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(54) **Title:**
GIP RECEPTOR-ACTIVE GLUCAGON COMPOUNDS

(57) **Abstract:**
Glucagon peptides with increased GIP activity are provided, optionally with GLP-I and/or glucagon activity. In some embodiments, C-terminally extended glucagon peptides comprising an amino acid sequence substantially similar to native glucagon are provided herein.

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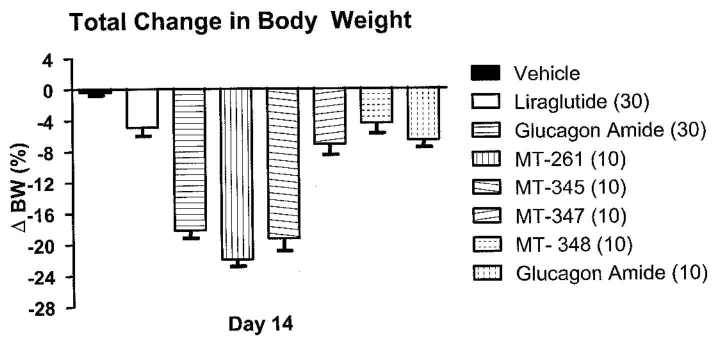
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

[Continued on next page]

(54) **Title**: GIP RECEPTOR-ACTIVE GLUCAGON COMPOUNDS

FIGURE 36



(57) **Abstract**: Glucagon peptides with increased GIP activity are provided, optionally with GLP-I and/or glucagon activity. In some embodiments, C-terminally extended glucagon peptides comprising an amino acid sequence substantially similar to native glucagon are provided herein.

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GIP RECEPTOR-ACTIVE GLUCAGON COMPOUNDS

CROSS REFERENCE TO RELATED APPLICATIONS:

This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application Serial No. 61/187,578 filed June 16, 2009, the entirety of which is hereby incorporated by reference herein.

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. GLP-1(7-36) amide or GLP-1(7-37) acid are biologically potent forms of GLP-1, that demonstrate essentially equivalent activity at the GLP-1 receptor.

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 or exceeds 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.

GLP-1 has different biological activities compared to glucagon. Its actions include stimulation of insulin synthesis and secretion, inhibition of glucagon secretion, and inhibition of food intake. GLP-1 has been shown to reduce hyperglycemia (elevated glucose levels) in diabetics. Exendin-4, a peptide from lizard venom that shares about 50% amino acid identity with GLP-1, activates the GLP-1 receptor and likewise has been shown to reduce hyperglycemia in diabetics.

There is also evidence that GLP-1 and exendin-4 may reduce food intake and promote weight loss, an effect that would be beneficial not only for diabetics but also for patients suffering from obesity. Patients with obesity have a higher risk of diabetes, hypertension, hyperlipidemia, cardiovascular disease, and musculoskeletal diseases.

Accordingly, there remains a need for alternative and preferably improved methods for treating diabetes and obesity.

SUMMARY

As described herein, analogs of glucagon peptides which exhibit agonist activity at the GIP receptor are provided by the invention. Methods of using such analogs are additionally provided herein.

Native glucagon (SEQ ID NO: 1) does not activate the GIP receptor and typically, native glucagon exhibits essentially 0% (e.g., less than 0.001%, less than 0.0001%) activity of native GIP at the GIP receptor. Native glucagon also has about 1% of the activity of native-GLP-1 at the GLP-1 receptor. Modifications to the native glucagon sequence (SEQ ID NO: 1) are described herein which produce analogs of glucagon peptides that can exhibit potent glucagon activity equivalent to or better than the activity of native glucagon (SEQ ID NO: 1), potent GIP activity equivalent to or better than the activity of native GIP (SEQ ID NO: 675), and/or potent GLP-1 activity equivalent to or better than the activity of native GLP-1. GLP-1(7-36) amide (SEQ ID NO: 52) or GLP-1(7-37) (acid) (SEQ ID NO: 50) are biologically potent forms of GLP-1, that demonstrate essentially equivalent activity at the GLP-1 receptor.

The data described herein show that analogs of glucagon having both GIP activity and GLP-1 activity are particularly advantageous for inducing weight loss or preventing weight gain, as well as for treating hyperglycemia, including diabetes. This activity is particularly unexpected in view of teachings in the art that antagonizing GIP is desirable for reducing daily food intake and body weight, and increasing insulin sensitivity and energy expenditure. (Irwin et al., *Diabetologia* 50: 1532-1540 (2007); and Althage et al., *J Biol Chem*, e-publication on April 17, 2008).

Accordingly, in some embodiments, the analogs of glucagon peptides described herein exhibit an EC50 for GIP receptor activation activity of about 100nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. In some embodiments, the analogs described herein exhibit an EC50 at the GIP receptor that is about 0.001 nM, 0.01 nM, or 0.1 nM. In some embodiments, the analogs described herein exhibit an EC50 at the GIP receptor that is no more than about 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 8 nM, 10 nM, 15 nM, 20 nM, 25 nM, 30 nM, 40 nM, 50 nM, 75 nM, or 100 nM. In some embodiments, the analogs exhibit an EC50 for glucagon receptor activation of about 100nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. In some embodiments, the analogs described herein exhibit an EC50 at the glucagon receptor that is about 0.001 nM, 0.01 nM, or 0.1 nM. In some embodiments, the EC50 at the glucagon receptor is no more than about 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 8 nM, 10 nM, 15 nM, 20 nM, 25 nM, 30 nM, 40 nM, 50 nM, 75 nM, or 100 nM. In some embodiments, the analogs exhibit an EC50 for GLP-1 receptor activation of about 100 nM or less, or about 75, 50, 25, 10, 8, 6, 5, 4, 3, 2 or 1 nM or less. In some embodiments, the analogs described herein exhibit an EC50 at the GLP-1 receptor that is about 0.001 nM, 0.01 nM, or 0.1 nM. In some embodiments, the EC50 at the GLP-1 receptor is no more than about 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 8 nM, 10 nM, 15 nM, 20 nM, 25 nM, 30 nM, 40 nM, 50 nM, 75 nM, or 100 nM. Receptor activation can be measured by *in vitro* assays measuring cAMP induction in HEK293 cells over-expressing the receptor, e.g. assaying HEK293 cells co-transfected with DNA encoding the receptor and a luciferase gene linked to cAMP responsive element as described in Example 14.

In some embodiments, the analogs exhibit at least about 0.005%, 0.0075%, 0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%,1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175% or 200% or higher activity at the GIP receptor relative to native GIP (GIP potency). In some embodiments, the analogs described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000% activity at the GIP receptor relative to native GIP. In some embodiments, the analogs exhibit at least about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 350%, 400%, 450%, or 500% or higher activity at the glucagon receptor relative to native glucagon (glucagon potency). In some embodiments, the analogs described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000% activity at the glucagon receptor relative to native glucagon. In some embodiments, the analogs exhibit at least about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175% or 200% or higher activity at the GLP-1 receptor relative to native GLP-1 (GLP-1 potency). In some embodiments, the analogs described herein exhibit no more than 1000%, 10,000%, 100,000%, or 1,000,000% activity at the GLP-1 receptor relative to native GLP-1.

In certain embodiments, the analogs and glucagon peptides described herein exhibit the indicated % activity, when lacking a hydrophilic moiety, e.g., PEG, but exhibit a decreased % activity (e.g., about a 10-fold decrease in activity), when comprising a hydrophilic moiety, e.g., PEG. Accordingly, in some embodiments, the analogs exhibit the aforementioned % activity levels when lacking a hydrophilic moiety and exhibit about a 10-fold decrease in activity when comprising a hydrophilic moiety. An analog's activity at a receptor relative to a native ligand of the receptor is calculated as the inverse ratio of EC50s for the analog vs. the native ligand.

Thus, one aspect of the invention provides analogs that exhibit activity at both the glucagon receptor and the GIP receptor ("glucagon/GIP co-agonists"). These analogs have lost native glucagon's selectivity for glucagon receptor compared to GIP receptor. In some embodiments, the EC50 of the analog at the GIP receptor is less than about 50-fold, 40-fold, 30-fold or 20-fold different (higher or lower) from its

EC50 at the glucagon receptor. In some embodiments, the GIP potency of the analog is less than about 500-, 450-, 400-, 350-, 300-, 250-, 200-, 150-, 100-, 75-, 50-, 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its glucagon potency. In some embodiments, the ratio of the EC50 of the analog at the GIP receptor divided by the EC50 of the analog at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC50 at the GIP receptor divided by the EC50 at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency of the analog compared to the glucagon potency of the analog is less than about 500, 450, 400, 350, 300, 250, 200, 150, 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, GLP-1 activity has been significantly reduced or destroyed, e.g., by an amino acid modification at position 7, e.g., substitution with 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.

Another aspect of the invention provides analogs that exhibit activity at the glucagon, GIP and GLP-1 receptors (“glucagon/GIP/GLP-1 tri-agonists”). These analogs have lost native glucagon's selectivity for the glucagon receptor compared to both the GLP-1 and GIP receptors. In some embodiments, the EC50 of the analog at the GIP receptor is less than about 5000-fold, 2500-fold, 1000-fold, 750-fold, 500-fold, 250-fold, 100-fold, 50-fold, 40-fold, 30-fold or 20-fold different (higher or lower) from its respective EC50s at the glucagon and GLP-1 receptors. In some embodiments, the GIP potency of the analog is less than about 1000-, 750-, 500-, 450-, 400-, 350-, 300-, 250-, 200-, 150-, 100-, 75-, 50-, 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its glucagon and GLP-1 potencies. In some embodiments, the ratio of the EC50 of the tri-agonist at the GIP receptor divided by the EC50 of the tri-agonist at the GLP-1 receptor is less than about 10,000, 7500, 5000, 2500, 1000, 750, 500, 250, 100, 75, 60, 50, 40, 30, 20, 15, 10, 5, or 1. In some embodiments, the ratio of the EC50 at the GLP-1 receptor divided by the EC50 at the GIP receptor is about 5, 4, 3, 2, or 1 or less than about 1 (e.g., less than about 0.00001, 0.0001, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the EC50 at the GLP-1 receptor is greater than about 0.1 (e.g., greater than about 0.25, greater than about 0.5, greater than about 0.75, greater than about 1). In some embodiments, the ratio of the GIP potency of the tri-agonist compared to the GLP-1 potency of the tri-agonist is less than about 1000, 750, 500, 250, 100, 75, 60, 50, 40, 30, 20, 15, 10, 5, or 1. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the GLP-1 receptor is about 5, 4, 3, 2, or 1, or less than about 1 (e.g., less than about 0.0001, 0.001, 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In related embodiments, the ratio of the EC50 of the tri-agonist at the GIP receptor divided by the EC50 of the tri-agonist at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC50 at the GIP receptor divided by the EC50 at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency of the tri-agonist compared to the glucagon potency of the tri-agonist is less than about 500, 450, 400, 350, 300, 250, 200, 150, 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the EC50 of the tri-agonist at the GLP-1 receptor divided by the EC50 of the tri-agonist at the glucagon receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the EC50 at the GLP-1 receptor divided by the EC50 at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GLP-1 potency of the tri-agonist compared to the glucagon potency of the tri-agonist is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5. In some embodiments, the ratio of the potency at the GLP-1 receptor divided by the potency at the glucagon receptor is about 1 or less than about 1 (e.g., about 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2).

Yet another aspect of the invention provides analogs that exhibit activity at the GLP-1 and GIP receptors, but in which the glucagon activity has been significantly reduced or destroyed ("GIP/GLP-1 co-agonists"), e.g., by an amino acid modification at position 3. For example, substitution at this position with an acidic, basic, or a hydrophobic amino acid (glutamic acid, ornithine, norleucine) reduces glucagon activity. In some embodiments, the EC50 of the analog at the GIP receptor is less than about 1000-fold, 750-fold, 500-fold, 250-fold, 100-fold, 50-fold, 40-fold, 30-fold or 20-fold different (higher or lower) from its EC50 at the GLP-1 receptor. In some embodiments, the GIP potency of the analog is less than about 1000-, 750-, 500-, 250-, 100-, 25-, 20-, 15-, 10-, or 5-fold different (higher or lower) from its GLP-1 potency. In some embodiments these analogs have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1% but less than about 10%. In some embodiments, the ratio of the EC50 of the analog at the GIP receptor divided by the EC50 of the analog at the GLP-1 receptor is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5, and no less than 1. In some embodiments, the ratio of the GIP potency of the analog compared to the GLP-1 potency of the analog is less than about 100, 75, 60, 50, 40, 30, 20, 15, 10, or 5, and no less than 1. In some embodiments, the ratio of the EC50 of the co-agonist at the GIP receptor divided by the EC50 of the co-agonist at the GLP-1 receptor is less than about 10,000, 7500, 5000, 2500, 1000, 750, 500, 250, 100, 75, 60, 50, 40, 30, 20, 15, 10, 5, or 1. In some embodiments, the ratio of the EC50 at the GLP-1 receptor divided by the EC50 at the GIP receptor is about 5, 4, 3, 2, or 1 or less than about 1 (e.g., less than about 0.00001, 0.0001, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2). In some embodiments, the ratio of the GIP potency of the co-agonist compared to the GLP-1 potency of the co-agonist is less than about 1000, 750, 500, 250, 100, 75, 60, 50, 40, 30, 20, 15, 10, 5, or 1. In some embodiments, the ratio of the potency at the GIP receptor divided by the potency at the GLP-1 receptor is about 5, 4, 3, 2, or 1, or less than about 1 (e.g., less than about 0.0001, 0.001, 0.01, 0.013, 0.0167, 0.02, 0.025, 0.03, 0.05, 0.067, 0.1, 0.2).

A further aspect of the invention provides analogs that exhibit activity at the GIP receptor, in which the glucagon and GLP-1 activity have been significantly reduced or destroyed ("GIP agonist glucagon peptides"), e.g., by amino acid modifications at positions 3 and 7. In some embodiments these analogs have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1%, 0.5%, or 1% but less than about 1%, 5%, or 10%. In some embodiments these analogs also have about 10% or less of the activity of native GLP-1 at the GLP-1 receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1%, 0.5%, or 1% but less than about 1%, 5%, or 10%.

In some aspects of the invention, the analogs which exhibit agonist activity at the GIP receptor comprise SEQ ID NO: 1 with at least one amino acid modification and an extension of 1 to 21 amino acids (e.g., 5 to 18, 7 to 15, 9 to 12 amino acids) C-terminal to the amino acid at position 29 of the analog.

In certain aspects, the analogs comprise at least one amino acid modification and up to 15 amino acid modifications (e.g., no more than 15 amino acid modifications, no more than 10 amino acid modifications). For example, the analog can comprise SEQ ID NO: 1 with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acid modifications. In certain embodiments, the analogs comprise at least one amino acid modification at up to 10 amino acid modifications and additional conservative amino acid modifications. In further aspects, at least one of the amino acid modifications confers a stabilized alpha helix structure in the C-terminal portion of the analog. Modifications which achieve a stabilized alpha helix structure are described herein. See, for example, the teachings under the section entitled *Stabilization of the alpha helix/Intramolecular bridges*.

Analogs comprising an acylated or alkylated C-terminal extension or a C-terminal extension comprising 1-6 positive-charged amino acids unexpectedly exhibited an increased agonist activity at the GIP receptor. Thus, in certain aspects, at least one of the amino acids of the extension located at any of positions 37-43 (according to the numbering of SEQ ID NO: 1) comprises an acyl or alkyl group which is non-native to a naturally-occurring amino acid, i.e., the extension is acylated or alkylated. In some embodiments, the acyl or alkyl group is attached directly to the amino acid, e.g., via the side chain of the amino acid. In other embodiments, the acyl or alkyl group is attached to the amino acid via a spacer (e.g., an amino acid, a dipeptide, a tripeptide, a hydrophilic bifunctional spacer, a hydrophobic bifunctional spacer). Suitable amino acids comprising an acyl or alkyl group, as well as suitable acyl groups and alkyl groups, are described herein. See, for example, the teachings under the sections entitled *Acylation* and *Alkylation*.

In other embodiments, 1-6 amino acids (e.g., 1-2, 1-3, 1-4, 1-5 amino acids) of the extension are positive-charged amino acids, e.g., amino acids of Formula IV, such as, for example, Lys. As used herein, the term “positive-charged amino acid” refers to any amino acid, naturally-occurring or non-naturally occurring, comprising a positive charge on an atom of its side chain at a physiological pH (e.g., pH 6.8 to 8.0, pH 7.0 to 7.7). In certain aspects, the positive-charged amino acids are located at any of positions 37, 38, 39, 40, 41, 42, and 43. In specific embodiments, a positive-charged amino acid is located at position 40.

In other instances, the extension is acylated or alkylated as described herein and comprises 1-6 positive charged amino acids as described herein.

In yet other embodiments, the analogs which exhibit agonist activity at the GIP receptor comprises (i) SEQ ID NO: 1 with at least one amino acid modification, (ii) an extension of 1 to 21 amino acids (e.g., 5 to 18, 7 to 15, 9 to 12 amino acids) C-terminal to the amino acid at position 29 of the analog, and (iii) an amino acid comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid which is located outside of the C-terminal extension (e.g., at any of positions 1-29). In some embodiments, the analog comprises an acylated or alkylated amino acid at position 10. In particular aspects, the acyl or alkyl group is a C4 to C30 fatty acyl or C4 to C30 alkyl group. In some embodiments, the acyl or alkyl group is attached via a spacer, e.g., an amino acid, dipeptide, tripeptide, hydrophilic bifunctional spacer, hydrophobic bifunctional spacer). In certain aspects, the analog comprises an amino acid modification which stabilizes the alpha helix, such as a salt bridge between a Glu at position 16 and a Lys at position 20, or an alpha, alpha-disubstituted amino acid at any one, two, three, or more of positions 16, 20, 21, and 24. In specific aspects, the analog additionally comprises amino acid modifications which confer DPP-IV protease resistance. Analogs comprising further amino acid modifications are contemplated herein.

In certain embodiments, the analogs having GIP receptor activity exhibit at least 0.1% (e.g., at least 0.5%, 1%, 2%, 5%, 10%, 15%, or 20%) activity of native GIP at the GIP receptor. In some embodiments, the analogs exhibit more than 20% (e.g., more than 50%, more than 75%, more than 100%, more than 200%, more than 300%, more than 500%) activity of native GIP at the GIP receptor. In some embodiments, the analog exhibits appreciable agonist activity at one or both of the GLP-1 and glucagon receptors. In some aspects, the selectivity for these receptors (GIP receptor and GLP-1 receptor and/or glucagon receptor) are within 100-fold. For example, the selectivity for the GLP-1 receptor of the analogs having GIP receptor activity can be less than 100-fold, within 50-fold, within 25 fold, within 15 fold, within 10 fold) the selectivity for the GIP receptor and/or the glucagon receptor.

As described herein, high potency glucagon agonist analogs are provided that also exhibit increased activity at the glucagon receptor, and in further embodiments exhibit enhanced biophysical stability and/or aqueous solubility. In addition, in accordance with another aspect of the invention, glucagon agonist analogs are provided that have lost native glucagon's selectivity for the glucagon receptor verses the GLP-1 receptor, and thus represent co-agonists of those two receptors. Selected amino acid modifications within the glucagon analogs can control the relative activity of the analog at the GLP-1 receptor verses the glucagon receptor. Thus, yet another aspect of the invention provides glucagon co-agonist analogs that have higher activity at the glucagon receptor versus the GLP-1 receptor, glucagon co-agonist analogs that have approximately equivalent activity at both receptors, and glucagon co-agonist analogs that have higher activity at the GLP-1 receptor versus the glucagon receptor. The latter category of co-agonist can be engineered to exhibit little or no activity at the glucagon receptor, and yet retain ability to activate the GLP-1 receptor with the same or better potency than native GLP-1. Any of these analogs may also include modifications that confer enhanced biophysical stability and/or aqueous solubility.

Glucagon analogs that demonstrate co-agonism at the glucagon and GLP-1 receptors are advantageous for several applications. First of all the use of glucagon to treat hypoglycemia may overcompensate for low blood glucose levels and result in excess blood glucose levels. If a glucagon/GLP-1 receptor co-agonist is administered, the additional GLP-1 stimulation may buffer the glucagon agonist effect to prevent excessive glucose blood levels resulting from treatment of hypoglycemia.

In addition as described herein, glucagon co-agonist analogs of the invention may be used to control hyperglycemia, or to induce weight loss or prevent weight gain, when administered alone or in combination with other anti-diabetic or anti-obesity treatments. Another compound that induces weight loss is oxyntomodulin, a naturally occurring digestive hormone found in the small intestine (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: 27 (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: 27), the invention in some embodiments also specifically contemplates analogs and uses of analogs lacking the 8 contiguous carboxy amino acids of SEQ ID NO: 27.

The compounds can be customized by amino acid modifications to regulate the GLP-1 activity of the peptide, and thus the glucagon analogs of the present can be tailored to treat a particular condition or disease. More particularly, glucagon analogs are provided herein wherein each analog displays a characteristic relative level of activity at the respective glucagon and GLP-1 receptors. For example, modifications can be made to each peptide to produce a glucagon peptide having anywhere from at least about 1% (including at least about 1.5%, 2%, 5%, 7%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%) to about 200% or higher activity at the GLP-1 receptor relative to native GLP-1 and anywhere from at least about 1% (including about 1.5%, 2%, 5%, 7%, 10%, 20%, 30%, 40%, 50%, 60%, 75%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 350%, 400%, 450%) to about 500% or higher activity at the glucagon receptor relative to native glucagon. In some embodiments, the glucagon peptides described herein exhibit no more than about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon at the glucagon receptor. In some embodiments, the glucagon peptides described herein exhibit no more than about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native GLP-1 at the GLP-1 receptor. The amino acid sequence of native glucagon is SEQ ID NO: 1, the amino acid sequence of GLP-1(7-36)amide is SEQ ID NO: 52, and the amino acid sequence of GLP-1(7-37)acid is SEQ ID NO: 50. In exemplary embodiments, a glucagon peptide may exhibit at least 10% of the activity of native glucagon at the glucagon receptor and at least 50% of the activity of native GLP-1 at the GLP-1 receptor, or at least 40% of the activity of native glucagon at the glucagon receptor and at least 40% of the activity of native GLP-1 at the GLP-1 receptor, or at least 60% of the activity of native glucagon at the glucagon receptor and at least 60% of the activity of native GLP-1 at the GLP-1 receptor.

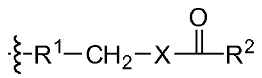
Selectivity of a glucagon peptide for the glucagon receptor versus the GLP-1 receptor can be described as the relative ratio of glucagon/GLP-1 activity (the peptide's activity at the glucagon receptor relative to native glucagon, divided by the peptide's activity at the GLP-1 receptor relative to native GLP-1). For example, a glucagon peptide that exhibits 60% of the activity of native glucagon at the glucagon receptor and 60% of the activity of native GLP-1 at the GLP-1 receptor has a 1:1 ratio of glucagon/GLP-1 activity. Exemplary ratios of glucagon/GLP-1 activity include about 1:1, 1.5:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1, or about 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, or 1:1.5. As an example, a glucagon/GLP-1 activity ratio of 10:1 indicates a 10-fold selectivity for the glucagon receptor versus the GLP-1 receptor. Similarly, a GLP-1/glucagon activity ratio of 10:1 indicates a 10-fold selectivity for the GLP-1 receptor versus the glucagon receptor.

In accordance with one embodiment, analogs of glucagon are provided that have enhanced potency and optionally improved solubility and stability. In one embodiment, enhanced glucagon potency is provided by 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. In one embodiment the enhanced potency glucagon agonist comprises a peptide 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 or a glucagon agonist analog of SEQ ID NO: 5. In accordance with one embodiment a glucagon analog protein having enhanced potency at the glucagon receptor relative to wild type glucagon is provided wherein the peptide comprises the sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 or SEQ ID NO: 10, wherein the glucagon peptide retains its selectivity for the glucagon receptor relative to the GLP-1 receptors.

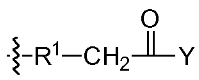
Glucagon receptor activity can be reduced, maintained, or enhanced by an amino acid modification at position 3, e.g. substitution of the naturally occurring glutamine at position 3. In one embodiment, substitution of the amino acid at position 3 with an acidic, basic, or hydrophobic amino acid (glutamic acid, ornithine, norleucine) has been shown to substantially reduce or destroy glucagon receptor activity. The analogs that are substituted with, for example, glutamic acid, ornithine, or norleucine have about 10% or less of the activity of native glucagon at the glucagon receptor, e.g. about 1-10%, or about 0.1-10%, or greater than about 0.1% but less than about 10%, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor. For example, exemplary analogs described herein have about 0.5%, about 1% or about 7% of the activity of native glucagon, while exhibiting at least 20% of the activity of GLP-1 at the GLP-1 receptor.

