. In certain embodiments, the hydrophilic bifunctional spacer comprises two or more reactive groups, e.g., an amine, a hydroxyl, a thiol, and a carboxyl group or any combinations thereof. In certain embodiments, the hydrophilic bifunctional spacer

comprises a hydroxyl group and a carboxylate. In other embodiments, the hydrophilic bifunctional spacer comprises an amine group and a carboxylate. In other embodiments, the hydrophilic bifunctional spacer comprises a thiol group and a carboxylate. In specific embodiments, the spacer comprises an amino poly(alkyloxy)carboxylate. In this regard, the spacer can comprise, for example, $NH_2(CH_2CH_2O)_n(CH_2)_mCOOH$, wherein m is any integer from 1 to 6 and n is any integer from 2 to 12, such as, e.g., 8-amino-3,6-dioxaoctanoic acid, which is commercially available from Peptides International, Inc. (Louisville, KY).

In some embodiments, the spacer is a hydrophobic bifunctional spacer. Hydrophobic bifunctional spacers are known in the art. See, e.g., *Bioconjugate Techniques*, G. T. Hermanson (Academic Press, San Diego, CA, 1996), which is incorporated by reference in its entirety. In certain embodiments, the hydrophobic bifunctional spacer comprises two or more reactive groups, e.g., an amine, a hydroxyl, a thiol, and a carboxyl group or any combinations thereof. In certain embodiments, the hydrophobic bifunctional spacer comprises a hydroxyl group and a carboxylate. In other embodiments, the hydrophobic bifunctional spacer comprises an amine group and a carboxylate. In other embodiments, the hydrophobic bifunctional spacer comprises a thiol group and a carboxylate. Suitable hydrophobic bifunctional spacers comprising a carboxylate, and a hydroxyl group or a thiol group are known in the art and include, for example, 8-hydroxyoctanoic acid and 8-mercaptooctanoic acid.

In some embodiments, the bifunctional spacer is not a dicarboxylic acid comprising an unbranched, methylene of 1-7 carbon atoms between the carboxylate groups. In some embodiments, the bifunctional spacer is a dicarboxylic acid comprising an unbranched, methylene of 1-7 carbon atoms between the carboxylate groups.

The spacer (e.g., amino acid, dipeptide, tripeptide, hydrophilic or hydrophobic bifunctional spacer) in specific embodiments is 3 to 10 atoms (e.g., 6 to 10 atoms, (e.g., 6, 7, 8, 9, or 10 atoms) in length. In more specific embodiments, the spacer is about 3 to 10 atoms (e.g., 6 to 10 atoms) in length and the acyl group is a C12 to C18 fatty acyl group, e.g., C14 fatty acyl group, C16 fatty acyl group, such that the total length of the spacer and acyl group is 14 to 28 atoms, e.g., about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 atoms. In some embodiments, the length of the spacer and acyl group is 17 to 28 (e.g., 19 to 26, 19 to 21) atoms.

In accordance with certain foregoing embodiments, the bifunctional spacer can be a synthetic or naturally occurring amino acid (including, but not limited to, any of those described herein) comprising an amino acid backbone that is 3 to 10 atoms in length (e.g., 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, and 8-aminooctanoic acid). Alternatively, the spacer can be a dipeptide or tripeptide spacer having a peptide backbone that is 3 to 10 atoms (e.g., 6 to 10 atoms) in length. Each amino acid of the dipeptide or tripeptide spacer can be the same as or different from the other amino acid(s) of the dipeptide or tripeptide and can be independently selected from the group consisting of: naturally-occurring and/or non-naturally occurring amino acids, including, for example, any of the D or L isomers of the naturally-occurring amino acids (Ala, Cys, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Arg, Ser, Thr, Val, Trp, Tyr), or any D or L isomers of the non-naturally occurring amino acids selected from the group consisting of: β-alanine (β-Ala), N-α-methyl-alanine (Me-Ala), aminobutyric acid (Abu), γ-aminobutyric acid (γ-Abu), aminohexanoic acid (ε-Ahx), aminoisobutyric acid (Aib), aminomethylpyrrole carboxylic acid, aminopiperidinecarboxylic acid, aminoserine (Ams), aminotetrahydropyran-4-carboxylic acid, arginine N-methoxy-N-methyl amide, β-aspartic acid (β-Asp), azetidine carboxylic acid, 3-(2-benzothiazolyl)alanine, α-*tert*-butylglycine, 2-amino-5-ureido-n-valeric acid (citrulline, Cit), β-Cyclohexylalanine (Cha), acetamidomethyl-cysteine, diaminobutanoic acid (Dab), diaminopropionic acid (Dpr), dihydroxyphenylalanine (DOPA), dimethylthiazolidine (DMTA), γ-Glutamic acid (γ-Glu), homoserine (Hse), hydroxyproline (Hyp), isoleucine N-methoxy-N-methyl amide, methyl-isoleucine (MeIle), isonipecotic acid (Isn), methyl-leucine (MeLeu), methyl-lysine, dimethyl-lysine, trimethyl-lysine, methanoproline, methionine-sulfoxide (Met(O)), methionine-sulfone (Met(O_2)), norleucine (Nle), methyl-norleucine (Me-Nle), norvaline (Nva), ornithine (Orn), para-aminobenzoic acid (PABA), penicillamine (Pen), methylphenylalanine (MePhe), 4-Chlorophenylalanine (Phe(4-Cl)), 4-fluorophenylalanine (Phe(4-F)), 4-nitrophenylalanine (Phe(4-NO_2)), 4-cyanophenylalanine ((Phe(4-CN)), phenylglycine (Phg), piperidinylalanine, piperidinylglycine, 3,4-dehydroproline, pyrrolidinylalanine, sarcosine (Sar), selenocysteine (Sec), O-Benzyl-phosphoserine, 4-amino-3-hydroxy-6-methylheptanoic acid (Sta), 4-amino-5-cyclohexyl-3-hydroxypentanoic acid

(ACHPA), 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA), 1,2,3,4,-tetrahydro-isoquinoline-3-carboxylic acid (Tic), tetrahydropyranglycine, thienylalanine (Thi), O-benzyl-phosphotyrosine, O-Phosphotyrosine, methoxytyrosine, ethoxytyrosine, O-(bis-dimethylamino-phosphono)-tyrosine, tyrosine sulfate tetrabutylamine, methyl-valine (MeVal), and alkylated 3-mercaptopropionic acid.

In some embodiments, the spacer comprises an overall negative charge, e.g., comprises one or two negatively charged amino acids. In some embodiments, the dipeptide is not any of the dipeptides of general structure A-B, wherein A is selected from the group consisting of Gly, Gln, Ala, Arg, Asp, Asn, Ile, Leu, Val, Phe, and Pro, wherein B is selected from the group consisting of Lys, His, Trp. In some embodiments, the dipeptide spacer is selected from the group consisting of: Ala-Ala, β-Ala- β-Ala, Leu-Leu, Pro-Pro, γ-aminobutyric acid- γ-aminobutyric acid, and γ-Glu- γ-Glu.

In some exemplary embodiments, the glucagon peptide is modified to comprise an acyl group by acylation of an amine, hydroxyl, or thiol of a spacer, which spacer is attached to a side chain of an amino acid at position 10, 20, 24, or 29, or at the C-terminal amino acid of the glucagon peptide.

In yet more specific embodiments, the acyl group is attached to the amino acid at position 10 of the glucagon peptide and the length of the spacer and acyl group is 14 to 28 atoms. The amino acid at position 10, in some aspects, is an amino acid of Formula I, e.g., Lys, or a disubstituted amino acid related to Formula I. In more specific embodiments, the glucagon peptide lacks an intramolecular bridge, e.g., a covalent intramolecular bridge. The glucagon peptide, for example, can be a peptide comprising one or more alpha, alpha-disubstituted amino acids, e.g., AIB, for stabilizing the alpha helix of the peptide. As shown herein, such peptides comprising an acylated spacer covalently attached to the side chain of the amino acid at position 10 exhibit enhanced potency at both the GLP-1 and glucagon receptors.

Suitable methods of peptide acylation via amines, hydroxyls, and thiols are known in the art. See, for example, Example 19 (for methods of acylating through an amine), Miller, *Biochem Biophys Res Commun* 218: 377-382 (1996); Shimohigashi and Stammer, *Int J Pept Protein Res* 19: 54-62 (1982); and Previero et al., *Biochim Biophys Acta* 263: 7-13 (1972) (for methods of acylating through a hydroxyl); and San and Silvius, *J Pept Res* 66: 169-180 (2005) (for methods of acylating through a

thiol); *Bioconjugate Chem.* "Chemical Modifications of Proteins: History and Applications" pages 1, 2-12 (1990); Hashimoto et al., *Pharmacuetical Res.* "Synthesis of Palmitoyl Derivatives of Insulin and their Biological Activity" Vol. 6, No: 2 pp.171-176 (1989).

The acyl group of the acylated glucagon peptide can be of any size, e.g., any length carbon chain, and can be linear or branched. In some specific embodiments of the invention, the acyl group is a C4 to C30 fatty acid. For example, the acyl group can be any of a C4 fatty acid, C6 fatty acid, C8 fatty acid, C10 fatty acid, C12 fatty acid, C14 fatty acid, C16 fatty acid, C18 fatty acid, C20 fatty acid, C22 fatty acid, C24 fatty acid, C26 fatty acid, C28 fatty acid, or a C30 fatty acid. In some embodiments, the acyl group is a C8 to C20 fatty acid, e.g., a C14 fatty acid or a C16 fatty acid.

In an alternative embodiment, the acyl group is a bile acid. The bile acid can be any suitable bile acid, including, but not limited to, cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurocholic acid, glycocholic acid, and cholesterol acid.

In some embodiments of the invention, the glucagon peptide is modified to comprise an acyl group by acylation of a long chain alkane by the glucagon peptide. In specific aspects, the long chain alkane comprises an amine, hydroxyl, or thiol group (e.g. octadecylamine, tetradecanol, and hexadecanethiol) which reacts with a carboxyl group, or activated form thereof, of the glucagon peptide. The carboxyl group, or activated form thereof, of the glucagon peptide can be part of a side chain of an amino acid (e.g., glutamic acid, aspartic acid) of the glucagon peptide or can be part of the peptide backbone.

In certain embodiments, the glucagon peptide is modified to comprise an acyl group by acylation of the long chain alkane by a spacer which is attached to the glucagon peptide. In specific aspects, the long chain alkane comprises an amine, hydroxyl, or thiol group which reacts with a carboxyl group, or activated form thereof, of the spacer. Suitable spacers comprising a carboxyl group, or activated form thereof, are described herein and include, for example, amino acids, dipeptides, tripeptides, hydrophilic bifunctional spacers and hydrophobic bifunctional spacers.

As used herein, the term “activated form of a carboxyl group” refers to a carboxyl group with the general formula R(C=O)X, wherein X is a leaving group and

R is the glucagon peptide or the spacer. For example, activated forms of a carboxyl groups may include, but are not limited to, acyl chlorides, anhydrides, and esters. In some embodiments, the activated carboxyl group is an ester with a N-hydroxysuccinimide ester (NHS) leaving group.

With regard to these aspects of the invention, in which a long chain alkane is acylated by the glucagon peptide or the spacer, the long chain alkane may be of any size and can comprise any length of carbon chain. The long chain alkane can be linear or branched. In certain aspects, the long chain alkane is a C4 to C30 alkane. For example, the long chain alkane can be any of a C4 alkane, C6 alkane, C8 alkane, C10 alkane, C12 alkane, C14 alkane, C16 alkane, C18 alkane, C20 alkane, C22 alkane, C24 alkane, C26 alkane, C28 alkane, or a C30 alkane. In some embodiments, the long chain alkane comprises a C8 to C20 alkane, e.g., a C14 alkane, C16 alkane, or a C18 alkane.

Also, in some embodiments, an amine, hydroxyl, or thiol group of the glucagon peptide is acylated with a cholesterol acid. In a specific embodiment, the glucagon peptide is linked to the cholesterol acid through an alkylated des-amino Cys spacer, i.e., an alkylated 3-mercaptopropionic acid spacer.

The acylated glucagon peptides described herein can be further modified to comprise a hydrophilic moiety. In some specific embodiments the hydrophilic moiety can comprise a polyethylene glycol (PEG) chain. The incorporation of a hydrophilic moiety can be accomplished through any suitable means, such as any of the methods described herein. In this regard, the acylated glucagon peptide can comprise SEQ ID NO: 1, including any of the modifications described herein, in which at least one of the amino acids at position 10, 20, 24, and 29 comprise an acyl group and at least one of the amino acids at position 16, 17, 21, 24, or 29, a position within a C-terminal extension, or the C-terminal amino acid are modified to a Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the side chain of the amino acid is covalently bonded to a hydrophilic moiety (e.g., PEG). In some embodiments, the acyl group is attached to position 10, optionally via a spacer comprising Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the hydrophilic moiety is incorporated at a Cys residue at position 24.

Alternatively, the acylated glucagon peptide can comprise a spacer, wherein the spacer is both acylated and modified to comprise the hydrophilic moiety. Nonlimiting examples of suitable spacers include a spacer comprising one or more

amino acids selected from the group consisting of Cys, Lys, Orn, homo-Cys, and Ac-Phe.

Alkylation

In accordance with some embodiments, the glucagon peptide is modified to comprise an alkyl group, e.g., an alkyl group which is not naturally-occurring on an amino acid (e.g., an alkyl group which is non-native to a naturally-occurring amino acid). Without being held to any particular theory, it is believed that alkylation of glucagon peptides will achieve similar, if not the same, effects as acylation of the glucagon peptides, e.g., a prolonged half-life in circulation, a delayed onset of action, an extended duration of action, an improved resistance to proteases, such as DPP-IV, and increased potency at the GLP-1 and glucagon receptors.

Alkylation can be carried out at any position within the glucagon peptide, including any of positions 1-29, a position within a C-terminal extension, or the C-terminal amino acid, provided that the glucagon activity is retained. Nonlimiting examples include positions 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28, or 29. The alkyl group can be covalently linked directly to an amino acid of the glucagon peptide, or indirectly to an amino acid of the glucagon peptide via a spacer, wherein the spacer is positioned between the amino acid of the glucagon peptide and the alkyl group. Glucagon peptides may be alkylated at the same amino acid position where a hydrophilic moiety is linked, or at a different amino acid position. Nonlimiting examples include alkylation at position 10 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28 or 29, within a C-terminal extension, or at the C-terminus (e.g., through adding a C-terminal Cys).

In a specific aspect of the invention, the glucagon peptide is modified to comprise an alkyl group by direct alkylation of an amine, hydroxyl, or thiol of a side chain of an amino acid of the glucagon peptide. In some embodiments, alkylation is at position 10, 20, 24, or 29. In this regard, the alkylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO : 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or thiol. In some specific

embodiments of the invention, the direct alkylation of the glucagon peptide occurs through the side chain amine, hydroxyl, or thiol of the amino acid at position 10.

In some embodiments, the amino acid comprising a side chain amine is an amino acid of Formula I. In some exemplary embodiments, the amino acid of Formula I, is the amino acid wherein n is 4 (Lys) or n is 3 (Orn).

In other embodiments, the amino acid comprising a side chain hydroxyl is an amino acid of Formula II. In some exemplary embodiments, the amino acid of Formula II is the amino acid wherein n is 1 (Ser).

In yet other embodiments, the amino acid comprising a side chain thiol is an amino acid of Formula III. In some exemplary embodiments, the amino acid of Formula III is the amino acid wherein n is 1 (Cys).

In yet other embodiments, the amino acid comprising a side chain amine, hydroxyl, or thiol is a disubstituted amino acid comprising the same structure of Formula I, Formula II, or Formula III, except that the hydrogen bonded to the alpha carbon of the amino acid of Formula I, Formula II, or Formula III is replaced with a second side chain.

In one embodiment of the invention, the alkylated glucagon peptide comprises a spacer between the peptide and the alkyl group. In some embodiments, the glucagon peptide is covalently bound to the spacer, which is covalently bound to the alkyl group. In some exemplary embodiments, the glucagon peptide is modified to comprise an alkyl group by alkylation of an amine, hydroxyl, or thiol of a spacer, which spacer is attached to a side chain of an amino acid at position 10, 20, 24, or 29 of the glucagon peptide. The amino acid to which the spacer is attached can be any amino acid (e.g., a singly α-substituted amino acid or an α,α-disubstituted amino acid) comprising a moiety which permits linkage to the spacer. For example, an amino acid comprising a side chain NH_2, –OH, or –COOH (e.g., Lys, Orn, Ser, Asp, or Glu) is suitable. In this respect, the alkylated glucagon peptide can comprise the amino acid sequence of SEQ ID NO: 1, or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, with at least one of the amino acids at positions 10, 20, 24, and 29 modified to any amino acid comprising a side chain amine, hydroxyl, or carboxylate.

In some embodiments, the spacer is an amino acid comprising a side chain amine, hydroxyl, or thiol or a dipeptide or tripeptide comprising an amino acid comprising a side chain amine, hydroxyl, or thiol.

When alkylation occurs through an amine group of a spacer, the alkylation can occur through the alpha amine of an amino acid or a side chain amine. In the instance in which the alpha amine is alkylated, the amino acid of the spacer can be any amino acid. For example, the amino acid of the spacer can be a hydrophobic amino acid, e.g., Gly, Ala, Val, Leu, Ile, Trp, Met, Phe, Tyr, 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, 8-aminooctanoic acid. Alternatively, the amino acid of the spacer can be an acidic residue, e.g., Asp and Glu, provided that the alkylation occurs on the alpha amine of the acidic residue. In the instance in which the side chain amine of the amino acid of the spacer is alkylated, the amino acid of the spacer is an amino acid comprising a side chain amine, e.g., an amino acid of Formula I (e.g., Lys or Orn). In this instance, it is possible for both the alpha amine and the side chain amine of the amino acid of the spacer to be alkylated, such that the glucagon peptide is dialkylated. Embodiments of the invention include such dialkylated molecules.

When alkylation occurs through a hydroxyl group of a spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula II. In a specific exemplary embodiment, the amino acid is Ser.

When alkylation occurs through a thiol group of spacer, the amino acid or one of the amino acids of the dipeptide or tripeptide can be an amino acid of Formula III. In a specific exemplary embodiment, the amino acid is Cys.

In some embodiments, the spacer is a hydrophilic bifunctional spacer. In certain embodiments, the hydrophilic bifunctional spacer comprises two or more reactive groups, e.g., an amine, a hydroxyl, a thiol, and a carboxyl group or any combinations thereof. In certain embodiments, the hydrophilic bifunctional spacer comprises a hydroxyl group and a carboxylate. In other embodiments, the hydrophilic bifunctional spacer comprises an amine group and a carboxylate. In other embodiments, the hydrophilic bifunctional spacer comprises a thiol group and a carboxylate. In a specific embodiment, the spacer comprises an amino poly(alkyloxy)carboxylate. In this regard, the spacer can comprise, for example, $NH_2(CH_2CH_2O)_n(CH_2)_mCOOH$, wherein m is any integer from 1 to 6 and n is any

integer from 2 to 12, such as, e.g., 8-amino-3,6-dioxaoctanoic acid, which is commercially available from Peptides International, Inc. (Louisville, KY).

In some embodiments, the spacer is a hydrophobic bifunctional spacer. In certain embodiments, the hydrophobic bifunctional spacer comprises two or more reactive groups, e.g., an amine, a hydroxyl, a thiol, and a carboxyl group or any combinations thereof. In certain embodiments, the hydrophobic bifunctional spacer comprises a hydroxyl group and a carboxylate. In other embodiments, the hydrophobic bifunctional spacer comprises an amine group and a carboxylate. In other embodiments, the hydrophobic bifunctional spacer comprises a thiol group and a carboxylate. Suitable hydrophobic bifunctional spacers comprising a carboxylate, and a hydroxyl group or a thiol group are known in the art and include, for example, 8-hydroxyoctanoic acid and 8-mercaptooctanoic acid.

The spacer (e.g., amino acid, dipeptide, tripeptide, hydrophilic or hydrophobic bifunctional spacer) in specific embodiments is 3 to 10 atoms (e.g., 6 to 10 atoms, (e.g., 6, 7, 8, 9, or 10 atoms)) in length. In more specific embodiments, the spacer is about 3 to 10 atoms (e.g., 6 to 10 atoms) in length and the alkyl is a C12 to C18 alkyl group, e.g., C14 alkyl group, C16 alkyl group, such that the total length of the spacer and alkyl group is 14 to 28 atoms, e.g., about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 atoms. In some embodiments, the length of the spacer and alkyl is 17 to 28 (e.g., 19 to 26, 19 to 21) atoms.

In accordance with certain foregoing embodiments, the bifunctional spacer can be a synthetic or non-naturally occurring amino acid comprising an amino acid backbone that is 3 to 10 atoms in length (e.g., 6-amino hexanoic acid, 5-aminovaleric acid, 7-aminoheptanoic acid, and 8-aminooctanoic acid). Alternatively, the spacer can be a dipeptide or tripeptide spacer having a peptide backbone that is 3 to 10 atoms (e.g., 6 to 10 atoms) in length. The dipeptide or tripeptide spacer can be composed of naturally-occurring and/or non-naturally occurring amino acids, including, for example, any of the amino acids taught herein. In some embodiments, the spacer comprises an overall negative charge, e.g., comprises one or two negatively charged amino acids. In some embodiments, the dipeptide spacer is selected from the group consisting of: Ala-Ala, β-Ala- β-Ala, Leu-Leu, Pro-Pro, γ-aminobutyric acid- γ-aminobutyric acid, and γ-Glu- γ-Glu.

Suitable methods of peptide alkylation via amines, hydroxyls, and thiols are known in the art. For example, a Williamson ether synthesis can be used to form an ether linkage between a hydroxyl group of the glucagon peptide and the alkyl group. Also, a nucleophilic substitution reaction of the peptide with an alkyl halide can result in any of an ether, thioether, or amino linkage.