In another embodiment, the naturally occurring glutamine at position 3 of the glucagon peptide can be substituted with a glutamine analog without a substantial loss of activity at the glucagon receptor, and in some cases, with an enhancement of glucagon receptor activity. 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: 601 or SEQ ID NO: 602) 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.

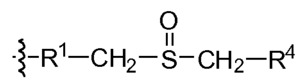
In some embodiments, the glutamine analog is a naturally occurring or a non-naturally occurring amino acid comprising a side chain of Structure I, II or III:



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 of Structure I, 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 of Structure II, R^1 is CH_2, Y is NHR^4, and R^4 is CH_3 (methylglutamine, Q(Me)); In exemplary embodiments of Structure III, 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: 595, SEQ ID NO: 601 SEQ ID NO: 603, SEQ ID NO: 604, SEQ ID NO: 605, and SEQ ID NO: 606.

In another embodiment analogs of glucagon are provided that have enhanced or retained potency at the glucagon receptor relative to the native glucagon peptide, but also have greatly enhanced activity at the GLP-1 receptor. Glucagon normally has about 1% of the activity of native-GLP-1 at the GLP-1 receptor, while GLP-1 normally has less than about 0.01% of the activity of native glucagon at the glucagon receptor. 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. In one embodiment, these glucagon analogs comprise a sequence of SEQ ID NO: 20 wherein the carboxy terminal amino acid has an amide group in place of the carboxylic acid group found on the native amino acid. These glucagon analogs have strong activity at both the glucagon and GLP-1 receptors and thus act as co-agonists at both receptors. In accordance with one embodiment a glucagon and GLP-1 receptor co-agonist is provided wherein the peptide comprises the sequence of SEQ ID NO: 20, wherein the amino acid at position 28 is Asn or Lys and the amino acid at position 29 is Thr-amide.

Enhanced activity at the GLP-1 receptor is also provided by stabilizing the alpha-helix structure in the C-terminal portion of glucagon (around amino acids 12-29), through formation of an intramolecular bridge between the side chains of two amino acids that are separated by three intervening amino acids, i.e., an amino acid at position "i" and an amino acid at position "i+4", wherein i is any integer between 12 and 25, by two intervening amino acids, i.e., an amino acid at position "j" and an amino acid at position "j+3," wherein j is any integer between 12 and 27, or by six intervening amino acids, i.e., an amino acid at position "k" and an amino acid at position "k+7," wherein k is any integer between 12 and 22. In exemplary embodiments, the bridge or linker is about 8 (or about 7-9) atoms in length and forms between side chains of amino acids at positions 12 and 16, or at positions 16 and 20, or at positions 20 and 24, or at positions 24 and 28. The side chains of these amino acids can be linked to one another through non-covalent bonds, e.g., hydrogen-bonding or ionic interactions, such as the formation of salt bridges, or by covalent bonds.

In accordance with one embodiment a glucagon agonist is provided comprising a glucagon peptide of SEQ ID NO: 20, wherein a lactam ring is formed between the side chains of a lysine residue, located at position 12, 20 or 28, and a glutamic acid residue, located at position 16 or 24, wherein the two amino acids of the glucagon peptide whose side chains participate in forming the lactam ring are spaced from one another by three intervening amino acids. In accordance with one embodiment the lactam bearing glucagon analog comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18. In one embodiment the carboxy terminal amino acid of the lactam bearing peptide comprises an amide group or an ester group in place of the terminal carboxylic acid. In one embodiment a glucagon peptide of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18 further comprises an additional amino acid covalently bound to the carboxy terminus of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 or SEQ ID NO: 18. In a further embodiment a glucagon peptide is provided comprising a sequence selected from the group consisting of SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68 and SEQ ID NO: 69 further comprises an additional amino acid covalently bound to the carboxy terminus of SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68 and SEQ ID NO: 69. In one embodiment the amino acid at position 28 is asparagine or lysine and the amino acid at position 29 is threonine.

In some specific embodiments, stabilization of the alpha helix structure in the C-terminal portion of the glucagon agonist peptide is achieved through the formation of a covalent 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 are used to stabilize the alpha helix.

Enhanced activity at the GLP-1 receptor is also provided by stabilizing the alpha-helix structure in the C-terminal portion of the glucagon peptide (around amino acids 12-29) through introduction of one or more α, α-disubstituted amino acids at positions that retain the desired activity. In some aspects, stabilization of the alpha-helix is accomplished in this manner without purposeful introduction of an intramolecular bridge such as a salt bridge or covalent bond. Such peptides may be 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 may be considered herein as a peptide lacking a covalent intramolecular bridge. 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 the glucagon peptide with amino iso-butyric acid (AIB) enhances GLP-1 activity, in the absence of a salt bridge or lactam. In some embodiments, one, two, three or more of positions 16, 20, 21 or 24 are substituted with AIB.

Enhanced activity at the GLP-1 and glucagon receptors for glucagon analog peptides lacking an intramolecular bridge (e.g., a covalent intramolecular bridge) is provided by the addition of an acyl or alkyl group to the side chain of the amino acid at position 10 of the peptide. In some aspects, the acyl or alkyl group is not naturally-occurring on an amino acid. In specific aspects, the acyl or alkyl group is non-native to any naturally-occuring amino acid. In some embodiments, the acyl group is a fatty acyl group, e.g., a C4 to C30 fatty acyl group. For example, provided herein is a glucagon analog peptide lacking a covalent intramolecular bridge comprising AIB at position 16 and a C14, C16, or C18 fatty acyl group covalently attached to a Lys residue at position 10. Also provided is a glucagon analog peptide lacking an intramolecular bridge (e.g., a covalent intramolecular bridge) comprising AIB at positions 2 and 16 and a C14, C16, or C18 fatty acyl group covalently attached to a Lys residue at position 10. Such acylated glucagon analog peptides lacking an intramolecular bridge (e.g., a covalent intramolecular bridge) may be pegylated as further described herein.

A further enhancement in GLP-1 activity and glucagon activity for acylated glucagon analog peptides lacking an intramolecular bridge (e.g., an intramolecular bridge) may be achieved by incorporating a spacer between the acyl or alkyl group and the side chain of the amino acid at position 10. In accordance with some embodiments, the spacer (e.g., an amino acid, a dipeptide, a tripeptide, a hydrophilic bifunctional spacer, or a hydrophobic bifunctional spacer) 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 GLP-1 activity and glucagon activity for acylated or alkylated peptides lacking an intramolecular bridge (e.g., a covalent intramolecular bridge) are further described herein.

For example, provided herein is a non-native glucagon peptide that differs from SEQ ID NO: 1 by no more than 10 amino acid modifications, comprising an acyl group or alkyl group, wherein the acyl or alkyl group is attached to a spacer and the spacer is attached to the side chain of an amino acid at position 10 of the glucagon peptide, wherein, when said glucagon peptide lacks a hydrophilic moiety, e.g., PEG, said glucagon peptide exhibits 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%, about 100%, about 150%, about 200%, about 400%, about 500% or more) of the activity of native GLP-1 at the GLP-1 receptor. In some embodiments, the glucagon peptide exhibits at least 0.5% (e.g., at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 20%) of the activity of native glucagon at the glucagon receptor, when the glucagon peptide lacks a hydrophilic moiety, e.g., PEG. In some embodiments, the glucagon peptides described above may exhibit any of the above indicated activities and no more than 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon at the glucagon receptor. In some embodiments, the glucagon peptides described above may exhibit any of the above indicated activities and no more than 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native GLP-1 at the GLP-1 receptor.

Enhanced activity at the GLP-1 receptor is also provided by an amino acid modification at position 20. In one embodiment, the glutamine at position 20 is replaced with another hydrophilic amino acid having a side chain that is either charged or has an ability to hydrogen-bond, and is at least about 5 (or about 4-6) atoms in length, for example, lysine, citrulline, arginine, or ornithine.

GLP-1 activity may be reduced by comprising (i) a C-terminal alpha carboxylate group, (ii) a substitution of the Thr at position 7 with an amino acid lacking a hydroxyl group, e.g., Abu or Ile, (iii) a 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 (iv) a combination thereof.

Any of the modifications described above which increase or decrease glucagon receptor activity and which increase GLP-1 receptor activity can be applied individually or in combination. Combinations of the modifications that increase GLP-1 receptor activity may provide higher GLP-1 activity than any of such modifications taken alone. For example, the invention provides glucagon analogs that comprise modifications at position 16, at position 20, and at the C-terminal carboxylic acid group, optionally with a covalent bond between the amino acids at positions 16 and 20; glucagon analogs that comprise modifications at position 16 and at the C-terminal carboxylic acid group; glucagon analogs that comprise modifications at positions 16 and 20, optionally with a covalent bond between the amino acids at positions 16 and 20; and glucagon analogs that comprise modifications at position 20 and at the C-terminal carboxylic acid group; optionally with the proviso that the amino acid at position 12 is not Arg; or optionally with the proviso that the amino acid at position 9 is not Glu.

Other modifications at position 1 or 2, as described herein, 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, 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 may reduce glucagon activity, sometimes significantly; surprisingly, this reduction in glucagon activity can be restored by stabilizing the alpha-helix in the C-terminal portion of glucagon, e.g. through a covalent bond between amino acids at positions "i" and "i+4", e.g., 12 and 16, 16 and 20, or 20 and 24. In some embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In some embodiments, this covalent bond is an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods 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.

Glucagon peptides with GLP-1 activity that contain a non-conservative substitution of His at position 1 with a large, aromatic amino acid (e.g., Tyr) can retain GLP-1 activity provided that the alpha-helix is stabilized via an intramolecular bridge, e.g. through a covalent bond between amino acids at positions "i" and "i+4", e.g., 12 and 16, 16 and 20, or 20 and 24. In some embodiments, this covalent bond is a lactam bridge between a glutamic acid at position 16 and a lysine at position 20. In some embodiments, this covalent bond is an intramolecular bridge other than a lactam bridge. For example, suitable covalent bonding methods 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 further exemplary embodiments, any of the foregoing compounds 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 another embodiment the solubility of the glucagon peptides disclosed herein are enhanced by the covalent linkage of a hydrophilic moiety to the peptide. In one embodiment the hydrophilic moiety is a polyethylene glycol (PEG) chain, optionally linked to the peptide at one or more of positions 16, 17, 21, 24, 29, 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. In other embodiments, an amino acid modified to comprise a hydrophilic group is added to the peptide at the C-terminal amino acid. In one embodiment the peptide co-agonist comprises a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, 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 the side chain of an amino acid residue at one of position 16, 17, 21 or 24 of said glucagon peptide further comprises a polyethylene glycol chain, having 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 another embodiment the solubility of any of the preceding glucagon analogs can be improved by amino acid substitutions and/or additions that introduce a charged amino acid into the C-terminal portion of the peptide, preferably 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, preferably 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 acids, and/or in a further embodiment one to three charged amino acids are also added to the C-terminus of the peptide. 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 one embodiment an analog of the peptide of SEQ ID NO: 20 is provided wherein the analog differs from SEQ ID NO: 20 by 1 to 2 amino acid substitutions at positions 17-26, and in one embodiment the analog differs from the peptide of SEQ ID NO: 20 by an amino acid substitution at position 20.

In accordance with some embodiments, the glucagon peptides disclosed herein are modified by truncation of the C-terminus by one or two amino acid residues. Such modified glucagon peptides, as shown herein, retain similar activity and potency at the glucagon receptor and GLP-1 receptor. In this regard, the glucagon peptides can comprise amino acids 1-27 or 1-28 of the native glucagon peptide (SEQ ID NO: 1), optionally with any of the additional modifications described herein.

In accordance with one embodiment the glucagon peptides disclosed herein are modified by the addition of a second peptide to the carboxy terminus of the glucagon peptide, for example, SEQ ID NO: 26, SEQ ID NO: 27 or SEQ ID NO: 28. In one embodiment a glucagon peptide having a peptide sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, and SEQ ID NO: 69 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: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In a further embodiment, in glucagon peptides which comprise the C-terminal extension, the threonine at position 29 of the native glucagon peptide is replaced with a glycine. A glucagon analog having a glycine substitution for threonine at position 29 and comprising the carboxy terminal extension of SEQ ID NO: 26 is four times as potent at the GLP-1 receptor as native glucagon modified to comprise the carboxy terminal extension of SEQ ID NO: 26. Potency at the GLP-1 receptor can be further enhanced by an alanine substitution for the native arginine at position 18.

Any of the glucagon peptides disclosed herein can be modified to comprise an acyl group or alkyl group, e.g., a C4 to C30 acyl or alkyl group. In some aspects, the acyl group or alkyl group is non-native to any naturally-occurring amino acid. Acylation or alkylation can increase the half-life of the glucagon peptides in circulation. Acylation or alkylation 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. As shown herein, the activity at the glucagon receptor and GLP-1 receptor of the glucagon peptide is maintained, if not substantially enhanced, after acylation. Further, the potency of the acylated glucagon peptides were comparable to the unacylated versions of the glucagon peptides, if not substantially enhanced. 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 spacer, 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.

Thus, as disclosed herein high potency glucagon analogs or glucagon co-agonist analogs are provided that also exhibit improved solubility and/or stability. An exemplary high potency glucagon analog exhibits at least about 200% of the activity of native glucagon at the glucagon receptor, and optionally is soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8, or between 6 and 9, or between 7 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. As another example, an exemplary glucagon co-agonist analog exhibits greater than about 40% or greater than about 60% activity at both the glucagon and the GLP-1 receptors (at a ratio between about 1:3 and 3:1, or between about 1:2 and 2:1), is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25◦C. Another exemplary glucagon co-agonist analog exhibits about 175% or more of the activity of native glucagon at the glucagon receptor and about 20% or less of the activity of native GLP-1 at the GLP-1 receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25◦C. Yet another exemplary glucagon co-agonist analog exhibits about 10% or less of the activity of native glucagon at the glucagon receptor and at least about 20% of the activity of native GLP-1 at the GLP-1 receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25◦C. Yet another exemplary glucagon co-agonist analog exhibits about 10% or less but above 0.1% , 0.5% or 1% of the activity of native glucagon at the glucagon receptor and at least about 50%, 60%, 70%, 80%, 90% or 100% or more of the activity of native GLP-1 at the GLP-1 receptor, is optionally soluble at a concentration of at least 1 mg/mL at a pH between 6 and 8 or between 6 and 9, or between 7 and 9 (e.g. pH 7), and optionally retains at least 95% of the original peptide after 24 hours at 25◦C. In some embodiments, the glucagon peptides exhibit no more than about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native GLP-1 at the GLP-1 receptor. 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).

Any one of the following peptides is excluded from the compounds of the invention, although any of the following peptides comprising one or more further modifications thereto as described herein exhibiting the desired GLP-1 or co-agonist activity, pharmaceutical compositions, kits, and treatment methods using such compounds may be included in the invention: The peptide of SEQ ID NO: 1 with an [Arg12] substitution and with a C-terminal amide; The peptide of SEQ ID NO: 1 with [Arg12,Lys20] substitutions and with a C-terminal amide; The peptide of SEQ ID NO: 1 with [Arg12,Lys24] substitutions and with a C-terminal amide; The peptide of SEQ ID NO: 1 with [Arg12,Lys29] substitutions and with a C-terminal amide; The peptide of SEQ ID NO: 1 with a [Glu9] substitution; The peptide of SEQ ID NO: 1 missing His1, with [Glu9, Glu16, Lys29] substitutions and C-terminal amide; The peptide of SEQ ID NO: 1 with [Glu9, Glu16, Lys29] substitutions and with a C-terminal amide; The peptide of SEQ ID NO: 1 with [Lys13, Glu17] substitutions linked via lactam bridge and with a C-terminal amide; The peptide of SEQ ID NO: 1 with [Lys17, Glu21] substitutions linked via lactam bridge and with a C-terminal amide; The peptide of SEQ ID NO: 1 missing His1, with [Glu20, Lys24] substitutions linked via lactam bridge.

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. The compounds of the present invention 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. 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 or treating hypoglycemia using a pre-formulated aqueous composition of glucagon peptides 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 one embodiment the glucagon peptide is pegylated at position 21 or 24 of the glucagon peptide and the PEG chain has a molecular weight of about 500 to about 5,000 Daltons. In one embodiment the modified glucagon solution is prepackaged in a device that is used to administer the composition to the patient suffering from hypoglycemia.

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, wherein the glucagon peptide is pegylated with a PEG chain having a molecular weight selected from the range of about 5,000 to about 40,000 Daltons

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.

Metabolic Syndrome, also known as metabolic syndrome X, insulin resistance syndrome or Reaven's syndrome, is a disorder that affects over 50 million Americans. Metabolic Syndrome is typically characterized by a clustering of at least three or more of the following risk factors: (1) abdominal obesity (excessive fat tissue in and around the abdomen), (2) atherogenic dyslipidemia (blood fat disorders including high triglycerides, low HDL cholesterol and high LDL cholesterol that enhance the accumulation of plaque in the artery walls), (3) elevated blood pressure, (4) insulin resistance or glucose intolerance, (5) prothrombotic state (e.g. high fibrinogen or plasminogen activator inhibitor-1 in blood), and (6) pro-inflammatory state (e.g. elevated C-reactive protein in blood). Other risk factors may include aging, hormonal imbalance and genetic predisposition.

Metabolic Syndrome is associated with an increased the risk of coronary heart disease and other disorders related to the accumulation of vascular plaque, such as stroke and peripheral vascular disease, referred to as atherosclerotic cardiovascular disease (ASCVD). Patients with Metabolic Syndrome may progress from an insulin resistant state in its early stages to full blown type II diabetes with further increasing risk of ASCVD. Without intending to be bound by any particular theory, the relationship between insulin resistance, Metabolic Syndrome and vascular disease may involve one or more concurrent pathogenic mechanisms including impaired insulin-stimulated vasodilation, insulin resistance-associated reduction in NO availability due to enhanced oxidative stress, and abnormalities in adipocyte-derived hormones such as adiponectin (Lteif and Mather, Can. J. Cardiol. 20 (suppl. B):66B-76B (2004)).

According to the 2001 National Cholesterol Education Program Adult Treatment Panel (ATP III), any three of the following traits in the same individual meet the criteria for Metabolic Syndrome: (a) abdominal obesity (a waist circumference over 102 cm in men and over 88 cm in women); (b) serum triglycerides (150 mg/dl or above); (c) HDL cholesterol (40 mg/dl or lower in men and 50 mg/dl or lower in women); (d) blood pressure (130/85 or more); and (e) fasting blood glucose (110 mg/dl or above). According to the World Health Organization (WHO), an individual having high insulin levels (an elevated fasting blood glucose or an elevated post meal glucose alone) with at least two of the following criteria meets the criteria for Metabolic Syndrome: (a) abdominal obesity (waist to hip ratio of greater than 0.9, a body mass index of at least 30 kg/m^2, or a waist measurement over 37 inches); (b) cholesterol panel showing a triglyceride level of at least 150 mg/dl or an HDL cholesterol lower than 35 mg/dl; (c) blood pressure of 140/90 or more, or on treatment for high blood pressure). (Mathur, Ruchi, "Metabolic Syndrome," ed. Shiel, Jr., William C., MedicineNet.com, May 11, 2009).

For purposes herein, if an individual meets the criteria of either or both of the criteria set forth by the 2001 National Cholesterol Education Program Adult Treatment Panel or the WHO, that individual is considered as afflicted with Metabolic Syndrome.

Without being bound to any particular theory, glucagon peptides described herein are useful for treating Metabolic Syndrome. Accordingly, the invention provides a method of preventing or treating Metabolic Syndrome, or reducing one, two, three or more risk factors thereof, in a subject, comprising administering to the subject a glucagon peptide described herein in an amount effective to prevent or treat Metabolic Syndrome, or the risk factor thereof.

Nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver disease ranging from simple fatty liver (steatosis), to nonalcoholic steatohepatitis (NASH), to cirrhosis (irreversible, advanced scarring of the liver). All of the stages of NAFLD have in common the accumulation of fat (fatty infiltration) in the liver cells (hepatocytes). Simple fatty liver is the abnormal accumulation of a certain type of fat, triglyceride, in the liver cells with no inflammation or scarring. In NASH, the fat accumulation is associated with varying degrees of inflammation (hepatitis) and scarring (fibrosis) of the liver. The inflammatory cells can destroy the liver cells (hepatocellular necrosis). In the terms "steatohepatitis" and "steatonecrosis", *steato* refers to fatty infiltration, hepatitis refers to inflammation in the liver, and necrosis refers to destroyed liver cells. NASH can ultimately lead to scarring of the liver (fibrosis) and then irreversible, advanced scarring (cirrhosis). Cirrhosis that is caused by NASH is the last and most severe stage in the NAFLD spectrum. (Mendler, Michel, "Fatty Liver: Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH)," ed. Schoenfield, Leslie J., MedicineNet.com, August 29, 2005).

Alcoholic Liver Disease, or Alcohol-Induced Liver Disease, encompasses three pathologically distinct liver diseases related to or caused by the excessive consumption of alcohol: fatty liver (steatosis), chronic or acute hepatitis, and cirrhosis. Alcoholic hepatitis can range from a mild hepatitis, with abnormal laboratory tests being the only indication of disease, to severe liver dysfunction with complications such as jaundice (yellow skin caused by bilirubin retention), hepatic encephalopathy (neurological dysfunction caused by liver failure), ascites (fluid accumulation in the abdomen), bleeding esophageal varices (varicose veins in the esophagus), abnormal blood clotting and coma. Histologically, alcoholic hepatitis has a characteristic appearance with ballooning degeneration of hepatocytes, inflammation with neutrophils and sometimes Mallory bodies (abnormal aggregations of cellular intermediate filament proteins). Cirrhosis is characterized anatomically by widespread nodules in the liver combined with fibrosis. (Worman, Howard J., “Alcoholic Liver Disease”, Columbia University Medical Center website).

Without being bound to any particular theory, glucagon peptides described herein are useful for the treatment of Alcoholic Liver Disease, NAFLD, or any stage thereof, including, for example, steatosis, steatohepatitis, hepatitis, hepatic inflammation, NASH, cirrhosis, or complications thereof. Accordingly, the invention provides a method of preventing or treating Alcoholic Liver Disease, NAFLD, or any stage thereof, in a subject comprising administering to a subject a glucagon peptide described herein in an amount effective to prevent or treat Alcoholic Liver Disease, NAFLD, or the stage thereof. Such treatment methods include reduction in one, two, three or more of the following: liver fat content, incidence or progression of cirrhosis, incidence of hepatocellular carcinoma, signs of inflammation, e.g. abnormal hepatic enzyme levels (e.g., aspartate aminotransferase AST and/or alanine aminotransferase ALT, or LDH), elevated serum ferritin, elevated serum bilirubin, and/or signs of fibrosis, e.g. elevated TGF-beta levels. In preferred embodiments, the glucagon peptides are used treat patients who have progressed beyond simple fatty liver (steatosis) and exhibit signs of inflammation or hepatitis. Such methods may result, for example, in reduction of AST and/or ALT levels.

In yet another embodiment a method of treating hyperglycemia, or 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. In one embodiment either method comprises administering an effective amount of a composition comprising a glucagon agonist selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, 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 another embodiment, the method comprises administering an effective amount of a composition comprising a glucagon agonist, wherein the glucagon agonist comprising a glucagon peptide selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID

NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, and SEQ ID NO: 69, 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: 26, SEQ ID NO: 27 or SEQ ID NO: 28. In further embodiments, methods of treating diabetes involving co-administering a conventional dose or a reduced dose of insulin and a glucagon peptide of the invention are provided. Methods of treating diabetes with a glucagon peptide of the invention, without co-administering insulin are also provided.

In yet another aspect, the invention provides novel methods for treating hyperglycemia and novel methods for decreasing appetite or promoting body weight loss that involve administration of a glucagon/GLP-1 co-agonist molecule (including pharmaceutically acceptable salts thereof) that activates both the glucagon receptor and the GLP-1 receptor. Agonism, i.e., activation, of both the glucagon and GLP-1 receptors provides an unexpected improvement compared to GLP-1 agonism alone in treating hyperglycemia. Thus, the addition of glucagon agonism provides an unexpected additive or synergistic effect, or other unexpected clinical benefit(s). Administration with a conventional dose of insulin, a reduced dose of insulin, or without insulin is contemplated according to such methods. Agonism of the glucagon receptor also has an unexpected beneficial effect compared to GLP-1 agonism alone in promoting weight loss or preventing weight gain.