The alkyl group of the alkylated glucagon peptide can be of any size, e.g., any length carbon chain, and can be linear or branched. In some embodiments of the invention, the alkyl group is a C4 to C30 alkyl. For example, the alkyl group can be any of a C4 alkyl, C6 alkyl, C8 alkyl, C10 alkyl, C12 alkyl, C14 alkyl, C16 alkyl, C18 alkyl, C20 alkyl, C22 alkyl, C24 alkyl, C26 alkyl, C28 alkyl, or a C30 alkyl. In some embodiments, the alkyl group is a C8 to C20 alkyl, e.g., a C14 alkyl or a C16 alkyl.

In some specific embodiments, the alkyl group comprises a steroid moiety of a bile acid, e.g., cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurocholic acid, glycocholic acid, and cholesterol acid.

In some embodiments of the invention, the glucagon peptide is modified to comprise an alkyl group by reacting a nucleophilic, long chain alkane with the glucagon peptide, wherein the glucagon peptide comprises a leaving group suitable for nucleophilic substitution. In specific aspects, the nucleophilic group of the long chain alkane comprises an amine, hydroxyl, or thiol group (e.g. octadecylamine, tetradecanol, and hexadecanethiol). The leaving group of the glucagon peptide can be part of a side chain of an amino acid or can be part of the peptide backbone. Suitable leaving groups include, for example, N-hydroxysuccinimide, halogens, and sulfonate esters.

In certain embodiments, the glucagon peptide is modified to comprise an alkyl group by reacting the nucleophilic, long chain alkane with a spacer which is attached to the glucagon peptide, wherein the spacer comprises the leaving group. In specific aspects, the long chain alkane comprises an amine, hydroxyl, or thiol group. In certain embodiments, the spacer comprising the leaving group can be any spacer discussed herein, e.g., amino acids, dipeptides, tripeptides, hydrophilic bifunctional spacers and hydrophobic bifunctional spacers further comprising a suitable leaving group.

With regard to these aspects of the invention, in which a long chain alkane is alkylated by the glucagon peptide or the spacer, the long chain alkane may be of any

size and can comprise any length of carbon chain. The long chain alkane can be linear or branched. In certain aspects, the long chain alkane is a C4 to C30 alkane. For example, the long chain alkane can be any of a C4 alkane, C6 alkane, C8 alkane, C10 alkane, C12 alkane, C14 alkane, C16 alkane, C18 alkane, C20 alkane, C22 alkane, C24 alkane, C26 alkane, C28 alkane, or a C30 alkane. In some embodiments, the long chain alkane comprises a C8 to C20 alkane, e.g., a C14 alkane, C16 alkane, or a C18 alkane.

Also, in some embodiments, alkylation can occur between the glucagon peptide and a cholesterol moiety. For example, the hydroxyl group of cholesterol can displace a leaving group on the long chain alkane to form a cholesterol-glucagon peptide product.

The alkylated glucagon peptides described herein can be further modified to comprise a hydrophilic moiety. In some specific embodiments the hydrophilic moiety can comprise a polyethylene glycol (PEG) chain. The incorporation of a hydrophilic moiety can be accomplished through any suitable means, such as any of the methods described herein. In this regard, the alkylated glucagon peptide can comprise SEQ ID NO: 1 or a modified amino acid sequence thereof comprising one or more of the amino acid modifications described herein, in which at least one of the amino acids at position 10, 20, 24, and 29 comprise an alkyl group and at least one of the amino acids at position 16, 17, 21, 24, and 29, a position within a C-terminal extension or the C-terminal amino acid are modified to a Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the side chain of the amino acid is covalently bonded to a hydrophilic moiety (e.g., PEG). In some embodiments, the alkyl group is attached to position 10, optionally via a spacer comprising Cys, Lys, Orn, homo-Cys, or Ac-Phe, and the hydrophilic moiety is incorporated at a Cys residue at position 24.

Alternatively, the alkylated glucagon peptide can comprise a spacer, wherein the spacer is both alkylated and modified to comprise the hydrophilic moiety. Nonlimiting examples of suitable spacers include a spacer comprising one or more amino acids selected from the group consisting of Cys, Lys, Orn, homo-Cys, and Ac-Phe.

Modifications that reduce GLP-1 activity

In certain embodiments, the glucagon peptide, or analog thereof, comprises an amino acid modification which selectively reduces GLP-1 activity. For example, the

acylated or alkylated glucagon peptide, or analog thereof, comprises a C-terminal alpha carboxylate group; a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile; deletion of the amino acid(s) C-terminal to the amino acid at position 27 or 28 (e.g., deletion of the amino acid at position 28, deletion of the amino acid at positions 28 and 29) to yield a peptide 27 or 28 amino acids in length, or a combination thereof.

Conjugates

The present disclosure also encompasses other conjugates in which glucagon peptides of the invention are linked, optionally via covalent bonding and optionally via a linker, to a conjugate moiety. Linkage can be accomplished by covalent chemical bonds, physical forces such electrostatic, hydrogen, ionic, van der Waals, or hydrophobic or hydrophilic interactions. A variety of non-covalent coupling systems may be used, including biotin-avidin, ligand/receptor, enzyme/substrate, nucleic acid/nucleic acid binding protein, lipid/lipid binding protein, cellular adhesion molecule partners; or any binding partners or fragments thereof which have affinity for each other.

The peptide can be linked to conjugate moieties via direct covalent linkage by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of these targeted amino acids. Reactive groups on the peptide or conjugate moiety include, e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group. Derivatizing agents include, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art. Alternatively, the conjugate moieties can be linked to the peptide indirectly through intermediate carriers, such as polysaccharide or polypeptide carriers. Examples of polysaccharide carriers include aminodextran. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier.

Cysteinyl residues are most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid, chloroacetamide to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are

derivatized by reaction with bromotrifluoroacetone, alpha-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

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

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

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

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

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

residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), deamidation of asparagine or glutamine, acetylation of the N-terminal amine, and/or amidation or esterification of the C-terminal carboxylic acid group.

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

Exemplary conjugate moieties that can be linked to any of the glucagon peptides described herein include but are not limited to a heterologous peptide or polypeptide (including for example, a plasma protein), a targeting agent, an immunoglobulin or portion thereof (e.g. variable region, CDR, or Fc region), a diagnostic label such as a radioisotope, fluorophore or enzymatic label, a polymer including water soluble polymers, or other therapeutic or diagnostic agents. In one embodiment a conjugate is provided comprising a glucagon peptide of the present invention and a plasma protein, wherein the plasma protein is selected from the group consisting of albumin, transferin, fibrinogen and globulins. In one embodiment the plasma protein moiety of the conjugate is albumin or transferin. In some embodiments, the linker comprises a chain of atoms from 1 to about 60, or 1 to 30 atoms or longer, 2 to 5 atoms, 2 to 10 atoms, 5 to 10 atoms, or 10 to 20 atoms long. In some embodiments, the chain atoms are all carbon atoms. In some embodiments, the chain atoms in the backbone of the linker are selected from the group consisting of C, O, N, and S. Chain atoms and linkers may be selected according to their expected

solubility (hydrophilicity) so as to provide a more soluble conjugate. In some embodiments, the linker provides a functional group that is subject to cleavage by an enzyme or other catalyst or hydrolytic conditions found in the target tissue or organ or cell. In some embodiments, the length of the linker is long enough to reduce the potential for steric hindrance. If the linker is a covalent bond or a peptidyl bond and the conjugate is a polypeptide, the entire conjugate can be a fusion protein. Such peptidyl linkers may be any length. Exemplary linkers are from about 1 to 50 amino acids in length, 5 to 50, 3 to 5, 5 to 10, 5 to 15, or 10 to 30 amino acids in length. Such fusion proteins may alternatively be produced by recombinant genetic engineering methods known to one of ordinary skill in the art.

As noted above, in some embodiments, the glucagon peptides are conjugated, e.g., fused to an immunoglobulin or portion thereof (e.g. variable region, CDR, or Fc region). Known types of immunoglobulins (Ig) include IgG, IgA, IgE, IgD or IgM. The Fc region is a C-terminal region of an Ig heavy chain, which is responsible for binding to Fc receptors that carry out activities such as recycling (which results in prolonged half-life), antibody dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC).

For example, according to some definitions the human IgG heavy chain Fc region stretches from Cys226 to the C-terminus of the heavy chain. The "hinge region" generally extends from Glu216 to Pro230 of human IgG1 (hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by aligning the cysteines involved in cysteine bonding). The Fc region of an IgG includes two constant domains, CH2 and CH3. The CH2 domain of a human IgG Fc region usually extends from amino acids 231 to amino acid 341. The CH3 domain of a human IgG Fc region usually extends from amino acids 342 to 447. References made to amino acid numbering of immunoglobulins or immunoglobulin fragments, or regions, are all based on Kabat et al. 1991, Sequences of Proteins of Immunological Interest, U.S. Department of Public Health, Bethesda, Md. In a related embodiments, the Fc region may comprise one or more native or modified constant regions from an immunoglobulin heavy chain, other than CH1, for example, the CH2 and CH3 regions of IgG and IgA, or the CH3 and CH4 regions of IgE.

Suitable conjugate moieties include portions of immunoglobulin sequence that include the FcRn binding site. FcRn, a salvage receptor, is responsible for recycling

immunoglobulins and returning them to circulation in blood. The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister et al. 1994, Nature 372:379). The major contact area of the Fc with the FcRn is near the junction of the CH2 and CH3 domains. Fc-FcRn contacts are all within a single Ig heavy chain. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the CH_2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain.

Some conjugate moieties may or may not include FcγR binding site(s). FcγR are responsible for ADCC and CDC. Examples of positions within the Fc region that make a direct contact with FcγR are amino acids 234-239 (lower hinge region), amino acids 265-269 (B/C loop), amino acids 297-299 (C'/E loop), and amino acids 327-332 (F/G) loop (Sondermann et al., Nature 406: 267-273, 2000). The lower hinge region of IgE has also been implicated in the FcRI binding (Henry, et al., Biochemistry 36, 15568-15578, 1997). Residues involved in IgA receptor binding are described in Lewis et al., (J Immunol. 175:6694-701, 2005). Amino acid residues involved in IgE receptor binding are described in Sayers et al. (J Biol Chem. 279(34):35320-5, 2004).

Amino acid modifications may be made to the Fc region of an immunoglobulin. Such variant Fc regions comprise at least one amino acid modification in the CH3 domain of the Fc region (residues 342-447) and/or at least one amino acid modification in the CH2 domain of the Fc region (residues 231-341). Mutations believed to impart an increased affinity for FcRn include T256A, T307A, E380A, and N434A (Shields et al. 2001, J. Biol. Chem. 276:6591). Other mutations may reduce binding of the Fc region to FcγRI, FcγRIIA, FcγRIIB, and/or FcγRIIIA without significantly reducing affinity for FcRn. For example, substitution of the Asn at position 297 of the Fc region with Ala or another amino acid removes a highly conserved N-glycosylation site and may result in reduced immunogenicity with concomitant prolonged half-life of the Fc region, as well as reduced binding to FcγRs (Routledge et al. 1995, Transplantation 60:847; Friend et al. 1999, Transplantation 68:1632; Shields et al. 1995, J. Biol. Chem. 276:6591). Amino acid modifications at positions 233-236 of IgG1 have been made that reduce binding to FcγRs (Ward and Ghetie 1995, Therapeutic Immunology 2:77 and Armour et al. 1999, Eur. J. Immunol. 29:2613). Some exemplary amino acid substitutions are described in US Patents 7,355,008 and 7,381,408, each incorporated by reference herein in its entirety.

The present disclosure also encompasses glucagon fusion peptides or proteins wherein a second peptide or polypeptide has been fused to a terminus, e.g., the carboxy terminus of the glucagon peptide. Exemplary candidates for C-terminal fusion include SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide.

Stabilization of the Alpha-Helix Structure

Reduction in glucagon activity upon modification of the amino acids at position 1 and/or position 2 of the glucagon peptide can be restored by stabilization of the alpha-helix structure in the C-terminal portion of the glucagon peptide (around amino acids 12-29). The alpha helix structure can be stabilized by, e.g., formation of a covalent or non-covalent intramolecular bridge, substitution and/or insertion of amino acids around positions 12-29 with an alpha helix-stabilizing amino acid (e.g., an α,α-disubstituted amino acid).

In some embodiments, an intramolecular bridge is formed between two amino acid side chains to stabilize the three dimensional structure of the carboxy terminal portion (e.g., amino acids 12-29) of the glucagon peptide. The two amino acid side chains can be linked to one another through non-covalent bonds, e.g., hydrogen-bonding, ionic interactions, such as the formation of salt bridges, or by covalent bonds. When the two amino acid side chains are linked to one another through one or more covalent bonds, the peptide may be considered herein as comprising a covlent intramolecular bridge. When the two amino acid side chains are linked to one another through non-covalent bonds, e.g., hydrogen bonds, ionic interactions, the peptide may be considered herein as comprising a non-covalent intramolecular bridge.

In some embodiments, the intramolecular bridge is formed between two amino acids that are 3 amino acids apart, e.g., amino acids at positions i and i+4, wherein i is any integer between 12 and 25 (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 25). More particularly, the side chains of the amino acid pairs 12 and 16, 16 and 20 , 20 and 24 or 24 and 28 (amino acid pairs in which i = 12, 16, 20, or 24) are linked to one another and thus stabilize the glucagon alpha helix. Alternatively, i can be 17.

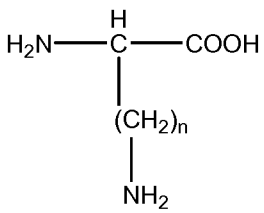
In some specific embodiments, wherein the amino acids at positions i and i+4 are joined by an intramolecular bridge, the size of the linker is about 8 atoms, or about 7-9 atoms.

In other embodiments, the intramolecular bridge is formed between two amino acids that are two amino acids apart, e.g., amino acids at positions j and j+3, wherein j is any integer between 12 and 26 (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 26). In some specific embodiments, j is 17.

In some specific embodiments, wherein amino acids at positions j and j+3 are joined by an intramolecular bridge, the size of the linker is about 6 atoms, or about 5 to 7 atoms.

In yet other embodiments, the intramolecular bridge is formed between two amino acids that are 6 amino acids apart, e.g., amino acids at positions k and k+7, wherein k is any integer between 12 and 22 (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, and 22). In some specific embodiments, k is 12, 13, or 17. In an exemplary embodiment, k is 17.

Examples of amino acid pairings that are capable of covalently bonding to form a six-atom linking bridge include Orn and Asp, Glu and an amino acid of Formula I, wherein n is 2, and homoglutamic acid and an amino acid of Formula I, wherein n is 1, wherein Formula I is:



wherein n = 1 to 4

[Formula I]

Examples of amino acid pairing that are capable of covalently bonding to form a seven-atom linking bridge include Orn-Glu (lactam ring); Lys-Asp (lactam); or Homoser-Homoglu (lactone). Examples of amino acid pairings that may form an eight-atom linker include Lys-Glu (lactam); Homolys-Asp (lactam); Orn-Homoglu (lactam); 4-aminoPhe-Asp (lactam); or Tyr-Asp (lactone). Examples of amino acid pairings that may form a nine-atom linker include Homolys-Glu (lactam); Lys-Homoglu (lactam); 4-aminoPhe-Glu (lactam); or Tyr-Glu (lactone). Any of the side chains on these amino acids may additionally be substituted with additional chemical groups, so long as the three-dimensional structure of the alpha-helix is not disrupted.

One of ordinary skill in the art can envision alternative pairings or alternative amino acid analogs, including chemically modified derivatives, that would create a stabilizing structure of similar size and desired effect. For example, a homocysteine-homocysteine disulfide bridge is 6 atoms in length and may be further modified to provide the desired effect. Even without covalent linkage, the amino acid pairings described above or similar pairings that one of ordinary skill in the art can envision may also provide added stability to the alpha-helix through non-covalent bonds, for example, through formation of salt bridges or hydrogen-bonding interactions.

The size of a lactam ring can vary depending on the length of the amino acid side chains, and in one embodiment the lactam is formed by linking the side chains of a lysine amino acid to a glutamic acid side chain. Further exemplary embodiments include the following pairings, optionally with a lactam bridge: Glu at position 12 with Lys at position 16; native Lys at position 12 with Glu at position 16; Glu at position 16 with Lys at position 20; Lys at position 16 with Glu at position 20; Glu at position 20 with Lys at position 24; Lys at position 20 with Glu at position 24; Glu at position 24 with Lys at position 28; Lys at position 24 with Glu at position 28. Alternatively, the order of the amide bond in the lactam ring can be reversed (e.g., a lactam ring can be formed between the side chains of a Lys12 and a Glu16 or alternatively between a Glu 12 and a Lys16).

Intramolecular bridges other than a lactam bridge can be used to stabilize the alpha helix of the glucagon analog peptides. In one embodiment, the intramolecular bridge is a hydrophobic bridge. In this instance, the intramolecular bridge optionally is between the side chains of two amino acids that are part of the hydrophobic face of the alpha helix of the glucagon analog peptide. For example, one of the amino acids joined by the hydrophobic bridge can be the amino acid at position 10, 14, and 18.

In one specific aspect, olefin metathesis is used to cross-link one or two turns of the alpha helix of the glucagon peptide using an all-hydrocarbon cross-linking system. The glucagon peptide in this instance can comprise α-methylated amino acids bearing olefinic side chains of varying length and configured with either R or S stereochemistry at the i and i+4 or i+7 positions. For example, the olefinic side can can comprise $(CH_2)n$, wherein n is any integer between 1 to 6. In one embodiment, n is 3 for a cross-link length of 8 atoms. Suitable methods of forming such intramolecular bridges are described in the art. See, for example, Schafmeister et al.,

J. Am. Chem. Soc. 122: 5891-5892 (2000) and Walensky et al., *Science* 305: 1466-1470 (2004). Alternatively, the glucagon peptide can comprise *O*-allyl Ser residues located on adjacent helical turns, which are bridged together via ruthenium-catalyzed ring closing metathesis. Such procedures of cross-linking are described in, for example, Blackwell et al., *Angew, Chem., Int. Ed.* 37: 3281-3284 (1998).

In another specific aspect, use of the unnatural thio-dialanine amino acid, lanthionine, which has been widely adopted as a peptidomimetic of cystine, is used to cross-link one turn of the alpha helix. Suitable methods of lanthionine-based cyclization are known in the art. See, for instance, Matteucci et al., *Tetrahedron Letters* 45: 1399-1401 (2004); Mayer et al., *J. Peptide Res.* 51: 432-436 (1998); Polinsky et al., *J. Med. Chem.* 35: 4185-4194 (1992); Osapay et al., *J. Med. Chem.* 40: 2241-2251 (1997); Fukase et al., *Bull. Chem. Soc. Jpn.* 65: 2227-2240 (1992); Harpp et al., *J. Org. Chem.* 36: 73-80 (1971); Goodman and Shao, *Pure Appl. Chem.* 68: 1303-1308 (1996); and Osapay and Goodman, *J. Chem. Soc. Chem. Commun.* 1599-1600 (1993).

In some embodiments, α, ω-diaminoalkane tethers, e.g., 1,4-diaminopropane and 1,5-diaminopentane) between two Glu residues at positions i and i+7 are used to stabilize the alpha helix of the glucagon peptide. Such tethers lead to the formation of a bridge 9-atoms or more in length, depending on the length of the diaminoalkane tether. Suitable methods of producing peptides cross-linked with such tethers are described in the art. See, for example, Phelan et al., *J. Am. Chem. Soc.* 119: 455-460 (1997).

In yet another embodiment of the invention, a disulfide bridge is used to cross-link one or two turns of the alpha helix of the glucagon peptide. Alternatively, a modified disulfide bridge in which one or both sulfur atoms are replaced by a methylene group resulting in an isosteric macrocyclization is used to stabilize the alpha helix of the glucagon peptide. Suitable methods of modifying peptides with disulfide bridges or sulfur-based cyclization are described in, for example, Jackson et al., *J. Am. Chem. Soc.* 113: 9391-9392 (1991) and Rudinger and Jost, *Experientia* 20: 570-571 (1964).

In yet another embodiment, the alpha helix of the glucagon peptide is stabilized via the binding of metal atom by two His residues or a His and Cys pair positioned at i and i+4. The metal atom can be, for example, Ru(III), Cu(II), Zn(II),

or Cd(II). Such methods of metal binding-based alpha helix stabilization are known in the art. See, for example, Andrews and Tabor, *Tetrahedron* 55: 11711-11743 (1999); Ghadiri et al., *J. Am. Chem. Soc.* 112: 1630-1632 (1990); and Ghadiri et al., *J. Am. Chem. Soc.* 119: 9063-9064 (1997).

The alpha helix of the glucagon peptide can alternatively be stabilized through other means of peptide cyclizing, which means are reviewed in Davies, *J. Peptide. Sci.* 9: 471-501 (2003). The alpha helix can be stabilized via the formation of an amide bridge, thioether bridge, thioester bridge, urea bridge, carbamate bridge, sulfonamide bridge, and the like. For example, a thioester bridge can be formed between the C-terminus and the side chain of a Cys residue. Alternatively, a thioester can be formed via side chains of amino acids having a thiol (Cys) and a carboxylic acid (e.g., Asp, Glu). In another method, a cross-linking agent, such as a dicarboxylic acid, e.g. suberic acid (octanedioic acid), etc. can introduce a link between two functional groups of an amino acid side chain, such as a free amino, hydroxyl, thiol group, and combinations thereof.

In accordance with one embodiment, the alpha helix of the glucagon peptide is stabilized through the incorporation of hydrophobic amino acids at positions i and i+4. For instance, i can be Tyr and i+4 can be either Val or Leu; i can be Phe and i+4 can be Cys or Met; I can be Cys and i+4 can be Met; or i can be Phe and i+4 can be Ile. It should be understood that, for purposes herein, the above amino acid pairings can be reversed, such that the indicated amino acid at position i could alternatively be located at i+4, while the i+4 amino acid can be located at the i position.