Exemplary glucagon/GLP-1 co-agonist molecules include glucagon peptides of the invention, GLP-1 analogs that activate both GLP-1 and glucagon receptors, fusions of glucagon and GLP-1, or fusions of glucagon analogs and GLP-1 analogs, or chemically modified derivatives thereof. Alternatively, a compound that activates the glucagon receptor can be co-administered with a compound that activates the GLP-1 receptor (such as a GLP-1 analog, an exendin-4 analog, or derivatives thereof). The invention also contemplates co-administration of a glucagon agonist analog with a GLP-1 agonist analog.

Such methods for treating hyperglycemia and/or for decreasing appetite or promoting body weight loss include administration of a glucagon analog with a modification at position 12 (e.g. Arg12), optionally in combination with modifications at position 16 and/or 20. The methods of the invention also include administration of glucagon analogs comprising an intramolecular bridge between the side chains of two amino acids within the region of amino acids 12 and 29 that are separated by three intervening amino acids, e.g. positions 12 and 16, positions 13 and 17 (e.g,. Lys13 Glu17 or Glu13 Lys17), positions 16 and 20, positions 17 and 21 (e.g. Lys17 Glu 21 or Glu17 Lys 21), positions 20 and 24, or positions 24 and 28, with the optional proviso that the amino acid at position 9 is not Glu, and optionally including a C-terminal amide or ester.

In accordance with one embodiment excluded from such glucagon/GLP-1 co-agonist molecules are any glucagon analogs or GLP-1 analogs in the prior art known to be useful in such a method. In another embodiment peptides described in U.S. Patent No. 6,864,069 as acting as both a GLP-1 agonist and a glucagon antagonist for treating diabetes are also excluded as glucagon/GLP-1 co-agonist molecules. In another embodiment, excluded is the use of glucagon antagonists to treat diabetes, such as the antagonists described in Unson et al., *J. Biol. Chem.*, 264:789-794 (1989), Ahn et al., *J. Med. Chem.*, 44:3109-3116 (2001), and Sapse et al., *Mol. Med.*, 8(5):251-262 (2002). In a further embodiment oxyntomodulin or a glucagon analog that contains the 8 C-terminal amino acids of oxyntomodulin (SEQ ID NO: 27) are also excluded as glucagon/GLP-1 co-agonist molecules.

Such methods for treating hyperglycemia are expected to be useful for a variety of types of hyperglycemia, including 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. Such methods for reducing appetite or promoting loss of body weight are expected to be useful in reducing body weight, preventing weight gain, or treating 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.

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

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 receptor mediated cAMP induction by glucagon analogs. More particularly, Fig. 3A compares induction of the glucagon receptor by glucagon analogs E16, K20 ●, E15, E16 ▲, E16, K20 ▼, E15, E16 ◀, E16 ▶ and Gluc-NH_2 ■

Fig. 4A and 4B represents data showing receptor mediated cAMP induction by glucagon analogs. More particularly, Fig. 4A compares induction of the glucagon receptor by glucagon analogs Gluc-NH_2 ●, E16Gluc-NH_2 ▲, E3, E16 Gluc-NH_2 ▼, Orn3, E16 Gluc-NH_2 ◀ and Nle3, E16 Gluc-NH_2, ▶ relative to native glucagon ■, whereas Fig. 4B compares induction of the GLP-1 receptor by glucagon analogs Gluc-NH_2 ●, E16 Gluc-NH_2 ▲, E3, E16Gluc-NH_2 ▼, Orn3, E16 Gluc-NH_2 ◀ and Nle3, E16 Gluc-NH_2, ▶ relative to native GLP-1 ■.

Fig. 5A and 5B represents data showing receptor mediated cAMP induction by glucagon analogs. More particularly, Fig. 5A compares induction of the glucagon receptor by glucagon analogs (E16, K20 Gluc-NH_2 ●(5nM, stock solution), E15, E16 Gluc-NH_2 ▲(5nM, stock solution), E16, K20 Gluc-NH_2 ▼(10nM, stock solution), E15, E16 Gluc-NH_2 ◀ (10nM, stock solution) and E16 Gluc-NH_2 ▶) relative to glucagon-NH_2 (■), whereas Fig. 5B compares induction of the GLP-1 receptor by glucagon analogs (E16, K20 Gluc-NH_2 ●, E15, E16 Gluc-NH_2 ▲, and E16 Gluc-NH_2, ▶) relative to GLP-1 (■) and glucagon-NH_2 (□).

Fig. 6A and 6B represents data showing receptor mediated cAMP induction by glucagon analogs. More particularly, Fig. 6A compares induction of the glucagon receptor by glucagon analogs (Gluc-NH_2 ●, K12E16-NH_2 lactam ▲, E16K20-NH_2 lactam ▼, K20E24-NH_2 lactam ◀ and E24K28-NH_2 lactam ▶) relative to glucagon (■), whereas Fig. 6B compares induction of the GLP-1 receptor by glucagon analogs (Gluc-NH_2 ●, K12E16-NH_2 lactam ▲, E16K20-NH_2 lactam ▼, K20E24-NH_2 lactam ◀ and E24K28-NH_2 lactam ▶) relative to GLP-1 (■).

Fig. 7A and 7B represents data showing receptor mediated cAMP induction by glucagon analogs. More particularly, Fig. 7A compares induction of the glucagon receptor by glucagon analogs (Gluc-NH_2 ●, E16 Gluc-NH_2, ▲, K12, E16 Gluc-NH_2 lactam ▼, E16, K20 Gluc-NH_2 ◀ and E16, K20 Gluc-NH_2 lactam ▶) relative to glucagon (■), whereas Fig. 7B compares induction of the GLP-1 receptor by glucagon analogs (Gluc-NH_2 ●, E16 Gluc-NH_2, ▲, K12, E16 Gluc-NH_2 lactam ▼, E16, K20 Gluc-NH_2 ◀ and E16, K20 Gluc-NH_2 lactam ▶) relative to GLP-1 (■).

Figs. 8A-8F represent data showing receptor mediated cAMP induction by glucagon analogs at the glucagon receptor (Figs. 8A, 8C and 8E) or the GLP-1 receptor (Figs. 8B, 8C and 8F) wherein hE = homoglutamic acid and hC = homocysteic acid.

Fig. 9A and 9B: represent data showing receptor mediated cAMP induction by GLP (17-26) glucagon analogs, wherein amino acid positions 17-26 of native glucagon (SEQ ID NO: 1) have been substituted with the amino acids of positions 17-26 of native GLP-1 (SEQ ID NO: 50). More particularly, Fig. 9A compares induction of the glucagon receptor by the designated GLP (17-26) glucagon analogs, and Fig. 9B compares induction of the GLP-1 receptor by the designated GLP (17-26) glucagon analogs.

Figs. 10A-E: are graphs providing *in vivo* data demonstrating the ability of the glucagon peptides of the present invention to induce weight loss in mice injected subcutaneously with the indicated amounts of the respective compounds. Sequence Identifiers for the glucagon peptide listed in Figs 10A -10E are as follows, for Fig. 10A: Chimera 2 Aib2 C24 40K PEG (SEQ ID NO: 486), Aib2 C24 Chimera 2 40K lactam (SEQ ID NO: 504) and Aib2 E16 K20 Gluc-NH2 Lac 40K (SEQ ID NO: 528); Fig. 10B: Aib2 C24 Chi 2 lactam 40K (SEQ ID NO: 504), DMIA1 C24 Chi 2 Lactam 40K (SEQ ID NO: 505), Chimera 2 DMIA1 C24 40K (SEQ ID NO: 519), and Chimera 2 Aib2 C24 40K (SEQ ID NO: 486), wherein the number at the end of the sequence designates the dosage used, either 70 or 350 nmol/kg; Fig. 10C: AIB2 w/ lactam C24 40K (SEQ ID NO: 504), AIB2 E16 K20 w/ lactam C24 40K (SEQ ID NO: 528), DMIA1 E16 K20 w/ lactam C24 40K (SEQ ID NO: 510), DMIA1 E16 K20 w/ lactam CEX 40K (SEQ ID NO: 513) and DMIA1 E16 K20 w/o lactam CEX 40K (SEQ ID NO: 529); Fig. 10D: AIB2 w lactam C24 40K (SEQ ID NO: 504), AIB2 E16 K20 w lactam C24 40K (SEQ ID NO: 528), DMIA1 E16 K20 w lactam C24 40K (SEQ ID NO: 510) and DMIA1 E16 K20 w lactam/Cex C24 40K (SEQ ID NO: 513), wherein the number at the end of the sequence designates the dosage used, either 14 or 70 nmol/kg/wk; Fig. 10E: AIB2 w/o lactam C24 40K (SEQ ID NO: 486), Chi 2 AIB2 C24 CEX 40K (SEQ ID NO: 533), AIB2 E16 A18 K20 C24 40K (SEQ ID NO: 492), AIB2 w/o lactam CEX G29 C40 40K (SEQ ID NO: 488), AIB2 w/o lactam CEX C40 C41-2 (SEQ ID NO: 532), AIB2 w/o lactam CEX C24 C40- 2 (SEQ

ID NO: 531) and AIB2 w/o lactam C24 60K (SEQ ID NO: 498), wherein the designation 40K or 60K represents the molecular weight of the polyethylene chain attached to the glucagon peptide.

Figures 11-13 are graphs providing *in vivo* data demonstrating the ability of acylated glucagon peptides to induce weight loss (Figure 11), reduce food intake (Figure 12), and reduce blood glucose levels (Figure 13) in mice injected subcutaneously with the indicated amounts of the compounds.

Figures 14A and 14B represent data showing glucagon and GLP-1 receptor mediated cAMP induction, respectively, by glucagon analogs.

Figure 15 represents a graph of blood glucose (mg/dL) as a function of time (mins) in DIO mice treated with 2 nmol/kg of vehicle only (triangles), Chimera-2 AIB^2, K^{10}-C8 Cys^{24}-40kD PEG (open squares), or Chimera-2 AIB^2, K^{10}-C16 Cys^{24}-40kD PEG (inverted triangles) followed by glucose challenge 15 mins after administration of the peptide.

Figure 16 represents a graph of blood glucose (mg/dL) as a function of time (mins) in DIO mice treated with 20 nmol/kg of vehicle only (triangles), Chimera-2 AIB^2, K^{10}-C8 Cys^{24}-40kD PEG (open squares), or Chimera-2 AIB^2, K^{10}-C16 Cys^{24}-40kD PEG (inverted triangles) followed by glucose challenge 15mins after administration of the peptide.

Figure 17 represents a graph of blood glucose (mg/dL) as a function of time (mins) of DIO mice treated with 70 nmol/kg of vehicle only (inverted triangles), Chimera-2 AIB^2, K^{10}-C8 Cys^{24}-40kD PEG (open triangles), Chimera-2 AIB^2, K^{10}-C16 Cys^{24}-40kD PEG (diamonds), or Chimera-2 AIB^2, Cys^{24}-40kD PEG (open squares) followed by glucose challenge 15mins after administration of the peptide.

Figure 18 represents a graph of blood glucose (mg/dL) as a function of time (mins) of DIO mice treated with 70 nmol/kg of vehicle only (inverted triangles), Chimera-2 AIB^2, K^{10}-C8 Cys^{24}-40kD PEG (open triangles), Chimera-2 AIB^2, K^{10}-C16 Cys^{24}-40kD PEG (diamonds), or Chimera-2 AIB^2, Cys^{24}-40kD PEG (open squares) followed by glucose challenge 24 hours after administration of the peptide.

Figure 19 represents a graph of the change in body weight (%) as a function of time (days) in DIO mice treated with 15 or 70 nmol/kg of vehicle only (diamonds with solid line); Chimera-2 AIB^2, Cys^{24}-40kD PEG (15 nmol/kg, open diamonds with dotted line; 70 nmol/kg, open triangles with solid line); Chimera-2 AIB^2, K^{10}-C8 Cys^{24}-40kD PEG (15 nmol/kg, closed triangle with dotted line; 70 nmol/kg, closed triangle with solid line); Chimera-2 AIB^2, K^{10}-C16 Cys^{24}-40kD Peg (15nmol/kg, inverted triangle with dotted line; 70 nmol/kg; inverted triangle with solid line).

Figure 20 represents a graph of the total change in body weight (%) in mice 14 days after QW injections of 10, 20, 40, or 80 nmol/kg Peptide A K^{10}-C_{14} or 20 nmol/kg Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD or a vehicle control

Figure 21 represents a graph of the blood glucose levels (mg/dL) in response to a glucose injection of mice injected with 10, 20, 40, or 80 nmol/kg Peptide A K^{10}-C_{14} or 20 nmol/kg Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD or a vehicle control 24 hours prior to the glucose injection.

Figure 22 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 23 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 24 represents a graph of the change in blood glucose (mg/dL; Day 7 levels minus Day 0 levels) 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 25 represents a graph of the mean residue ellipticity as a function of wavelength (nm) for Peptide X-PEG or Peptide Y-PEG in 10 mM Phosphate (pH 5.9) either with or without 10% TFE.

Figure 26 represents a graph of the % cAMP produced in response to Glucagon, GLP-1, Peptide X, Peptide X-PEG, Peptide Y, or Peptide Y-PEG binding to either the glucagon receptor (left) or GLP-1 receptor (right) as a function of peptide concentration (nM).

Figure 27 represents a collection of graphs which demonstrate the *in vivo* effects on A) body weight, B) fat mass, C) food intake, and D) fasting blood glucose levels in diet induced obese mice treated for one week with vehicle control, Peptide X-PEG, or Peptide Y-PEG. More specifically, Figure 27A represents a graph of the % change in body weight (BW) as a function of time (days), Figure 27 B represents a graph of the % change in fat mass as measured on Day 7 (as compared to initial fat mass measurements), Figure 27C represents a graph of the total food intake (g) over the course of the study as measured on Day 7, and Figure 27D represents a graph of the change in blood glucose (mg/dL) as measured on Day 7 (in comparison to initial blood glucose levels).

Figure 28 represents a collection of graphs which demonstrate the *in vivo* effects on body weight (Figures 28A and 28C) and fasting blood glucose levels (Figures 28B and 28D) in mice treated with either Peptide X-PEG (Figures 28A and 28B) or Peptide Y-PEG (Figures 28C and 28D) at varying doses (nmol/kg/week).

Figure 29 represents a collection of graphs showing the *in vivo* effects on A) body weight (BW), B) body fat mass, C) overall food intake, D) energy expenditure, E) respiratory quotient, F) locomotor activity, G) fasting blood glucose, H) glucose tolerance, and I) total plasma insulin levels in diet induced obese mice treated for one month with a vehicle control, Peptide X-PEG, or Peptide Y-PEG.

Figure 30 represents a collection of graphs showing the *in vivo* Week 3 effects on calorimetric measurements of A) food intake, B) total energy expenditure, C) total respiratory quotient, D) locomotor activity, E) total locomotor activity, F) area under the curve ipGTT, G) plasma C-peptide levels, H) PEPCK/HPRT fold expression, and I) G6P/HPRT fold expression levels in diet induced obese mice treated for one month with a vehicle control, Peptide X-PEG, or Peptide Y-PEG

Figure 31 represents a collection of graphs demonstrating the *in vivo* effects on plasma A) cholesterol, B) cholesterol FPLC, C) triglycerides, D) leptin., E) resistin, and F) adiponectin in diet induced obese mice treated for one month with a vehicle control, Peptide X-PEG, or Peptide Y-PEG.

Figure 32 represents a collection of graphs demonstrating the *in vivo* effects on A) BAT UCP-1 expression levels and B) white adipose tissue as reflected by phosphorylation of hormone sensitive lipase (pHSL) in mice treated with a vehicle control, Peptide X-PEG, or Peptide Y-PEG

Figure 33 represents a collection of graphs demonstrating the *in vivo* effects of a vehicle control, Peptide X-PEG, or Peptide Y-PEG in DIO rats on A) body weight and B) fat mass. Figure 33C represents a graph of the relative expression of CD68 to TFIIB as quantitatively assessed by real-time RT-PCR in epidiymal adipose tissue isolated from mice treated for two weeks with Peptide Y-PEG, Peptide X-PEG, or vehicle. Data are presented as relative CD68 mRNA expression normalized to TF1IB mRNA expression and expressed as mean ± SEM.

Figures 34A to 34F represent a collection of graphs demonstrating the *in vivo* effects on body weight (BW; 34A and 34B), fat mass (34C), food intake (34D), and blood glucose levels (34E and 34F) in GLP-1-R knock out mice treated with a vehicle control, Peptide X-PEG, or Peptide Y-PEG.

Figures 35A to 35C represent a series of graphs demonstrating the in vivo effects on body weight (35A), blood glucose (35B), and fat mass (35C) in DIO mice treated with vehicle control, Peptide V, or Peptide U.

Figure 36 represents a graph of the % total change in body weight of mice injected with vehicle, liraglutide, glucagon amide, MT-261, MT-345, MT-347, or MT-348 at the dose indicated in () as measured on Day 14 of the study.

Figure 37 represents a graph of the % total change in body weight of mice injected with vehicle, MT-278, MT-358, MT-261, MT-297, or MT-364 as measured on Day 7 of the study.

Figure 38 represents a graph of the % total change in body weight of mice injected with vehicle only, Peptide 83, Peptide 900, Peptide 901, or MT-364, as measured on Day 7 of the study.

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. An alternative desired effect for the co-agonist analogs of the present disclosure would include treating hyperglycemia, e.g., as measured by a change in blood glucose level closer to normal, or inducing weight loss/preventing weight gain, e.g., as measured by reduction in body weight, or preventing or reducing an increase in body weight, or normalizing body fat distribution. 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 "peptide" encompasses a sequence of 3 or more amino acids and typically less than 50 amino acids, wherein the amino acids are naturally occurring 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.

As used herein, the terms "polypeptide" and "protein" are terms that are used interchangeably to refer to a polymer of amino acids, without regard to the length of the polymer. Typically, polypeptides and proteins have a polymer length that is greater than that of "peptides."

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, deletions or post translational modifications (e.g., methylation, acylation, ubiquitination, intramolecular covalent bonding such as lactam bridge formation, PEGylation, 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 14.

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 14.

As used herein a "glucagon agonist analog" is a glucagon peptide comprising a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15, or an analog of such a sequence that has been modified to include one or more conservative amino acid substitutions at one or more of positions 2, 5, 7, 10, 11, 12, 13, 14, 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. 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 a "glucagon co-agonist" is a glucagon peptide that exhibits activity at the glucagon receptor of at least about 10% to about 500% or more relative to native glucagon and also exhibits activity at the GLP-1 receptor of about at least 10% to about 200% or more relative to native GLP-1.

As used herein a "glucagon/GLP-1 co-agonist molecule” is a molecule that exhibits activity at the glucagon receptor of at least about 10% relative to native glucagon and also exhibits activity at the GLP-1 receptor of at least about 10% relative to native GLP-1.

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: 52), GLP-1(7-37)acid (consisting of the sequence of SEQ ID NO: 50) 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.

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 and esters:
   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 chain" or "PEG chain", 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 chain" or "PEG chain" 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 chain 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 chain 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 "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.

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

The invention provides glucagon peptides with increased or decreased activity at the glucagon receptor, or the GLP-1 receptor, or at both receptors. The invention also provides glucagon peptides with altered selectivity for the glucagon receptor versus the GLP-1 receptor.

Increased activity at the glucagon receptor is provided by an amino acid modification at position 16 of native glucagon (SEQ ID NO: 1) as described herein.

Maintained or increased activity at the glucagon receptor is also provided by an amino acid modification at position 3 of native glucagon with a glutamine analog (e.g. (Dab(Ac)).

Reduced activity at the glucagon receptor is provided, e.g., by substitution of the amino acid at position 3 with an acidic, basic, or hydrophobic amino acid as described herein.

Increased 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.

Increased activity at the GLP-1 receptor is provided by modifications that stabilize the alpha helix in the C-terminal portion of glucagon (e.g. around residues 12-29). In some embodiments, such modifications permit formation of an intramolecular bridge between the side chains of two amino acids that are separated by three intervening amino acids, for example, positions 12 and 16, or 16 and 20, or 20 and 24, as described herein. In other embodiments, such modifications include insertion or substitution modifications that introduce one or more α, α-disubstituted amino acids, e.g. AIB at one or more of positions 16, 20, 21 or 24.

Increased activity at the GLP-1 and glucagon receptors for peptides lacking an intramolecular bridge, e.g., a covalent intramolecular brige, is provided by covalently attaching an acyl or alkyl group to to the side chain of the amino acid at position 10 of the peptide, wherein the acyl or alkyl group is non-native to the amino acid at position 10. Further increased activity at the GLP-1 and glucagon receptors for such peptides lacking an intramolecular bridge, e.g., a covalent intramolecular bridge, may be achieved by incorporating a spacer between the acyl or alkyl group and the side chain of the amino acid at position 10. Suitable spacers are described herein and include, but not limited to spacers that are 3 to 10 atoms in length.

Increased activity at the GLP-1 receptor is provided by an amino acid modification at position 20 as described herein.

Increased activity at the GLP-1 receptor is provided in glucagon analogs comprising the C-terminal extension of SEQ ID NO: 26. GLP-1 activity in such analogs comprising SEQ ID NO: 26 can be further increased by modifying the amino acid at position 18, 28 or 29, or at position 18 and 29, as described herein.

Restoration of glucagon activity which has been reduced by amino acid modifications at positions 1 and 2 is provided by a covalent bond between the side chains of two amino acids that are separated by three intervening amino acids, for example, positions 12 and 16, or 16 and 20, or 20 and 24, as described herein.

A further modest increase in GLP-1 potency is provided by modifying the amino acid at position 10 to be Trp.

Any of the modifications described above which increase or decrease glucagon receptor activity and which increase GLP-1 receptor activity can be applied individually or in combination. Any of the modifications described above can also be combined with other modifications that confer other desirable properties, such as increased solubility and/or stability and/or duration of action. Alternatively, any of the modifications described above can be combined with other modifications that do not substantially affect solubility or stability or activity. Exemplary modifications include but are not limited to:

(A) Improving solubility, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. Such a charged amino acid can be introduced 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. Such modifications increase solubility, e.g. provide 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.

(B) Increasing solubility and duration of action or half-life in circulation by addition of a hydrophilic moiety such as a polyethylene glycol chain, as described herein, e.g. at position 16, 17, 20, 21, 24 or 29, or at the C-terminal amino acid of the peptide.

(C) Increasing , by modification of the aspartic acid at position 15, for example, by deletion or substitution with glutamic acid, homoglutamic acid, cysteic acid or homocysteic acid. Such modifications can reduce degradation or cleavage 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.

(D) Increasing stability by modification of the methionine at position 27, for example, by substitution with leucine or norleucine. Such modifications can reduce oxidative degradation. Stability can also be increased by modification of the Gln at position 20 or 24, e.g. by substitution with Ala, Ser, Thr, or AIB. Such modifications can reduce degradation that occurs through deamidation of Gln. Stability can be increased by modification of Asp at position 21, e.g. by substitution with Glu. Such modifications can reduce degradation that occurs through dehydration of Asp to form a cyclic succinimide intermediate followed by isomerization to iso-aspartate.

(E) Increasing resistance to dipeptidyl peptidase IV (DPP IV) cleavage by modification of the amino acid at position 1 or 2 as described herein.

(F) Conservative or non-conservative substitutions, additions or deletions that do not affect 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; deletions at one or more of positions 27, 28 or 29; or a deletion of amino acid 29 optionally combined with a C-terminal amide or ester in place of the C-terminal carboxylic acid group;

(G) Adding C-terminal extensions as described herein;

(H) Increasing half-life in circulation and/or extending the duration of action and/or delaying the onset of action, for example, through acylation or alkylation of the glucagon peptide, as described herein;

(I) Homodimerization or heterodimerization as described herein.

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 amino acid modifications relative to the native glucagon sequence.

Other modifications include substitution of His at position 1 with a large, aromatic amino acid (e.g., Tyr, Phe, Trp or amino-Phe);

Ser at position 2 with Ala;

substitution of Tyr at position 10 with Val or Phe;

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.

One embodiment disclosed herein 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-

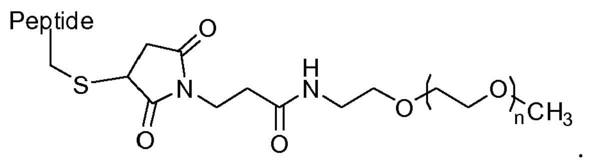
Met-Asn-Thr (SEQ ID NO: 1) to enhance the peptide's potency at the glucagon receptor. Surprisingly, applicants have discovered that the normally occurring serine at position 16 of native glucagon (SEQ ID NO: 1) can be substituted with select acidic amino acids to enhance the potency of glucagon, in terms of its ability to stimulate cAMP synthesis in a validated *in vitro* model assay (see Example 14). More particularly, this substitution enhances the potency of the analog at least 2-fold, 4-fold, 5-fold, and up to 10-fold greater at the glucagon receptor. This substitution also enhances the analog's activity at the GLP-1 receptor at least 5-fold, 10-fold, or 15-fold relative to native glucagon, but selectivity is maintained for the glucagon receptor over the GLP-1 receptor.

In accordance with one embodiment the serine residue at position 16 of native glucagon is substituted with an amino acid selected from the group consisting of glutamic acid, glutamine, homoglutamic acid, homocysteic acid, threonine or glycine. In accordance with one embodiment the serine residue at position 16 of native glucagon is substituted with an amino acid selected from the group consisting of glutamic acid, glutamine, homoglutamic acid and homocysteic acid, and in one embodiment the serine residue is substituted with glutamic acid. In one embodiment the glucagon peptide having enhanced specificity for the glucagon receptor comprises the peptide of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or a glucagon agonist analog thereof, wherein the carboxy terminal amino acid retains its native carboxylic acid group. In accordance with one embodiment a glucagon agonist comprising the sequence of NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-COOH (SEQ ID NO: 10) is provided, wherein the peptide exhibits approximately fivefold enhanced potency at the glucagon receptor, relative to native glucagon as measured by the *in vitro* cAMP assay of Example 14.

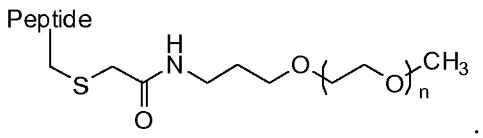
Hydrophilic moieties

The glucagon peptides of the present invention can be further modified to improve the peptide's solubility and stability in aqueous solutions at physiological pH, while retaining the high biological activity relative to native glucagon. 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 (.beta.-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. 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.

In accordance with one embodiment, introduction of hydrophilic groups at positions 17, 21, and 24 of the peptide of SEQ ID NO: 9 or SEQ ID NO: 10 are anticipated to improve the solubility and stability of the high potency glucagon analog in solutions having a physiological pH. Introduction of such groups also increases duration of action, e.g. as measured by a prolonged half-life in circulation. Suitable hydrophilic moieties include any water soluble polymers known in the art, including PEG, homo- or co-polymers of PEG, a monomethyl-substituted polymer of PEG (mPEG), or polyoxyethylene glycerol (POG). In accordance with one embodiment the hydrophilic group comprises a polyethylene (PEG) chain. More particularly, in one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 6 or SEQ ID NO: 7 wherein a PEG chain is covalently linked to the side chains of amino acids present at positions 21 and 24 of the glucagon peptide and the carboxy terminal amino acid of the peptide has the carboxylic acid group.

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, Omethylisourea, 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 form 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 CH2 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.

Fusion protein and Terminal extension

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. More particularly, the fusion glucagon peptide may comprise a glucagon agonist of SEQ ID NO: 55, SEQ ID NO: 9 or SEQ ID NO: 10 further comprising an amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS), SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 (KRNR) linked to amino acid 29 of the glucagon peptide. In one embodiment the amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS), SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 (KRNR) is bound to amino acid 29 of the glucagon peptide through a peptide bond. Applicants have discovered that in glucagon fusion peptides comprising the C-terminal extension peptide of Exendin-4 (e.g., SEQ ID NO: 26 or SEQ ID NO: 29), substitution of the native threonine residue at position 29 with glycine dramatically increases GLP-1 receptor activity. This amino acid substitution can be used in conjunction with other modifications disclosed herein to enhance the affinity of the glucagon analogs for the GLP-1 receptor. For example, the T29G substitution can be combined with the S16E and N20K amino acid substitutions, optionally with a lactam bridge between amino acids 16 and 20, and optionally with addition of a PEG chain as described herein. In one embodiment a glucagon/GLP-1 receptor co-agonist is provided, comprising the sequence of SEQ ID NO: 64. In one embodiment the glucagon peptide portion of the glucagon fusion peptide is selected from the group consisting of SEQ ID NO: 55, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5 wherein a PEG chain, when present at positions 17, 21, 24, or the C-terminal amino acid, or at both 21 and 24, is selected from the range of 500 to 40,000 Daltons. More particularly, in one embodiment the glucagon peptide segment is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 63, wherein the PEG chain is selected from the range of 500 to 5,000. In one embodiment the glucagon peptide is a fusion peptide comprising the sequence of SEQ ID NO: 55 and SEQ ID NO: 65 wherein the peptide of SEQ ID NO: 65 is linked to the carboxy terminus of SEQ ID NO: 55.

Charge neutral C-terminus

In accordance with one embodiment, an additional chemical modification of the glucagon peptide of SEQ ID NO: 10 bestows increased GLP-1 receptor potency to a point where the relative activity at the glucagon and GLP-1 receptors is virtually equivalent. Accordingly, in one embodiment a glucagon/GLP-1 receptor co-agonist is provided wherein the terminal amino acid of the glucagon peptides of the present invention have an amide group in place of the carboxylic acid group that is present on the native amino acid. The relative activity of the glucagon analog at the respective glucagon and GLP-1 receptors can be adjusted by further modifications to the glucagon peptide to produce analogs demonstrating about 40% to about 500% or more of the activity of native glucagon at the glucagon receptor and about 20% to about 200% or more of the activity of native GLP-1 at the GLP-1 receptor, e.g. 50-fold, 100-fold or more increase relative to the normal activity of glucagon at the GLP-1 receptor. In some embodiments, the glucagon peptides described herein exhibit up to about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native glucagon at the glucagon receptor. In some embodiments, the glucagon peptides described herein exhibit up to about 100%, 1000%, 10,000%, 100,000%, or 1,000,000% of the activity of native GLP-1 at the GLP-1 receptor.

Stabilization of the alpha helix/Intramolecular bridges

In a further embodiment glucagon analogs are provided that exhibit enhanced GLP-1 receptor agonist activity wherein an intramolecular bridge is formed between two amino acid side chains to stabilize the three dimensional structure of the carboxy terminus of the 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.

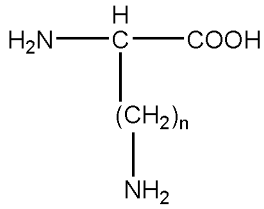
In some specific embodiments, wherein the amino acids at positions i and i+4 are joined by an intramolecular bridge, the size of the linker is about 8 atoms, or about 7-9 atoms.

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.

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.

In accordance with one embodiment a glucagon analog is provided that exhibits glucagon/GLP-1 receptor co-agonist activity wherein the analog comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11, 47, 48 and 49. In one embodiment the side chains are covalently bound to one another, and in one embodiment the two amino acids are bound to one another to form a lactam ring. The size of the 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.

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). In accordance with one embodiment a glucagon analog of SEQ ID NO: 45 is provided wherein at least one lactam ring is formed between the side chains of an amino acid pair selected from the group consisting of amino acid pairs 12 and 16, 16 and 20 , 20 and 24 or 24 and 28. In one embodiment a glucagon/GLP-1 receptor co-agonist is provided wherein the co-agonist comprises a glucagon peptide analog of SEQ ID NO: 20 wherein the peptide comprises an intramolecular lactam bridge formed between amino acid positions 12 and 16 or between amino acid positions 16 and 20. In one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising the sequence of SEQ ID NO: 20, wherein an intramolecular lactam bridge is formed between amino acid positions 12 and 16, between amino acid positions 16 and 20, or between amino acid positions 20 and 24 and the amino acid at position 29 is glycine, wherein the sequence of SEQ ID NO: 29 is linked to the C-terminal amino acid of SEQ ID NO: 20. In a further embodiment the amino acid at position 28 is aspartic acid.

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 yet another embodiment of the invention, the glucagon peptide with enhanced GLP-1 activity comprises (a) one or more substitutions within amino acid positions 12-29 with an α, α-disubstituted amino acid and optionally, (b) a C-terminal amide. In some aspects, it is to be appreciated that such glucagon peptides specifically lack an intramolecular bridge, e.g., a covalent intramolecular bridge, that stabilizes the alpha-helix in the C-terminal portion of glucagon (around positions 12-29). In some embodiments, one, two, three, four or more of positions 16, 17, 18, 19, 20, 21, 24 or 29 of glucagon is substituted with an α, α-disubstituted amino acid, e.g., 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). For example, substitution of position 16 with AIB enhances GLP-1 activity, 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). In some embodiments, one, two, three or more of positions 16, 20, 21 or 24 are substituted with AIB. Such a glucagon peptide may further comprise one or more of the other modifications described herein, including, but not limited to, acylation, alkylation, pegylation, deletion of 1-2 amino acids at the C-terminus, addition of and/or substitution with charged amino acids at the C-terminus, replacement of the C-terminal carboxylate with an amide, addition of a C-terminal extension, and conservative and/or non-conservative amino acid substitutions, such as substitution of Met at position 27 with Leu or Nle, substitution of Asp at position 15 with Glu (or like amino acid), substitution at position 1 and/or 2 with amino acids which achieve DPP-IV protease resistance, substitution of Ser at position 2 with Ala, substitution of Tyr at position 10 with Val or Phe, substitution of Lys at position 12 with Arg, substitution of Ser at position 16 with Thr or AIB, substitution of Gln at position 20 and/or 24 with Asp, Glu, or AIB, substitution of Ser at position 16 with Glu or Thr, Arg at position 18 with Ala, Gln at position 20 with Lys, Asp at position 21 with Glu, and Gln at position 24 with Asn or Cys. In some embodiments, the foregoing glucagon peptide comprises a Gln or Gly at position 29 or addition of a C-terminal extension, e.g., GGPSSGAPPPS (SEQ ID NO: 26) C-terminal to the amino acid at position 28. In a specific aspect, the glucagon peptide comprises one or more of an amide group in place of the C-terminal carboxylate, an acyl group, e.g., a C16 fatty acid, and a hydrophilic moiety, e.g., a polyethylene glycol (PEG).

Also, in another specific aspect, the glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 1-25, 30-64, and 66-555 comprising no more than ten modifications relative to SEQ ID NO: 1 and comprising one or more amino acid substitutions with AIB at positions 16, 20, 21, and/or 24, wherein the peptide lacks an intramolecular bridge, e.g., a covalent intramolecular bridge, between the side chains of two amino acids of the peptide. Accordingly, in a more specific aspect, the glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 556-561.

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 the amino acid at position 10 of the glucagon peptide. In specific embodiments, the acyl or alkyl group is not naturally occurring on an amino acid. In certain aspects, the acyl or alkyl group is non-native to the amion acid at position 10. In exemplary embodiments, the glucagon peptide lacking an intramolecular bridge comprises the amino acid sequence of any of SEQ ID NOs: 556-561 and an acyl or alkyl group covalently attached to the side chain of the amino acid at position 10 of the glucagon peptide. 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.

Modification at position 1

In accordance with one embodiment of the invention, the glucagon peptide with enhanced GLP-1 activity comprises (a) an amino acid substitution of His at position 1 with a large, aromatic amino acid and (b) an intramolecular bridge that stabilizes that alpha-helix in the C-terminal portion of the molecule (e.g. around positions 12-29). In a specific embodiment, the amino acid at position 1 is Tyr, Phe, Trp, amino-Phe, nitro-Phe, chloro-Phe, sulfo-Phe, 4-pyridyl-Ala, methyl-Tyr, or 3-amino Tyr. In a specific aspect, the intramolecular bridge is between the side chains of two amino acids that are separated by three intervening amino acids, i.e., between the side chains of amino acids i and i+4. In some embodiments, the intramolecular bridge is a lactam bridge. In a more specific embodiment of the invention, the glucagon peptide comprises a large, aromatic amino acid at position 1 and a lactam bridge between the amino acids at positions 16 and 20 of the peptide. Such a glucagon peptide may further comprise one or more (e.g., two, three, four, five or more) of the other modifications described herein. For example, the glucagon peptide can comprise an amide in place of the C-terminal carboxylate. Accordingly, in one embodiment, the glucagon peptide comprises that amino acid sequence of SEQ ID NO: 555.

Acylation

In accordance with one embodiment, the glucagon peptide comprises an acyl group, e.g., an acyl group which is non-native to a naturally-occurring amino acid. The acyl group causes the peptide to have one or more of (i) a prolonged half-life in circulation, (ii) a delayed onset of action, (iii) an extended duration of action, (iv) an improved resistance to proteases, such as DPP-IV, and (v) increased potency at the GLP-1 receptor, GIP receptor, and/or glucagon receptor. As shown herein, acylated glucagon peptides do not exhibit decreased activity at the glucagon receptor, GIP receptor, and/or, GLP-1 receptor in comparison to the corresponding unacylated glucagon peptide. Rather, in some instances, acylated glucagon peptides actually exhibit increased activity at the GLP-1 receptor, GIP receptor, and/or glucagon receptor. 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.

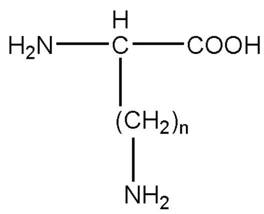
Acylation can be carried out at any position within the glucagon peptide, including any of positions 1-29, a position within a C-terminal extension, or the C-terminal amino acid, provided that glucagon and/or GLP-1 activity and/or GIP activit is retained, if not enhanced. Nonlimiting examples include positions 5, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 27, 28, 29, 30, 37, 38, 39, 40, 41, 42, or 43 (according to the numbering of the amino acids of SEQ ID NO: 1). In specific embodiments, acylation occurs at position 10 or 40 of the glucagon peptide and the glucagon peptide lacks an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam bridge) and comprises a C-terminal extension. Such acylated peptides lacking an intramolecular bridge and comprising a C-terminal extension demonstrate enhanced activity at the GLP-1, GIP, and glucagon receptors as compared to the corresponding non-acylated peptides. 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 or 40 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28, 29, or 40 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, 29, or 40. 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, 29, or 40 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 or 40.

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

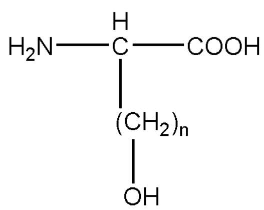


wherein n = 1 to 4

[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:

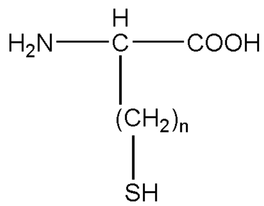


wherein n = 1 to 4

[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.

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. The amino acid to which the spacer is attached can be any amino acid (e.g., a singly or doubly α-substituted 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, 29, and 40 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, and 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 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. 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 bifunctional spacer, 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, 29, or 40, 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 or 40 of the glucagon peptide and the length of the spacer and acyl group is 14 to 28 atoms. The amino acid at position 10 or 40, 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. Accordingly, the acylated glucagon peptide can comprise the amino acid sequence of any of SEQ ID NOs: 555-561 and 610-612, the AIB-containing peptides of Tables 20 and 28, or of SEQ ID NOs: 657-669.

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, bifunctional spacers, e.g., 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 alkylated des-amino Cys spacer can be, for example, a des-amino-Cys spacer comprising a dodecaethylene glycol moiety.

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, 29, and 40 comprise an acyl group and at least one of the amino acids at position 16, 17, 21, 24, 29, 40, 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 or 40, 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.

In a specific aspect of the invention, the acylated glucagon peptide comprises the amino acid sequence of any of SEQ ID NOs: 534-544, 546-549 and 657-669.

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-occuring 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 positions 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, 29, 30, 37, 38, 39, 40, 41, 42, or 43 according to the numbering of the amino acids of SEQ ID NO: 1. 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 40 and pegylation at one or more positions in the C-terminal portion of the glucagon peptide, e.g., position 24, 28, 29, 40, 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, 29, or 40. 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, 29, or 40 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 40.

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, 29, or 40 of the glucagon peptide. The amino acid to which the spacer is attached can be any 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, 29, or 40 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, and 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 is 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 hydropholic bifunctional spacer comprises an amine group and a carboxylate. In other embodiments, the hydropholic 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 bifunctional spacer, 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, 29, or 40 comprise an alkyl group and at least one of the amino acids at position 16, 17, 21, 24, 29, 40, 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 40, 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.

C-terminal truncation

In some embodiments, the glucagon peptides described herein are further modified by truncation or deletion of one or two amino acids of the C-terminus of the glucagon peptide (i.e., position 29 and/or 28) without affecting activity and/or potency at the glucagon and GLP-1 receptors. In this regard, the glucagon peptide can comprise amino acids 1-27 or 1-28 of the native glucagon peptide (SEQ ID NO: 1), optionally with one or more modifications described herein.

In one embodiment, the truncated glucagon agonist peptide comprises SEQ ID NO: 550 or SEQ ID NO: 551. In another embodiment, the truncated glucagon agonist peptide comprises SEQ ID NO: 552 or SEQ ID NO: 553.

Charged C-terminal residues

The solubility of the glucagon peptide of SEQ ID NO: 20 can be further improved, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of glucagon peptide of SEQ ID NO: 20, preferably at a position C-terminal to position 27. Such a charged amino acid can be introduced 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. Alternatively, solubility can also be enhanced by covalently linking hydrophilic moieties, such as polyethylene glycol, to the peptide.

Exemplary Embodiments

In accordance with one embodiment, a glucagon analog is provided comprising the sequence of SEQ ID NO: 55, wherein said analog differs from SEQ ID NO: 55 by 1 to 3 amino acids, selected from positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21, 24, 27, 28, and 29, wherein said glucagon peptide exhibits at least 20% of the activity of native GLP-1 at the GLP-1 receptor.

In accordance with one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising the sequence:

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 33) wherein the Xaa at position 15 is selected from the group of amino acids consisting of Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr, Gly or an acidic amino acid, and R is COOH or $CONH_2$, with the proviso that when position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In one embodiment the glucagon/GLP-1 receptor co-agonist comprises the sequence of SEQ ID NO: 33 wherein the amino acid at position 28 is aspartic acid and the amino acid at position 29 is glutamic acid. In another embodiment the amino acid at position 28 is the native asparagine, the amino acid at position 29 is glycine and the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 65 is covalently linked to the carboxy terminus of SEQ ID NO: 33.

In one embodiment a co-agonist is provided comprising the sequence of SEQ ID NO: 33 wherein an additional acidic amino acid added to the carboxy terminus of the peptide. In a further embodiment the carboxy terminal amino acid of the glucagon analog has an amide in place of the carboxylic acid group of the natural amino acid. In one embodiment the glucagon analog comprises a sequence selected from the group consisting of SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44.

In accordance with one embodiment a glucagon peptide analog of SEQ ID NO: 33 is provided, wherein said analog differs from SEQ ID NO: 33 by 1 to 3 amino acids, selected from positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21 and 27, with the proviso that when the amino acid at position 16 is serine, either position 20 is lysine, or a lactam bridge is formed between the amino acid at position 24 and either the amino acid at position 20 or position 28. In accordance with one embodiment the analog differs from SEQ ID NO: 33 by 1 to 3 amino acids selected from positions 1, 2, 3, 21 and 27. In one embodiment the glucagon peptide analog of SEQ ID NO: 33 differs from that sequence by 1 to 2 amino acids, or in one embodiment by a single amino acid, selected form positions 1, 2, 3, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21 and 27, with the proviso that when the amino acid at position 16 is serine, either position 20 is lysine, or a lactam bridge is formed between the amino acid at position 24 and either the amino acid at position 20 or position 28.

In accordance with another embodiment a relatively selective GLP-1 receptor agonist is provided comprising the sequence NH2-His-Ser-Xaa-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 53) wherein the Xaa at position 3 is selected from the group of amino acids consisting of Glu, Orn or Nle, the Xaa at position 15 is selected from the group of amino acids consisting of Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr, Gly or an acidic amino acid, and R is COOH, $CONH_2$, SEQ ID NO: 26 or SEQ ID NO: 29, with the proviso that when position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In one embodiment the amino acid at position 3 is glutamic acid. In one embodiment the acidic amino acid substituted at position 28 and/or 29 is aspartic acid or glutamic acid. In one embodiment the glucagon peptide, including a co-agonist peptide, comprises the sequence of SEQ ID NO: 33 further comprising an additional acidic amino acid added to the carboxy terminus of the peptide. In a further embodiment the carboxy terminal amino acid of the glucagon analog has an amide in place of the carboxylic acid group of the natural amino acid.

In accordance with one embodiment a glucagon/GLP-1 receptor co-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-Xaa-Arg-Arg-Ala-Xaa-Asp-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 34), wherein the Xaa at position 15 is selected from the group of amino acids consisting of Asp, Glu, cysteic acid, homoglutamic acid and homocysteic acid, Xaa at position 16 is selected from the group of amino acids consisting of Ser, Glu, Gln, homoglutamic acid and homocysteic acid, the Xaa at position 20 is Gln or Lys, the Xaa at position 24 is Gln or Glu and the Xaa at position 28 is Asn, Asp or Lys, R is COOH or $CONH_2$, the Xaa at position 29 is Thr or Gly, and R is COOH, $CONH_2$, SEQ ID NO: 26 or SEQ ID NO: 29, with the proviso that when position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In one embodiment R is $CONH_2$, the Xaa at position 15 is Asp, the Xaa at position 16 is selected from the group of amino acids consisting of Glu, Gln, homoglutamic acid and homocysteic acid, the Xaas at positions 20 and 24 are each Gln the Xaa at position 28 is Asn or Asp and the Xaa at position 29 is Thr. In one embodiment the Xaas at positions 15 and 16 are each Glu, the Xaas at positions 20 and 24 are each Gln, the Xaa at position 28 is Asn or Asp, the Xaa at position 29 is Thr and R is $CONH_2$.

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, 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 activity at the glucagon receptor. In 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 another embodiment the amino acid at position 20 is substituted with Lys, Arg, Orn or Citrullene and/or position 21 is substituted with Glu, homoglutamic acid or homocysteic acid.

In one embodiment a glucagon analog of SEQ ID NO: 20 is provided wherein 1 to 6 amino acids, selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 21, 27, 28 or 29 of the analog differ from the corresponding amino acid of SEQ ID NO: 1, with the proviso that when the amino acid at position 16 is serine, position 20 is Lys, or alternatively when position 16 is serine the position 24 is Glu and either position 20 or position 28 is Lys. In accordance with another embodiment a glucagon analog of SEQ ID NO: 20 is provided wherein 1 to 3 amino acids selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 20, 21, 27, 28 or 29 of the analog differ from the corresponding amino acid of SEQ ID NO: 1. In another embodiment, a glucagon analog of SEQ ID NO: 8, SEQ ID NO: 9 or SEQ ID NO: 11 is provided wherein 1 to 2 amino acids selected from positions 1, 2, 5, 7, 10, 11, 13, 14, 17, 18, 19, 20 or 21 of the analog differ from the corresponding amino acid of SEQ ID NO: 1, and in a further embodiment the one to two differing amino acids represent conservative amino acid substitutions relative to the amino acid present in the native glucagon sequence (SEQ ID NO: 1). In one embodiment a glucagon peptide of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 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, 13, 14, 17, 18, 19, 20, 21, 27 or 29. In one embodiment the substitutions at positions 2, 5, 7, 10, 11, 13, 14, 16, 17, 18, 19, 20, 21, 27 or 29 are conservative amino acid substitutions.