In accordance with other embodiments of the invention, the alpha helix is stabilized through incorporation (either by amino acid substitution or insertion) of one or more alpha helix-stabilizing amino acids at the C-terminal portion of the glucagon peptide (around amino acids 12-29). In a specific embodiment, the alpha helix-stabilizing amino acid is an α, α-disubstitued amino acid, including, but not limited to any of amino iso-butyric acid (AIB), an amino acid disubstituted with the same or a different group selected from methyl, ethyl, propyl, and n-butyl, or with a cyclooctane or cycloheptane (e.g., 1-aminocyclooctane-1-carboxylic acid). In some embodiments, one, two, three, four or more of positions 16, 17, 18, 19, 20, 21, 24 or 29 of the glucagon peptide is substituted with an α, α-disubstituted amino acid. In a specific embodiment, one, two, three or all of positions 16, 20, 21, and 24 are substituted with

AIB. For example, the glucagon peptide can comprise a substitution of position 16 with AIB in the absence of an intramolecular bridge, e.g., a non-covalent intramolecular bridge (e.g., a salt bridge) or a covalent intramolecular bridge (e.g., a lactam). Such peptides lacking an intramolecular bridge are advantageously easy to prepare.

In accordance with some embodiments, the glucagon peptide lacking an intramolecular bridge comprises one or more substitutions within amino acid positions 12-29 with an α, α-disubstituted amino acid and an acyl or alkyl group covalently attached to the side chain of an amino acid of the glucagon peptide, e.g., the amino acid at position 10 of the glucagon peptide. In specific embodiments, the acyl or alkyl group is non-native to a naturally occurring amino acid. In certain aspects, the acyl or alkyl group is non-native to the amino acid at position 10. Such acylated or alkylated glucagon peptides lacking an intramolecular bridge exhibit enhanced activity at the GLP-1 and glucagon receptors as compared to the non-acylated counterpart peptides. Further enhancement in activity at the GLP-1 and glucagon receptors can be achieved by the acylated glucagon peptides lacking an intramolecular bridge by incorporating a spacer between the acyl or alkyl group and the side chain of the amino acid at position 10 of the peptide. Acylation and alkylation, with or without incorporating spacers, are further described herein.

In specific embodiments, the acylated or alkylated glucagon peptide, or analog thereof, further comprises a modification which selectively reduces activity at the GLP-1 receptor. For example, the acylated or alkylated glucagon peptide, or analog thereof, comprises one or a combination of: a C-terminal alpha carboxylate, a deletion of the amino acids C-terminal to the amino acid at position 27 or 28 (e.g., deletion of the amino acid at position 29, deletion of the amino acids at positions 28 and 29), a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile.

Examples of embodiments

In accordance with one embodiment the native glucagon peptide of SEQ ID NO: 1 is modified by the substitution of the native amino acid at position 28 and/or 29 with a negatively charged amino acid (e.g., aspartic acid or glutamic acid) and optionally the addition of a negatively charged amino acid (e.g., aspartic acid or glutamic acid) to the carboxy terminus of the peptide. In an alternative embodiment

the native glucagon peptide of SEQ ID NO: 1 is modified by the substitution of the native amino acid at position 29 with a positively charged amino acid (e.g., lysine, arginine or histidine) and optionally the addition of one or two positively charged amino acid (e.g., lysine, arginine or histidine) on the carboxy terminus of the peptide. In accordance with one embodiment a glucagon analog having improved solubility and stability is provided wherein the analog comprises the amino acid sequence of SEQ ID NO: 34 with the proviso that at least one amino acids at position, 28, or 29 is substituted with an acidic amino acid and/or an additional acidic amino acid is added at the carboxy terminus of SEQ ID NO: 34. In one embodiment the acidic amino acids are independently selected from the group consisting of Asp, Glu, cysteic acid and homocysteic acid.

In accordance with one embodiment a glucagon agonist having improved solubility and stability is provided wherein the agonist comprises the amino acid sequence of SEQ ID NO: 33, wherein at least one of the amino acids at positions 27, 28 or 29 is substituted with a non-native amino acid residue (i.e. at least one amino acid present at position 27, 28 or 29 of the analog is an acid amino acid different from the amino acid present at the corresponding position in SEQ ID NO: 1). In accordance with one embodiment a glucagon agonist is provided comprising the sequence of SEQ ID NO: 33 with the proviso that when the amino acid at position 28 is asparagine and the amino acid at position 29 is threonine, the peptide further comprises one to two amino acids, independently selected from the group consisting of Lys, Arg, His, Asp or Glu, added to the carboxy terminus of the glucagon peptide.

It has been reported that certain positions of the native glucagon peptide can be modified while retaining at least some of the activity of the parent peptide. Accordingly, applicants anticipate that one or more of the amino acids located at positions at positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28 or 29 of the peptide of SEQ ID NO: 11 can be substituted with an amino acid different from that present in the native glucagon peptide, and still retain the enhanced potency, physiological pH stability and biological activity of the parent glucagon peptide. For example, in accordance with one embodiment the methionine residue present at position 27 of the native peptide is changed to leucine or norleucine to prevent oxidative degradation of the peptide.

In one embodiment a glucagon analog of SEQ ID NO: 33 is provided wherein 1 to 6 amino acids, selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 1. In accordance with another embodiment a glucagon analog of SEQ ID NO: 33 is provided wherein 1 to 3 amino acids selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 1. In another embodiment, a glucagon analog of SEQ ID NO: 7, SEQ ID NO: 8 or SEQ ID NO: 34 is provided wherein 1 to 2 amino acids selected from positions 1, 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21 or 24 of the analog differ from the corresponding amino acid of SEQ ID NO: 1, and in a further embodiment those one to two differing amino acids represent conservative amino acid substitutions relative to the amino acid present in the native sequence (SEQ ID NO: 1). In one embodiment a glucagon peptide of SEQ ID NO: 11 or SEQ ID NO: 13 is provided wherein the glucagon peptide further comprises one, two or three amino acid substitutions at positions selected from positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27 or 29. In one embodiment the substitutions at positions 2, 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 27 or 29 are conservative amino acid substitutions.

In one embodiment a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 1 wherein the analog differs from SEQ ID NO: 1 by having an amino acid other than serine at position 2 and by having an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 1. In one embodiment the acidic amino acid is aspartic acid or glutamic acid. In one embodiment a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13 or SEQ ID NO: 32 is provided wherein the analog differs from the parent molecule by a substitution at position 2. More particularly, position 2 of the analog peptide is substituted with an amino acid selected from the group consisting of D-serine, alanine, D-alanine, glycine, n-methyl serine and amino isobutyric acid.

In another embodiment a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 1 wherein the analog differs from SEQ ID NO: 1 by having an amino acid other than histidine at position 1 and by having an acidic amino acid substituted for the native amino acid at position 28 or 29 or an acidic amino acid added to the carboxy terminus of the peptide of SEQ ID NO: 1. In one embodiment

the acidic amino acid is aspartic acid or glutamic acid. In one embodiment a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13 or SEQ ID NO: 32 is provided wherein the analog differs from the parent molecule by a substitution at position 1. More particularly, position 1 of the analog peptide is substituted with an amino acid selected from the group consisting of DMIA, D-histidine, desaminohistidine, hydroxyl-histidine, acetyl-histidine and homo-histidine.

In accordance with one embodiment the modified glucagon peptide comprises a sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 32. In a further embodiment a glucagon peptide is provided comprising a sequence of SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13 or SEQ ID NO: 32 further comprising one to two amino acids, added to the C-terminus of SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 13 or SEQ ID NO: 32, wherein the additional amino acids are independently selected from the group consisting of Lys, Arg, His, Asp Glu, cysteic acid or homocysteic acid. In one embodiment the additional amino acids added to the carboxy terminus are selected from the group consisting of Lys, Arg, His, Asp or Glu or in a further embodiment the additional amino acids are Asp or Glu.

In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 7 or a glucagon agonist analog thereof. In one embodiment the peptide comprising a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13. In another embodiment the peptide comprising a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 10 and SEQ ID NO: 11. In one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 8, SEQ ID NO: 10 and SEQ ID NO: 11 further comprising an additional amino acid, selected from the group consisting of Asp and Glu, added to the C-terminus of the glucagon peptide. In one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 11 or SEQ ID NO: 13, and in a further embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 11.

In accordance with one embodiment a glucagon agonist is provided comprising a modified glucagon peptide selected from the group consisting of:

NH_2-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Xaa-Xaa-Xaa-R (SEQ ID NO: 34),

NH_2-His-Ser-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-Asp-Thr-R (SEQ ID NO: 11) and

NH_2-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Xaa-Tyr-Leu-Glu-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asp-Thr-R (SEQ ID NO: 13)

wherein Xaa at position 15 is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid, the Xaa at position 28 is Asn or an acidic amino acid and the Xaa at position 29 is Thr or an acidic amino acid and R is an acidic amino acid, COOH or $CONH_2$, with the proviso that an acidic acid residue is present at one of positions 28, 29 or 30. In one embodiment R is COOH, and in another embodiment R is $CONH_2$.

The present disclosure also encompasses glucagon fusion peptides wherein a second peptide has been fused to the C-terminus of the glucagon peptide to enhance the stability and solubility of the glucagon peptide. More particularly, the fusion glucagon peptide may comprise a glucagon agonist analog comprising a glucagon peptide NH_2-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Xaa-Xaa-Xaa-R (SEQ ID NO: 34), wherein R is an acidic amino acid or a bond and an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to the carboxy terminal amino acid of the glucagon peptide. In one embodiment the glucagon peptide is selected from the group consisting of SEQ ID NO: 33, SEQ ID NO: 7 or SEQ ID NO: 8 further comprising an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to the carboxy terminal amino acid of the glucagon peptide. In one embodiment the glucagon fusion peptide comprises SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6 or a glucagon agonist analog thereof, further comprising an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide. In accordance with one embodiment the fusion peptide further comprises a PEG chain linked to an amino acid at position 16, 17, 21, 24, 29, within a C-terminal extension, or at the C-terminal amino acid, wherein the PEG chain is selected from the range of 500 to 40,000 Daltons. In one embodiment the amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) is bound to amino acid 29 of the glucagon peptide through a peptide bond. In one embodiment the glucagon peptide

portion of the glucagon fusion peptide comprises a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 13. In one embodiment the glucagon peptide portion of the glucagon fusion peptide comprises the sequence of SEQ ID NO: 11 or SEQ ID NO: 13, wherein a PEG chain is linked at position 21, 24, 29, within a C-terminal extension or at the C-terminal amino acid, respectively.

In another embodiment the glucagon peptide sequence of the fusion peptide comprises the sequence of SEQ ID NO: 11, further comprising an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide. In one embodiment the glucagon fusion peptide comprises a sequence selected from the group consisting of SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26. Typically the fusion peptides of the present invention will have a C-terminal amino acid with the standard carboxylic acid group. However, analogs of those sequences wherein the C-terminal amino acid has an amide substituted for the carboxylic acid are also encompassed as embodiments. In accordance with one embodiment the fusion glucagon peptide comprises a glucagon agonist analog selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 13, further comprising an amino acid sequence of SEQ ID NO: 23 (GPSSGAPPPS-$CONH_2$) linked to amino acid 29 of the glucagon peptide.

The glucagon agonists of the present invention can be further modified to improve the peptide's solubility and stability in aqueous solutions while retaining the biological activity of the glucagon peptide. In accordance with one embodiment, introduction of hydrophilic groups at one or more positions selected from positions 16, 17, 20, 21, 24 and 29 of the peptide of SEQ ID NO: 11, or a glucagon agonist analog thereof, are anticipated to improve the solubility and stability of the pH stabilize glucagon analog. More particularly, in one embodiment the glucagon peptide of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 13, or SEQ ID NO: 32 is modified to comprise one or more hydrophilic groups covalently linked to the side chains of amino acids present at positions 21 and 24 of the glucagon peptide.

In accordance with one embodiment, the glucagon peptide of SEQ ID NO: 11 is modified to contain one or more amino acid substitution at positions 16, 17, 20, 21, 24 and/or 29, wherein the native amino acid is substituted with an amino acid having

a side chain suitable for crosslinking with hydrophilic moieties, including for example, PEG. The native peptide can be substituted with a naturally occurring amino acid or a synthetic (non-naturally occurring) amino acid. Synthetic or non-naturally occurring amino acids refer to amino acids that do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein.

In one embodiment, a glucagon agonist of SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 13 is provided wherein the native glucagon peptide sequence has been modified to contain a naturally occurring or synthetic amino acid in at least one of positions 16, 17, 21, 24, 29, within a C-terminal extension or at the C-terminal amino acid of the native sequence, wherein the amino acid substitute further comprises a hydrophilic moiety. In one embodiment the substitution is at position 21 or 24, and in a further embodiment the hydrophilic moiety is a PEG chain. In one embodiment the glucagon peptide of SEQ ID NO: 11 is substituted with at least one cysteine residue, wherein the side chain of the cysteine residue is further modified with a thiol reactive reagent, including for example, maleimido, vinyl sulfone, 2-pyridylthio, haloalkyl, and haloacyl. These thiol reactive reagents may contain carboxy, keto, hydroxyl, and ether groups as well as other hydrophilic moieties such as polyethylene glycol units. In an alternative embodiment, the native glucagon peptide is substituted with lysine, and the side chain of the substituting lysine residue is further modified using amine reactive reagents such as active esters (succinimido, anhydride, etc) of carboxylic acids or aldehydes of hydrophilic moieties such as polyethylene glycol. IN one embodiment the glucagon peptide is selected form the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

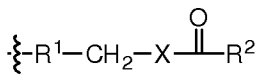
In accordance with one embodiment the pegylated glucagon peptide comprises two or more polyethylene chains covalently bound to the glucagon peptide wherein the total molecular weight of the glucagon chains is about 1,000 to about 5,000 Daltons. In one embodiment the pegylated glucagon agonist comprises a peptide of SEQ ID NO: 6, wherein a PEG chain is covalently linked to the amino acid residue at position 21 and at position 24, and wherein the combined molecular weight of the two PEG chains is about 1,000 to about 5,000 Daltons. In another embodiment the pegylated glucagon agonist comprises a peptide of SEQ ID NO: 6, wherein a PEG

chain is covalently linked to the amino acid residue at position 21 and at position 24, and wherein the combined molecular weight of the two PEG chains is about 5,000 to about 20,000 Daltons.

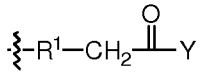
The polyethylene glycol chain may be in the form of a straight chain or it may be branched. In accordance with one embodiment the polyethylene glycol chain has an average molecular weight selected from the range of about 500 to about 40,000 Daltons. In one embodiment the polyethylene glycol chain has a molecular weight selected from the range of about 500 to about 5,000 Daltons. In another embodiment the polyethylene glycol chain has a molecular weight of about 20,000 to about 40,000 Daltons.

Any of the glucagon peptides described above may be further modified to include a covalent or non-covalent intramolecular bridge or an alpha helix-stabilizing amino acid within the C-terminal portion of the glucagon peptide (amino acid positions 12-29). In accordance with one embodiment, the glucagon peptide comprises any one or more of the modifications discussed above in addition to an amino acid substitution at positions 16, 20, 21, or 24 (or a combination thereof) with an α,α-disubstituted amino acid, e.g., AIB. In accordance with another embodiment, the glucagon peptide comprises any one or more modifications discussed above in addition to an intramolecular bridge, e.g., a lactam, between the side chains of the amino acids at positions 16 and 20 of the glucagon peptide.

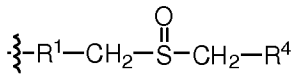
In accordance with some embodiments, the glucagon peptide comprises the amino acid sequence of SEQ ID NO: 77, wherein the Xaa at position 3 is an amino acid comprising a side chain of Structure I, II, or III:



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, X is NH or Y is NHR^4. In some embodiments, R^1 is C_{0-2} alkyl or C_1 heteroalkyl. In some embodiments, R^2 is NHR^4 or C_1 alkyl. In some embodiments, R^4 is H or C^1 alkyl. In exemplary embodiments, 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, 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, an amino acid comprising a side chain of Structure III is provided where, R^1 is CH_2 and R^4 is H (methionine-sulfoxide, M(O)); In specific embodiments, 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.

In certain embodiments, the glucagon peptide is an analog of the glucagon peptide of SEQ ID NO: 77. In specific aspects, the analog comprises any of the amino acid modifications described herein, including, but not limited to: a substitution of Asn at position 28 with a charged amino acid; a 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; a substitution at position 28 with Asn, Asp, or Glu; a substitution at position 28 with Asp; a substitution at position 28 with Glu; a substitution of Thr at position 29 with a charged amino acid; a 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; a substitution at position 29 with Asp, Glu, or Lys; a substitution at position 29 with Glu; a insertion of 1-3 charged amino acids after position 29; an insertion after position 29 of Glu or Lys; an insertion after position 29 of Gly-Lys or Lys-Lys; and a combination thereof.

In certain embodiments, the analog of the glucagon peptide of SEQ ID NO: 77 comprises an α,α-disubstituted amino acid, such as AIB, at one, two, three, or all of positions 16, 20, 21, and 24.

In certain embodiments, the analog of the glucagon peptide of SEQ ID NO: 77 comprises one or more of the following: substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), substitution of Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile; substitution of Tyr at position 10 with Phe or Val; 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 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; deletion of amino acids at positions 27-29; deletion of amino acids at positions 28-29; deletion of the amino acid at positions 29; addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly, or a combination thereof.

In accordance with specific embodiments, the glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 62-67 and 69-74.

In certain embodiments, the analog of the glucagon peptide comprising SEQ ID NO: 77 comprises a hydrophilic moiety, e.g., PEG, covalently linked to the amino acid at any of positions 16, 17, 20, 21, 24, and 29 or at the C-terminal amino acid.

In certain embodiments, the analog of the glucagon peptide comprising SEQ ID NO: 77 comprises an amino acid comprising a side chain covalently attached, optionally, through a spacer, to an acyl group or an alkyl group, which acyl group or alkyl group is non-native to a naturally-occurring amino acid. The acyl group in some embodiments is a C4 to C30 fatty acyl group. In other embodiments, the alkyl group is a C4 to C30 alkyl. In specific aspects, the acyl group or alkyl group is covalently

attached to the side chain of the amino acid at position 10. In some embodiments, the amino acid at position 7 is Ile or Abu.

Use

As described in detail in the Examples, the glucagon agonists of the present invention have enhanced biophysical stability and aqueous solubility in solutions of phisiological pH, while retaining or demonstrating enhanced bioactivity relative to the native peptide. Accordingly, the glucagon agonists of the present invention are believed to be suitable for any use that has previously been described for the native glucagon peptide. Therefore, the modified glucagon peptides described herein can be used to treat hypoglycemia, to increase blood glucose level, to induce temporary paralysis of the gut for radiological uses, to reduce and maintain body weight, as adjunctive therapy with insulin, or to treat other metabolic diseases that result from low blood levels of glucagon.

The glucagon peptides described herein also are expected to be used to reduce or maintain body weight, or to treat hyperglycemia, or to reduce blood glucose level, or to normalize blood glucose level, and/or to stabilize blood glucose level. "Normalizing" blood level means that the blood glucose level is returned to normal (e.g,, lowering blood glucose level if it is higher than normal, or raising blood glucose level if it is lower than normal). "Stabilizing" blood glucose level means reducing the maximal variation in blood glucose level over a period of time, e.g., 8 hours, 16 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days or 1 week. For example, administration of glucagon peptide causes the blood glucose level over time to be maintained closer to the normal range of glucose values than it would be in the absence of administration of glucagon peptide.

The glucagon peptides of the invention may be administered alone or in combination with other anti-diabetic or anti-obesity agents. Anti-diabetic agents known in the art or under investigation include insulin, sulfonylureas, such as tolbutamide (Orinase), acetohexamide (Dymelor), tolazamide (Tolinase), chlorpropamide (Diabinese), glipizide (Glucotrol), glyburide (Diabeta, Micronase, Glynase), glimepiride (Amaryl), or gliclazide (Diamicron); meglitinides, such as repaglinide (Prandin) or nateglinide (Starlix); biguanides such as metformin (Glucophage) or phenformin; thiazolidinediones such as rosiglitazone (Avandia),

pioglitazone (Actos), or troglitazone (Rezulin), or other PPARγ inhibitors; alpha glucosidase inhibitors that inhibit carbohydrate digestion, such as miglitol (Glyset), acarbose (Precose/Glucobay); exenatide (Byetta) or pramlintide; Dipeptidyl peptidase-4 (DPP-4) inhibitors such as vildagliptin or sitagliptin; SGLT (sodium-dependent glucose transporter 1) inhibitors; glucokinase activators (GKA); glucagon receptor antagonists (GRA); or FBPase (fructose 1,6-bisphosphatase) inhibitors.

Anti-obesity agents known in the art or under investigation include appetite suppressants, including phenethylamine type stimulants, phentermine (optionally with fenfluramine or dexfenfluramine), diethylpropion (Tenuate®), phendimetrazine (Prelu-2®, Bontril®), benzphetamine (Didrex®), sibutramine (Meridia®, Reductil®); rimonabant (Acomplia®), other cannabinoid receptor antagonists; oxyntomodulin; fluoxetine hydrochloride (Prozac); Qnexa (topiramate and phentermine), Excalia (bupropion and zonisamide) or Contrave (bupropion and naltrexone); or lipase inhibitors, similar to xenical (Orlistat) or Cetilistat (also known as ATL-962), or GT 389-255.

One aspect of the present disclosure is directed to a pre-formulated aqueous solution of the presently disclosed glucagon agonist for use in treating hypoglycemia. The improved stability and/or solubility of the agonist compositions described herein allow for the preparation of pre-formulated aqueous solutions of glucagon for rapid administration and treatment of hypoglycemia. Accordingly, in one embodiment a solution comprising a glucagon agonist of the present invention is provided for administration to a patient suffering from hypoglycemia. In one embodiment a solution comprising a pegylated glucagon agonist as disclosed herein is provided for administration to a patient suffering from hypoglycemia, wherein the total molecular weight of the PEG chains linked to the pegylated glucagon agonist is between about 500 to about 5,000 Daltons. In one embodiment the pegylated glucagon agonist comprises a peptide selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19, and glucagon agonist analogs of thereof, wherein the side chain of an amino acid residue at position 16, 17, 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid of said glucagon peptide is covalently bound to the polyethylene glycol chain. In one embodiment, the pegylated glucagon agonist comprises the peptide of SEQ ID NO: 16, wherein the amino acid residue at position 21 of the peptide is

covalently linked to polyethylene glycol. In one embodiment, the pegylated glucagon agonist comprises the peptide of SEQ ID NO: 17, wherein the amino acid residue at position 24 of the peptide is covalently linked to polyethylene glycol.