In accordance with one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising a variant of the sequence of SEQ ID NO 33, wherein 1 to 10 amino acids selected from positions 16, 17, 18, 20, 21, 23, 24, 27, 28 and 29, respectively, of the variant differ from the corresponding amino acid of SEQ ID NO: 1. In accordance with one embodiment a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by one or more amino acid substitutions selected from the group consisting of Gln17, Ala18, Glu21, Ile23, Ala24, Val27 and Gly29. In accordance with one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising variants of the sequence of SEQ ID NO 33, wherein 1 to 2 amino acids selected from positions 17-26 of the variant differ from the corresponding amino acid of SEQ ID NO: 1. In accordance with one embodiment a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution selected from the group consisting of Gln17, Ala18, Glu21, Ile23 and Ala24. In accordance with one embodiment a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution at position 18 wherein the substituted amino acid is selected from the group consisting of Ala, Ser, Thr, and Gly. In accordance with one embodiment a variant of the sequence of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by an amino acid substitution of Ala at position 18. Such variations are encompassed by SEQ ID NO: 55. In another embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising variants of the sequence of SEQ ID NO 33, wherein 1 to 2 amino acids selected from positions 17-22 of the variant differ from the corresponding amino acid of SEQ ID NO: 1, and in a further embodiment a variant of SEQ ID NO 33 is provided wherein the variant differs from SEQ ID NO: 33 by 1or 2 amino acid substitutions at positions 20 and 21. In accordance with one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising the sequence: NH2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Xaa-Xaa-Arg-Arg-Ala-Xaa-Xaa-Phe-Val-Xaa-Trp-Leu-Met-Xaa-Xaa-R (SEQ ID NO: 51), wherein the

Xaa at position 15 is Asp, Glu, cysteic acid, homoglutamic acid or homocysteic acid, the Xaa at position 16 is Ser, Glu, Gln, homoglutamic acid or homocysteic acid, the Xaa at position 20 is Gln, Lys, Arg, Orn or citrulline, the Xaa at position 21 is Asp, Glu, homoglutamic acid or homocysteic acid, the Xaa at position 24 is Gln or Glu, the Xaa at position 28 is Asn, Lys or an acidic amino acid, the Xaa at position 29 is Thr or an acid amino acid and R is COOH or $CONH_2$. In one embodiment R is $CONH_2$. In accordance with one embodiment a glucagon/GLP-1 receptor co-agonist is provided comprising a variant of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 47, SEQ ID NO: 48 or SEQ ID NO: 49, wherein the variant differs from said sequence by an amino acid substitution at position 20. In one embodiment the amino acid substitution is selected form the group consisting of Lys, Arg, Orn or citrulline for position 20.

In one embodiment a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 34 wherein the analog differs from SEQ ID NO: 34 by having an amino acid other than serine at position 2. In one embodiment the serine residue is substituted with aminoisobutyric acid, D-alanine, and in one embodiment the serine residue is substituted with aminoisobutyric acid. Such modifications suppresses cleavage by dipeptidyl peptidase IV while retaining the inherent potency of the parent compound (e.g. at least 75, 80, 85, 90, 95% or more of the potentcy of the parent compound). In one embodiment the solubility of the analog is increased, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In another embodiment the analog further comprises 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: 34.

In one embodiment the glucagon analogs disclosed herein are further modified at position 1 or 2 to reduce susceptibility to cleavage by dipeptidyl peptidase IV. In one embodiment a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 is provided wherein the analog differs from the parent molecule by a substitution at position 2 and exhibits reduced susceptibility (i.e., resistance) to cleavage by dipeptidyl peptidase IV. More particularly, 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, 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, N-methyl serine and aminoisobutyric acid. In one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 21 or SEQ ID NO: 22.

In one embodiment a glucagon analog of SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 is provided wherein the analog differs from the parent molecule by a substitution at position 1 and exhibits reduced susceptibility (i.e., resistance) to cleavage by dipeptidyl peptidase IV. More particularly, 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. In another embodiment a glucagon agonist is provided comprising an analog peptide of SEQ ID NO: 34 wherein the analog differs from SEQ ID NO: 34 by having an amino acid other than histidine at position 1. In one embodiment the solubility of the analog is increased, for example, by introducing one, two, three or more charged amino acid(s) to the C-terminal portion of native glucagon, preferably at a position C-terminal to position 27. In exemplary embodiments, one, two, three or all of the charged amino acids are negatively charged. In another embodiment the analog further comprises 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: 34. In one embodiment the acidic amino acid is aspartic acid or glutamic acid.

In one embodiment the glucagon/GLP-1 receptor co-agonist comprises a sequence of SEQ ID NO: 20 further comprising an additional carboxy terminal extension of one amino acid or a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In the embodiment wherein a single amino acid is added to the carboxy terminus of SEQ ID NO: 20, the amino acid is typically selected from one of the 20 common amino acids, and in one embodiment the additional carboxy terminus amino acid has an amide group in place of the carboxylic acid of the native amino acid. In one embodiment the additional amino acid is selected from the group consisting of glutamic acid, aspartic acid and glycine.

In an alternative embodiment a glucagon/GLP-1 receptor co-agonist is provided wherein the peptide comprises at least one lactam ring formed between the side chain of a glutamic acid residue and a lysine residue, wherein the glutamic acid residue and a lysine residue are separated by three amino acids. In one embodiment the carboxy terminal amino acid of the lactam bearing glucagon peptide has an amide group in place of the carboxylic acid of the native amino acid. More particularly, in one embodiment a glucagon and GLP-1 co-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-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 66)

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Lys-Asp-Phe-Val-Gln-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 67)

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Xaa-Xaa-R (SEQ ID NO: 68)

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-Glu-Trp-Leu- Met-Lys-Xaa-R (SEQ ID NO: 69)

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Asn-Thr-R (SEQ ID NO: 16)

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Glu-Trp-Leu- Met-Lys-Thr-R (SEQ ID NO: 17)

NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Lys-Asp-Phe-Val-Glu-Trp-Leu- Met-Lys-Thr-R (SEQ ID NO: 18)

wherein Xaa at position 28 = Asp, or Asn, the Xaa at position 29 is Thr or Gly, R is selected from the group consisting of COOH, $CONH_2$, glutamic acid, aspartic acid, glycine, SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28, and a lactam bridge is formed between Lys at position 12 and Glu at position 16 for SEQ ID NO: 66, between Glu at position 16 and Lys at position 20 for SEQ ID NO: 67, between Lys at position 20 and Glu at position 24 for SEQ ID NO: 68, between Glu at position 24 and Lys at position 28 for SEQ ID NO: 69, between Lys at position 12 and Glu at position 16 and between Lys at position 20 and Glu at position 24 for SEQ ID NO: 16, between Lys at position 12 and Glu at position 16 and between Glu at position 24 and Lys at position 28 for SEQ ID NO: 17 and between Glu at position 16 and Lys at position 20 and between Glu at position 24 and Lys at position 28 for SEQ ID NO: 18. In one embodiment R is selected from the group consisting of COOH, $CONH_2$, glutamic acid, aspartic acid, glycine, the amino acid at position 28 is Asn, and the amino acid at position 29 is threonine. In one embodiment R is $CONH_2$, the amino acid at position 28 is Asn and the amino acid at position 29 is threonine. In another embodiment R is selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 29 and SEQ ID NO: 65 and the amino acid at position 29 is glycine.

In a further embodiment the glucagon/GLP-1 receptor co-agonist is selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18, wherein the peptide further comprises an additional carboxy terminal extension of one amino acid or a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In one embodiment the terminal extension comprises the sequence of SEQ ID NO: 26, SEQ ID NO: 29 or SEQ ID NO: 65 and the glucagon peptide comprises the sequence of SEQ ID NO: 55. In one embodiment the glucagon/GLP-1 receptor co-agonist comprises the sequence of SEQ ID NO: 33 wherein the amino acid at position 16 is glutamic acid, the amino acid at position 20 is lysine, the amino acid at position 28 is asparagine and the amino acid sequence of SEQ ID No: 26 or SEQ ID NO: 29 is linked to the carboxy terminus of SEQ ID NO: 33.

In the embodiment wherein a single amino acid is added to the carboxy terminus of SEQ ID NO: 20, the amino acid is typically selected from one of the 20 common amino acids, and in one embodiment the amino acid has an amide group in place of the carboxylic acid of the native amino acid. In one embodiment the additional amino acid is selected from the group consisting of glutamic acid and aspartic acid and glycine. In the embodiments wherein the glucagon agonist analog further comprises a carboxy terminal extension, the carboxy terminal amino acid of the extension, in one embodiment, ends in an amide group or an ester group rather than a carboxylic acid.

In another embodiment the glucagon/GLP-1 receptor co-agonist comprises the sequence: NH_2-His-Ser-Gln-Gly-Thr-Phe- Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Glu-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-Xaa-$CONH_2$ (SEQ ID NO: 19), wherein the Xaa at position 30 represents any amino acid. In one embodiment Xaa is selected from one of the 20 common amino acids, and in one embodiment the amino acid is glutamic acid, aspartic acid or glycine. The solubility of this peptide can be further improved by covalently linking a PEG chain to the side chain of amino acid at position 17, 21, 24 or 30 of SEQ ID NO: 19. In a further embodiment the peptide comprises an additional carboxy terminal extension of a peptide selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28. In accordance with one embodiment the glucagon/GLP-1 receptor co-agonist comprises the sequence of SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32.

Additional site specific modifications internal to the glucagon sequence of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 and SEQ ID NO: 64 can be made to yield a set of glucagon agonists that possess variable degrees of GLP-1 agonism. Accordingly, peptides that possess virtually identical *in vitro* potency at each receptor have been prepared and characterized. Similarly, peptides with tenfold selectively enhanced potency at each of the two receptors have been identified and characterized. As noted above substitution of the serine residue at position 16 with glutamic acid enhances the potency of native glucagon at both the Glucagon and GLP-1 receptors, but maintains approximately a tenfold selectivity for the glucagon receptor. In addition by substituting the native glutamine at position 3 with glutamic acid (SEQ ID NO: 22) generates a glucagon analog that exhibits approximately a tenfold selectivity for the GLP-1 receptor.

The solubility of the glucagon/GLP-1 co-agonist peptides can be further enhanced in aqueous solutions at physiological pH, while retaining the high biological activity relative to native glucagon by the introduction of hydrophilic groups at positions 16, 17, 21, and 24 of the peptide, or by the addition of a single modified amino acid (i.e., an amino acid modified to comprise a hydrophilic group) at the carboxy terminus of the glucagon/GLP-1 co-agonist peptide. In accordance with one embodiment the hydrophilic group comprises a polyethylene (PEG) chain. More particularly, in one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 or SEQ ID NO: 18 wherein a PEG chain is covalently linked to the side chain of an amino acids at position 16, 17, 21, 24, 29 or the C-terminal amino acid of the glucagon peptide, with the proviso that when the peptide comprises SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 or SEQ ID NO: 13 the polyethylene glycol chain is covalently bound to an amino acid residue at position 17, 21 or 24, when the peptide comprises SEQ ID NO: 14 or SEQ ID NO: 15 the polyethylene glycol chain is covalently bound to an amino acid residue at position 16, 17 or 21, and when the peptide comprises SEQ ID NO: 16, SEQ ID NO: 17 or SEQ ID NO: 18 the polyethylene glycol chain is covalently bound to an amino acid residue at position 17 or 21.

In one embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 11, SEQ ID NO: 12 or SEQ ID NO: 13, wherein a PEG chain is covalently linked to the side chain of an amino acids at position 17, 21, 24, or the C-terminal amino acid of the glucagon peptide, and the carboxy terminal amino acid of the peptide has an amide group in place of the carboxylic acid group of the native amino acid. In one embodiment the glucagon/GLP-1 receptor co-agonist peptide comprises a sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, 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 is covalently linked to the side chain of an amino acid at position 17, 21 or 24 of SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 19, or at position 16, 17 or 21 of SEQ ID NO: 14 and SEQ ID NO: 15 or at position 17 or 21 of SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18 of the glucagon peptide. In another embodiment the glucagon/GLP-1 receptor co-agonist peptide comprises the sequence of SEQ ID NO: 11 or SEQ ID NO: 19, wherein a

PEG chain is covalently linked to the side chain of an amino acids at position 17, 21 or 24 or the C-terminal amino acid of the glucagon peptide.

In accordance with one embodiment, and subject to the proviso limitations described in the preceding paragraphs, the glucagon co-agonist peptide is modified to contain one or more amino acid substitution at positions 16, 17, 21, 24, or 29 or the C-terminal amino acid, 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. Alternatively, the amino acid having a side chain suitable for crosslinking with hydrophilic moieties, including for example, PEG, can be added to the carboxy terminus of any of the glucagon analogs disclosed herein. In accordance with one embodiment an amino acid substitution is made in the glucagon/GLP-1 receptor co-agonist peptide at a position selected from the group consisting of 16, 17, 21, 24, or 29 replacing the native amino acid with an amino acid selected from the group consisting of lysine, cysteine, ornithine, homocysteine and acetyl phenylalanine, wherein the substituting amino acid further comprises a PEG chain covalently bound to the side chain of the amino acid. In one embodiment a glucagon peptide selected form the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, 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 is further modified to comprise a PEG chain is covalently linked to the side chain of an amino acid at position 17 or 21 of the glucagon peptide. In one embodiment the pegylated glucagon/GLP-1 receptor co-agonist further comprises the sequence of SEQ ID NO: 26, SEQ ID NO: 27 or SEQ ID NO: 29.

In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55 or SEQ ID NO: 56, further comprising a C-terminal extension of SEQ ID NO: 26, SEQ ID NO: 29 or SEQ ID NO: 65 linked to the C-terminal amino acid of SEQ ID NO: 55 or SEQ ID NO: 56, and optionally further comprising a PEG chain covalently linked to the side chain of an amino acids at position 17, 18, 21, 24 or 29 or the C-terminal amino acid of the peptide. In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55 or SEQ ID NO: 56, wherein a PEG chain is covalently linked to the side chain of an amino acids at position 21 or 24 of the glucagon peptide and the peptide further comprises a C-terminal extension of SEQ ID NO: 26, or SEQ ID NO: 29.

In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 55, or SEQ ID NO: 33 or SEQ ID NO: 34, wherein an additional amino acid is added to the carboxy terminus of SEQ ID NO: 33 or SEQ ID NO: 34, and a PEG chain is covalently linked to the side chain of the added amino acid. In a further embodiment, the pegylated glucagon analog further comprises a C-terminal extension of SEQ ID NO: 26 or SEQ ID NO: 29 linked to the C-terminal amino acid of SEQ ID NO: 33 or SEQ ID NO: 34. In another embodiment the glucagon peptide comprises the sequence of SEQ ID NO: 19, wherein a PEG chain is covalently linked to the side chain of the amino acid at position 30 of the glucagon peptide and the peptide further comprises a C-terminal extension of SEQ ID NO: 26 or SEQ ID NO: 29 linked to the C-terminal amino acid of SEQ ID NO: 19.

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 10,000 Daltons. In one embodiment the polyethylene glycol chain has an average molecular weight selected from the range of about 1,000 to about 5,000 Daltons. In an alternative embodiment the polyethylene glycol chain has an average molecular weight selected from the range of about 10,000 to about 20,000 Daltons. 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 consisting of SEQ ID NO: 5 or a glucagon agonist analog of SEQ ID NO: 5, 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 certain exemplary embodiments, the glucagon peptide comprises the amino acid sequence of SEQ ID NO: 1 with up to ten amino acid modifications and comprises an amino acid at position 10 which is acylated or alkylated. In some embodiments, the amino acid at position 10 is acylated or alkylated with a C4 to C30 fatty acid. In certain aspects, the amino acid at position 10 comprises an acyl group or an alkyl group which is non-native to a naturally-occurring amino acid.

In certain embodiments, the glucagon peptide comprising an amino acid at position 10 which is acylated or alkylated comprises a stabilized alpha helix. Accordingly, in certain aspects, the glucagon peptide comprises an acyl or alkyl group as described herein and an intramolecular bridge, e.g., a covalent intramolecular bridge (e.g., a lactam bridge) between 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. Alternatively or additionally, the glucagon peptide comprises an acyl or alkyl group as described herein and one, two, three or more of positions 16, 20, 21 and/or 24 of the glucagon peptide are substituted with an α, α-disubstituted amino acid, e.g., AIB. In some instances, the non-native glucagon peptide comprises Glu at position 16 and Lys at position 20, wherein optionally a lactam bridge lnkes the Glu and the Lys, and, optionally, the glucagon peptide further comprises one or more modifications selected from the group consisting of: Gln at position 17, Ala at position 18, Glu at position 21, Ile at position 23, and Ala at position 24.

Also, in any of the embodiments, wherein the glucagon peptide comprises an amino acid at position 10 which is acylated or alkylated, the glucagon peptide can further comprise a C-terminal amide in lieu of the C-terminal alpha carboxylate.

In some embodiments, the glucagon peptide comprising an acyl or alkyl group as described herein further comprises an amino acid substitution at position 1, at position 2, or at positions 1 and 2, wherein the amino acid substitution(s) achieve DPP-IV protease resistance. For example, the His at position 1 may be 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. Alternatively or additionally, the Ser at position 2 may be substituted with an amino acid selected from the group consisting of: D-serine, alanine, D-alanine, valine, glycine, N-methyl serine, N-methyl alanine, and amino isobutyric acid.

The glucagon peptide comprising the amino acid at position 10 which is acylated or alkylated as described herein can comprise any amino acid sequence which is substantially related to SEQ ID NO: 1. For instance, the glucagon peptide comprises SEQ ID NO: 1 with up to 10 amino acid modifications (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 modifications). In certain embodiments, the amino acid sequence of the acylated or alkylated glucagon peptide is greater than 25% identical to SEQ ID NO: 1

(e.g., greater than 30%, 35%, 40%, 50%, 60%, 70% 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or nearly 100% identical to SEQ ID NO: 1). In certain specific embodiments, the glucagon peptide is one which comprises SEQ ID NOs: 55 with an amino acid at position 10 acylated or alkylated as described herein. The glucagon peptide can be any of SEQ ID NOs: 55, 55 with 1 or 2 amino acid modifications, 2-4, 9-18, 20, 23-25, 33, 40-44, 53, 56, 61, 62, 64, 66-514, and 534.

The acyl or alkyl group of these embodiments may be any acyl or alkyl group described herein. For example, the acyl group may be a C4 to C30 (e.g., C8 to C24) fatty acyl group and the alkyl group may be a C4 to C30 (e.g., C8 to C24) alkyl group.

The amino acid to which the acyl or alkyl group is attached may be any of the amino acids described herein, e.g., an amino acid of any of Formula I (e.g., Lys), Formula II, and Formula III.

In some embodiments, the acyl group or alkyl group is directly attached to the amino acid at position 10. In some embodiments, the acyl or alkyl group is attached to the amino acid at position 10 via a spacer, such as, for example, a spacer which is 3 to 10 atoms in length, e.g., an amino acid or dipeptide. Suitable spacers for purposes of attaching an acyl or alkyl group are described herein.

In some embodiments of the invention, an analog of a glucagon peptide, which analog exhibits agonist activity at the GIP receptor, is provided. The analog in certain embodiments comprises the amino acid sequence of SEQ ID NO: 1 with at least one amino acid modification (optionally, up to 15 amino acid modifications), and an extension of 1 to 21 amino acids C-terminal to the amino acid at position 29 of the analog.

In certain aspects, the analogs comprise at least one amino acid modification and up to 15 amino acid modifications (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 amino acid modifications, up to 10 amino acid modifications). In certain embodiments, the analogs comprise at least one amino acid modification at up to 10 amino acid modifications and additional conservative amino acid modifications. Conservative amino acid modifications are described herein.

In some aspects, at least one of the amino acid modifications confers a stabilized alpha helix structure in the C-terminal portion of the analog. Modifications which achieve a stabilized alpha helix structure are described herein. See, for example, the teachings under the section entitled *Stabilization of the alpha helix/Intramolecular bridges*. In some aspects, the analog comprises an intramolecular bridge (e.g., a covalent intramolecular bridge, a non-covalent intramolecular bridge) between the side chains of two amino acids of the analog. In certain aspects, an intramolecular bridge links the side chains of the amino acids at positions i and i+4, wherein i is 12, 13, 16, 17, 20, or 24. In other aspects, an intramolecular bridge connects the side chains of the amino acids at positions j and j+3, wherein j is 17, or at positions k and k+7" wherein k is any integer between 12 and 22. In certain embodiments, the intramolecular bridge is a covalent intramolecular bridge, e.g., a lactam bridge. In specific aspects, the lactam bridge connects the side chains of the amino acids at positions 16 and 20. In particular aspects, one of the amino acids at positions 16 and 20 is a positive-charged amino acid and the other is a negative-charged amino acid. For example, the analog can comprise a lactam bridge connecting the side chains of a Glu at position 16 and a Lys at position 20. In other aspects, the negative-charged amino acid and the positive-charged amino acid form a salt bridge. In this instance, the intramolecular bridge is a non-covalent intramolecular bridge.

In particular aspects, the amino acid modification which confers a stabilized alpha helix is an insertion or substitution of an amino acid of SEQ ID NO: 1 with an α,α-disubstituted amino acid. Suitable α,α-disubstituted amino acids for purposes of stabilizing the alpha helix are described herein and include, for example, AIB. In some aspects, one, two, three, or more of the amino acids at positions 16, 20, 21, and 24 of SEQ ID NO: 1 are substituted with an α,α-disubstituted amino acid, e.g., AIB. In particular embodiments, the amino acid at position 16 is AIB.

The analog which exhibits agonist activity at the GIP receptor can comprise additional modifications, such as any of those described herein. For instance, the amino acid modifications may increase or decrease activity at one or both of the GLP-1 receptor and glucagon receptor. The amino acid modifications may increase stability of the peptide, e.g., increase resistance to DPP-IV protease degradation, stabilize the bond between amino acids 15 and 16. The amino acid modifications may increase the solubility of the peptide and/or alter the time of action of the analog at any of the GIP, glucagon, and GLP-1 receptors. A combination of any of these types of modifications may be present in the analogs which exhibit agonist activity at the GIP receptor.

Accordingly, in some aspects, the analog comprises the amino acid sequence of SEQ ID NO: 1 with one or more of: Gln at position 17, Ala at position 18, Glu at position 21, Ile at position 23, and Ala, Asn, or Cys at position 24, or conservative amino acid substitutions thereof. In some aspects, the analog comprises a C-terminal amide in place of the C-terminal alpha carboxylate. In certain embodiments, the analog comprises an amino acid substitution at position 1, position 2, or positions 1 and 2, which substitution(s) achieve DPP-IV protease resistance. Suitable amino acid substitutions are described herein. For example, DMIA at position 1 and/or d-Ser or AIB at position 2.

In some embodiments, the analog is modified at positions 27 and/or 28, and optionally at position 29. For example, the Met at position 27 is substituted with a large aliphatic amino acid, optionally Leu, the Asn at position 28 is substituted with a small aliphatic amino acid, optionally Ala, and the Thr at position 29 is substituted with a small aliphatic amino acid, optionally Gly. Without being bound to any particular theory, it is believed that substitution with LAG at positions 27-29 provides increased GIP activity relative to the native MNT sequence of SEQ ID NO: 1 at those positions. In some aspects, the amino acid at position 1 is an amino acid comprising an imidazole ring, e.g., His, analogs of His, and the analog is modified at positions 27 and/or 28, and optionally at position 29, as described herein.

Additionally or alternatively, the analog may comprise one or a combination of: (a) Ser at position 2 substituted with Ala; (b) Gln at position 3 substituted with Glu or a glutamine analog; (c) Thr at position 7 substituted with a Ile; (d) Tyr at position 10 substituted with Trp or an amino acid comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid; (e) Lys at position 12 substituted with Ile; (f) Asp at position 15 substituted with Glu; (g) Ser at position 16 substituted with Glu; (h) Gln at position 20 substituted with Ser, Thr, Ala, AIB; (i) Gln at position 24 substituted with Ser, Thr, Ala, AIB; (j) Met at position 27 substituted with Leu or Nle; (k) Asn at position 29 substituted with a charged amino acid, optionally, Asp or Glu; and (l) Thr at position 29 substituted with Gly or a charged amino acid, optionally, Asp or Glu.

In certain aspects, the analog does not comprise an amino acid modification at position 1 which modification confers GIP agonist activity. In some aspects, the amino acid at position 1 is not a large, aromatic amino acid, e.g., Tyr. In some embodiments, the amino acid at position 1 is an amino acid comprising an imidazole ring, e.g., His, analogs of His. In certain embodiments, the analog is not any of the compounds disclosed in U.S. Patent Application No. 61/151,349. In certain aspects, the analog comprises the amino acid sequence of any of SEQ ID NOs: 657-669. In certain aspects, the analog comprises a modified amino acid sequence of any of SEQ ID NOs: 657-669 in which the amino acid at position 12 is Ile and/or the amino acid at position 27 is Leu and/or the amino acid at position 28 is Ala. In some aspects, the analog comprises the amino acid sequence of any of SEQ ID NOs: 676, 677, 679, 680

With regard to the analogs which exhibit agonist activity at the GIP receptor, the analog comprises an extension of 1-21 amino acids (e.g., 5-19, 7-15, 9-12 amino acids). The extension of the analog may comprise any amino acid sequence, provided that the extension is 1 to 21 amino acids. In some aspects, the extension is 7 to 15 amino acids and in other aspects, the extension is 9 to 12 amino acids. In some embodiments, the extension comprises (i) the amino acid sequence of SEQ ID NO: 26 or 674, (ii) an amino acid sequence which has high sequence identity (e.g., at least 80%, 85%, 90%, 95%, 98%, 99%) with the amino acid sequence of SEQ ID NO: 26 or 674, or (iii) the amino acid sequence of (i) or (ii) with one or more conservative amino acid modifications.

In some embodiments, at least one of the amino acids of the extension is acylated or alkylated. The amino acid comprising the acyl or alkyl group may be located at any position of extension of the analog. In certain embodiments, the acylated or alkylated amino acid of the extension is located at one of positions 37, 38, 39, 40, 41, or 42 (according to the numbering of SEQ ID NO: 1) of the analog. In certain embodiments, the acylated or alkylated amino acid is located at position 40 of the analog.

In exemplary embodiments, the acyl or alkyl group is an acyl or alkyl group which is non-native to a naturally-occurring amino acid. For example, the acyl or alkyl group may be a C4 to C30 (e.g., C12 to C18) fatty acyl group or C4 to C30 (e.g., C12 to C18) alkyl. The acyl or alkyl group may be any of those discussed herein.

In some embodiments, the acyl or alkyl group is attached directly to the amino acid, e.g., via the side chain of the amino acid. In other embodiments, the acyl or alkyl group is attached to the amino acid via a spacer (e.g., an amino acid, a dipeptide, a tripeptide, a hydrophilic bifunctional spacer, a hydrophobic bifunctional spacer). In certain aspects, the spacer is 3 to 10 atoms in length. In some embodiments, the spacer is an amino acid or dipeptide comprising one or two of 6-aminohexanoic acid, Ala, Pro, Leu, beta-Ala, gamma-Glu (e.g., gamma-Glu-gamma-Glu). In particular aspects, the total length of the spacer is 14 to 28 atoms.

Also, in exemplary embodiments, the amino acid to which the acyl or alkyl group is attached may be any of those described herein, including, for example, an amino acid of Fomula I, II, or III. The amino acid which is acylated or alkylated may be a Lys, for example. Suitable amino acids comprising an acyl or alkyl group, as well as suitable acyl groups, alkyl groups, and spacers are described herein. See, for example, the teachings under the sections entitled *Acylation* and *Alkylation*.

In other embodiments, 1-6 amino acids (e.g., 1-2, 1-3, 1-4, 1-5 amino acids) of the extension are positive-charged amino acids, e.g., amino acids of Formula IV, such as, for example, Lys. As used herein, the term "positive-charged amino acid" refers to any amino acid, naturally-occurring or non-naturally occurring, comprising a positive charge on an atom of its side chain at a physiological pH. In certain aspects, the positive-charged amino acids are located at any of positions 37, 38, 39, 40, 41, 42, and 43. In specific embodiments, a positive-charged amino acid is located at position 40.

In other instances, the extension is acylated or alkylated as described herein and comprises 1-6 positive charged amino acids as described herein.

In yet other embodiments, the analogs which exhibit agonist activity at the GIP receptor comprises (i) SEQ ID NO: 1 with at least one amino acid modification, (ii) an extension of 1 to 21 amino acids (e.g., 5 to 18, 7 to 15, 9 to 12 amino acids) C-terminal to the amino acid at position 29 of the analog, and (iii) an amino acid comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid which is located outside of the C-terminal extension (e.g., at any of positions 1-29). In some embodiments, the analog comprises an acylated or alkylated amino acid at position 10. In particular aspects, the acyl or alkyl group is a C4 to C30 fatty acyl or C4 to C30 alkyl group. In some embodiments, the acyl or alkyl group is attached via a spacer, e.g., an amino acid, dipeptide, tripeptide, hydrophilic bifunctional spacer, hydrophobic bifunctional spacer). In certain aspects, the analog comprises an amino acid modification which stabilizes the alpha helix, such as a salt bridge between a Glu at position 16 and a Lys at position 20, or an alpha, alpha-disubstituted amino acid at any one, two, three, or more of positions 16, 20, 21, and 24. In specific aspects, the analog additionally comprises amino acid modifications which confer DPP-IV protease resistance, e.g., DMIA at position 1, AIB at position 2. Analogs comprising further amino acid modifications are contemplated herein.

In certain embodiments, the analogs having GIP receptor activity exhibit at least 0.1% (e.g., at least 0.5%, 1%, 2%, 5%, 10%, 15%, or 20%) activity of native GIP at the GIP receptor when the analog lacks a hydrophilic moiety, e.g., PEG. In some embodiments, the analogs exhibit more than 10%, (e.g., more than 20%, more than 50%, more than 75%, more than 100%, more than 200%, more than 300%, more than 500%) activity of native GIP at the GIP receptor. In some embodiments, the analog exhibits appreciable agonist activity at one or both of the GLP-1 and glucagon receptors. In some aspects, the potency and/or selectivity for these receptors (GIP receptor and GLP-1 receptor and/or glucagon receptor) are within 1000-fold, 750-fold, 500-fold, 250-fold, or 100-fold (higher or lower). For example, the selectivity for the GLP-1 receptor of the analogs having GIP receptor activity can be less than 1000-fold, 500-fold, 100-fold, within 50-fold, within 25 fold, within 15 fold, within 10 fold) (higher or lower) the selectivity for the GIP receptor and/or the glucagon receptor.

In any of the aspects described herein, the invention may exclude any of the peptides disclosed in International Application Publication No. WO 2010/011439, International Application Publication No. WO 2008/101017, or International Application Publication No. WO 2009/155258.

Uses

As described in detail in the Examples, the glucagon agonists of the present invention have enhanced biophysical stability and aqueous solubility while 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. Accordingly, the modified glucagon peptides described herein can be used to treat hypoglycemia or to increase blood glucose level, to induce temporary paralysis of the gut for radiological uses, or 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.

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 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. In one embodiment a solution comprising a pegylated glucagon agonist 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: 23, SEQ ID NO: 24, and SEQ ID NO: 25, and glucagon agonist analogs of SEQ ID NO: 23, SEQ ID NO: 24, and SEQ ID NO: 25, or a pegylated lactam derivative of glucagon comprising the sequence of SEQ ID NO: 20, wherein the side chain of an amino acid residue of said glucagon peptide is covalently bound to the polyethylene glycol chain.

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 delivery device.

Surprisingly, 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: 23, SEQ ID NO: 24, and SEQ ID NO: 25, or a glucagon agonist analog thereof, or a pegylated lactam derivative of glucagon comprising the sequence of SEQ ID NO: 20 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 or prolonged time of activity but without loss of the bioactivity. Accordingly, such compounds can be administered 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 17, 21, 24 or 21 and 24. In one embodiment the composition is an aqueous solution comprising insulin and the glucagon analog. In embodiments where the glucagon peptide comprises the sequence of SEQ ID NO: 24 or SEQ ID NO: 25 the PEG chain is covalently bound at position 21 or 24 of the glucagon peptide. 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, SEQ ID NO: 13 SEQ ID NO: 14 and SEQ ID NO: 15. 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 or 24. In one embodiment the glucagon peptide is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15. 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. The first modified peptide comprises a sequence selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24 and SEQ ID NO: 25, optionally linked to a PEG chain of about 500 to about 5,000 Daltons, and the second peptide comprises a sequence selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24 and SEQ ID NO: 25, covalently linked to a 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 17, 21 or 24 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: 27 (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: 27 (KRNRNNIA) or SEQ ID NO: 28. These compounds can be administered to individuals to induce weight loss or prevent weight gain. In accordance with one embodiment a glucagon agonist analog of SEQ ID NO: 33 or SEQ ID NO: 20, further comprising the amino acid sequence of SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 linked to amino acid 29 of the glucagon peptide, is administered to individuals to induce weight loss or prevent weight gain. More particularly, the glucagon peptide comprises a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14 and SEQ ID NO: 15, further comprising the amino acid sequence of SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 linked to amino acid 29 of the glucagon peptide.

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 bioactivity of glucagon. In one embodiment the terminal ten amino acids of Exendin-4 (i.e., the sequence of SEQ ID NO: 26 (GPSSGAPPPS)) are linked to the carboxy terminus of a glucagon peptide of the present disclosure. These fusion proteins are anticipated to have pharmacological activity for suppressing appetite and inducing weight loss/weight maintenance. In accordance with one embodiment a glucagon agonist analog of SEQ ID NO: 33 or SEQ ID NO: 20, further comprising the amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS) or SEQ ID NO: 29 linked to amino acid 29 of the glucagon peptide, is administered to individuals to induce weight loss or prevent weight gain. More particularly, the glucagon peptide comprises a sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 55 and SEQ ID NO: 56 further comprising the amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS) or SEQ ID NO: 29 linked to amino acid 29 of the glucagon peptide. In one embodiment the administered glucagon peptide analog comprises the sequence of SEQ ID NO: 64.

Multimers

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: 3 and SEQ ID NO: 4). The dimer can be a homodimer or alternatively can be a heterodimer. In certain embodiments, the linker connecting the two (or more) glucagon peptides 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.

In one embodiment the dimer comprises a homodimer of a glucagon fusion peptide wherein the glucagon peptide portion comprises SEQ ID NO: 11 or SEQ ID NO: 20 and an amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS), SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 (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 or 24 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: 8, SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11 and the second glucagon peptide comprises SEQ ID NO: 20. 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 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: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and the second glucagon peptide comprise SEQ ID NO: 11, 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: 11, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18 and the second glucagon peptide is independently selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18, 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: 20 and the second glucagon peptide is independently selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9 and SEQ ID NO: 11. In one embodiment the dimer is formed between two peptides wherein each peptide comprises the amino acid sequence of SEQ ID NO: 11.

Kits

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 a modified glucagon peptide selected from the group consisting of 1) a glucagon peptide comprising the sequence of SEQ ID NO: 20, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO:11; 2) a glucagon fusion peptide comprising a glucagon agonist analog of SEQ ID NO: 11, SEQ ID NO: 20 or SEQ ID NO: 55, and an amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS), SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 (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: 51, further comprising an amino acid sequence of SEQ ID NO: 26 (GPSSGAPPPS), SEQ ID NO: 27 (KRNRNNIA) or SEQ ID NO: 28 (KRNR) linked to amino acid 29 of the glucagon peptide, wherein the PEG chain covalently bound to position 17, 21 or 24 has a molecular weight of about 500 to about 40,000 Daltons. In one embodiment the kit comprise a glucagon/GLP-1 co-agonist wherein the peptide comprises a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18.

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 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 an 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.

Pharmaceutical Formulations

In accordance with one embodiment a pharmaceutical composition is provided wherein the composition comprises a glucadon peptide 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 buffering agents to achieve a physiological compatible pH. The buffering agents may include any compounds capabale 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).

Position 3 Modification

Any of the glucagon peptides, including glucagon analogs, glucagon agonist analogs, glucagon co-agonists, and glucagon/GLP-1 co-agonist molecules, described herein may be modified to contain a modification at position 3, e.g., Gln substituted with Glu, to produce a peptide with high selectivity, e.g., tenfold selectivity, for the GLP-1 receptor as compared to the selectivity for the glucagon receptor.

Any of the glucagon peptides, including glucagon analogs, glucagon agonist analogs, glucagon co-agonists, and glucagon/GLP-1 co-agonist molecules, described herein may be modified to contain a modification at position 3, e.g.,Gln substituted with a glutamine analog (e.g. Dab(Ac)), without a substantial loss of activity at the glucagon receptor, and in some cases, with an enhancement of glucagon receptor activity.

Preparation Methods

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 0.2mmole 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 30-70ml 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: 35)

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: 36)

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

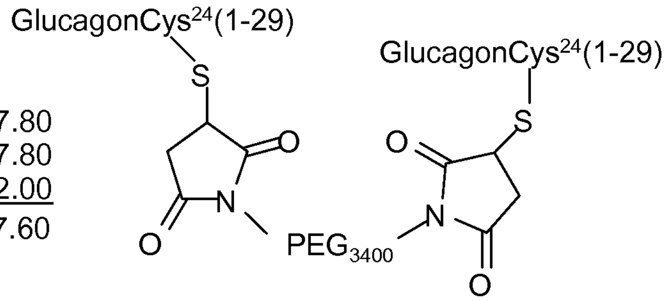
SEQ ID NO: 38

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.

3457.80
3457.80
3572.00
10487.60



EXAMPLE 11

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.

HSQGTFTSDYSKYLDERRAQDFVQWLMNT-NH2 (12-16 Lactam; SEQ ID NO: 12)

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). Lys(Cl-Z) was used 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 12

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:

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

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: 26 and Glucagon-Cex R^{12} represents SEQ ID NO: 39.

Table 1 Solubility date 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

EXAMPLE 13

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 14

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). Effective 50% concentrations were calculated by using Origin software (OriginLab, Northampton, MA. Results are shown in Figs. 3-9 and in Tables 2 through 10.

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

Table 4
cAMP Induction by E16 Glucagon Analogs

Percent Potency Relative to Native Ligand		
Peptide	**GRec**	**GLP-1Rec**
E16 Gluc-NH2	187.2	17.8
Glucagon	100.0	0.8
Gluc-NH2	43.2	4.0
NLeu3, E16 Gluc-NH2	7.6	20.6
E3, E16 Gluc-NH2	1.6	28.8
Orn3, E16 Gluc-NH2	0.5	0.1
GLP-1	<0.1	100

Table 5
cAMP Induction by E16 Glucagon Analogs

Percent Potency Relative to Native Ligand		
Peptide	GRec	GLP-1Rec
E16 Gluc-NH2	187.2	17.8
E15, E16 Gluc-NH2	147.0	9.2
E16, K20 Gluc-NH2	130.1	41.5
Gluc-NH2	43.2	4.0

Table 6
EC50 values for cAMP Induction by E16 Glucagon Analogs

	Glucagon Receptor			**GLP-1 Receptor**		
Peptide	**EC50 (nM)**	StDev	n	**EC50 (nM)**	StDev	n
Glucagon	**0.28**	0.14	10	**4.51**	N/A	1
Glucagon-NH2	**0.53**	0.33	8	**1.82**	0.96	5
E16 Gluc-NH2	**0.07**	0.07	10	**0.16**	0.14	10
E16, G30 Gluc-NH2	**0.41**	0.36	5	**0.24**	0.10	5
E16, G30 Gluc-Cex	**0.51**	0.46	5	**1.19**	0.86	5
GLP-1	**2214**	N/A	1	**0.03**	0.02	9

Table 7
EC50 values for cAMP Induction by E16 Glucagon Analogs

	Glucagon Receptor			**GLP-1 Receptor**		
Peptide	**EC50 (nM)**	StDev	n	**EC50 (nM)**	StDev	n
E16 Glucagon NH2	**0.07**	0.07	10	**0.16**	0.14	10
$hCSO_3$16 Glucagon-NH2	**0.25**	0.12	2	**0.19**	0.02	2
hE16 Glucagon-NH2	**0.17**	0.08	2	**0.25**	0.03	2
H16 Glucagon-NH2	**0.45**	0.3	2	**0.38**	0.11	2
Q16 Glucagon-NH2	**0.22**	0.1	2	**0.39**	0.08	2
D16 Glucagon-NH2	**0.56**	0.15	2	**0.93**	0.28	2
(S16) Glucagon-NH2	**0.53**	0.33	8	**1.82**	0.96	5

Table 8
EC50 values for cAMP Induction by E16 Glucagon Analogs

	Glucagon Receptor			GLP-1 Receptor		
Peptide	**EC50 (nM)**	StD	n	**EC50 (nM)**	StDev	n
E16 Glucagon NH2	**0.07**	0.07	10	**0.16**	0.14	10
T16 Glucagon NH2	**0.10**	0.02	3	**1.99**	0.48	3
G16 Glucagon NH2	**0.10**	0.01	3	**2.46**	0.60	3
Glucagon NH2	**0.53**	0.33	4	**1.82**	0.96	5
GLP-1	2214	N/A	1	**0.03**	0.02	9

E16 Gluc NH_2 was 4-fold more potent at the glucagon receptor relative to G16-COOH and T16 Gluc NH_2,when the compounds were tested side by side.

Table 9
cAMP Induction by E16/Lactam Glucagon Analogs

Percent Potency Relative to Native Ligand		
Peptide	GRec	GLP-1Rec
E24K28 Gluc-NH2 Lac	196.4	12.5
E16K20 Gluc-NH2 Lac	180.8	63.0
K12E16 Gluc-NH2 Lac	154.2	63.3
K20E24 Gluc-NH2 Lac	120.2	8.1
E16 Gluc-NH2	187.2	17.8
E16, K20 Gluc-NH2	130.1	41.5
Glucagon	100.0	0.8
Gluc-NH2	43.2	4.0

Table 10
cAMP Induction by GLP-1 17-26 Glucagon Analogs

	Glucagon Receptor		GLP-1 Receptor	
Peptide	EC50(nM)	StD	EC50(nM)	StD
GLP-1			0.023	0.002
Gluc-NH2	0.159	0.023		
E16 GLP-1			0.009	0.000
E16 Glucagon-NH2	0.072	0.007		
E16 GLP(17-26)Glu(27-29)-NH2	0.076	0.004	0.014	0.001
E16 GLP(17-29)-NH2	0.46	0.023	0.010	0.000
E16 GLP(17-29)-NH2 E24, K28	0.23	0.020	0.007	
E16 GLP(17-29)-NH2 E24, K28 Lactam	0.16	0.017	0.007	0.000

EXAMPLE 15

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 16

The following glucagon peptides are constructed generally as described above in Examples 1-11:

In all of the following sequences, "a" means a C-terminal amide.

HSQGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 70)
HSQGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 71)
HSQGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 72)
HSQGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 73)
HSQGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 74)
HSQGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 75)
HSQGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 76)
HSQGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 77)
HSQGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 78)
HSQGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 79)
HSQGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 80)
HSQGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 81)

HSQGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 82)

HSQGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 83)

HSQGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 84)

HSQGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 85)

HSQGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 86)

X1SQGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 87)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 88)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 89)
X1SQGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 90)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 91)
X1SQGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 92)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 93)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 94)
X1SQGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 95)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 96)
X1SQGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 97)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 98)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 99)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 100)
X1SQGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 101)
X1SQGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 102)
X1SQGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 103)
Wherein in the preceding sequences, X1 = (Des-amino)His

HX2QGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 104)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 105)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 106)
HX2QGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 107)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 108)
HX2QGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 109)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 110)

HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 111)
HX2QGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 112)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 113)
HX2QGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 114)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 115)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 116)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 117)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 118)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 119)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 120)
Wherein in the preceding sequences X2 = Aminoisobutyric acid

HX2QGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 121)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 122)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 123)
HX2QGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 124)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 125)
HX2QGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 126)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 127)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 128)
HX2QGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 129)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 130)
HX2QGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 131)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 132)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 133)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 134)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 135)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 136)
HX2QGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 137)
Wherein in the preceding sequences X2 = (D-Ala)

HSEGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 138)
HSEGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 139)
HSEGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 140)

HSEGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 141)

HSEGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 142)

HSEGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 143)

HSEGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 144)

HSEGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 145)

HSEGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 146)

HSEGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 147)

HSEGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 148)

HSEGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 149)

HSEGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 150)

HSEGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 151)

HSEGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 152)

HSEGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 153)

HSEGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 154)

X1SEGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 155)

X1SEGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 156)

X1SEGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 157)

X1SEGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 158)

X1SEGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 159)

X1SEGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 160)

X1SEGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 161)

X1SEGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 162)

X1SEGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 163)

X1SEGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 164)

X1SEGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 165)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 166)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 167)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 168)
X1SEGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 169)
X1SEGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 170)
X1SEGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 171)
Wherein in the preceding sequences X1 = (Des-amino)His

HX2EGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 172)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 173)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 174)
HX2EGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 175)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 176)
HX2EGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 177)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 178)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 179)
HX2EGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 180)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 181)
HX2EGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 182)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 183)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 184)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 185)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 186)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 187)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 188)
Wherein in the preceding sequences X2 = Aminoisobutyric acid

HX2EGT FTSDY SKYLD ERRAQ DFVQW LMNTa (SEQ ID NO: 189)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (SEQ ID NO: 190)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 191)
HX2EGT FTSDY SKYLD ERRAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 192)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 193)
HX2EGT FTSDY SKYLD KRRAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 194)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (SEQ ID NO: 195)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 196)
HX2EGT FTSDY SKYLD ERAAQ DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 197)

HX2EGT FTSDY SKYLD ERAAK DFVQW LMNTa (lactam @ 12-16; SEQ ID NO: 198)
HX2EGT FTSDY SKYLD KRAAE DFVQW LMNTa (lactam @ 16-20; SEQ ID NO: 199)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (SEQ ID NO: 200)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 12-16; SEQ ID NO: 201)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMNTa (lactam @ 16-20; SEQ ID NO: 202)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (SEQ ID NO: 203)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 12-16; SEQ ID NO: 204)
HX2EGT FTSDY SKYLD EQAAK EFIAW LVKGa (lactam @ 16-20; SEQ ID NO: 205)
Wherein in the preceding sequences X2 = (D-Ala)

HSQGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 206)
HSQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 207)
HSQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 208)
HSQGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 209)
HSQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 210)
HSQGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 211)
HSQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 212)
HSQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 213)
HSQGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 214)
HSQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 215)
HSQGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 216)
HSQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 217)
HSQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 218)
HSQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 219)
HSQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 220)
HSQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 221)
HSQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 222)

X1SQGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 223)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 224)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 225)
X1SQGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 226)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 227)
X1SQGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 228)
X1SQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 229)
X1SQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 230)
X1SQGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 231)

X1SQGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 232)
X1SQGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 233)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 234)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 235)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 236)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 237)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 238)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 239)
Wherein in the preceding sequences X1 = (Des-amino)His; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 240)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 241)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 242)
HX2QGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 243)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 244)
HX2QGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 245)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 246)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 247)
HX2QGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 248)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 249)
HX2QGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 250)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 251)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 252)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 253)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 254)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 255)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 256)
Wherein in the preceding sequences X2 = Aminoisobutyric acid; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 257)

HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 258)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 259)
HX2QGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 260)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 261)
HX2QGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 262)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 263)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 264)
HX2QGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 265)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 266)
HX2QGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 267)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 268)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 269)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 270)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 271)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 272)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 273)

Wherein in the preceding sequences X2 = (D-Ala); and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSEGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 274)
HSEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 275)
HSEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 276)
HSEGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 277)
HSEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 278)
HSEGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 279)
HSEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 280)
HSEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 281)
HSEGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 282)
HSEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 283)
HSEGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 284)

HSEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 285)
HSEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 286)
HSEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 287)
HSEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 288)
HSEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 289)
HSEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 290)

X1SEGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 291)
X1SEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 292)
X1SEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 293)
X1SEGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 294)
X1SEGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 295)
X1SEGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 296)
X1SEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 297)
X1SEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 298)
X1SEGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 299)
X1SEGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 300)
X1SEGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 301)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 302)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 303)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 304)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 305)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 306)
X1SEGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 307)

Wherein in the preceding sequences X1 = (Des-amino)His; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2EGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 308)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 309)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 310)
HX2EGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 311)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 312)
HX2EGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 313)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 314)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 315)

HX2EGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 316)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 317)
HX2EGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 318)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 319)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 320)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 321)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 322)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 323)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 324)

Wherein in the preceding sequences X2 = Aminoisobutyric acid; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2EGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (SEQ ID NO: 325)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 326)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 327)
HX2EGT FTSDY SKYLD ERRAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 328)
HX2EGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 329)
HX2EGT FTSDY SKYLD KRRAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 330)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 331)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 332)
HX2EGT FTSDY SKYLD ERAAQ DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 333)
HX2EGT FTSDY SKYLD ERAAK DFVC*W LMNTa (lactam @ 12-16; SEQ ID NO: 334)
HX2EGT FTSDY SKYLD KRAAE DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 335)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 336)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 12-16; SEQ ID NO: 337)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 338)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (SEQ ID NO: 339)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 12-16; SEQ ID NO: 340)
HX2EGT FTSDY SKYLD EQAAK EFIC*W LVKGa (lactam @ 16-20; SEQ ID NO: 341)

Wherein in the preceding sequences X2 = (D-Ala); and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSQGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 342)
HSQGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 343)
HSQGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 344)
HSQGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 345)
X1SQGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 346)
X1SQGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 347)
X1SQGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 348)
X1SQGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 349)
Wherein X1 = (Des-amino)His; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 350)
HX2QGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 351)
HX2QGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 352)
HX2QGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 353)
Wherein X2 = Aminoisobutyric acid; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 354)
HX2QGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 355)
HX2QGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 356)
HX2QGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 357)
Wherein X2 = (D-Ala); and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSEGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 358)
HSEGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 359)
HSEGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 360)
HSEGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 361)
X1SEGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 362)