The treatment methods in accordance with the present invention including but not limited to treatment of hypoglycemia, may comprise the steps of administering the presently disclosed glucagon agonists to a patient using any standard route of administration, including parenterally, such as intravenously, intraperitoneally, subcutaneously or intramuscularly, intrathecally, transdermally, rectally, orally, nasally or by inhalation. In one embodiment the composition is administered subcutaneously or intramuscularly. In one embodiment, the composition is administered parenterally and the glucagon composition is prepackaged in a syringe. In another embodiment, the composition is prepackaged in an inhaler or other aerosolized drug deliver device. Advantageously, the aqueous stable glucagon analogs disclosed herein exhibit superior stability and solubility in aqueous buffers in the broadest pH range used for pharmacological purposes, relative to native glucagon. The use of the stabilized glucagon analogs disclosed herein allow for the preparation and storage of glucagon agonist solutions at physiological pH for long periods of time.

Applicants have discovered that pegylated glucagon peptides can be prepared that retain the parent peptide's bioactivity and specificity. However, increasing the length of the PEG chain, or attaching multiple PEG chains to the peptide, such that the total molecular weight of the linked PEG is greater than 5,000 Daltons, begins to delay the time action of the modified glucagon. In accordance with one embodiment, a glucagon peptide of SEQ ID NO: 11 or SEQ ID NO: 13, or a glucagon agonist analog thereof, is provided wherein the peptide comprises one or more polyethylene glycol chains, wherein the total molecular weight of the linked PEG is greater than 5,000 Daltons, and in one embodiment is greater than 10,000 Daltons, but less than 40,000 Daltons. Such modified glucagon peptides have a delayed time of activity but without loss of the bioactivity. Accordingly, such compounds can be administered prophylactically to extend the effect of the administered glucagon peptide.

Glucagon peptides that have been modified to be covalently bound to a PEG chain having a molecular weight of greater than 10,000 Daltons can be administered in conjunction with insulin to buffer the actions of insulin and help to maintain stable

blood glucose levels in diabetics. The modified glucagon peptides of the present disclosure can be co-administered with insulin as a single composition, simultaneously administered as separate solutions, or alternatively, the insulin and the modified glucagon peptide can be administered at different time relative to one another. In one embodiment the composition comprising insulin and the composition comprising the modified glucagon peptide are administered within 12 hours of one another. The exact ratio of the modified glucagon peptide relative to the administered insulin will be dependent in part on determining the glucagon levels of the patient, and can be determined through routine experimentation.

In accordance with one embodiment a composition is provided comprising insulin and a modified glucagon peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and glucagon agonist analogs thereof, wherein the modified glucagon peptide further comprises a polyethylene glycol chain covalently bound to an amino acid side chain at position 16, 17, 21, 24, 29, within a C-terminal extension, or at the C-terminal amino acid. In one embodiment the composition is an aqueous solution comprising insulin and the glucagon analog. In embodiments wherein the glucagon peptide comprises the sequence of SEQ ID NO: 11 or SEQ ID NO: 13, the peptide may further comprise a polyethylene glycol chain covalently bound to an amino acid side chain at position 16, 17, 21, 24, 29, within a C-terminal extension, or at the C-terminal amino acid. In one embodiment the molecular weight of the PEG chain of the modified glucagon peptide is greater than 10,000 Daltons. In one embodiment the pegylated glucagon peptide comprises a peptide selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO: 13 wherein the side chain of an amino acid residue at position 21 or 24 of said glucagon peptide is covalently bound to the polyethylene glycol chain. In one embodiment the polyethylene glycol chain has a molecular weight of about 10,000 to about 40,000.

In accordance with one embodiment the modified glucagon peptides disclosed herein are used to induce temporary paralysis of the intestinal tract. This method has utility for radiological purposes and comprises the step of administering an effective amount of a pharmaceutical composition comprising a pegylated glucagon peptide, a glucagon peptide comprising a c-terminal extension or a dimer of such peptides. In one embodiment the glucagon peptide comprises a sequence 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, SEQ ID NO: 12 and SEQ ID NO: 13. In one embodiment the glucagon peptide further comprises a PEG chain, of about 1,000 to 40,000 Daltons is covalently bound to an amino acid residue at position 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid. In one embodiment the glucagon peptide is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 and SEQ ID NO: 17. In one embodiment the PEG chain has a molecular weight of about 500 to about 5,000 Daltons.

In a further embodiment the composition used to induce temporary paralysis of the intestinal tract comprises a first modified glucagon peptide and a second modified glucagon peptide, wherein the first modified peptide comprises a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 13, optionally linked to a PEG chain of about 500 to about 5,000 Daltons, and the second peptide comprises a covalently linked PEG chain of about 10,000 to about 40,000 Daltons. In this embodiment the PEG chain of each peptide is covalently bound to an amino acid residue at either position 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid, of the respective peptide, and independent of one another.

Oxyntomodulin, a naturally occurring digestive hormone found in the small intestine, has been reported to cause weight loss when administered to rats or humans (see Diabetes 2005;54:2390-2395). 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: 23 (KRNRNNIA). Accordingly, applicants believe that the bioactivity of oxyntomodulin can be retained (i.e. appetite suppression and induced weight loss/weight maintenance), while improving the solubility and stability of the compound and improving the pharmacokinetics, by substituting the glucagon peptide portion of oxyntomodulin with the modified glucagon peptides disclosed herein. In addition applicants also believe that a truncated oxyntomodulin molecule comprising a glucagon peptide of the invention, having the terminal four amino acids of oxyntomodulin removed, will also be effective in suppressing appetite and inducing weight loss/weight maintenance.

Accordingly, the present invention also encompasses the modified glucagon peptides of the present invention that have a carboxy terminal extension of SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22. In accordance with one embodiment a glucagon agonist analog of SEQ ID NO: 33, further comprising the amino acid sequence of SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 linked to amino acid 29 of the glucagon peptide, is administered to individuals to induce weight loss or prevent weight gain. In accordance with one embodiment a glucagon agonist analog of SEQ ID NO: 11 or SEQ ID NO: 13, further comprising the amino acid sequence of SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 linked to amino acid 29 of the glucagon peptide, is administered to individuals to induce weight loss or prevent weight gain. In another embodiment a method of reducing weight gain or inducing weight loss in an individual comprises administering an effective amount of a composition comprising a glucagon agonist comprising a glucagon peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5, 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: 24 (KRNRNNIA) or SEQ ID NO: 25, and wherein a PEG chain of about 1,000 to 40,000 Daltons is covalently bound to an amino acid residue at position 21 and/or 24. In one embodiment the glucagon peptide segment of the glucagon agonist is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19, wherein a PEG chain of about 1,000 to 40,000 Daltons is covalently bound to an amino acid residue at position 16, 17, 21, 24, or 29, within a C-terminal extension, or at the C-terminal amino acid.

Exendin-4 is a peptide made up of 39 amino acids. It is a powerful stimulator of a receptor known as GLP-1. This peptide has also been reported to suppress appetite and induce weight loss. Applicants have found that the terminal sequence of Exendin-4 when added at the carboxy terminus of glucagon improves the solubility and stability of glucagon without compromising the bioactivy of glucagon. In one embodiment the terminal ten amino acids of Exendin-4 (i.e. the sequence of SEQ ID NO: 20 (GPSSGAPPPS)) are linked to the carboxy terminus of a glucagon peptide of the present disclosure. In specific aspects, the sequence of SEQ ID NO: 20 is linked to the C-terminus of the glucagon peptide and the amino acid at position 29 is either

Thr or Gly. These fusion proteins are anticipated to have pharmacological activity for suppressing appetite and inducing weight loss/weight maintenance. In one embodiment the terminal amino acid of the SEQ ID NO: 20 extension comprises an amide group in place of the carboxy group (i.e., SEQ ID NO: 23) and this sequence is linked to the carboxy terminus of a glucagon peptide of the present disclosure.

In one embodiment a method of reducing weight gain or inducing weight loss in an individual comprises administering an effective amount of a composition comprising a glucagon agonist comprising a glucagon peptide of 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 (GPSSGAPPPS) or SEQ ID NO: 23. In one embodiment the glucagon peptide is 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, SEQ ID NO: 12 and SEQ ID NO: 13 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 (GPSSGAPPPS) or SEQ ID NO: 23. In one embodiment the glucagon peptide of the glucagon agonist is selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO: 13. In one embodiment the glucagon peptide segment of the fusion peptide is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 and SEQ ID NO: 17, wherein the molecular weight of the PEG chain is selected from the range of 500 to 40,000 Daltons. More particularly, in one embodiment the glucagon peptide of the fusion peptide is selected from the group consisting of SEQ ID NO: 16 and SEQ ID NO: 17 wherein the molecular weight of the PEG chain is selected from the range of 1,000 to 5,000.

In another embodiment a composition is administered to a patient to suppress appetite, prevent weight gain and/or induce weight loss by the administration of a pharmaceutical composition comprising a first pegylated glucagon peptide and a second pegylated glucagon peptide, wherein the first and second peptide are fusion peptides comprising a c-terminal peptide extension comprising SEQ ID NO: 20 (GPSSGAPPPS) or SEQ ID NO: 23. The first pegylated glycogen peptide comprising a covalently linked PEG of about 500 to about 10,000 Daltons and the

second pegylated glucagon peptide comprising a covalently linked PEG chain of about 10,000 to about 40,000 Daltons.

The present disclosure also encompasses multimers of the modified glucagon peptides disclosed herein. Two or more of the modified glucagon peptides can be linked together using standard linking agents and procedures known to those skilled in the art. For example, dimers can be formed between two modified glucagon peptides through the use of bifunctional thiol crosslinkers and bi-functional amine crosslinkers, particularly for the glucagon peptides that have been substituted with cysteine, lysine ornithine, homocysteine or acetyl phenylalanine residues (e.g. SEQ ID NO: 4 and SEQ ID NO: 5). The dimer can be a homodimer or alternatively can be a heterodimer. In one embodiment the dimer comprises a homodimer of a glucagon fusion peptide wherein the glucagon peptide portion comprises an agonist analog of SEQ ID NO: 11 and an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide. In another embodiment the dimer comprises a homodimer of a glucagon agonist analog of SEQ ID NO: 11, wherein the glucagon peptide further comprises a polyethylene glycol chain covalently bound to position 21, 24, 29, within a C-terminal extension, or at the C-terminal amino acid of the glucagon peptide.

In accordance with one embodiment a dimer is provided comprising a first glucagon peptide bound to a second glucagon peptide via a linker, wherein the first glucagon peptide comprises a peptide selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11 and the second glucagon peptide comprises SEQ ID NO: 33. Furthermore, with regards to the second glucagon peptide, when the amino acid at position 28 is asparagine and the amino acid at position 29 is threonine, the second glucagon peptide further comprises one to two amino acids (independently selected from the group consisting of Lys, Arg, His, Asp or Glu), added to the carboxy terminus of the second glucagon glucagon peptide, and pharmaceutically acceptable salts of said glucagon polypeptides.

In accordance with another embodiment a dimer is provided comprising a first glucagon peptide bound to a second glucagon peptide via a linker, wherein said first glucagon peptide is 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: 8, SEQ ID NO: 9, SEQ ID NO:

10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13 and the second glucagon peptide is independently selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO: 13, and pharmaceutically acceptable salts of said glucagon polypeptides. In one embodiment the first glucagon peptide is selected from the group consisting of SEQ ID NO: 7 and the second glucagon peptide is independently selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13. In one embodiment the dimer is formed between two peptides wherein each peptide comprising the amino acid sequence of SEQ ID NO: 11.

In accordance with one embodiment a pharmaceutical composition is provided wherein the composition comprises a glucagon agonist analog of the present disclosure, or pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. The pharmaceutical composition can comprise any pharmaceutically acceptable ingredient, including, for example, acidifying agents, additives, adsorbents, aerosol propellants, air displacement agents, alkalizing agents, anticaking agents, anticoagulants, antimicrobial preservatives, antioxidants, antiseptics, bases, binders, buffering agents, chelating agents, coating agents, coloring agents, desiccants, detergents, diluents, disinfectants, disintegrants, dispersing agents, dissolution enhancing agents, dyes, emollients, emulsifying agents, emulsion stabilizers, fillers, film forming agents, flavor enhancers, flavoring agents, flow enhancers, gelling agents, granulating agents, humectants, lubricants, mucoadhesives, ointment bases, ointments, oleaginous vehicles, organic bases, pastille bases, pigments, plasticizers, polishing agents, preservatives, sequestering agents, skin penetrants, solubilizing agents, solvents, stabilizing agents, suppository bases, surface active agents, surfactants, suspending agents, sweetening agents, therapeutic agents, thickening agents, tonicity agents, toxicity agents, viscosity-increasing agents, water-absorbing agents, water-miscible cosolvents, water softeners, or wetting agents.

In some embodiments, the pharmaceutical composition comprises any one or a combination of the following components: acacia, acesulfame potassium, acetyltributyl citrate, acetyltriethyl citrate, agar, albumin, alcohol, dehydrated alcohol, denatured alcohol, dilute alcohol, aleuritic acid, alginic acid, aliphatic polyesters, alumina, aluminum hydroxide, aluminum stearate, amylopectin, α-amylose, ascorbic acid, ascorbyl palmitate, aspartame, bacteriostatic water for injection, bentonite, bentonite magma, benzalkonium chloride, benzethonium chloride, benzoic acid,

benzyl alcohol, benzyl benzoate, bronopol, butylated hydroxyanisole, butylated hydroxytoluene, butylparaben, butylparaben sodium, calcium alginate, calcium ascorbate, calcium carbonate, calcium cyclamate, dibasic anhydrous calcium phosphate, dibasic dehydrate calcium phosphate, tribasic calcium phosphate, calcium propionate, calcium silicate, calcium sorbate, calcium stearate, calcium sulfate, calcium sulfate hemihydrate, canola oil, carbomer, carbon dioxide, carboxymethyl cellulose calcium, carboxymethyl cellulose sodium, β-carotene, carrageenan, castor oil, hydrogenated castor oil, cationic emulsifying wax, cellulose acetate, cellulose acetate phthalate, ethyl cellulose, microcrystalline cellulose, powdered cellulose, silicified microcrystalline cellulose, sodium carboxymethyl cellulose, cetostearyl alcohol, cetrimide, cetyl alcohol, chlorhexidine, chlorobutanol, chlorocresol, cholesterol, chlorhexidine acetate, chlorhexidine gluconate, chlorhexidine hydrochloride, chlorodifluoroethane (HCFC), chlorodifluoromethane, chlorofluorocarbons (CFC)chlorophenoxyethanol, chloroxylenol, corn syrup solids, anhydrous citric acid, citric acid monohydrate, cocoa butter, coloring agents, corn oil, cottonseed oil, cresol, m-cresol, o-cresol, p-cresol, croscarmellose sodium, crospovidone, cyclamic acid, cyclodextrins, dextrates, dextrin, dextrose, dextrose anhydrous, diazolidinyl urea, dibutyl phthalate, dibutyl sebacate, diethanolamine, diethyl phthalate, difluoroethane (HFC), dimethyl-β-cyclodextrin, cyclodextrin-type compounds such as Captisol®, dimethyl ether, dimethyl phthalate, dipotassium edentate, disodium edentate, disodium hydrogen phosphate, docusate calcium, docusate potassium, docusate sodium, dodecyl gallate, dodecyltrimethylammonium bromide, edentate calcium disodium, edtic acid, eglumine, ethyl alcohol, ethylcellulose, ethyl gallate, ethyl laurate, ethyl maltol, ethyl oleate, ethylparaben, ethylparaben potassium, ethylparaben sodium, ethyl vanillin, fructose, fructose liquid, fructose milled, fructose pyrogen-free, powdered fructose, fumaric acid, gelatin, glucose, liquid glucose, glyceride mixtures of saturated vegetable fatty acids, glycerin, glyceryl behenate, glyceryl monooleate, glyceryl monostearate, self-emulsifying glyceryl monostearate, glyceryl palmitostearate, glycine, glycols, glycofurol, guar gum, heptafluoropropane (HFC), hexadecyltrimethylammonium bromide, high fructose syrup, human serum albumin, hydrocarbons (HC), dilute hydrochloric acid, hydrogenated vegetable oil, type II, hydroxyethyl cellulose, 2-hydroxyethyl-β-cyclodextrin, hydroxypropyl cellulose, low-substituted hydroxypropyl cellulose, 2-

hydroxypropyl-β-cyclodextrin, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate, imidurea, indigo carmine, ion exchangers, iron oxides, isopropyl alcohol, isopropyl myristate, isopropyl palmitate, isotonic saline, kaolin, lactic acid, lactitol, lactose, lanolin, lanolin alcohols, anhydrous lanolin, lecithin, magnesium aluminum silicate, magnesium carbonate, normal magnesium carbonate, magnesium carbonate anhydrous, magnesium carbonate hydroxide, magnesium hydroxide, magnesium lauryl sulfate, magnesium oxide, magnesium silicate, magnesium stearate, magnesium trisilicate, magnesium trisilicate anhydrous, malic acid, malt, maltitol, maltitol solution, maltodextrin, maltol, maltose, mannitol, medium chain triglycerides, meglumine, menthol, methylcellulose, methyl methacrylate, methyl oleate, methylparaben, methylparaben potassium, methylparaben sodium, microcrystalline cellulose and carboxymethylcellulose sodium, mineral oil, light mineral oil, mineral oil and lanolin alcohols, oil, olive oil, monoethanolamine, montmorillonite, octyl gallate, oleic acid, palmitic acid, paraffin, peanut oil, petrolatum, petrolatum and lanolin alcohols, pharmaceutical glaze, phenol, liquified phenol, phenoxyethanol, phenoxypropanol, phenylethyl alcohol, phenylmercuric acetate, phenylmercuric borate, phenylmercuric nitrate, polacrilin, polacrilin potassium, poloxamer, polydextrose, polyethylene glycol, polyethylene oxide, polyacrylates, polyethylene-polyoxypropylene-block polymers, polymethacrylates, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene stearates, polyvinyl alcohol, polyvinyl pyrrolidone, potassium alginate, potassium benzoate, potassium bicarbonate, potassium bisulfite, potassium chloride, postassium citrate, potassium citrate anhydrous, potassium hydrogen phosphate, potassium metabisulfite, monobasic potassium phosphate, potassium propionate, potassium sorbate, povidone, propanol, propionic acid, propylene carbonate, propylene glycol, propylene glycol alginate, propyl gallate, propylparaben, propylparaben potassium, propylparaben sodium, protamine sulfate, rapeseed oil, Ringer's solution, saccharin, saccharin ammonium, saccharin calcium, saccharin sodium, safflower oil, saponite, serum proteins, sesame oil, colloidal silica, colloidal silicon dioxide, sodium alginate, sodium ascorbate, sodium benzoate, sodium bicarbonate, sodium bisulfite, sodium chloride, anhydrous sodium citrate, sodium citrate dehydrate, sodium chloride, sodium cyclamate, sodium edentate, sodium dodecyl sulfate, sodium lauryl sulfate,

sodium metabisulfite, sodium phosphate, dibasic, sodium phosphate, monobasic, sodium phosphate, tribasic, anhydrous sodium propionate, sodium propionate, sodium sorbate, sodium starch glycolate, sodium stearyl fumarate, sodium sulfite, sorbic acid, sorbitan esters (sorbitan fatty esters), sorbitol, sorbitol solution 70%, soybean oil, spermaceti wax, starch, corn starch, potato starch, pregelatinized starch, sterilizable maize starch, stearic acid, purified stearic acid, stearyl alcohol, sucrose, sugars, compressible sugar, confectioner's sugar, sugar spheres, invert sugar, *Sugartab*, Sunset Yellow FCF, synthetic paraffin, talc, tartaric acid, tartrazine, tetrafluoroethane (HFC), theobroma oil, thimerosal, titanium dioxide, alpha tocopherol, tocopheryl acetate, alpha tocopheryl acid succinate, beta-tocopherol, delta-tocopherol, gamma-tocopherol, tragacanth, triacetin, tributyl citrate, triethanolamine, triethyl citrate, trimethyl-β-cyclodextrin, trimethyltetradecylammonium bromide, tris buffer, trisodium edentate, vanillin, type I hydrogenated vegetable oil, water, soft water, hard water, carbon dioxide-free water, pyrogen-free water, water for injection, sterile water for inhalation, sterile water for injection, sterile water for irrigation, waxes, anionic emulsifying wax, carnauba wax, cationic emulsifying wax, cetyl ester wax, microcrystalline wax, nonionic emulsifying wax, suppository wax, white wax, yellow wax, white petrolatum, wool fat, xanthan gum, xylitol, zein, zinc propionate, zinc salts, zinc stearate, or any excipient in the *Handbook of Pharmaceutical Excipients*, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000), which is incorporated by reference in its entirety. *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), which is incorporated by reference in its entirety, discloses various components used in formulating pharmaceutically acceptable compositions and known techniques for the preparation thereof. Except insofar as any conventional agent is incompatible with the pharmaceutical compositions, its use in pharmaceutical compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The pharmaceutical formulations disclosed herein may be designed to be short-acting, fast-releasing, long-acting, or sustained-releasing as described below. The pharmaceutical formulations may also be formulated for immediate release, controlled release or for slow release. The instant compositions may further comprise, for example, micelles or liposomes, or some other encapsulated form, or

may be administered in an extended release form to provide a prolonged storage and/or delivery effect. The disclosed pharmaceutical formulations may be administered according to any regime including, for example, daily (1 time per day, 2 times per day, 3 times per day, 4 times per day, 5 times per day, 6 times per day), every two days, every three days, every four days, every five days, every six days, weekly, bi-weekly, every three weeks, monthly, or bi-monthly.

In some embodiments, the foregoing component(s) may be present in the pharmaceutical composition at any concentration, such as, for example, at least A, wherein A is 0.0001% w/v, 0.001% w/v, 0.01% w/v, 0.1% w/v, 1% w/v, 2% w/v, 5% w/v, 10% w/v, 20% w/v, 30% w/v, 40% w/v, 50% w/v, 60% w/v, 70% w/v, 80% w/v, or 90% w/v. In some embodiments, the foregoing component(s) may be present in the pharmaceutical composition at any concentration, such as, for example, at most B, wherein B is 90% w/v, 80% w/v, 70% w/v, 60% w/v, 50% w/v, 40% w/v, 30% w/v, 20% w/v, 10% w/v, 5% w/v, 2% w/v, 1% w/v, 0.1% w/v, 0.001% w/v, or 0.0001%. In other embodiments, the foregoing component(s) may be present in the pharmaceutical composition at any concentration range, such as, for example from about A to about B. In some embodiments, A is 0.0001% and B is 90%.

The pharmaceutical compositions may be formulated to achieve a physiologically compatible pH. In some embodiments, the pH of the pharmaceutical composition may be at least 5, at least 5.5, at least 6, at least 6.5, at least 7, at least 7.5, at least 8, at least 8.5, at least 9, at least 9.5, at least 10, or at least 10.5 up to and including pH 11, depending on the formulation and route of administration. In certain embodiments, the pharmaceutical compositions may comprise one or more buffering agents to achieve a physiological compatible pH. The buffering agents may include any compounds capable of buffering at the desired pH such as, for example, phosphate buffers (e.g. PBS), triethanolamine, Tris, bicine, TAPS, tricine, HEPES, TES, MOPS, PIPES, cacodylate, MES, and others. In certain embodiments, the strength of the buffer is at least 0.5 mM, at least 1 mM, at least 5 mM, at least 10 mM, at least 20 mM, at least 30 mM, at least 40 mM, at least 50 mM, at least 60 mM, at least 70 mM, at least 80 mM, at least 90 mM, at least 100 mM, at least 120 mM, at least 150 mM, or at least 200 mM. In some embodiments, the strength of the buffer is no more than 300 mM (e.g. at most 200 mM, at most 100 mM, at most 90 mM, at

most 80 mM, at most 70 mM, at most 60 mM, at most 50 mM, at most 40 mM, at most 30 mM, at most 20 mM, at most 10 mM, at most 5 mM, at most 1 mM).

In one embodiment the pharmaceutical composition comprises a 1 mg/ml concentration of the glucagon agonist analog and 10-50 mM Triethanolamine at pH 7.0-8.5, or 6-9, or 7-9. In one embodiment the pharmaceutical composition comprises a 1 mg/ml concentration of the glucagon agonist analog and 20 mM Triethanolamine at pH 8.5.

The modified glucagon peptides of the present invention can be provided in accordance with one embodiment as part of a kit. In one embodiment a kit for administering a glucagon agonist to a patient in need thereof is provided wherein the kit comprises any of the glucagon peptides of the invention in aqueous solution. Exemplary glucagon peptides for inclusion in such kits include a glucagon peptide selected from the group consisting of 1) a glucagon peptide comprising the sequence of SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO:11 or SEQ ID NO: 13 or SEQ ID NO: 33; 2) a glucagon fusion peptide comprising a glucagon agonist analog of SEQ ID NO: 11 or SEQ ID NO: 13 or SEQ ID NO: 33, and an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide; and 3) a pegylated glucagon peptide of SEQ ID NO: 11 or SEQ ID NO: 13 or SEQ ID NO: 33, further comprising an amino acid sequence of SEQ ID NO: 20 (GPSSGAPPPS), SEQ ID NO: 21 (KRNRNNIA) or SEQ ID NO: 22 (KRNR) linked to amino acid 29 of the glucagon peptide, wherein the PEG chain covalently bound to position 16, 17, 21, 24 or 29, within a C-terminal extension, or at the C-terminal amino acid has a molecular weight of about 500 to about 40,000 Daltons. In one embodiment the kit is provided with a device for administering the glucagon composition to a patient, e.g. syringe needle, pen device, jet injector or other needle-free injector. The kit may alternatively or in addition include one or more of a variety of containers, *e.g.*, vials, tubes, bottles, single or multi-chambered pre-filled syringes, cartridges, infusion pumps (external or implantable), jet injectors, pre-filled pen devices and the like, optionally containing the glucagon peptide in a lyophilized form or in aqueous solution. Preferably, the kits will also include instructions for use. In accordance with one embodiment the device of the kit is an aerosol dispensing device, wherein the composition is prepackaged within the aerosol device. In another embodiment the kit comprises a syringe and a

needle, and in one embodiment the sterile glucagon composition is prepackaged within the syringe.

The compounds of this invention may be prepared by standard synthetic methods, recombinant DNA techniques, or any other methods of preparing peptides and fusion proteins. Although certain non-natural amino acids cannot be expressed by standard recombinant DNA techniques, techniques for their preparation are known in the art. Compounds of this invention that encompass non-peptide portions may be synthesized by standard organic chemistry reactions, in addition to standard peptide chemistry reactions when applicable.

EXAMPLES

General Synthesis Protocol:

Glucagon analogs were synthesized using HBTU-activated "Fast Boc" single coupling starting from 0.2 mmole of Boc Thr(OBzl)Pam resin on a modified Applied Biosystem 430 A peptide synthesizer. Boc amino acids and HBTU were obtained from Midwest Biotech (Fishers, IN). Side chain protecting groups used were: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(pMeBzl), His(Bom), Lys(2Cl-Z), Ser(OBzl), Thr(OBzl), Tyr(2Br-Z), and Trp(CHO). The side-chain protecting group on the N-terminal His was Boc.

Each completed peptidyl resin was treated with a solution of 20% piperdine in dimethylformamide to remove the formyl group from the tryptophan. Liquid hydrogen fluoride cleavages were performed in the presence of p-cresol and dimethyl sulfide. The cleavage was run for 1 hour in an ice bath using an HF apparatus (Penninsula Labs). After evaporation of the HF, the residue was suspended in diethyl ether and the solid materials were filtered. Each peptide was extracted into 30-70 ml aqueous acetic acid and a diluted aliquot was analyzed by HPLC [Beckman System Gold, 0.46x5cm Zorbax C8, 1ml/min, 45C, 214nm, A buffer =0.1%TFA, B=0.1%TFA/90%acetonitrile, gradient of 10% to 80%B over 10min].

Purification was done on a FPLC over a 2.2 x 25 cm Kromasil C18 column while monitoring the UV at 214nm and collecting 5 minute fractions. The homogeneous fractions were combined and lyophilized to give a product purity of >95%. The correct molecular mass and purity were confirmed using MALDI-mass spectral analysis.

General Pegylation Protocol: (Cys-maleimido)

Typically, the glucagon Cys analog is dissolved in phosphate buffered saline (5-10mg/ml) and 0.01M ethylenediamine tetraacetic acid is added (10-15% of total volume). Excess (2-fold) maleimido methoxyPEG reagent (Nektar) is added and the reaction stirred at room temp while monitoring reaction progress by HPLC. After 8-24hrs, the reaction mixture, is acidified and loaded onto a preparative reverse phase column for purification using 0.1%TFA/acetonitrile gradient. The appropriate fractions were combined and lyophilized to give the desired pegylated analogs.

EXAMPLE 1

Synthesis of Glucagon Cys^{17}(1-29) and Similar MonoCys Analogs

0.2mmole Boc Thr(OBzl) Pam resin (SynChem Inc) in a 60ml reaction vessel and the following sequence was entered and run on a modified Applied Biosystems 430A Peptide Synthesizer using FastBoc HBTU-activated single couplings.

HSQGTFTSDYSKYLDSCRAQDFVQWLMNT (SEQ ID NO: 27)

The following side chain protecting groups were used: Arg(Tos), Asp(OcHex), Asn(Xan), Cys(pMeBzl), Glu(OcHex), His(Boc), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Trp(CHO), and Tyr(Br-Z). The completed peptidyl resin was treated with 20% piperidine/dimethylformamide to remove the Trp formyl protection then transferred to an HF reaction vessel and dried in vacuo. 1.0ml p-cresol and 0.5 ml dimehyl sulfide were added along with a magnetic stir bar. The vessel was attached to the HF apparatus (Pennisula Labs), cooled in a dry ice/methanol bath, evacuated, and aprox. 10ml liquid hydrogen fluoride was condensed in. The reaction was stirred in an ice bath for 1hr then the HF was removed in vacuo. The residue was suspended in ethyl ether; the solids were filtered, washed with ether, and the peptide extracted into 50 ml aqueous acetic acid. An analytical HPLC was run [0.46 x 5 cm Zorbax C8, 1 ml/min, 45C, 214nm, A buffer of 0.1%TFA, B buffer of 0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.] with a small sample of the cleavage extract. The remaining extract was loaded onto a 2.2 x 25cm Kromasil C18 preparative reverse phase column and an acetonitrile gradient was run using a Pharmacia FPLC system. 5min fractions were collected while monitoring the UV at 214nm (2.0A). A=0.1%TFA, B=0.1%TFA/50%acetonitrile. Gradient = 30%B to 100%B over 450min.

The fractions containing the purest product (48-52) were combined frozen, and lyophilized to give 30.1mg. An HPLC analysis of the product demonstrated a purity of >90% and MALDI mass spectral analysis demonstrated the desired mass of 3429.7. Glucagon Cys^{21}, Glucagon Cys^{24}, and Glucagon Cys^{29} were similarly prepared.

EXAMPLE 2

Synthesis of Glucagon-Cex and Other C-Terminal Extended Analogs.

285mg (0.2mmole) methoxybenzhydrylamine resin (Midwest Biotech) was placed in a 60ml reaction vessel and the following sequence was entered and run on a modified Applied Biosystems 430A peptide synthesizer using FastBoc HBTU-activated single couplings.

HSQGTFTSDYSKYLDSRRAQDFVQWLMNTGPSSGAPPPS (SEQ ID NO: 28)

The following side chain protecting groups were used: Arg(Tos), Asp(OcHex), Asn(Xan), Cys(pMeBzl), Glu(OcHex), His(Boc), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Trp(CHO), and Tyr(Br-Z). The completed peptidyl resin was treated with 20% piperidine/dimethylformamide to remove the Trp formyl protection then transferred to HF reaction vessel and dried in vacuo. 1.0ml p-cresol and 0.5 ml dimehyl sulfide were added along with a magnetic stir bar. The vessel was attached to the HF apparatus (Pennisula Labs), cooled in a dry ice/methanol bath, evacuated, and aprox. 10ml liquid hydrogen fluoride was condensed in. The reaction was stirred in an ice bath for 1hr then the HF was removed in vacuo. The residue was suspended in ethyl ether; the solids were filtered, washed with ether, and the peptide extracted into 50 ml aqueous acetic acid. An analytical HPLC was run [0.46 x 5 cm Zorbax C8, 1 ml/min, 45C, 214nm, A buffer of 0.1%TFA, B buffer of 0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.] on an aliquot of the cleavage extract. The extract was loaded onto a 2.2 x 25cm Kromasil C18 preparative reverse phase column and an acetonitrile gradient was run for elution using a Pharmacia FPLC system. 5min fractions were collected while monitoring the UV at 214nm (2.0A). A=0.1%TFA, B=0.1%TFA/50%acetonitrile. Gradient = 30%B to 100%B over 450min. Fractions 58-65 were combined, frozen and lyophilized to give 198.1mg.

HPLC analysis of the product showed a purity of greater than 95%. MALDI mass spectral analysis showed the presence of the desired theoretical mass of 4316.7 with the product as a C-terminal amide. Oxyntomodulin and oxyntomodulin-KRNR were similarly prepared as the C-terminal carboxylic acids starting with the appropriately loaded PAM-resin.

EXAMPLE 3

Glucagon Cys^{17} Mal-PEG-5K

15.1mg of Glucagon Cys^{17}(1-29) and 27.3mg methoxy poly(ethyleneglycol) maleimide avg. M.W.5000 (mPEG-Mal-5000,Nektar Therapeutics) were dissolved in 3.5ml phosphate buffered saline (PBS) and 0.5ml 0.01M ethylenediamine tetraacetic acid (EDTA) was added. The reaction was stirred at room temperature and the progress of the reaction was monitored by HPLC analysis [0.46 x 5 cm Zorbax C8, 1ml/min,45C, 214nm (0.5A), A=0.1%TFA, B=0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.].

After 5 hours, the reaction mixture was loaded onto 2.2 x 25 cm Kromasil C18 preparastive reverse phase column. An acetonitrile gradient was run on a Pharmacia FPLC while monitoring the UV wavelength at 214nm and collecting 5 min fractions. A=0.1%TFA, B=0.1%TFA/50% acetonitrile, gradient= 30%B to 100%B over 450 min. The fractions corresponding to the product were combined, frozen and lyophilized to give 25.9 mg.

This product was analyzed on HPLC [0.46 x 5 cm Zorbax C8, 1 ml/min, 45C, 214nm (0.5A), A=0.1%TFA, B=0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.] which showed a purity of aprox. 90%. MALDI (matrix assisted laser desorption ionization) mass spectral analysis showed a broad mass range (typical of PEG derivatives) of 8700 to 9500. This shows an addition to the mass of the starting glucagon peptide (3429) of approximately 5,000 a.m.u.

EXAMPLE 4

Glucagon Cys^{21} Mal-PEG-5K

21.6mg of Glucagon Cys^{21}(1-29) and 24mg mPEG-MAL-5000 (Nektar Therapeutics) were dissolved in 3.5ml phosphate buffered saline (PBS) and 0.5ml 0.01M ethylene diamine tetraacetic acid (EDTA) was added. The reaction was stirred

at room temp. After 2hrs, another 12.7 mg of mPEG-MAL-5000 was added. After 8hrs, the reaction mixture was loaded onto a 2.2 x 25cm Vydac C18 preparative reverse phase column and an acetonitrile gradient was run on a Pharmacia FPLC at 4 ml/min while collecting 5min fractions. A=0.1%TFA, B=0.1%TFA/50%ACN. Gradient= 20% to 80%B over 450min.

The fractions corresponding to the appearance of product were combined frozen and lyophilized to give 34 mg. Analysis of the product by analytical HPLC [0.46 x 5 cm Zorbax C8, 1 ml/min, 45C, 214nm (0.5A), A=0.1%TFA, B=0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.] showed a homogeneous product that was different than starting glucagon peptide. MALDI (matrix assisted laser desorption ionization) mass spectral analysis showed a broad mass range (typical of PEG analogs) of 8700 to 9700. This shows an addition to the mass of the starting glucagon peptide (3470) of approximately 5,000 a.m.u.

EXAMPLE 5

Glucagon Cys^{24} Mal-PEG-5K

20.1mg Glucagon C^{24}(1-29) and 39.5mg mPEG-Mal-5000 (Nektar Therapeutics) were dissolved in 3.5ml PBS with stirring and 0.5 ml 0.01M EDTA was added. The reaction was stirred at room temp for 7 hrs, then another 40 mg of mPEG-Mal-5000 was added. After approximately 15 hr, the reaction mixture was loaded onto a 2.2 x 25 cm Vydac C18 preparative reverse phase column and an acetontrile gradient was run using a Pharmacia FPLC. 5 min. fractions were collected while monitoring the UV at 214nm (2.0A). A buffer = 0.1%TFA, B buffer = 0.1%TFA/50%ACN, gradient = 30%B to 100%B over 450min. The fractions corresponding to product were combined, frozen and lyophilized to give 45.8mg. MALDI mass spectral analysis showed a typical PEG broad signal with a maximum at 9175.2 which is approximately 5,000 a.m.u. more than Glucagon C^{24} (3457.8).

EXAMPLE 6

Glucagon Cys^{24} Mal-PEG-20K

25.7mg of Glucagon Cys^{24}(1-29) and 40.7mg mPEG-Mal-20K (Nektar Therapeutics) were dissolved in 3.5ml PBS with stirring at room temp. and 0.5 ml 0.01M EDTA was added. After 6hrs, the ratio of starting material to product was

aprox. 60:40 as determined by HPLC. Another 25.1mg of mPEG-Mal-20K was added and the reaction allowed to stir another 16hrs. The product ratio had not significantly improved, so the reaction mixture was loaded onto a 2.2 x 25 cm Kromasil C18 preparative reverse phase column and purified on a Pharmacia FPLC using a gradient of 30%B to 100%B over 450min. A buffer =0.1%TFA, B buffer = 0.1%TFA/50%ACN, flow = 4ml/min, and 5 min fractions were collected while monitoring the UV at 214nm (2.0A). The fractions containing homogeneous product were combined, frozen and lyophilized to give 25.7 mg. Purity as determined by analytical HPLC was ~90%. A MALDI mass spectral analysis showed a broad peak from 23,000 to 27,000 which is approximately 20,000 a.m.u. more than starting Glucagon C^{24} (3457.8).

EXAMPLE 7

Glucagon Cys^{29} Mal-PEG-5K

20.0mg of Glucagon Cys^{29}(1-29) and 24.7 mg mPEG-Mal-5000 (Nektar Therapeutics) were dissolved in 3.5 ml PBS with stirring at room temperature and 0.5 ml 0.01M EDTA was added. After 4 hr, another 15.6 mg of mPEG-Mal-5000 was added to drive the reaction to completion. After 8 hrs, the reaction mixture was loaded onto a 2.2 x 25 cm Vydac C18 preparative reverse phase column and an acetonitrile gradient was run on a Pharmacia FPLC system. 5 min fractions were collected while monitoring the UV at 214nm (2.0A). A=0.1%TFA, B=0.1%TFA/50%ACN. Fractions 75-97 were combined frozen and lyophilized to give 40.0 mg of product that is different than recovered starting material on HPLC (fractions 58-63). Analysis of the product by analytical HPLC [0.46 x 5 cm Zorbax C8, 1 ml/min, 45C, 214nm (0.5A), A=0.1%TFA, B=0.1%TFA/90%ACN, gradient=10%B to 80%B over 10min.] showed a purity greater than 95%. MALDI mass spectral analysis showed the presence of a PEG component with a mass range of 8,000 to 10,000 (maximum at 9025.3) which is 5,540 a.m.u. greater than starting material (3484.8).

EXAMPLE 8
Glucagon Cys^{24} (2-butyrolactone)

To 24.7mg of Glucagon Cys^{24}(1-29) was added 4ml 0.05M ammonium bicarbonate/50%acetonitrile and 5.5 ul of a solution of 2-bromo-4-hydroxybutyric acid-γ-lactone (100ul in 900ul acetonitrile). After 3hrs of stirring at room temperature, another 105 ul of lactone solution was added to the reaction mixture which was stirred another 15hrs. The reaction mixture was diluted to 10ml with 10% aqueous acetic acid and was loaded onto a 2.2 x 25 cm Kromasil C18 preparative reverse phase column. An acetonitrile gradient (20%B to 80%B over 450min) was run on a Pharmacia FPLC while collecting 5min fractions and monitoring the UV at 214nm (2.0A). Flow =4ml/min, A=0.1%TFA, B=0.1%TFA/50%ACN. Fractions 74-77 were combined frozen and lyophilized to give 7.5mg. HPLC analysis showed a purity of 95% and MALDI mass spect analysis showed a mass of 3540.7 or 84 mass units more than starting material. This result is consistent with the addition of a single butyrolactone moiety.

EXAMPLE 9
Glucagon Cys^{24}(S-carboxymethyl)

18.1mg of Glucagon Cys^{24}(1-29) was dissolved in 9.4ml 0.1M sodium phosphate buffer (pH=9.2) and 0.6ml bromoacetic acid solution (1.3mg/ml in acetonitrile) was added. The reaction was stirred at room temperature and the reaction progress was followed by analytical HPLC. After 1hr another 0.1ml bromoacetic acid solution was added. The reaction was stirred another 60min. then acidified with aqueous acetic acid and was loaded onto a 2.2 x 25cm Kromasil C18 preparative reverse phase column for purification. An acetonitrile gradient was run on a Pharmacia FPLC (flow = 4ml/min) while collecting 5min fractions and monitoring the UV at 214nm (2.0A). A=0.1%TFA, B=0.1%TFA/50%ACN. Fractions 26-29 were combined frozen and lyophilized to give several mg of product. Analytical HPLC showed a purity of 90% and MALDI mass spectral analysis confirmed a mass of 3515 for the desired product.



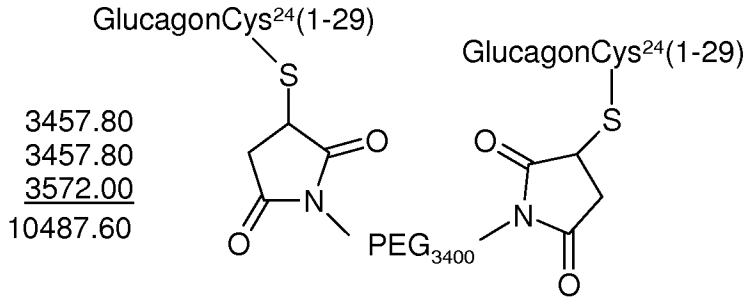
Molecular Weight =3515.87
Exact Mass =3512
Molecular Formula =C153H224N42O50S2

SEQ ID NO: 30

EXAMPLE 10

Glucagon Cys^{24} maleimido,PEG-3.4K-dimer

16mg Glucagon Cys^{24} and 1.02mg Mal-PEG-Mal-3400, poly(ethyleneglycol)-bis-maleimide avg. M.W. 3400, (Nektar Therpeutics) were dissolved in 3.5 phosphate buffered saline and 0.5ml 0.01M EDTA and the reaction was stirred at room temperature. After 16hrs, another 16mg of Glucagon Cys^{24} was added and the stirring continued. After approximately 40hrs, the reaction mixture was loaded onto a Pharmcia PepRPC 16/10 column and an acetonitrile gradient was run on a Pharmacia FPLC while collecting 2min fractions and monitoring the UV at 214nm (2.0A). Flow=2ml/min, A=0.1%TFA, B=0.1%TFA/50%ACN. Fractions 69-74 were combined frozen and lyophilized to give 10.4mg. Analytical HPLC showed a purity of 90% and MALDI mass spectral analysis shows a component in the 9500-11,000 range which is consistent with the desired dimer.