X1SEGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 363)
X1SEGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 364)
X1SEGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 365)
Wherein X1 = (Des-amino)His; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2EGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 366)
HX2EGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 367)
HX2EGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 368)
HX2EGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 369)
Wherein X2 = (D-Ala) ; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2EGT FTSDY SKYLD C*RRAK DFVQW LMNTa (SEQ ID NO: 370)
HX2EGT FTSDY SKYLD C*RAAK DFVQW LMNTa (SEQ ID NO: 371)
HX2EGT FTSDY SKYLD C*QAAK EFIAW LMNTa (SEQ ID NO: 372)
HX2EGT FTSDY SKYLD C*QAAK EFIAW LVKGa (SEQ ID NO: 373)
Wherein X2 = (D-Ala) ; and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSQGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 374)
HSQGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 375)
HSQGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 376)
HSQGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 377)
HSQGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 378)
HSQGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 379)
HSQGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 380)
HSQGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 381)
HSQGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 382)

HSQGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 383)
HSQGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 384)
HSQGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 385)
HSQGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 386)
HSQGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 387)

X1SQGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 388)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 389)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 390)
X1SQGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 391)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 392)
X1SQGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 393)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 394)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 395)
X1SQGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 396)
X1SQGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 397)
X1SQGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 398)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 399)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 400)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 401)
Wherein in the preceding sequences X1 = (Des-amino)His

HX2QGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 402)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 403)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 404)
HX2QGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 405)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 406)
HX2QGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 407)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 408)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 409)
HX2QGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 410)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 411)
HX2QGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 412)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 413)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 414)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 415)
Wherein in the preceding sequences X2 = Aminoisobutyric acid

HX2QGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 416)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 417)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 418)
HX2QGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 419)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 420)
HX2QGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 421)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 422)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 423)
HX2QGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 424)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 425)
HX2QGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 426)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 427)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 428)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 429)
Wherein in the preceding sequences X2 = (D-Ala)

HSEGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 430)
HSEGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 431)
HSEGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 432)
HSEGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 433)
HSEGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 434)
HSEGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 435)
HSEGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 436)
HSEGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 437)
HSEGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 438)
HSEGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 439)
HSEGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 440)
HSEGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 441)
HSEGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 442)
HSEGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 443)

X1SEGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 444)
X1SEGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 445)
X1SEGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 446)
X1SEGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 447)

X1SEGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 448)
X1SEGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 449)
X1SEGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 450)
X1SEGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 451)
X1SEGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 452)
X1SEGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 453)
X1SEGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 454)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 455)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 456)
X1SEGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 457)
Wherein in the preceding sequences X1 = (Des-amino)His

HX2EGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 458)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 459)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 460)
HX2EGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 461)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 462)
HX2EGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 463)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 464)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 465)
HX2EGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 466)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 467)
HX2EGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 468)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 469)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 470)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 471)
Wherein in the preceding sequences X2 = Aminoisobutyric acid

HX2EGT FTSDY SKYLD ERRAQ DFVQW LMDTa (SEQ ID NO: 472)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (SEQ ID NO: 473)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 474)
HX2EGT FTSDY SKYLD ERRAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 475)
HX2EGT FTSDY SKYLD ERRAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 476)
HX2EGT FTSDY SKYLD KRRAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 477)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (SEQ ID NO: 478)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 479)

HX2EGT FTSDY SKYLD ERAAQ DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 480)
HX2EGT FTSDY SKYLD ERAAK DFVQW LMDTa (lactam @ 12-16; SEQ ID NO: 481)
HX2EGT FTSDY SKYLD KRAAE DFVQW LMDTa (lactam @ 16-20; SEQ ID NO: 482)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (SEQ ID NO: 483)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 12-16; SEQ ID NO: 484)
HX2EGT FTSDY SKYLD EQAAK EFIAW LMDTa (lactam @ 16-20; SEQ ID NO: 485)
Wherein in the preceding sequences X2 = (D-Ala)

The following glucagon peptides with a GLP-1/glucagon activity ratio of about 5 or more are also constructed generally as described above in Examples 1-11. Generally, in these peptides, AIB at position 2 provides DPP IV resistance but also significantly reduces glucagon activity.

HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 486)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNC*a (SEQ ID NO: 487)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNGG PSSGA PPPSC*a (SEQ ID NO: 488)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNGG PSSGA PPPSC*a (lactam @ 16-20; SEQ ID NO: 489)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNGG PSSGA PPPSa (SEQ ID NO: 490)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNGG PSSGA PPPSa (lactam @ 16-20; SEQ ID NO: 491)
Wherein in the preceding sequences X2=AIB, and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNTa (SEQ ID NO: 492)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNC*a (SEQ ID NO: 493)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNGG PSSGA PPPSC*a (SEQ ID NO: 494)
HX2QGT FTSDY SKYLD ERAAK DFVQW LMNGG PSSGA PPPSC*a (lactam @ 16-20; SEQ ID NO: 495)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNGG PSSGA PPPSa (SEQ ID NO: 496)
HX2QGT FTSDY SKYLD ERAAK DFVC*W LMNGG PSSGA PPPSa (lactam @ 16-20; SEQ ID NO: 497)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 498)

HX2QGT FTSDY SKYLD ERRAK DFVQW LMNC*a (SEQ ID NO: 499)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNGG PSSGA PPPSC*a (SEQ ID NO: 500)
HX2QGT FTSDY SKYLD ERRAK DFVQW LMNGG PSSGA PPPSC*a (lactam @ 16-20; SEQ ID NO: 501)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNGG PSSGA PPPSa (SEQ ID NO: 502)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNGG PSSGA PPPSa (lactam @ 16-20; SEQ ID NO: 503)
Wherein in the preceding sequences X2=AIB, and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

The following glucagon peptides which are GLP-1/glucagon co-agonists are also constructed generally as described above in Examples 1-11. Formation of a lactam bridge between amino acids 16 and 20 restores the reduction in glucagon activity caused by the substitution at position 2.

HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 504)
Wherein in the preceding sequence X2=AIB, and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

X1SQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (lactam @ 16-20; SEQ ID NO: 505)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMNC*a (lactam @ 16-20; SEQ ID NO: 506)
X1SQGT FTSDY SKYLD EQAAK EFIAW LMNGG PSSGA PPPSC*a (lactam @ 16-20; SEQ ID NO: 507)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMNGG PSSGA PPPSC*a (lactam @ 16-20; SEQ ID NO: 508)
X1SQGT FTSDY SKYLD EQAAK EFIC*W LMNGG PSSGA PPPSa (lactam @ 16-20; SEQ ID NO: 509)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 510)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 511)
X1SQGT FTSDY SKYLD ERRAK DFVQW LMNC*a (lactam @ 16-20; SEQ ID NO: 512)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNGG PSSGA PPPSa (lactam @ 16-20; SEQ ID NO: 513)

Wherein in the preceding sequences X1=DMIA (alpha, alpha-dimethyl imidiazole acetic acid), and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSQGT FTSDY SKYLD EQAAK EFIC*W LMNTa (optionally with lactam @ 16-20; SEQ ID NO: 514)

Wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 517)
HX2QGT FTSDY SKYLD ERRAK DFVC*W LMNTa (lactam @ 16-20; SEQ ID NO: 528)
HX2QGT FTSDY SKYLD ERRAK EFIC*W LMNGG PSSGA PPPSC*a (SEQ ID NO: 531)
HX2QGT FTSDY SKYLD EQAAK EFIAW LMNGG PSSGA PPPSC*C*a (SEQ ID NO: 532)
HX2QGT FTSDY SKYLD EQAAK EFIC*W LMNGG PSSGA PPPSa (SEQ ID NO: 533)

Wherein in the preceding sequence X2=AIB, and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSQGT FTSDYSKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 518)
X1SQGT FTSDYSKYLD EQAAK EFIC*W LMNTa (SEQ ID NO: 519)
X1SQGT FTSDYSKYLD EQAAK EFIAW LMNC*a (SEQ ID NO: 520)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNGG PSSGA PPPSa (SEQ ID NO: 529)
X1SQGT FTSDY SKYLD ERRAK DFVC*W LMNTa (SEQ ID NO: 530)

Wherein in the preceding sequences X1=DMIA (alpha, alpha-dimethyl imidiazole acetic acid), and wherein the C* is a Cys, or a Cys attached to a hydrophilic polymer, or alternatively the C* is a Cys attached to a polyethylene glycol of about 20 kD average weight, or alternatively the C* is a Cys attached to a polyethylene glycol of about 40 kD average weight.

HSQGT FTSDYSKYLD SRRAQ DFVQW LMNTGPSSGAPPPSa (SEQ ID NO: 521)

HSQGT FTSDYSKYLD SRRAQ DFVQW LMNGGPSSGAPPPSa (SEQ ID NO: 522)
HSQGT FTSDYSKYLD SRRAQ DFVQW LMKGGPSSGAPPPSa (SEQ ID NO: 523)
HSQGT FTSDYSKYLD SRRAQ DFVQW LVKGGPSSGAPPPSa (SEQ ID NO: 524)
HSQGT FTSDYSKYLD SRRAQ DFVQW LMDGGPSSGAPPPSa (SEQ ID NO: 525)
HSQGT FTSDYSKYLD ERRAK DFVQW LMDGGPSSGAPPPSa (SEQ ID NO: 526)
HAEGT FTSDV SSYLE GQAAK EFIAW LVKGGa (SEQ ID NO: 527)

X1X2QGT FTSDY SKYLD ERX5AK DFVX3W LMNX4 (SEQ ID NO: 61)
wherein
X1=His, D-histidine, desaminohistidine, hydroxyl-histidine, acetyl-histidine, homo-histidine or alpha, alpha-dimethyl imidiazole acetic acid (DMIA) N-methyl histidine, alpha-methyl histidine, or imidazole acetic acid,
X2=Ser, D-serine, Ala, Val, glycine, N-methyl serine or aminoisobutyric acid (AIB), N-methyl alanine and D-alanine.
X3=Ala, Gln or Cys-PEG
X4=Thr-CONH2 or Cys-PEG or GGPSSGAPPPS (SEQ ID NO: 515) or GGPSSGAPPPSC-PEG (SEQ ID NO: 516)
Provided that when X3 is Cys-PEG, X4 is not Cys-PEG or GGPSSGAPPPSC-PEG (SEQ ID NO: 516), and when X2=Ser, X1 is not His.
X5=Ala or Arg

X1X2QGT FTSDY SKYLD EQ X5AK EFI X3W LMNX4 (SEQ ID NO: 62)
wherein
X1=His, D-histidine, desaminohistidine, hydroxyl-histidine, acetyl-histidine, homo-histidine or alpha, alpha-dimethyl imidiazole acetic acid (DMIA), N-methyl histidine, alpha-methyl histidine, or imidazole acetic acid
X2=Ser, D-serine, Ala, Val, glycine, N-methyl serine or aminoisobutyric acid (AIB), N-methyl alanine and D-alanine.
X3=Ala, Gln or Cys-PEG
X4=Thr-CONH2 or Cys-PEG or GGPSSGAPPPS (SEQ ID NO: 515) or GGPSSGAPPPSC-PEG (SEQ ID NO: 516)
Provided that when X3 is Cys-PEG, X4 is not Cys-PEG or GGPSSGAPPPSC-PEG (SEQ ID NO: 516), and when X2=Ser, X1 is not His.
X5=Ala or Arg

HSEGT FTSDY SKYLD EQAAK EFIAW LXNTa (SEQ ID NO: 554), wherein X at position 27 is Norleucine, wherein the amino acid at position 29 is amidated

Any of the preceding sequences can include additional modifications, e.g., 1, 2, 3, 4 or 5 modifications that do not destroy activity, including but not limited to W10 or R20 substitutions that can be used to enhance potency. Any of the preceding sequences can also be produced without the modifications that confer DPP IV resistance, i.e., in which the native His is at position 1 and the native Ser is at position 2. In addition, any of the preceding compounds may optionally be linked to a conjugate, such as a heterologous polypeptide, an immunoglobulin or a portion thereof (e.g. Fc region), a targeting agent, a diagnostic label, or a diagnostic or therapeutic agent.

EXAMPLE 17

The following glucagon peptides modified to comprise the c-terminal extension of SEQ ID NO: 26 linked to the carboxy terminus of the glucagon peptide were constructed generally as described above in Examples 1-11 and assayed for activity at the GLP-1 and glucagon receptors using the *in vitro* assay described in Example 14.

Table 11 represents the activity of various glucagon analogs at the glucagon and GLP-1 receptors. The data shows that for glucagon analogs comprising the c-terminal extension of SEQ ID NO: 26, amino acid substititions at positions 16, 20, 28 and 29 can impact the analogs activity at the GLP-1 receptor.

Table 11

Glucagon-Cex
Structure Activity Relationship

Glucagon Peptide	Glucagon Receptor		GLP-1 Receptor	
	EC50 (nM)	Relative Potency (%)	EC50 (nM)	Relative Potency (%)
-MNT^{29} (SEQ ID NO: 1)	0.086	100		
-MNTG^{30} PSSGAPPPS (SEQ ID NO: 521)	0.14	61	1.19	2
-MNGG^{30} PSSGAPPPS (SEQ ID NO: 522)	0.28	30	0.31	8

-MKGG^{30} PSSGAPPPS (SEQ ID NO: 523)	0.61	14	0.80	3
-VKGG^{30} PSSGAPPPS (SEQ ID NO: 524)	1.16	7	0.21	12
-MDGG^{30} PSSGAPPPS (SEQ ID NO: 525)	0.12	72	0.13	19
E^{16}K^{20}-MDGG^{30} PSSGAPPPS (SEQ ID NO: 526)	0.22	39	0.020	125
GLP-1-VKGG^{30} (SEQ ID NO: 527)			0.025	100

EXAMPLE 18

Table 12 represents *in vitro* data accumulated for various glucagon peptides comparing their relative activities at the glucagon and GLP-1 receptors.

Table 12: COMPARISON OF AGONISTS AND CO-AGONISTS w/ and w/o PEG

	% Potency Relative to Native	
CONTROLS	**GR**	**GL-1R**
Glucagon	100	0.78
GLP-1	<0.01	100

	Parent w/o PEG		Parent w/PEG	
	% Potency Relative to Native		% Potency Relative to Native	
AGONISTS	GR	GLP-1R	GR	GLP-1R
Chimera AIB2, Cys24 (SEQ ID NO: 486)	15.4	160.6	2.6	82.5
Chimera AIB2, Cys29 (SEQ ID NO: 487)	20.1	124.6	5.6	54.3
Chimera AIB2, Gly29,30 Cys40 Cex (SEQ ID NO: 488)	2.2	359.1	0.3	68.8
Chimera AIB2, Gly29,30 Cys40 Cex Lactam (SEQ ID NO: 489)	14.2	169.6	3.2	63.6
Chimera AIB2, Gly29,30 Cys24 Cex (SEQ ID NO: 490)	2.5	457.8	0.2	95.4
Chimera AIB2, Gly29,30 Cys24 Cex Lactam (SEQ ID NO: 491)	25.2	381.5	1.4	96.4
E16, K20AIB2, A18 Cys24 (SEQ ID NO: 492)	--	--	1.1	73.5
E16, K20AIB2, A18 Gly29,30 Cys24 Cex (SEQ ID NO: 496)	--	--	0.1	88.5
CO-AGONISTS	GR	GLP-1R	GR	GLP-1R
Chimera DMIA1, Cys24 Lactam (SEQ ID NO: 505)	160.7	82.5	19.1	12.5
Chimera AIB2, Cys24 Lactam (SEQ ID NO: 504)	114.2	230.4	9.2	38.0
Chimera DMIA1, Cys29 Lactam (SEQ ID NO: 506)	--	--	--	--
Chimera DMIA1, Gly29,30 Cys40 Cex Lactam (SEQ ID NO: 507)	--	--	--	--
E16, K20 DMIA1, Gly29,30 Cys40 Cex Lactam (SEQ ID NO: 508)	--	--	--	--
Chimera DMIA1, Gly29,30 Cys24 Cex Lactam (SEQ ID NO: 509)	--	--	--	--
E16, K20 DMIA1, Cys24 Lactam (SEQ ID NO: 510)	--	--	64.1	9.3

E16, K20 AIB2, Cys24 Lactam (SEQ ID NO: 517)	108.3	96.9	15.8	31.0
Chimera Cys24 (SEQ ID NO: 518)	--	--	19.8	29.3
E16, K20 DMIA1, Gly29,30 Cys24 Cex Lactam (SEQ ID NO: 513)	116.0	78.3	12.6	11.3
Chimera DMIA1, Cys29 (SEQ ID NO: 520)	--	--	5.3	27.3
Chimera DMIA1, Cys24 (SEQ ID NO: 519)	28.9	64.5	6.9	19.3

EXAMPLE 19

Acylated and/or PEGylated peptides were prepared as follows. Peptides were synthesized on a solid support resin using either a CS Bio 4886 Peptide Synthesizer or Applied Biosystems 430A Peptide Synthesizer. In situ neutralization chemistry was 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) was 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 removed FMOC/formyl groups. Coupling to the free ε-amino Lys residue was 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 resulted in removal of any side chain protecting groups and the N-terminal BOC group. Peptide resins were neutralized with 5% DIEA/DMF, dried, and then cleaved from the support using HF/p-cresol, 95:5, at 0°C for one hour. Following ether extraction, a 5% HOAc solution was used to solvate the crude peptide. A sample of the solution was then verified to contain the correct molecular weight peptide by ESI-MS. Correct peptides were 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 was used for the purification. Acylated peptide analogs generally completed elution by a buffer ratio of 20:80. Portions were pooled together and checked for purity on an analytical RP-HPLC. Pure fractions were lyophilized yielding white, solid peptides. Yields typically ranged from 10 mg to 100 mg depending on the synthesis.

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.) was 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 commenced for 4-6 hours and the reaction analyzed by analytical RP-HPLC. PEGylated products appeared distinctly from the starting material with decreased retention times. Purification was performed on a Vydac C4 column with conditions similar to those used for the initial peptide purification. Elution occurred around buffer ratios of 50:50. Fractions of pure PEGylated peptide were found and lyophilized. Yields were above 50%, varying per reaction.

Peptides were assayed for biological activity, by co-tranfecting HEK293 cells with either the glucagon receptor (GLUR) or GLP-1 receptor (GLP-1R) and a luciferase gene linked to a cAMP responsive element. The transfected cells were serum deprived by culturing for 16 hours in DMEM supplemented with 0.25% Bovine Growth Serum and then incubated for 5 hours with serial dilutions of the selected analogs and either Glucagon or GLP-1 as standards, respectively. Peptide absorbance readings were obtained from UV Absorbance measurements at 280 nm on a Genesys 6 Spectrophotometer (Thermo Electron Corporation). Beer's Law was used to calculate solution concentrations based on the number of tryptophan and tyrosine residues in each analog. At the end of the incubation, 100 μL LucLite luminescence substrate reagent was added to each well, the plate sealed and shaken, and placed into a Wallac Trilux luminescence counter for cAMP detection. Effective 50% concentrations (EC50) were calculated using Origin software (OriginLab, Northampton, MA).

Acylated glucagon-based co-agonist peptides were prepared. *In vitro* results for a selection of these peptides are shown in Table 13. Although the unacylated peptide, like native glucagon, was insoluble in phosphate-buffered saline solutions at 1 mg/mL concentrations, acylation was observed to enhance solubility of the peptide at neutral pH.

TABLE 13

Receptor Activation Curves and nM EC_{50} values for Acylated Peptides				
Peptide	**GLP-1 Receptor**	**N**	**Glucagon Receptor**	**N**
GLP-1	0.04	15	>100	3
E16 K20-glucagon-NH2	0.21	9	0.18	10
E16 K20-glucagon-NH2 with K^{10}-C_{16}	0.09	8	0.40	8
E16 K20-glucagon-NH2 with K^{10}-W-C_{16}	0.05	8	0.14	8
E16 K20-glucagon-NH2 with K^{10}-C_{18}	0.03	8	0.12	8
E16 K20-glucagon-NH2 with K^{10}-W-C_{18}	0.04	11	0.05	12
Glucagon	7.42	6	0.07	17

All four acylated peptides exhibited increased potency at the GLP-1 receptor. Inclusion of the tryptophan spacer provided better potency at the glucagon receptor. An acyl chain length of C18 is slightly preferred.

While acylation can extend the half-life of a peptide to hours or more, PEGylation with repeats in tens of kDa ranges can do even more. Peptides comprising both types of modifications were prepared. These peptides are expected to exhibit extended half-life in circulation, as well as resistance to DPP-IV and other proteases. *In vitro* results for a selection of these peptides are shown in Table 14.

TABLE 14

Receptor Activation Curves and nM EC_{50} values for Acylated, PEGylated Peptides				
Peptide	**GLP-1 Receptor**	**N**	**Glucagon Receptor**	**N**
GLP-1	0.04	15	>100	3
E16 K20-glucagon-NH2 (SEQ ID NO: 545)	0.21	9	0.18	10
E16 K20-glucagon-NH2 with K^{10}-W-C_{16} and C^{24}-40K PEG (SEQ ID NO: 546)	0.23	13	0.52	13
E16 K20-glucagon-NH2 with K^{10}-C_{18} and C^{24}-40K PEG (SEQ ID NO: 547)	0.15	12	0.84	13
E16 K20-glucagon-NH2 with K^{10}-W-C_{18} and C^{24}-40K PEG (SEQ ID NO: 548)	1.64	3	1.30	5
Glucagon (SEQ ID NO: 1)	7.42	6	0.07	17

Two of the three peptides retained their high potency at both the GLP-1 and glucagon receptors, with an EC50 of less than 1nM. The K^{10}-W-C_{18} acylated and PEGylated peptide exhibited about ten-fold potency losses at both receptors. This series of peptides shows that the position ten acylation is compatible with a PEGylation 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).

EXAMPLE 20

Various acylated glucagon co-agonist peptides were made as essentially described in Example 19 and tested for *in vivo* activity. Specifically, Peptide A (SEQ

ID NO:1 modified to contain AIB at position 2, Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, Cys at position 24, which Cys is bonded to a 40K PEG, and C-terminal amide) was further modified to comprise a Lys at position 10. The Lys10 was acylated with a C8 fatty acid chain, a C14 fatty acid chain, a C16 fatty acid chain, or a C18 fatty acid chain.

Activity at the GLP-1 receptor of each of the acylated peptides was assayed as described in Example 14 and compared to the activity of GLP-1(7-37)acid (SEQ ID NO: 50) as a control. The EC50 of each of the acylated peptides at the GLP-1 receptor shown in Table 15 is similar to the EC50 of the GLP-1 peptide.

TABLE 15

	GLP-1 Receptor Activation Potency EC_{50} (nM)
GLP-1	0.0222 ± 0.0002
Peptide A K^{10}-C_8	0.0174 ± 0.0004
Peptide A K^{10}-C_{14}	0.0168 ± 0.0004
Peptide A K^{10}-C_{16}	0.0127 ± 0.0003
Peptide A K^{10}-C_{18}	0.0118 ± 0.0002

The peptides were then tested *in vivo* by subcutaneously injecting diet-induced obesity (DIO) mice with various acylated and non-acylated peptides, or vehicle alone, QW (70 nmol/kg/week) for 2 weeks. 6 mice per group with initial average body weight of 44 g were tested. Body weight, body composition, food intake, and blood glucose levels were determined periodically.

As shown in Figure 11, the acylated peptides are able to cause weight loss to a similar extent than the non-acylated peptide. As shown in Figure 11, between about 7 and 12% weight loss is achieved within the first 3 days of treatment with the acylated peptides. As shown in Figure 12, the acylated peptides caused a decrease in food intake. Furthermore, as shown in Figure 13, the ad libitum blood glucose levels of the acylated peptides were reduced after 1 day of treatment.

EXAMPLE 21

The following acylated glucagon co-agonist peptides were made as essentially described in Example 19.

(A) “Chimera-2 Aib2 Lys10-C18 Cys24(40K)”: native glucagon amino acid sequence (SEQ ID NO: 1) comprising the following modifications: Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, and Ala at position 24, and a C-terminal amide (“Chimera 2”), further modified with AIB at position 2, a Lys10 acylated with a C18 fatty acid and a Cys at position 24 pegylated with a 40K PEG group;

(B) “Chimera-2 Aib2 Lys10-C16 Cys24(40K)”: Chimera 2 further modified with AIB at position 2, a Lys10 acylated with a C16 fatty acid and a Cys24 pegylated with a 40K PEG group;

(C) “Glucagon Lys10-C18 E16 K20 Cys24(40K)”: native glucagon amino acid sequence (SEQ ID NO: 1) comprising the following modifications: Glu at position 16, Lys at position 20, and C-terminal amide (“E16K20-glucagon-NH2”) was further modified with a Lys10 acylated with a C18 fatty acid and a Cys24 pegylated with a 40K PEG group;

(D) “Glucagon Lys10-TrpC16 E16 K20 Cys24(40K)”: E16K20-glucagon-NH2 was further modified with Lys10 linked to a Trp spacer which was acylated with a C16 fatty acid;

(E) “Glucagon Lys10-TrpC18 E16 K20 Cys24(40K)”: E16K20-glucagon-NH2 was further modified with Lys10 linked to a Trp spacer which was acylated with a C18 fatty acid.