EXAMPLE 11

Glucagon Solubility Assays:

A solution (1mg/ml or 3mg/ml) of glucagon (or an analog) is prepared in 0.01N HCl. 100ul of stock solution is diluted to 1ml with 0.01N HCl and the UV absorbance (276nm) is determined. The pH of the remaining stock solution is adjusted to pH7 using 200-250ul 0.1M Na_2HPO_4 (pH9.2). The solution is allowed to stand overnight at 4°C then centrifuged. 100ul of supernatant is then diluted to 1ml with 0.01N HCl, and the UV absorbance is determined (in duplicate).

The initial absorbance reading is compensated for the increase in volume and the following calculation is used to establish percent solubility:

$$\frac{\text{Final Absorbance}}{\text{Initial Absorbance}} \times 100 = \text{percent soluble}$$

Results are shown in Table 1 wherein Glucagon-Cex represents wild type glucagon (SEQ ID NO: 1) plus a carboxy terminal addition of SEQ ID NO: 20 and Glucagon-Cex R^{12} represents SEQ ID NO: 1 wherein the Lys at position 12 is substituted with Arg and a peptide of SEQ ID NO: 20 is added to the carboxy terminus.

Table 1 Solubility data for glucagon analogs

Analog	Percent Soluble
Glucagon	16
Glucagon-Cex, R12	104
Glucagon-Cex	87
Oxyntomodulin	104
Glucagon, Cys17PEG5K	94
Glucagon, Cys21PEG5K	105
Glucagon, Cys24PEG5K	133

The solubility the glucagon agonist analogs, D28, E29, E30, E15D28, D28E30, D28E29, was investigated using the same assay used for the compounds listed in Table 1. The data (shown in Figs. 3 & 4) demonstrates the superior solubility of the D28, E29, E30, E15D28, D28E30, D28E29 analogs relative to native glucagon at pH values of 5.5 and 7.0. The data presented in Fig. 3 represents the solubility measured after 60 hours at 25 °C, whereas the data presented in Fig. 4 represents the solubility measured after 24 hours at 25 °C and then 24 hours at 4 °C. Fig. 5

represents data regarding the maximum solubility of the glucagon analogs D28, D28E30 and E15D28.

EXAMPLE 12

Glucagon Receptor Binding Assay

The affinity of peptides to the glucagon receptor was measured in a competition binding assay utilizing scintillation proximity assay technology. Serial 3-fold dilutions of the peptides made in scintillation proximity assay buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% w/v bovine serum albumin) were mixed in 96 well white/clear bottom plate (Corning Inc., Acton, MA) with 0.05 nM (3-[^{125}I]-iodotyrosyl) Tyr10 glucagon (Amersham Biosciences, Piscataway, NJ), 1-6 micrograms per well, plasma membrane fragments prepared from cells over-expressing human glucagon receptor, and 1 mg/well polyethyleneimine-treated wheat germ agglutinin type A scintillation proximity assay beads (Amersham Biosciences, Piscataway, NJ). Upon 5 min shaking at 800 rpm on a rotary shaker, the plate was incubated 12h at room temperature and then read on MicroBeta1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Non-specifically bound (NSB) radioactivity was measured in the wells with 4 times greater concentration of "cold" native ligand than the highest concentration in test samples and total bound radioactivity was detected in the wells with no competitor. Percent specific binding was calculated as following: % Specific Binding = ((Bound-NSB)/(Total bound-NSB)) X 100. IC_{50} values were determined by using Origin software (OriginLab, Northampton, MA).

EXAMPLE 13

Functional Assay- cAMP Synthesis

The ability of glucagon analogs to induce cAMP was measured in a firefly luciferase-based reporter assay. HEK293 cells co-transfected with either glucagon- or GLP-1 receptor and luciferase gene linked to cAMP responsive element were serum deprived by culturing 16h in DMEM (Invitrogen, Carlsbad, CA) supplemented with 0.25% Bovine Growth Serum (HyClone, Logan, UT) and then incubated with serial dilutions of either glucagon, GLP-1 or novel glucagon analogs for 5 h at 37°C, 5% CO_2 in 96 well poly-D-Lysine-coated "Biocoat" plates (BD Biosciences, San Jose,

CA). At the end of the incubation 100 microliters of LucLite luminescence substrate reagent (Perkin-Elmer, Wellesley, MA) were added to each well. The plate was shaken briefly, incubated 10 min in the dark and light output was measured on MicroBeta-1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). The luminescent light output indicates activation of the luciferase reporter gene, which in turn is a measure of the activation of the receptor. Effective 50% concentrations ("EC50") were calculated by using Origin software (OriginLab, Northampton, MA. Results are shown in Tables 2 and 3. EC50 is the concentration of the peptide that produces 50% of the peptide's maximum activation response at the indicated receptor. A relatively lower EC50 indicates that a peptide is relatively more potent at that receptor, while a higher EC50 indicates that a peptide is less potent.

Table 2

cAMP Induction by Glucagon Analogs with C-Terminus Extension

Peptide	cAMP Induction			
	Glucagon Receptor		GLP-1 Receptor	
	EC_{50}, nM	N*	EC_{50}, nM	N
Glucagon	0.22 ± 0.09	14	3.85 ±1.64	10
GLP-1	2214.00 ± 182.43	2	0.04 ± 0.01	14
Glucagon Cex	0.25 ± 0.15	6	2.75 ± 2.03	7
Oxyntomodulin	3.25 ± 1.65	5	2.53 ± 1.74	5
Oxyntomodulin KRNR	2.77 ± 1.74	4	3.21 ± 0.49	2
Glucagon R12	0.41 ± 0.17	6	0.48 ± 0.11	5
Glucagon R12 Cex	0.35 ± 0.23	10	1.25 ± 0.63	10
Glucagon R12 K20	0.84 ± 0.40	5	0.82 ± 0.49	5
Glucagon R12 K24	1.00 ± 0.39	4	1.25 ± 0.97	5
Glucagon R12 K29	0.81 ± 0.49	5	0.41 ± 0.24	6
Glucagon Amide	0.26 ± 0.15	3	1.90 ± 0.35	2
Oxyntomodulin C24	2.54 ± 0.63	2	5.27 ± 0.26	2
Oxyntomodulin C24 PEG 20K	0.97 ± 0.04	1	1.29 ± 0.11	1

* - number of experiments

Table 3

cAMP Induction by Pegylated Glucagon Analogs

Peptide	cAMP Induction			
	Glucagon Receptor		GLP-1 Receptor	
	EC_{50}, nM	N*	EC_{50}, nM	N
Glucagon	0.33 ± 0.23	18	12.71 ±3.74	2
Glucagon C17 PEG 5K	0.82 ± 0.15	4	55.86 ± 1.13	2
Glucagon C21 PEG 5K	0.37 ± 0.16	6	11.52 ± 3.68	2
Glucagon C24 PEG 5K	0.22 ± 0.10	12	13.65 ± 2.95	4
Glucagon C29 PEG 5K	0.96 ± 0.07	2	12.71 ± 3.74	2
Glucagon C24 PEG 20K	0.08 ± 0.05	3	Not determined	
Glucagon C24 Dimer	0.10 ± 0.05	3	Not determined	
GLP-1	> 1000		0.05 ± 0.02	4

* - number of experiments

Data for additional glucagon analogs is presented in Figs. 6-9 and in Table 4

Table 4

Observed EC50s (nM) in Cells Overexpressing the Glucagon Receptor

	Test 1	Test 2	Test 3	Test 4
Glucagon standard	0.12	0.04	0.05	0.11
K29	0.35			0.22
K30	0.22	0.06		
K29, K30	0.89			
K30, K31		0.12		
D28		0.05		0.17
E28			0.14	
E29		0.05	0.04	
E30		0.04		
D28, E29			0.03	
D28, E30			0.05	
D28, E15			0.15	

Figure 11 displays data on cAMP induction at the glucagon and GLP-1 receptors for a glucagon analog with the following modifications: T16,A20,E21,A24,Nle27,D28, and

E29 (SEQ ID NO: 56). The data shows that a glucagon analog containing multiple modifications (seven substitutions) retains substantial glucagon activity.

EXAMPLE 14

Stability Assay for glucagon Cys-maleimido PEG analogs

Each glucagon analog was dissolved in water or PBS and an initial HPLC analysis was conducted. After adjusting the pH (4, 5, 6, 7), the samples were incubated over a specified time period at 37°C and re-analyzed by HPLC to determine the integrity of the peptide. The concentration of the specific peptide of interest was determined and the percent remaining intact was calculated relative to the initial analysis. Results for Glucagon Cys^{21}-maleimidoPEG$_{5K}$ are shown in Figs. 1 and 2.

EXAMPLE 15

Synthesis of Glucagon Lactams

285 mg (0.2 mmole) methoxybenzhydrylamine resin (Midwest Biotech) was added to a 60 mL reaction vessels and the following sequence was assembled on a modified Applied Biosystems 430A peptide synthesizer using Boc DEPBT-activated single couplings.

The following side chain protecting groups were used: Arg(Tos), Asp(OcHx), Asn(Xan), Glu(OFm), His(BOM), Lys(Fmoc), Ser(Bzl), Thr(Bzl), Trp(CHO), Tyr(Br-Z). As an example, Lys(Cl-Z) was used to protect the native Lys at position 12 if lactams were constructed from 16-20, 20-24, or 24-28. The completed peptidyl resin was treated with 20% piperidine/dimethylformamide for one hour with rotation to remove the Trp formyl group as well as the Fmoc and OFm protection from Lys12 and Glu16. Upon confirmation of removal by a positive ninhydrin test, the resin was washed with dimethylformamide, followed by dichloromethane and than again with dimethylformamide. The resin was treated with 520 mg (1 mmole) Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in dimethylformamide and diisopropylethylamine (DIEA). The reaction proceeded for 8-10 hours and the cyclization was confirmed by a negative ninhydrin reaction. The resin was washed with dimethylformamide, followed by dichloromethane and subsequently treated with trifluoroacetic acid for 10 minutes. The removal of the Boc

group was confirmed by a positive ninhydrin reaction. The resin was washed with dimethylformamide and dichloromethane and dried before being transferred to a hydrofluoric acid (HF) reaction vessel. 500 µL p-cresol was added along with a magnetic stir bar. The vessel was attached to the HF apparatus (Peninsula Labs), cooled in a dry ice/methanol bath, evacuated, and approximately 10 mL of liquid hydrofluoric acid was condensed into the vessel. The reaction was stirred for 1 hour in an ice bath and the HF was subsequently removed in vacuo. The residue was suspended in ethyl ether; the solids were filtered, washed with ether, and the peptide was solubilized with 150 mL 20% acetonitrile/1% acetic acid.

An analytical HPLC analysis of the crude solubilized peptide was conducted under the following conditions [4.6 X 30 mm Xterra C8, 1.50 mL/min, 220 nm, A buffer 0.1% TFA/10% ACN, B buffer 0.1% TFA/100% ACN, gradient 5-95%B over 15 minutes]. The extract was diluted twofold with water and loaded onto a 2.2 X 25 cm Vydac C4 preparative reverse phase column and eluted using an acetonitrile gradient on a Waters HPLC system (A buffer of 0.1% TFA/10% ACN, B buffer of 0.1% TFA/10% CAN and a gradient of 0-100% B over 120 minutes at a flow of 15.00 ml/min. HPLC analysis of the purified peptide demonstrated greater than 95% purity and electrospray ionization mass spectral analysis confirmed a mass of 3506 Da for the 12-16 lactam. Lactams from 16-20, 20-24, and 24-28 were prepared similarly.

EXAMPLE 16

Exemplary peptides

Glucagon peptides with the following sequences were constructed generally as described herein:

XSQGTFTSDYSKYLDERRAKDFVC*WLMNT (lactam @ 16-20) (SEQ ID NO: 40)

Wherein X=DMIA, C* is Cys linked to PEG

XSQGTFTSDYSKYLDERRAKDFVAWLMNC* (lactam @ 16-20) (SEQ ID NO: 41)

Wherein X=DMIA, Q24A, C* is Cys linked to PEG

X1SQGTFTSDYSKYLDERRAKDFVC*WLX2NT (lactam @ 16-20) (SEQ ID NO: 42)

Wherein X1=DMIA, X2 is Nle or Leu, C* is Cys linked to PEG X1SQGTFTSDYSKYLDERRAKDFVAWLX2NC* (lactam @ 16-20) (SEQ ID NO: 43)

Wherein X1=DMIA, Q24A, X2 is Nle or Leu, C* is Cys linked to PEG

Glucagon peptides with the following modifications from SEQ ID NO: 1 was constructed generally as described herein:

A20,A24,Nle27,D28 (SEQ ID NO: 44)

A20,A24,Nle27,D28,E29 (SEQ ID NO: 45)

A20,A24,Nle27,D28,E30 (SEQ ID NO: 46)

A20,A24,Nle27,E28,E29 (SEQ ID NO: 47)

A20,A24,Nle27,E28,E30 (SEQ ID NO: 48)

A20,A24,Nle27,E29,E30 (SEQ ID NO: 49)

A20,E21,A24,Nle27,D28 (SEQ ID NO: 50)

A20,E21,A24,Nle27,D28,E29 (SEQ ID NO: 51)

A20,E21,A24,Nle27,D28,E30 (SEQ ID NO: 52)

A20,E21,A24,Nle27,E28,E29 (SEQ ID NO: 53)

A20,E21,A24,Nle27,E28,E30 (SEQ ID NO: 54)

A20,E21,A24,Nle27,E29,E30 (SEQ ID NO: 55)

Alternatively, any of these peptides may comprise AIB20 and/or AIB24 instead of the A20 and/or A24 substitutions.

Any of these peptides may further comprise a T16 or AIB16 amino acid substitution. For example, T16,A20,E21,A24,Nle27,D28,E29 (SEQ ID NO: 56) was constructed.

A glucagon peptide with the following modifications from SEQ ID NO: 1 was constructed generally as described herein: DMIA1, E16, K20-glucagon-COOH (C24-PEG, E16 to K20 lactam). Its sequence is set forth below: DMIA-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Cys*-Trp-Leu-Met-Asn-Thr-COOH, in which the Cys* at position 24 was Cys attached to a PEG of about 40,000 dalton molecular weight, and in which the Glu at position 16 and the Lys at position 20 were linked via a lactam bridge (SEQ ID NO: 40). This peptide was tested for activity at the glucagon and

GLP-1 receptors generally according to Example 13. The peptide exhibited 27.7% of the potency of native glucagon and 1.1% of the potency of native GLP-1.

EXAMPLE 17

Preparation of acylated and/or PEGylated peptides

Acylated and/or PEGylated peptides are prepared as follows. Peptides are synthesized on a solid support resin using either a CS Bio 4886 Peptide Synthesizer or Applied Biosystems 430A Peptide Synthesizer. In situ neutralization chemistry is used as described by Schnolzer et al., *Int. J. Peptide Protein Res.* 40: 180-193 (1992). For acylated peptides, the target amino acid residue to be acylated (e.g., position ten) is substituted with an N ε -FMOC lysine residue. Treatment of the completed N-terminally BOC protected peptide with 20% piperidine in DMF for 30 minutes removes FMOC/formyl groups. Coupling to the free ε-amino Lys residue is achieved by coupling a ten-fold molar excess of either an FMOC-protected spacer amino acid (ex. FMOC-(N-BOC)-Tryptophan-OH) or acyl chain (ex. C17-COOH) and PyBOP or DEPBT coupling reagent in DMF/DIEA. Subsequent removal of the spacer amino acid's FMOC group is followed by repetition of coupling with an acyl chain. Final treatment with 100% TFA results in removal of any side chain protecting groups and the N-terminal BOC group. Peptide resins are neutralized with 5% DIEA/DMF, are dried, and then are cleaved from the support using HF/p-cresol, 95:5, at 0°C for one hour. Following ether extraction, a 5% HOAc solution is used to solvate the crude peptide. A sample of the solution is then verified to contain the correct molecular weight peptide by ESI-MS. Correct peptides are purified by RP-HPLC using a linear gradient of 10% CH3CN/0.1% TFA to 0.1% TFA in 100% CH3CN. A Vydac C18 22 mm x 250 mm protein column is used for the purification. Acylated peptide analogs generally complete elution by a buffer ratio of 20:80. Portions are pooled together and checked for purity on an analytical RP-HPLC. Pure fractions are lyophilized yielding white, solid peptides.

If a peptide comprises a lactam bridge and target residues to be acylated, acylation is carried out as described above upon addition of that amino acid to the peptide backbone.

For peptide pegylation, 40 kDa methoxy poly(ethylene glycol) maleimido-propionamide (Chirotech Technology Ltd.) is reacted with a molar equivalent of

peptide in 7M Urea, 50mM Tris-HCl buffer using the minimal amount of solvent needed to dissolve both peptide and PEG into a clear solution (generally less than 2 mL for a reaction using 2-3 mg peptide). Vigorous stirring at room temperature commences for 4-6 hours and the reaction is analyzed by analytical RP-HPLC. PEGylated products appear distinctly from the starting material with decreased retention times. Purification is performed on a Vydac C4 column with conditions similar to those used for the initial peptide purification. Elution typically occurs around buffer ratios of 50:50. Fractions of pure PEGylated peptide are collected and lyophilized.

Peptides are assayed for biological activity as described above in Example 13.

EXAMPLE 18

Changes in Serum Glucose Concentration in Beagle Dogs after Administration of Glucagon Analogs

Canine/Beagle dogs of 8-12 kg, being of 8-16 months of age and good health were used to determine the pharmacokinetics and pharmacodynamics of glucagon action. Every animal was fasted overnight and bled at the following time points after each dose: 0 hr. (pre-dose), 5, 10, 20, 30, 45, 60, 90, 120, 240 minutes post dose. Six animals were used for each dose group and approximately 1-2ml of whole blood was withdrawn at each time point. About 1.0 ml whole blood was added to K_2 EDTA tubes containing a sufficient volume of Trasylol (aprotinin) to yield at least 500 KIU/mL of whole blood. Approximately 500 uL plasma was collected after centrifuging the samples in a refrigerated centrifuge at about 1,500 – 3,000 x g for 10-15 min. The samples were transferred to plastic vials and stored frozen at -70°C, or below. The remaining 1.0 mL of whole blood was converted into serum by placing blood sample into an empty tube, letting sit at ambient temperature for 15-20 min, then centrifuging at 1,500 – 3,000 x g for 10-15 min. in a refrigerated centrifuge. The samples were transferred to plastic vials and stored frozen at -70°C, or below. Glucagon and the analogs were dissolved in 0.01N HCl at a concentration of 0.1667 mg/ml and the animals were dosed at 0.03 ml/kg.

The animals were administered a 0.005 mg/kg dose intramuscularly of either glucagon, a glucagon analog comprising glucagon with the sequence of SEQ ID NO: 31 linked to the carboxy terminus of glucagon (glucagon-CEX) or a glucagon analog

comprising an aspartic acid substitution at amino acid 28 (glucagon-Asp28) SEQ ID NO: 11. The resulting data is presented in Fig. 10.

EXAMPLE 19

The following peptides were made as essentially described above:

(A) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with an amino acid substitution at position 16 with AIB ("AIB 16 Glucagon");

(B) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with an amino acid substitution at position 19 with AIB ("AIB 19 Glucagon");

(C) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with an amino acid substitution at position 20 with AIB ("AIB 20 Glucagon");

(D) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with an amino acid substitution at position 21 with AIB ("AIB 21 Glucagon");

(E) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with an amino acid substitution at position 24 with AIB ("AIB 24 Glucagon");

(F) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with amino acid substitutions at positions 16 and 20 with AIB ("AIB 16, 20 Glucagon");

(G) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with amino acid substitutions at positions 16 and 24 with AIB ("AIB 16, 24 Glucagon");

(H) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with amino acid substitutions at positions 20 and 24 with AIB ("AIB 20, 24 Glucagon"); and

(I) A peptide comprising the amino acid sequence of SEQ ID NO: 1 with amino acid substitutions at positions 16, 20, and 24 with AIB ("AIB 16, 20, 24 Glucagon").

The peptides were assayed as essentially described in Example 13 and the results are shown in Table 5.

TABLE 5

Peptide	Glucagon Receptor			GLP-1 Receptor		
	Average	STDev	n	Average	STDev	n
GLP-1	**2878.03**	**2510.39**	7	**0.05**	**0.02**	#
Glucagon	**0.14**	**0.06**	14	**13.70**	**4.26**	#
AIB 16 Glucagon	**0.37**	**0.05**	2	**32.43**	**8.63**	4
AIB 19 Glucagon	**13.52**	**0.64**	3	**64.75**	**11.38**	4
AIB 20 Glucagon	**0.32**	**0.14**	6	**7.88**	**0.36**	2
AIB 21 Glucagon	**0.55**	**0.17**	6	**5.92**	**1.83**	2
AIB 24 Glucagon	**0.22**	**0.02**	3	**23.92**	**7.20**	4
AIB 16,20 Glucagon	**0.26**	**0.06**	2	**13.62**	**8.92**	2
AIB 16,24 Glucagon	**0.18**	**0.01**	2	**31.81**	**-**	1
AIB 20,24 Glucagon	**0.34**	**0.15**	2	**10.59**	**1.95**	2
AIB 16,20,24 Glucagon	**0.42**	**0.18**	2	**12.15**	**3.77**	2

EXAMPLE 20

The following glucagon analog peptides were made as essentially described herein:

"D28/E29 Glucagon" comprising the amino acid sequence of SEQ ID NO: 1 modified to comprise Asp at position 28 and Glu at position 29:

HSQGTFTSDYSKYLDSRRAQDFVQWLMDE

(SEQ ID NO: 75); and

"AIB16/D28/E29 Glucagon" comprising the amino acid sequence of SEQ ID NO: 75 further modified to comprise Aib at position 16:

HSQGTFTSDYSKYLDAibRRAQDFVQWLMDE (SEQ ID NO: 76).

The *in vitro* activity of each peptide at the glucagon receptor was tested as described in Example 13. The EC50 of the D28/E29 Glucagon peptide was 0.06 nM, while that of the AIB16/D28/E29 Glucagon peptide was 0.08 nM.

EXAMPLE 21

Peptides of Set A, each comprising the amino acid sequence of SEQ ID NO: 1 with the modifications listed in Table 6, are made as essentially described herein.

TABLE 6

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Gln	AIB	Gln	Gln	Met	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Gln	Gln	Met	Asn	Thr	CEX	Present
Gln	AIB	Ala	Ala	Met	Asn	Thr	CEX	Present
Gln	AIB	Ser	Ser	Met	Asn	Thr	CEX	Present
Gln	AIB	Thr	Thr	Met	Asn	Thr	CEX	Present
Gln	AIB	Gln	Gln	Leu	Asn	Thr	CEX	Present
Gln	AIB	Gln	Gln	Nle	Asn	Thr	CEX	Present
Gln	AIB	Gln	Gln	Met	Asp	Thr	CEX	Present
Gln	AIB	Gln	Gln	Met	Asn	Glu	CEX	Present
Gln	AIB	Gln	Gln	Met	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Met	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Thr	CEX	Present

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Glu	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Thr	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Glu	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Met	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Thr	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Glu	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Thr	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Glu	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Met	Asn	Thr	CEX	Present

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Thr	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Glu	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Thr	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Thr	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Glu	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Glu	CEX	Present
Gln	AIB	Gln	Gln	Met	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Gln	Gln	Met	Asn	Gly	CEX	Present
Gln	AIB	Ala	Ala	Met	Asn	Gly	CEX	Present
Gln	AIB	Ser	Ser	Met	Asn	Gly	CEX	Present
Gln	AIB	Thr	Thr	Met	Asn	Gly	CEX	Present
Gln	AIB	Gln	Gln	Leu	Asn	Gly	CEX	Present
Gln	AIB	Gln	Gln	Nle	Asn	Gly	CEX	Present
Gln	AIB	Gln	Gln	Met	Asp	Gly	CEX	Present

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Ala	Ala	Met	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Gly	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Gly	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Met	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Gly	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Gly	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Met	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Gly	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Gly	CEX	Present
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Gly	CEX	Present
Gln	AIB	Gln	Gln	Met	Asn	Thr	n/a	Absent

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Gln	Gln	Met	Asn	Thr	n/a	Absent
Gln	AIB	Ala	Ala	Met	Asn	Thr	n/a	Absent
Gln	AIB	Ser	Ser	Met	Asn	Thr	n/a	Absent
Gln	AIB	Thr	Thr	Met	Asn	Thr	n/a	Absent
Gln	AIB	Gln	Gln	Leu	Asn	Thr	n/a	Absent
Gln	AIB	Gln	Gln	Nle	Asn	Thr	n/a	Absent
Gln	AIB	Gln	Gln	Met	Asp	Thr	n/a	Absent
Gln	AIB	Gln	Gln	Met	Asn	Glu	n/a	Absent
Gln	AIB	Gln	Gln	Met	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Met	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Thr	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Glu	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Thr	n/a	Absent

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Glu	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Met	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Thr	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Glu	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Thr	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Glu	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Met	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Thr	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Glu	n/a	Absent

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Thr	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Thr	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Glu	n/a	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Glu	n/a	Absent
Gln	AIB	Gln	Gln	Met	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Gln	Gln	Met	Asn	Thr	Glu	Absent
Gln	AIB	Ala	Ala	Met	Asn	Thr	Glu	Absent
Gln	AIB	Ser	Ser	Met	Asn	Thr	Glu	Absent
Gln	AIB	Thr	Thr	Met	Asn	Thr	Glu	Absent
Gln	AIB	Gln	Gln	Leu	Asn	Thr	Glu	Absent
Gln	AIB	Gln	Gln	Nle	Asn	Thr	Glu	Absent
Gln	AIB	Gln	Gln	Met	Asp	Thr	Glu	Absent
Gln	AIB	Gln	Gln	Met	Asn	Glu	Glu	Absent
Gln	AIB	Gln	Gln	Met	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Met	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Thr	Glu	Absent

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Leu	Asn	Glu	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Ala	Ala	Nle	Asn	Glu	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Met	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Leu	Asn	Glu	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Ser	Ser	Nle	Asn	Glu	Glu	Absent

Amino Acid Substitution								C-terminal Extension-NH_2 (SEQ ID NO: 20)**
3	16	20	24	27	28	29	30*	
Dab(Ac)	AIB	Thr	Thr	Met	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Leu	Asn	Glu	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Thr	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Thr	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asp	Glu	Glu	Absent
Dab(Ac)	AIB	Thr	Thr	Nle	Asn	Glu	Glu	Absent

Peptides of a second set (Set B) are made with the same structures of the peptides of Set A, except that the peptides of Set B comprise a Cys at position 24 wherein the Cys residue is covalently attached to a 40 kDa PEG.

The peptides of Sets A and B are tested for *in vitro* activity at the glucagon receptor as essentially described in Example 13.

EXAMPLE 22

The following glucagon analog peptides comprising a backbone of Peptide J

HS-X-GTFTSDYSKYLDTRRAAEFVAWL(Nle)DE

(SEQ ID NO: 59)

or Peptide K

HS-X-GTFTSDYSKYLD(Aib)RRAADFVAWLMDE

(SEQ ID NO: 60)

with additional modification at position 3 were made by solid-phase peptide synthesis as essentially described herein. The peptides were tested for *in vitro* activity at the glucagon receptor as essentially described in Example 13. The EC50 (nM) of each peptide is shown in Table 7.

TABLE 7

Peptide Backbone	**Amino Acid at Position 3**	**SEQ ID NO:**	**EC_{50} at Glucagon Receptor (nM)**	**% activity***
J	Q	61	0.24	25%
J	C(Acm)	62	0.18	33%
J	Dab(Ac)	63	0.31	19%
J	Dap(urea)	64	0.48	13%
J	Q(Me)	65	0.48	13%
J	M(O)	66	0.91	7%
J	Orn(Ac)	67	0.92	7%
K	Q	68	0.39	15%
K	Dab(Ac)	69	0.07	86%
K	Q(Me)	70	0.11	55%

Q = glutamine; C(Acm) = acetamidomethyl-cysteine; Dab(Ac) = acetyldiaminobutanoic acid; Dap(urea) = carbamoyldiaminopropanoic acid; Q(Me) = methylglutamine; M(O) = methionine-sulfoxide; Orn(Ac) = acetylornithine.

As shown in Table 7, multiple amino acids could be placed at position 3 without a substantial loss of activity at the glucagon receptor, and, in some cases, the

modification actually increased the activity, e.g., Dab(Ac) and Q(Me) on the Peptide K backbone.

EXAMPLE 23

Glucagon analog peptides comprising Dab(Ac) at position 3 on various glucagon analog backbones were made as essentially described herein and the *in vitro* activity at the glucagon receptor was tested. The structures and activities of each peptide are shown in Table 8.

TABLE 8

Amino acid sequence	**SEQ ID NO:**	**EC_{50} (nM) at Glucagon Receptor**	**% activity***
Wildtype Glucagon	1	0.026	100
HSQGTFTSDYSKYLDSRRAQDFVQWLMDT	78	0.015	173
HSDab(Ac)GTFTSDYSKYLDAibRRAADFVAWLLDE	71	0.069	37
HSDab(Ac)GTFTSDYSKYLDAibRRAADFVAWLLDTGPSSGAPPPS amide	72	0.023	113
HSDab(Ac)GTFTSDYSKYLDAibRRASDFVSWLLDE	73	0.048	54
HSDab(Ac)GTFTSDYSKYLDAibRRATDFVTWLLDE	74	0.057	46

EXAMPLE 24

Peptides of a first set (Set A) comprising the amino acid sequence of SEQ ID NO: 1 with the modifications shown in Table 9 are made as essentially described herein:

TABLE 9

Amino acid at Position 3	Amino acid at Position 7	Amino acid at position 16	Position of Acylated Lys	Fatty acyl group attached to Lys	Amino acid at Position 28	Amino acid at Position 29	C-terminal extension (SEQ ID NO: 20)
Gln	Ile	AIB	10	C14	Asp	Thr	Absent
Gln	Ile	AIB	10	C14	Asn	Glu	Absent
Gln	Ile	AIB	10	C14	Asp	Glu	Absent
Gln	Ile	AIB	10	C14	Glu	Thr	Absent
Gln	Ile	AIB	10	C14	Glu	Glu	Absent
Gln	Ile	AIB	10	C14	Asp	Gly	Present
Dab(Ac)	Ile	AIB	10	C14	Asp	Thr	Absent
Dab(Ac)	Ile	AIB	10	C14	Asn	Glu	Absent
Dab(Ac)	Ile	AIB	10	C14	Asp	Glu	Absent
Dab(Ac)	Ile	AIB	10	C14	Glu	Thr	Absent
Dab(Ac)	Ile	AIB	10	C14	Glu	Glu	Absent
Dab(Ac)	Ile	AIB	10	C14	Asp	Gly	Present
Gln	Ile	AIB	30	C14	Asp	Thr	Absent
Gln	Ile	AIB	30	C14	Asn	Glu	Absent
Gln	Ile	AIB	30	C14	Asp	Glu	Absent
Gln	Ile	AIB	30	C14	Glu	Thr	Absent
Gln	Ile	AIB	30	C14	Glu	Glu	Absent
Gln	Ile	AIB	40	C14	Asp	Gly	Present
Dab(Ac)	Ile	AIB	30	C14	Asp	Thr	Absent
Dab(Ac)	Ile	AIB	30	C14	Asn	Glu	Absent

Amino acid at Position 3	Amino acid at Position 7	Amino acid at position 16	Position of Acylated Lys	Fatty acyl group attached to Lys	Amino acid at Position 28	Amino acid at Position 29	C-terminal extension (SEQ ID NO: 20)
Dab(Ac)	Ile	AIB	30	C14	Asp	Glu	Absent
Dab(Ac)	Ile	AIB	30	C14	Glu	Thr	Absent
Dab(Ac)	Ile	AIB	30	C14	Glu	Glu	Absent
Dab(Ac)	Ile	AIB	40	C14	Asp	Gly	Present
Gln	Thr	AIB	10	C14	Asn	deleted	Absent
Gln	Thr	AIB	10	C14	deleted	deleted	Absent
Dab(Ac)	Thr	AIB	10	C14	Asn	deleted	Absent
Dab(Ac)	Thr	AIB	10	C14	deleted	deleted	Absent

Peptides comprising the same structure of Set A, except that the acyl group is a C16 or a C18 fatty acyl group are made, as essentially described herein. The peptides comprising a C16 fatty acyl group comprised the peptides of Set B, while the peptides comprising a C18 fatty acyl group comprised the peptides of Set C.

Peptides of Set D comprising the same structures of the peptides of Sets A, B, and C, except that the fatty acyl groups are covalently attached to the side chain of the Lys residue at the indicated position via one of the following spacers, are made as described herein: γ-Glu-γ-Glu; β-Ala-β-Ala, Ala-Ala, 6-aminohexanoic acid, Leu-Leu, and Pro-Pro.

The peptides of Sets A-D are tested for *in vitro* activity at the glucagon receptor as described in Example 13 and the EC50 of each are compared to the activity of native glucagon at the glucagon receptor.

EXAMPLE 25

Several glucagon analogs lacking a covalent intramolecular bridge and comprising an AIB at position 2, an AIB at position 16, and a fatty acyl group attached via a spacer to a Lys residue at position 10 were made as essentially

described herein. The acylated glucagon analogs differed by the type of spacer, the presence or absence of pegylation, and/or by the size of the acyl group. The acylated glucagon analogs were tested for *in vitro* activity at the glucagon receptor and the GLP-1 receptor as essentially described in Example 13. A summary of the structure and *in vitro* activity at the glucagon and GLP-1 receptors of each peptide is shown in Tables 10 and 11.

TABLE 10

Glucagon analog backbone amino acid sequence: HXQGTFTSDKSKYLDXRRAQDFVQWLMNT-NH_2 wherein X = AIB (SEQ ID NO: 95)					
Peptide Name	SEQ ID NO:	Spacer	Size of Fatty Acyl Group	EC50 at Glucagon Receptor (nM)	EC50 at GLP-1 Receptor (nM)
wt glucagon	1	n/a	n/a	0.031 ± 0.014	
wt GLP-1		n/a	n/a		0.036 ± 0.010
26	96	None	None	0.653 ± 0.285	0.475 ± 0.046
50	97	None	C16	0.572 ± 0.084	0.291 ± 0.060
82	98	Ala-Ala	C16	0.024 ± 0.001	0.108 ± 0.018
83	99	γ-Glu- γ-Glu	C16	0.014 ± 0.002	0.043 ± 0.005
84	100	β-Ala-β-Ala	C16	0.011	0.004
85	101	6-amino-hexanoic acid	C16	0.010	0.005
86	102	Leu-Leu	C16	0.011	0.006
87	103	Pro-Pro	C16	0.017	0.009
77*	104	None	C14	21.94 ± 14.47	1.458 ± 0.132
78*	105	γ-Glu- γ-Glu	C14	0.319 ± 0.091	0.103 ± 0.023
81*	107	Ala-Ala	C14	0.597 ± 0.175	0.271 ± 0.019
79*	109	Ala-Ala	C16	0.102 ± 0.011	0.055 ± 0.001
80*	110	γ-Glu- γ-Glu	C16	0.108 ± 0.028	0.042 ± 0.008

* indicates that the peptide comprised a Cys residue at position 24 (in place of Gln) which Cys was covalently attached to a 40 kDa PEG group.

TABLE 11

Glucagon analog backbone amino acid sequence: HXQGTFTSDKSKYLDXRRAQDFVWLMNT-NH_2 wherein X = AIB (SEQ ID NO: 562)					
Peptide Name	SEQ ID NO:	Spacer	Size of Fatty Acyl Group	EC50 at Glucagon Receptor (nM)	EC50 at GLP-1 Receptor (nM)
wt glucagon	1	n/a	n/a	0.008 ± 0.003	
wt GLP-1		n/a	n/a		0.004 ± 0.001
77**	111	none	C14	0.144 ± 0.029	0.063 ± 0.012
78**	112	γ-Glu- γ-Glu	C14	0.009 ± 0.001	0.008 ± 0.001
81**	113	Ala-Ala	C14	0.027 ± 0.006	0.018 ± 0.001
80**	114	γ-Glu- γ-Glu	C16	0.006 ± 0.001	0.008 ± 0.001
79**	115	Ala-Ala	C16	0.010 ± 0.001	0.008 ± 0.001

** peptide comprising Cys at position 24 (in place of Gln) which Cys was not covalently attached to a PEG molecule

As shown in Tables 10 and 11, the peptides comprising a fatty acyl group attached via a spacer significantly increased their potency as compared to peptides comprising a fatty acyl group attached directly to the peptide backbone.

EXAMPLE 26

The glucagon analog peptides of SEQ ID NOs: 71, 76, and 78 were assayed for stability. All peptides comprised an Asp at position 28 and the peptides of SEQ ID NOs: 71 and 76 additionally comprised Glu at position 29. While the peptide of SEQ ID NO: 78 did not comprise any additional modifications, both the peptides of SEQ ID NOs: 71 and 76 comprised an AIB at position 16. The peptide of SEQ ID

NO: 71 furthermore comprised a Dab(Ac) at position 3, an Ala at positions 20 and 24, and a Leu at position 27.

The peptides were formulated in a solution at a peptide concentration of 1 mg/mL. Syringes were filled with one of the peptide solutions and adjusted to minimize contact with air. The syringes were maintained at 4, 25, 30, or 40 degrees Celsius. Analytical RP-HPLC (reverse phase-high performance liquid chromatography) was used to monitor potential chemical degradation at 0, 1, 2, 4, and 6 months with a UV detector at 280 nm. SEC (size exclusion chromatography) was used to assess the formation of any aggregates at 0, 1, 2, 4, and 6 months with a UV detector at 280 nm.

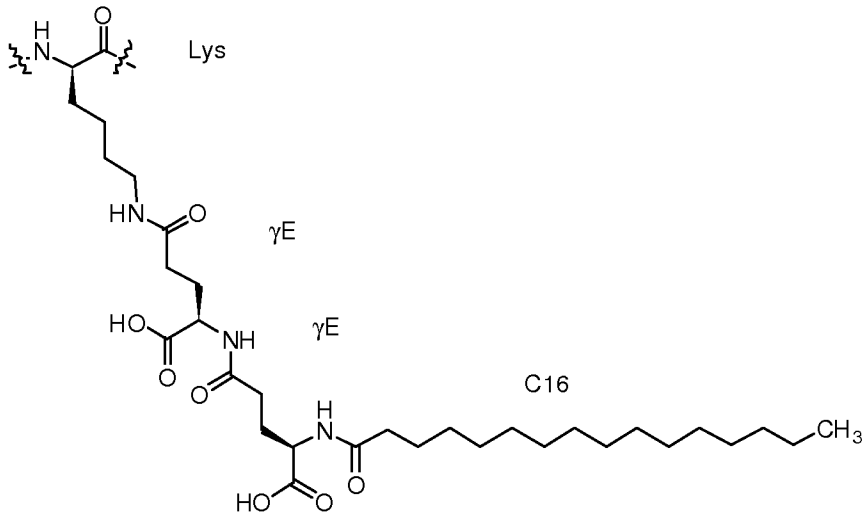
The integrated area of the peak based on UV absorbance of the peptides of SEQ ID NOs: 71, 76, and 78 as a function of time are shown in Figures 12-14, respectively. As shown in these figures, the stability of the peptides was greatest for the peptide of SEQ ID NO: 71 and was least for the peptide of SEQ ID NO: 78, although 90% of the peptide at 30 deg. C was detected after 2 months time. No significant loss in peak area was observed with SEQ ID NO: 71, indicating good chemical and biophysical stabilities of the peptide.

EXAMPLE 27

DIO mice (8 mice per group), each with an average bodyweight of 48.7 g, were subcutaneously injected daily for seven days with vehicle only, with 30 nmol/kg or 100 nmol/kg of an acylated glucagon analog peptide, or with the long-acting GLP-1 analog, Liraglutide (Novo Nordisk, Denmark). The acylated glucagon analogs were as follows:

"(C16) Glucagon Amide" comprised the amino acid sequence of wild-type glucagon (SEQ ID NO: 1) with the Tyr at position 10 modified to an acylated Lys residue, wherein the acylated Lys comprised a C16 fatty acyl group, and the C-terminal carboxylate replaced with an amide group ;

"γE-γE-C16 Glucagon Amide" comprised the same structure of C16 Glucagon Amide, except that the C16 fatty acyl group was attached to the Lys at position 10 through a gamma-Glu-gamma-Glu dipeptide spacer (see structure of acylated Lys below);



"AA-C16 Glucagon Amide" comprised the same structure of C16 Glucagon Amide, except that the C16 fatty acyl group was attached to the Lys at position 10 through an Ala-Ala dipeptide spacer; and

"βAβA-C16 Glucagon Amide" comprised the same structure of C16 Glucagon Amide, except that the C16 fatty acyl group was attached to the Lys at position 10 through an β-Ala-β-Ala dipeptide spacer.

The body weight of the mice was monitored daily and the total change in body weight (%) is shown in Figure 15. As shown in Figure 15, most of the acylated glucagon peptides at each dose caused a reduction in body weight. While Liraglutide demonstrated an approximate 12% decrease in body weight, the glucagon analog peptide γE-γE -C16 Glucagon Amide exhibited the greatest ability to cause weight loss in mice at the matched dose. Even the lower dose of γE-γE -C16 Glucagon Amide caused a substantial decrease in body weight.

The fat mass of the mice was measured on Day 7 of the study. As shown in Figure 16, the mice which were administered 100 nmol/kg γE-γE -C16 Glucagon Amide exhibited the lowest fat mass.

Blood glucose levels of the mice were also monitored during the course of the assay. As shown in Figure 17, the glucagon analog peptide γE-γE -C16 Glucagon Amide at the higher dose worked as well as Liraglutide to decrease blood glucose levels in mice.

EXAMPLE 28

Glucagon analog peptides were made by solid-phase peptide synthesis as described herein and were acylated at either position 10 or 30 of the peptide. The peptides and their structure were as follows:

"Peptide dS2E16K20K30-C14 Gluc Amide" comprised the amino acid sequence HXQGTFTSDYSKYLDERRAKDFVQWLMNTK-amide (SEQ ID NO: 79), wherein the X at position 2 is d-Ser, wherein the Lys at position 30 is acylated with a C14 fatty acyl group, and the C-terminal carboxylate is replaced with an amide;

"Peptide dS2K10(C14)E16K20-Gluc Amide" comprised the amino acid sequence HXQGTFTSDKSKYLDERRAKDFVQWLMNT-amide (SEQ ID NO: 80); wherein the X at position 2 is d-Ser, wherein the Lys at position 10 is acylated with a C14 fatty acyl group, and the C-terminal carboxylate is replaced with an amide;

"Peptide dS2E16K20K30-C16 Gluc Amide" comprised the amino acid sequence HXQGTFTSDYSKYLDERRAKDFVQWLMNTK-amide (SEQ ID NO: 81), wherein the X at position 2 is d-Ser, wherein the Lys at position 30 is acylated with a C16 fatty acyl group, and the C-terminal carboxylate is replaced with an amide;

"Peptide dS2K10(C16)E16K20-Gluc Amide" comprised the amino acid sequence HXQGTFTSDKSKYLDERRAKDFVQWLMNT-amide (SEQ ID NO: 82); wherein the X at position 2 is d-Ser, wherein the Lys at position 10 is acylated with a C16 fatty acyl group, and the C-terminal carboxylate is replaced with an amide;

"Peptide Chimera 2-AIB2-K10-acylated" comprised the amino acid sequence HXQGTFTSDKSKYLDEQAAKEFICWLMNT-amide (SEQ ID NO: 83); wherein the X at position 2 is AIB, the K at position 10 is acylated with a C18 fatty acyl group, Cys at position 24 comprises a 40 kDa PEG molecule, and the C-terminal carboxylate is replaced with an amide; and

"Peptide Chimera 2-AIB2-K30-acylated" comprised the amino acid sequence HXQGTFTSDYSKYLDEQAAKEFICWLMNTK-amide (SEQ ID NO: 84), wherein the X at position 2 is AIB, the K at position 30 is acylated with a C18 fatty acyl group, Cys at position 24 comprises a 40 kDa PEG molecule, and the C-terminal carboxylate is replaced with an amide.

The in vitro activity at the GLP-1 receptor and glucagon receptor of each peptide was tested as essentially described in Example 13. The results are shown in Table 12.

TABLE 12

Peptide Name	Position at which acyl group is found	EC50 at the glucagon receptor (nM)	EC50 at the GLP-1 receptor (nM)
Peptide dS2E16K20K30-C14 Gluc Amide	30	3.53	0.84
Peptide dS2K10(C14)E16K20-Gluc Amide	10	0.155	0.041
Peptide dS2E16K20K30-C16 Gluc Amide	30	4.89	3.05
Peptide dS2K10(C16)E16K20-Gluc Amide	10	0.076	0.041
Peptide Chimera 2-AIB2-K30-acylated	30	n/a	0.465
Peptide Chimera 2-AIB2-K10-acylated	10	n/a	0.007

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Claims:

1. A glucagon peptide with glucagon agonist activity, comprising (i) the amino acid sequence of SEQ ID NO: 1 with one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 substituted with an α,α-disubstituted amino acid.

2. The glucagon peptide of claim 1, comprising:

(a) amino isobutyric acid (AIB) at position 16 of the glucagon peptide,

(b) AIB at position 20 of the glucagon peptide,

(c) AIB at position 21 of the glucagon peptide,

(d) AIB at position 24 of the glucagon peptide,

(e) AIB at positions 16 and 20 of the glucagon peptide,

(f) AIB at positions 16 and 24 of the glucagon peptide,

(g) AIB at positions 20 and 24 of the glucagon peptide; or

(h) AIB at positions 16, 20, and 24 of the glucagon peptide.

3. The glucagon peptide of claim 2, comprising AIB at position 16 of the glucagon peptide.

4. An analog of the glucagon peptide of any of claims 1 to 3, comprising additional amino acid modifications, wherein the glucagon peptide comprises: (i) at least one charged amino acid C-terminal to the amino acid at position 27 of the glucagon peptide; (ii) a substitution of the amino acid at position 3 of the glucagon peptide with a glutamine analog; or (iii) both (i) and (ii).

5. The analog of the glucagon peptide of claim 4, comprising at least one amino acid modification selected from the group consisting of:

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;

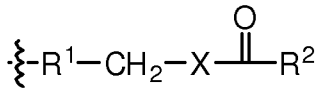
substitution at position 29 with Glu;

insertion of 1-3 charged amino acids after position 29;

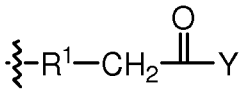
insertion after position 29 of Glu or Lys;

insertion after position 29 of Gly-Lys or Lys-Lys;

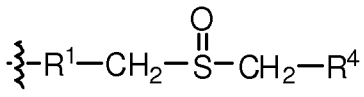
substitution of Gln at position 3 with an amino acid comprising a side chain of Structure I, II, or III:



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;

and a combination thereof.

6. The analog of claim 4 or 5, further comprising at least one amino acid modification selected from the group consisting of:

substitution of Gln at position 20 with Ala, Ser or Thr;

substitution of Gln at position 24 with Ala, Ser or Thr;

addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly;

and a combination thereof.

7. The analog of claim 6 comprising the amino acid sequence of any of SEQ ID NOs: 71-74.

8. The analog of any of claims 4 to 7, further comprising a hydrophilic moiety.

9. The analog of claim 8, wherein the hydrophilic moiety is covalently linked to any of amino acid positions 16, 17, 20, 21, 24, or 29, or at the C-terminal amino acid of the analog.

10. The analog of claim 8 or 9, wherein the hydrophilic moiety is covalently linked to Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine.

11. The analog of any of claims 8 to 10, wherein the hydrophilic moiety is a polyethylene glycol (PEG).

12. The analog of claim 11, wherein the PEG has a molecular weight of about 1,000 Daltons to about 40,000 Daltons.

13. The analog of claim 12, wherein the PEG has a molecular weight of about 20,000 Daltons to about 40,000 Daltons.

14. The analog of any of claims 4 to 13, further comprising (i) a substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), (ii) substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV), or (iii) both (i) and (ii).

15. The analog of claim 14, wherein the His at position 1 is substituted with D-His, N-methyl-His, alpha-methyl-His, imidazole acetic acid, des-amino-His, hydroxyl-His, acetyl-His, homo-His, or alpha, alpha-dimethyl imidiazole acetic acid (DMIA).

16. The analog of claim 15, wherein the His at position 1 is substituted with DMIA.

17. The analog of any of claims 14 to 16, wherein the Ser at position 2 is substituted with D-Ser, D-Ala, Gly, n-methyl-Ser, Val, or alpha, amino isobutyric acid (AIB).

18. The analog of any of claims 4 to 17, comprising an amino acid comprising a side chain covalently attached to an acyl group or an alkyl group, which acyl group or alkyl group is non-native to a naturally-occurring amino acid.

19. The analog of claim 18, wherein the amino acid to which the acyl group or alkyl group is attached is the amino acid at position 10 of the analog or the C-terminal amino acid.

20. The analog of claim 18 or 19, wherein the acyl group or alkyl group is attached to the side chain of the amino acid through a spacer.

21. The analog of any of claims 18 to 20, wherein the acyl group is a C_4 to C_{30} fatty acyl group or the alkyl group is a C_4 to C_{30} alkyl.

22. The analog of any of claims 4 to 21, further comprising an amino acid modification selected from the group consisting of:

substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group;

deletion of 1 or 2 amino acids C-terminal to the amino acid at position 27;

a C-terminal alpha carboxylate;

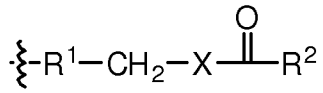
and a combination thereof.

23. The analog of claim 22, wherein the amino acid lacking a hydroxyl group is Ile or aminobutyric acid (Abu).

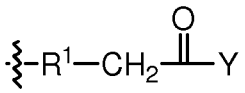
24. The analog of any of claims 4 to 23, further comprising an amino acid modification selected from the group consisting of:

substitution of Tyr at position 10 with Phe or Val;
substitution of Lys at position 12 with Arg;
substitution of Asp at position 15 with Glu;
substitution of Ser at position 16 with Thr;
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;
deletion of amino acids at positions 27-29;
deletion of amino acids at positions 28-29;
deletion of the amino acid at positions 29;
addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly;
and a combination thereof.

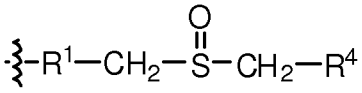
25. A glucagon peptide with glucagon agonist activity, comprising the amino acid sequence of SEQ ID NO: 77, wherein the Xaa at position 3 is an amino acid comprising a side chain of Structure I, II, or III:



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.

26. The glucagon peptide of claim 25, wherein X is NH or Y is NHR^4.

27. The glucagon peptide of claim 25 or 26, wherein R^1 is C_{0-2} alkyl or C_1 heteroalkyl.

28. The glucagon peptide of any of claims 25 to 27, wherein R^2 is NHR^4 or C_1 alkyl.

29. The glucagon peptide of any of claims 25 to 28, wherein R^4 is H or C^1 alkyl.

30. The glucagon peptide of claim 25, wherein the Xaa at position 3 is an amino acid comprising a side chain of:

(i) Structure I and R^1 is CH_2-S, X is NH, and R^2 is CH_3 (C(Acm);

(ii) Structure I and R^1 is CH_2, X is NH, R^2 is CH_3 (Dab(Ac)),

(iii) Structure I and R1 is C0 alkyl, X is NH, R2 is NHR4, and R4 is H (Dap(urea));

(iv) Structure II and R1 is CH2, Y is NHR4, and R4 is CH3 9 (Q(Me);

(v) Structure III and R1 is CH2 and R4 of Structure III is H (M(O)); or

(vi) Structure I and R^1 is CH_2-CH_2, X is NH, and R^2 is CH_3 (Orn(Ac)).

31. An analog of the glucagon peptide of any of claims 25 to 30, 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).

32. The analog of claim 31, wherein the α,α-disubstituted amino acid is AIB.

33. The analog of claim 32, wherein the AIB is at position 16 of the glucagon peptide.

34. The analog of any of claims 31 to 33, wherein the glucagon peptide comprises an amino acid modification selected from the group consisting of:

substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),
substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

substitution of Thr at position 7 with Abu or Ile;
substitution of Tyr at position 10 with Phe or Val;
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 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;
deletion of amino acids at positions 27-29;
deletion of amino acids at positions 28-29;
deletion of the amino acid at positions 29;
addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly,
substitution or addition of an amino acid comprising a side chain covalently attached to an acyl or alkyl group which is non-native to a naturally-occurring amino acid;
and a combination thereof.

35. The analog of claim 34, wherein the glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 62-67 and 69-74.

36. The analog of any of claims 31 to 35, comprising a hydrophilic moiety covalently linked to the amino acid at any of positions 16, 17, 20, 21, 24, and 29 or at the C-terminal amino acid.

37. The analog of claim 36, wherein the hydrophilic moiety is PEG.

38. The analog of any claims 34 to 37, wherein the acyl group or alkyl group is covalently attached to the side chain of the amino acid at position 10 or the C-terminal amino acid.

39. The analog of any of claims 34 to 38, wherein the acyl group is a C_4 to C_{30} fatty acyl group or the alkyl group is a C_4 to C_{30} alkyl.

40. The analog of any of claims 34 to 39, wherein the acyl or alkyl group is attached to the side chain of the amino acid through a spacer.

41. The analog of claim 40, wherein the glucagon peptide comprises an amino acid lacking a hydroxyl group at position 7.

42. The analog of claim 41, wherein the amino acid lacking a hydroxyl group is Abu or Ile.

43. 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 the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

wherein Z is selected from the group consisting of –COOH, -Asn-COOH, Asn-Thr-COOH, and Y-COOH, wherein Y is 1 to 2 amino acids, and

wherein a lactam bridge 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.

44. The glucagon peptide of claim 43, wherein the amino acids at positions i and i+4 of SEQ ID NO: 39 are Lys and Glu.

45. The glucagon peptide of claim 44, wherein the amino acid at position 16 is Glu and the amino acid at position 20 is Lys.

46. The glucagon peptide of any of claims 43 to 45, wherein 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).

47. The glucagon peptide of claim 46, wherein X1 is DMIA.

48. The glucagon peptide of any of claims 43 to 47, wherein X2 is selected from the group consisting of: D-Ser, D-Ala, Gly, n-methyl-Ser, Val, and alpha, amino isobutyric acid (AIB).

49. The glucagon peptide of any of claims 43 to 48, wherein Z is Asn-Thr-COOH.

50. The glucagon peptide of any of claims to 43 to 48, wherein Z is Asn-COOH.

51. The glucagon peptide of any of claims 43 to 48, wherein Z is –COOH.

52. The glucagon peptide of any of claims 43 to 51, wherein the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, or 29, or at the C-terminal amino acid.

53. The glucagon peptide of claim 52, wherein the hydrophilic moiety is covalently linked to Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine.

54. The glucagon peptide of claim 52 or 53, wherein the hydrophilic moiety is a polyethylene glycol (PEG).

55. The glucagon peptide of claim 54, wherein the PEG has a molecular weight of about 1,000 Daltons to about 40,000 Daltons.

56. The glucagon peptide of claim 54, wherein the PEG has a molecular weight of about 20,000 Daltons to about 40,000 Daltons.

57. The glucagon peptide of any of claims 43 to 56 that has an EC50 at the human glucagon receptor of about 10 nM or less, as determined in vitro

in an assay measuring cAMP induction in HEK293 cells over-expressing the glucagon receptor.

58. 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, or all of the amino acids at positions 16, 20, 21, and 24 of the glucagon peptide is substituted with an α, α-disubstituted amino acid,

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

optionally, wherein the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 16, 17, 20, 21, 24, or 29, or at the C-terminal amino acid.

59. The glucagon peptide of claim 58, comprising:

(a) AIB at position 16 of the glucagon peptide,

(b) AIB at position 20 of the glucagon peptide,

(c) AIB at position 21 of the glucagon peptide,

(d) AIB at position 24 of the glucagon peptide,

(e) AIB at positions 16 and 20 of the glucagon peptide,

(f) AIB at positions 16 and 24 of the glucagon peptide,

(g) AIB at positions 20 and 24 of the glucagon peptide, or

(h) AIB at positions 16, 20, and 24 of the glucagon peptide.

60. The glucagon peptide of claim 58 or 59, wherein 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).

61. The glucagon peptide of claim 60, wherein X1 is DMIA.

62. The glucagon peptide of any of claims 58 to 61, wherein X2 is selected from the group consisting of: D-Ser, D-Ala, Gly, n-methyl-Ser, Val, and alpha, amino isobutyric acid (AIB).

63. The glucagon peptide of any of claims 58 to 62, wherein Z is Asn-Thr-COOH.

64. The glucagon peptide of any of claims to 58 to 62, wherein Z is Asn-COOH.

65. The glucagon peptide of any of claims 58 to 62, wherein Z is –COOH.

66. The glucagon peptide of any of claims 58 to 65, wherein the hydrophilic moiety is covalently linked to Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine.

67. The glucagon peptide of any of claims 58 to 66, wherein the hydrophilic moiety is a polyethylene glycol (PEG).

68. The glucagon peptide of claim 67, wherein the PEG has a molecular weight of about 1,000 Daltons to about 40,000 Daltons.

69. The glucagon peptide of claim 67, wherein the PEG has a molecular weight of about 20,000 Daltons to about 40,000 Daltons.

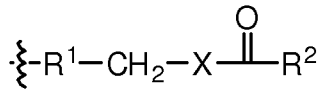
70. The glucagon peptide of any of claims 58 to 69 that has an EC50 at the human glucagon receptor of about 10 nM or less, as determined *in vitro* in an assay measuring cAMP induction in HEK293 cells over-expressing the glucagon receptor.

71. An analog of the glucagon peptide of claim 43 or claim 58 that has glucagon agonist activity and comprises a further amino acid modification selected from the group consisting of:

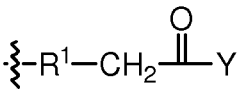
substitution of Tyr at position 10 with Phe or Val;
substitution of Lys at position 12 with Arg;
substitution of Asp at position 15 with Glu;
substitution of Ser at position 16 with Thr or AIB;
substitution of Gln at position 20 with Ala or AIB;
substitution of Asp at position 21 with Glu;
substitution of Gln at position 24 with Ala or AIB;
substitution of Met at position 27 with Leu or Nle;
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 of Glu or Lys;
insertion at position 31 of Lys;
or a combination thereof.

72. The analog of claim 71, further comprising one or a combination of the following amino acid modifications:

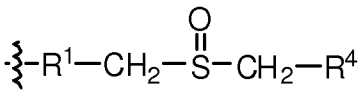
substitution of Gln at position 3 with an amino acid comprising a side chain of Structure I, II, or III:



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;

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, optionally in combination with a substitution of Thr at position 7 with Abu or Ile;

substitution of Gln at position 20 with Ala, Ser or Thr;

substitution of Gln at position 24 with Ala, Ser or Thr;

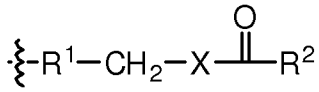
addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, wherein the amino acid at position 29 is Thr or Gly,

substitution or addition of an amino acid covalently attached to a hydrophilic moiety;

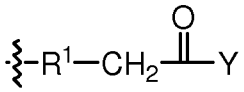
or a combination thereof.

73. An analog of the glucagon peptide of claim 43 or 58 that has glucagon agonist activity and comprises one or a combination of the following amino acid modifications:

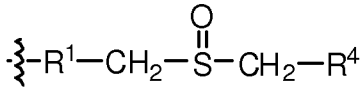
substitution of Gln at position 3 with an amino acid comprising a side chain of Structure I, II, or III:



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;

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, optionally in combination with a substitution of Thr at position 7 with Abu or Ile;

substitution of Gln at position 20 with Ala, Ser or Thr;

substitution of Gln at position 24 with Ala, Ser or Thr;

addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, 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.

74. An analog of the glucagon peptide of SEQ ID NO: 1 that has glucagon agonist activity and comprises

(a) at least one amino acid modification selected from the group consisting of:

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 after position 29 of Glu or Lys;

insertion after position 29 of Gly-Lys or Lys-Lys; or a combination thereof;

and at least one amino acid modification selected from Group A or Group B, or a combination thereof;

wherein Group A is an amino acid modification selected from the group consisting of substitution of Asp at position 15 with Glu, and substitution of Ser at position 16 with Thr or AIB; and

wherein Group B is an amino acid modification selected from the group consisting of:

substitution of His at position 1 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

substitution of Ser at position 2 with a non-native amino acid that reduces susceptibility of the glucagon peptide to cleavage by dipeptidyl peptidase IV (DPP-IV),

substitution of Tyr at position 10 with Phe or Val;

substitution of Lys at position 12 with Arg;

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;

deletion of amino acids at positions 27-29;

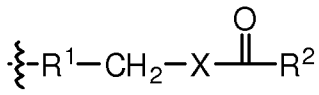
deletion of amino acids at positions 28-29;

deletion of the amino acid at positions 29;

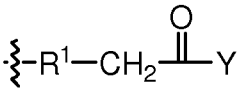
or a combination thereof.

75. The analog of claim 74, further comprising one or a combination of the following amino acid modifications:

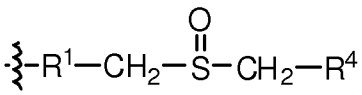
substitution of Gln at position 3 with an amino acid comprising a side chain of Structure I, II, or III:



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;

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, optionally in combination with a substitution of Thr at position 7 with Abu or Ile;

substitution of Gln at position 20 with Ala, Ser or Thr;

substitution of Gln at position 24 with Ala, Ser or Thr;

addition of the amino acid sequence of SEQ ID NO: 20 to the C-terminus, 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.

76. The analog of claim 74 or 75, wherein a lactam bridge 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.

77. The analog of any of claims 74 to 76, wherein one, two, three, or all of the amino acids at positions 16, 20, 21, and 24 of the analog is substituted with an α,α-disubstituted amino acid.

78. The analog of claim 77, wherein the α,α-disubstituted amino acid is AIB.

79. The analog of claim 78, comprising the amino acid sequence of SEQ ID NO: 76.

80. A glucagon peptide comprising the amino acid sequence of any of SEQ ID NOs: 40-56.

81. A glucagon peptide comprising the amino acid sequence of SEQ ID NO: 40.

82. The glucagon peptide or analog of any of the preceding claims, wherein, when the glucagon peptide, or analog thereof, lacks a hydrophilic moiety, the glucagon peptide, or analog thereof, exhibits at least about 20% of the activity of native glucagon at the glucagon receptor.

83. The glucagon peptide or analog of any of the preceding claims, wherein, when the glucagon peptide, or analog thereof, lacks a hydrophilic moiety, the glucagon peptide, or analog thereof, exhibits no more than about 0.5% of the activity of native GLP-1 at the GLP-1 receptor.

84. A dimer comprising two peptides bound via a linker, wherein at least one of the two peptides is a glucagon peptide of any of claims 1-3, 25-30, 43-70, and 80-83 or an analog of claim 4-24, 31-42, and 71-79.

85. The dimer of claim 84, wherein the dimer is a homodimer.

86. The dimer of claim 84 or 85, wherein the linker is selected from the group consisting of a bifunctional thiol crosslinker and a bio-functional amine crosslinker.

87. A conjugate comprising a conjugate moiety and a glucagon peptide of any of claims 1-3, 25-30, 43-70, and 80-83, an analog of claim 4-24, 31-42, and 71-79, a dimer of any of claims 84 to 86, or a combination thereof.

88. A fusion peptide comprising a glucagon peptide of any of claims 1-3, 25-30, 43-70, and 80-83, an analog of claim 4-24, 31-42, and 71-79, or a dimer of any of claims 84 to 86, fused to a peptide comprising the amino acid sequence of SEQ ID NO: 20.

89. A pharmaceutical composition comprising a glucagon peptide of any of claims 1-3, 25-30, 43-70, and 80-83, an analog of claim 4-24, 31-42, and 71-79, a dimer of any of claims 84 to 86, a conjugate of claim 87, a fusion peptide of claim 88, or a combination thereof, and a pharmaceutically acceptable carrier.

90. A kit for administering a glucagon agonist to a patient in need thereof, said kit comprising a pharmaceutical composition of claim 89 and a device for administering said pharmaceutical composition to the patient.

91. The kit of claim 90, wherein the device comprises a syringe and a needle, wherein the pharmaceutical composition is pre-packaged within the syringe.

92. A method of causing temporary paralysis of the intestinal tract in a patient in need thereof, said method comprising administering to the patient a pharmaceutical composition of claim 89 in an amount effective to cause temporary paralysis of the intestinal tract in the patient.

93. A method of reducing weight gain or inducing weight loss in a patient in need thereof, said method comprising administering to the patient a pharmaceutical composition of claim 89 in an amount effective to reduce weight gain or induce weigh loss in the patient.

94. A method of treating or preventing hypoglycemia in a patient in need thereof, comprising administering to the patient a pharmaceutical composition of claim 89 in an amount effective to treat or prevent hypoglycemia in the patient.

95. A method of stabilizing a blood glucose level in a patient in need thereof, wherein the patient is on a treatment regimen comprising administration of insulin, comprising administering to the patient a pharmaceutical composition of claim 89 in an amount effective to stabilize the blood glucose level of the patient.

96. The method of claim 95, wherein the pharmaceutical composition further comprises the insulin.