The acylated glucagon co-agonist peptides were tested for their activities at the Glucagon and GLP-1 receptors generally as described in Example 14. The EC50 at each of the glucagon receptor and the GLP-1 receptor in comparison to controls (GLP-1 (7-37) OH (amino acids 7-37 of GLP-1), Glucagon (1-29)OH (SEQ ID NO: 1), and Chimera 2 Cys24 (40K) (Chimera 2 with a 40K PEG on Cys 24)) are as shown in Table 16.

TABLE 16

	EC50 at Glucagon Receptor (nM)	EC50 at GLP-1 Receptor (nM)
GLP-1 (7-37) OH	>1000.00	0.04
Glucagon (1-29) OH	0.07	7.5
Chimera 2 Cys24 (40K)	2.83	0.04
Chimera-2 Aib2 Lys10-C18 Cys24(40K)	8.55	0.14
Chimera-2 Aib2 Lys10-C16 Cys24(40K)	17.41	0.05
Glucagon Lys10-C18 E16 K20 Cys24(40K)	0.84	0.15
Glucagon Lys10-TrpC16 E16 K20 Cys24(40K)	0.54	0.23
Glucagon Lys10-TrpC18 E16 K20 Cys24(40K)	1.29	1.64

EXAMPLE 22

The following acylated glucagon co-agonist peptides were made as essentially described in Example 19:

(A) Peptide A: native glucagon amino acid sequence (SEQ ID NO: 1) comprising the following modifications: Glu at position 16, Lys at position 20, and C-terminal amide ("E16K20-glucagon-NH2");

(B) Peptide B: E16K20-glucagon-NH2 further comprising a Lys10 acylated with a C16 fatty acid;

(C) Peptide C: E16K20-glucagon-NH2 further comprising a Lys10 acylated with a C18 fatty acid;

(D) Peptide D: E16K20-glucagon-NH2 further comprising a Lys10 linked to a Glu (a spacer residue) acylated with a C16 fatty acid;

(E) Peptide E: E16K20-glucagon-NH2 further comprising a Lys10 linked to a Trp (a spacer residue) acylated with a C18 fatty acid.

The activity of the peptides were assayed generally according to Example 14 and the EC50 at each of the glucagon receptor and the GLP-1 receptor are shown in Table 17.

TABLE 17

	EC_{50} at Glucagon Receptor (nM)	EC_{50} at GLP-1 Receptor (nM)
GLP-1 OH	>1000	0.037
Glucagon (1-29) OH (SEQ ID NO: 1)	0.098	10
Peptide A	0.203	0.188
Peptide B	0.236	0.125
Peptide C	0.086	0.032
Peptide D	0.062	0.056
Peptide E	0.044	0.031

EXAMPLE 23

A glucagon co-agonist peptide was made comprising the amino acid sequence of SEQ ID NO: 1 with the following modifications: Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, Ala at position 24, Val at position 27, Lys at position 28 and C-terminal amide ("Chimera 1"). C-terminally truncated versions of Chimera 1 were made by deleting the amino acid at position 29 of Chimera 1 ("Chi 1 (1-28)"), or by deleting amino acids at both positions 28 and 29 of Chimera 1 ("Chi 1 (1-27)").

A glucagon peptide comprising the amino acid sequence of SEQ ID NO: 1 with the following modifications: Glu at position 16, C-terminal amide ("E16 Gluc-NH2") was also C-terminally truncated, by deleting either the amino acid at position

29 (“E16 GlucNH2 (1-28)”) or by deleting amino acids at both positions 28 and 29 (“E16 GlucNH2 (1-27)”).

The activity at the glucagon receptor and the GLP-1 receptor of the truncated peptides, as well as the non-truncated peptides, were assayed for functional activity generally according to Example 14. Deletion of amino acids at positions 28 and 29 of the E16 GlucNH2 peptide or the Chimera 1 peptide did not significantly impact the activity of the peptide at the glucagon receptor. Deletion of amino acids at positions 28 and 29 of E16 GlucNH2 did not appreciably change the potency of the peptide at the GLP-1 receptor. Deletion of amino acids at positions 28 and 29 of Chimera 1 did not impact its activity at the GLP-1 receptor.

Deletion of the amino acid at position 29 of either the Chimera 1 peptide or the E16 GlucNH2 peptide did not significantly impact the activity at either the glucagon receptor or the GLP-1 receptor.

EXAMPLE 24

Diet-induced obesity (DIO) mice were injected intraperitoneally at the -15 min time point with 0.2, 2, 20, or 70 nmol/kg of one of the following:

(A) vehicle only,

(B) native glucagon amino acid sequence (SEQ ID NO: 1) comprising the following modifications: Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, and Ala at position 24, and a C-terminal amide (“Chimera 2”) further modified to comprise AIB at position 2 and Cys at position 24, which Cys is pegylated with a 40K PEG (“Chimera-2-AIB^{2} Cys^{24}-40kD”),

(C) Chimera 2 further modified to comprise AIB at position 2, Lys at position 10, which Lys is acylated with a C8 fatty acid, and Cys at position 24, which Cys is pegylated with a 40K PEG (“Chimera-2 AIB^{2} K^{10}-C8 Cys^{24}-40kD”), or

(D) Chimera 2 further modified to comprise AIB at position 2, Lys at position 10, which Lys is acylated with a C16 fatty acid, and Cys at position 24, which Cys is pegylated with a 40K PEG (“Chimera-2 AIB^{2} K^{10}-C16 Cys^{24}-40kD”).

A saline solution comprising 25% (v/v) glucose was injected at a dose of 1.5 g/kg of body weight at the 0 min time point. Blood glucose levels were measured at the -15, 0, 15, 30, 60, and 120 min time points.

Figures 15-17 show the blood glucose levels (mg/dL) of mice injected with 2, 20, and 70 nmol/kg, respectively, at the indicated time points. For all doses tested, Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD demonstrated the greatest ability to lower blood glucose in the mice. As shown in Figure 17, this peptide had similar activity as Chimera-2-AIB^2 Cys^{24}-40kD.

EXAMPLE 25

DIO mice were injected intraperitoneally at the -24 hr time point with 70 nmol/kg of one of the following:

(A) vehicle only,

(B) Chimera-2-AIB^2 Cys^{24}-40kD, as described above in Example 24,

(C) Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD, as described above in Example 24, or

(D) Chimera-2 AIB^2 K^{10}-C16 Cys^{24}-40kD, as described above in Example 24.

A saline solution comprising 25% (v/v) glucose was injected at a dose of 1.5 g/kg of body weight at the 0 min time point. Blood glucose levels were measured at the 0, 15, 30, 60, and 120 min time points.

Figure 18 demonstrates the blood glucose levels (mg/dL) of the mice at the indicated time points. All three peptides demonstrate significant activity at lowering blood glucose in the mice.

EXAMPLE 26

DIO mice were injected intraperitoneally with vehicle only or 15 or 70 nmol/kg of one of the following:

(A) Chimera-2-AIB^2 Cys^{24}-40kD, as described above in Example 24,

(B) Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD, as described above in Example 24, or

(C) Chimera-2 AIB^2 K^{10}-C16 Cys^{24}-40kD, as described above in Example 24.

Body weight was measured before injection and at 1, 3, 5, and 7 days post-injection.

Figure 19 demonstrates the % change of body weight for each group of mice. At both doses tested, Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD and Chimera-2-AIB^2 Cys^{24}-40kD demonstrate comparable ability to lower body weight. At the higher dose tested, Chimera-2 AIB^2 K^{10}-C16 Cys^{24}-40kD demonstrates significant ability to lower body weight

EXAMPLE 27

A peptide of SEQ ID NO: 555, comprising a Tyrosine at position 1 and a lactam bridge between E16 and K20, (and an amide in place of the C-terminal carboxylate) was synthesized as essentially described above and tested in vitro for activity at GLP-1 and glucagon receptors by Example 14. The EC50 of the peptide at each receptor is shown in Table 18.

TABLE 18

Receptor	**EC_{50} (nM)**	**Std. Dev**	**Relative Activity**
Glucagon	0.044	0.151	343.18%
GLP-1	0.062	0.062	100.00%

Relative activity is activity relative to the native hormone of the indicated receptor.

Based on these data, it was determined that the peptide of SEQ ID NOs: 555 was an exemplary glucagon/GLP-1 co-agonist peptide.

EXAMPLE 28

A peptide of SEQ ID NO: 1 (Glucagon(1-29)), a peptide of SEQ ID NO: 1 with an amide replacing the C-terminal carboxylate (Glucagon (1-29a)), and a peptide of SEQ ID NO: 1 with AIB at each of positions 2 and 16 and an amide replacing the C-terminal carboxylate (Glucagon(1-29a) Aib^2 Aib^{16}) were synthesized as essentially described above. These peptides were then tested in vitro for activity at the GLP-1 receptor and glucagon receptors by the methods described in Example 14. The EC50 of each peptide are shown in Table 19.

TABLE 19

	Glucagon Receptor		GLP-1 Receptor	
Peptide	EC_{50} (nM)	SD	EC_{50} (nM)	SD
Glucagon(1-29)	0.04	0.01	3.65	0.21
Glucagon (1-29a) Aib^2 Aib^{16}	0.09	0.02	0.10	0.01
Glucagon(1-29a)	ND	ND	0.50	0.05
GLP-1(1-31)OH	ND	ND	0.03	0.00

SD = standard deviation

EXAMPLE 29

The following peptides were synthesized as essentially described above:

(1) Glucagon(1-29), as described in Example 28,

(2) Glucagon(1-29a) Aib^2 Aib^{16} (as described in Example 28) with a Cys at position 24 and a Lys at position 10 covalently bonded to a Trp comprising a C16 fatty acid ("Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24}")

(3) Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24} in which the Cys comprises a 40 kD PEG group ("Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24}-40kD"),

(4) Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24} comprising Aib at position 20 ("Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Aib^{20} Cys^{24}), and

(5) Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Aib^{20} Cys^{24} in which the Cys comprises a 40 kD PEG group ("Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Aib^{20} Cys^{24}-40kD).

These peptides were then tested in vitro for activity at the GLP-1 receptor and glucagon receptors by the methods of Example 14. The EC50 of each peptide are shown in Table 20.

TABLE 20

	Glucagon Receptor		GLP-1 Receptor	
Peptide	EC_{50} (nM)	SD	EC_{50} (nM)	SD
Glucagon(1-29)	0.04	0.01		
Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24}	0.25	0.02	0.24	0.03
Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Cys^{24}-40K	0.29	0.03	0.19	0.02
Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Aib^{20} Cys^{24}	2.06	0.02	1.15	0.19
Glucagon (1-29a) Aib^2 Lys^{10}-Trp-C16 Aib^{16} Aib^{20} Cys^{24}-40K	2.37	0.24	0.60	0.06
GLP-1(1-31)OH			0.02	0.01

EXAMPLE 30

The *in vivo* effects of acylated and pegylated glucagon peptides were tested in DIO mice. Specifically, 6 groups of DIO mice (8 mice per group), each group having an average initial body weight of 58 g, were injected intraperitoneally with 10, 20, 40, or 80 nmol/kg of an acylated and pegylated glucagon peptide or a vehicle control once a week for 2 weeks. The acylated and pegylated glucagon peptides used in the study were Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD (as described in Example 26) and Peptide A K^{10}-C_{14} (as described in Example 20).

Changes in body weight of and food intake by the mice were measured 0, 1, 3, 5, 7, 8, 10, 12, and 14 days after injection. Blood glucose levels of the mice were monitored throughout the 14 days. Glucose tolerance tests were performed by injecting a 25% glucose in saline solution 1 hour or 24 hours after administration of the acylated or pegylated peptide and measuring blood glucose levels at -60, 0, 15, 30, 60, or 120 min after the glucose injection.

As shown in Figure 20, the total body weight of mice injected with 40 or 80 nmol/kg of acylated and pegylated Peptide A K^{10}-C_{14} was reduced as compared to mice injected with the vehicle control.

As shown in Figure 21, the blood glucose levels of mice injected with 20, 40, or 80 nmol/kg Peptide A K^{10}-C_{14} or with 20 nmol/kg Chimera-2 AIB^2 K^{10}-C8 Cys^{24}-40kD in response to a glucose injection are lowered in comparison to vehicle control.

EXAMPLE 31

Acylated glucagon analog peptides comprising or lacking a covalent intramolecular bridge were made by solid-phase synthesis and tested for *in vitro* activity at the glucagon and GLP-1 receptors. The EC50 (nM) at each receptor and the % activity of the peptide relative to the native peptide at the corresponding receptor is shown in Table 21.

TABLE 21

Peptide Name	SEQ ID NO:	EC_{50} at the GLP-1 receptor (nM)	% Activity of GLP-1	EC_{50} at the Glucagon receptor (nM)	% Activity of Glucagon
DMIA1, K10(C14), [E16/K20]-Gluc Amide	607	0.050	30%	0.027	203.7%
DMIA1, K10(C16), [E16/K20]-Gluc Amide	608	0.015	100%	0.014	392%
DMIA1, K10(C18), [E16/K20]-Gluc Amide	609	0.011	136%	0.13	42.3%
AIB2, AIB16, K10(C14) Gluc Amide	610	0.024	33.3%	0.044	77.3%
AIB2, AIB16, K10(C16) Gluc Amide	611	0.011	72.3%	0.020	170%
AIB2, AIB16, K10(C18) Gluc Amide	612	0.009	88.9%	0.016	212.5%
dS2, E16/K20, K10(C14) Gluc Amide	613	0.128	6.3%	0.155	21.9%
dS2, E16/K20, K10(C16) Gluc Amide	614	0.041	19.5%	0.076	44.7%
dS2, E16/K20, K10(C18) Gluc Amide	615	0.025	60%	0.028	196%

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 14. A summary of the structure and *in vitro* activity at the glucagon and GLP-1 receptors of each peptide is shown in Tables 22 and 23.

TABLE 22

Glucagon analog backbone amino acid sequence: HXQGTFTSDKSKYLDXRRAQDFVQWLMNT-NH_2 wherein X = AIB (SEQ ID NO: 562)					
Peptide Name	SEQ ID NO:	Spacer	Size of Fatty Acyl Group	EC_{50} at Glucagon Receptor (nM)	EC_{50} 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	637	None	None	0.653 ± 0.285	0.475 ± 0.046
50	563	None	C16	0.572 ± 0.084	0.291 ± 0.060
82	564	Ala-Ala	C16	0.024 ± 0.001	0.108 ± 0.018
83	565	γ-Glu- γ-Glu	C16	0.014 ± 0.002	0.043 ± 0.005
84	566	β-Ala-β-Ala	C16	0.011	0.004
85	567	6-amino-hexanoic acid	C16	0.010	0.005
86	568	Leu-Leu	C16	0.011	0.006
87	569	Pro-Pro	C16	0.017	0.009
77*	570	None	C14	21.94 ± 14.47	1.458 ± 0.132
78*	571	γ-Glu- γ-Glu	C14	0.319 ± 0.091	0.103 ± 0.023
81*	573	Ala-Ala	C14	0.597 ± 0.175	0.271 ± 0.019
79*	575	Ala-Ala	C16	0.102 ± 0.011	0.055 ± 0.001
80*	576	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 23

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	EC_{50} at Glucagon Receptor (nM)	EC_{50} 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**	616	none	C14	0.144 ± 0.029	0.063 ± 0.012
78**	617	γ-Glu- γ-Glu	C14	0.009 ± 0.001	0.008 ± 0.001
81**	618	Ala-Ala	C14	0.027 ± 0.006	0.018 ± 0.001
80**	619	γ-Glu- γ-Glu	C16	0.006 ± 0.001	0.008 ± 0.001
79**	620	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

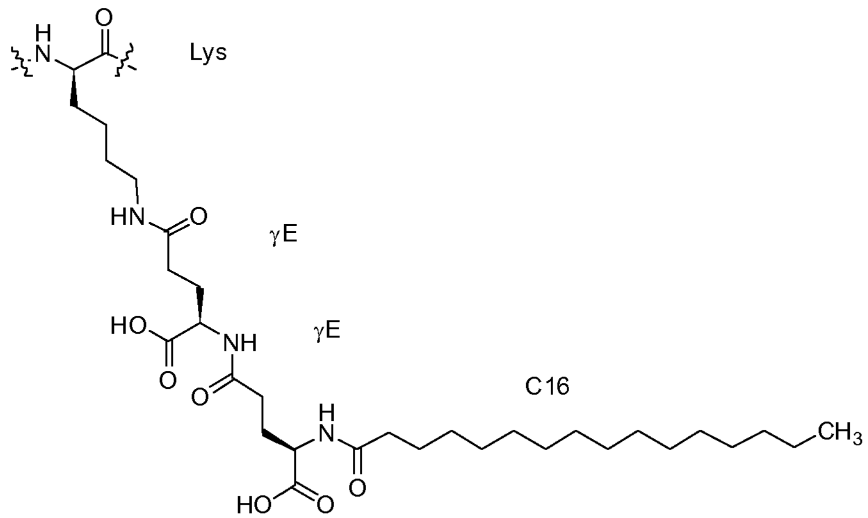
As shown in Tables 22 and 23, the peptides comprising a fatty acyl group attached via a spacer significantly increased their potency as compared to peptides comprising a fatty acyl group attached directly to the peptide backbone.

EXAMPLE 32

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 22. As shown in Figure 22, 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 23, 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 24, 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 33

Acylation of a glucagon analog peptide having GLP-1 activity was evalulated as follows. A non-acylated glucagon analog peptide comprising the structure of Chimera 2 with AIB at position 2 and Cys at position 24 (comprising a 40 kDa PEG molecule) was modified to comprise an acylated Lys residue at position 10. The non-acylated glucagon analog peptide comprised the amino acid sequence of SEQ ID NO: 580. The Lys at position 10 was acylated with a C8, C14, C16, or C18 fatty acyl group and the acylated peptides comprised the structures of SEQ ID NOs: 534-537, respectively. The *in vitro* activity at the GLP-1 receptor of the non-acylated peptide and acylated versions thereof were tested as essentially described herein. The EC50 at the GLP-1 receptor of each peptide is shown in Table 24.

TABLE 24

Glucagon analog peptide sequence HXQGTFTSDYSKYLDEQAAKEFICWLMNT-NH_2, wherein X = AIB (SEQ ID NO: 580)		
	EC_{50} (nM)	SD
GLP-1	0.026	0.003
Non-acylated Glucagon analog peptide (SEQ ID NO: 580)	0.095	0.015
C_8 acylated Glucagon analog peptide (SEQ ID NO: 534)	0.058	0.002
C_{14} acylated Glucagon analog peptide (SEQ ID NO: 535)	0.044	0.005
C_{16} acylated Glucagon analog peptide (SEQ ID NO: 536)	0.033	0.005
C_{18} acylated Glucagon analog peptide (SEQ ID NO: 537)	0.011	0.001

EXAMPLE 34

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: 581), 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: 582); 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: 583), 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: 584); 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: 585); 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: 586), 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.

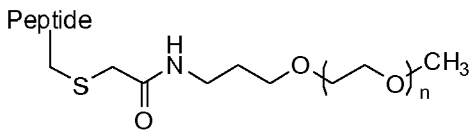
The in vitro activity at the GLP-1 receptor and glucagon receptor of each peptide was tested as essentially described in Example 14. The results are shown in Table 25.

TABLE 25

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
dS2K10(C16)E16K20-Gluc Amide	10	0.076	0.041
Peptide Chimera 2-AIB2-K10-acylated	30	N/A	0.465
Peptide Chimera 2-AIB2-K30-acylated	10	N/A	0.007

EXAMPLE 35

Solid-phase peptide synthesis was employed for the assembly of the sequence, XSQGTFTSDYSKYLDERRAKDFVCWLMNT-NH_2, wherein X=DMIA (SEQ ID NO: 587). After selective deprotection of the Glu at position 16 and the Lys at position 20, the peptide was cylized via a lactam bridge on resin. The crude peptide after cleavage was then purified by preparative RP-HPLC and characterized by MS (calc. for [M+H]: 3479.9 ; found 3480.9). PEGylation was conducted by mixing the peptide precursor and iodoacetyl-functioned 40k Da PEG (NOF)(1:1) in 7 M urea/ 50 mM Tris buffer, pH 8.5, at room temperature for 45 minutes to form a covalent, thioether bond between the PEG and a Cys of the peptide, as shown below

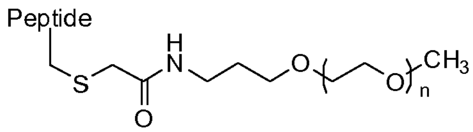


The PEGylated peptide was purified by preparative HPLC and the desired fractions were collected and lyophilized to yield a off-white powder. The product was confirmed by MALDI-TOF-MS (44000-46000, broad peak).

The *in vitro* activity at the GLP-1 receptor and glucagon receptor were tested as essentially described in Example 14. The EC50s at the GLP-1 receptor and glucagon receptor were 0.327 nM and 0.042 nM, respectively.

EXAMPLE 36

Solid-phase peptide synthesis was employed for the preparation of the peptide precursor, HXEGTFTSDYSKYLDEQAAKEFICWLMNT-NH_2, wherein X = AIB (SEQ ID NO: 589). The crude peptide was then purified by preparative RP-HPLC and characterized by MS (calc. for [M+H]: 3412.8 ; found 3413.9). PEGylation was conducted by mixing the peptide precursor and iodoacetyl-functioned 40k Da PEG (NOF)(1:1) in 7 M urea/ 50 mM Tris buffer, pH 8.5, at room temperature for 45 minutes to form a covalent, thioether bond between the PEG and a Cys of the peptide, as shown below



. The PEGylated peptide was purified by preparative HPLC and the desired fractions were collected and lyophilized to yield a off-white powder. The product was confirmed by MALDI-TOF-MS (44000-46000, broad peak).

The *in vitro* activity at the GLP-1 receptor and glucagon receptor were tested as essentially described in Example 14. The EC50s at the GLP-1 receptor and glucagon receptor were 0.027 nM and 33 nM, respectively.

EXAMPLE 37

The following glucagon analog peptides comprising a backbone of Peptide J

HS-X-GTFTSDYSKYLDTRRAAEFVAWL(Nle)DE

(SEQ ID NO: 591)

or Peptide K

HS-X-GTFTSDYSKYLD(Aib)RRAADFVAWLMDE

(SEQ ID NO: 592)

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 14. The EC50 (nM) of each peptide is shown in Table 26.

32993-212943

TABLE 26

Peptide Backbone	Amino Acid at Position 3	SEQ ID NO:	EC50 at Glucagon Receptor (nM)	% activity*
J	Q	593	0.24	25%
J	C(Acm)	594	0.18	33%
J	Dab(Ac)	595	0.31	19%
J	Dap(urea)	596	0.48	13%
J	Q(Me)	597	0.48	13%
J	M(O)	598	0.91	7%
J	Orn(Ac)	599	0.92	7%
K	Q	600	0.39	15%
K	Dab(Ac)	601	0.07	86%
K	Q(Me)	602	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 26, 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 38

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 27.

TABLE 27

Amino acid sequence	SEQ ID NO:	EC50 (nm) at Glucagon Receptor	% activity*
Wildtype Glucagon	1	0.026	100
HSQGTFTSDYSKYLDSRRAQDFVQWLMDT	642	0.015	173
HSDab(Ac)GTFTSDYSKYLDAibRRAADFVAWLLDE	603	0.069	37
HSDab(Ac)GTFTSDYSKYLDAibRRAADFVAWLLDTGPSSGAPPS amide	604	0.023	113
HSDab(Ac)GTFTSDYSKYLDAibRRASDFVSWLLDE	605	0.048	54
HSDab(Ac)GTFTSDYSKYLDAibRRATDFVTWLLDE	606	0.057	46

EXAMPLE 39

A first glucagon analog peptide (AIB2, AIB16, K10(C16) Gluc Amide) comprising SEQ ID NO: 1 with AIB at positions 2 and 16, Lys at position 10, wherein the Lys at position 10 was covalently attached to a C16 fatty acyl group, and an amide in place of the C-terminal carboxylate was made as essentially described herein. A second glucagon analog peptide (AIB2, AIB16, K10(C16), K30 Gluc Amide) having the same structure as the first glucagon analog peptide, except that a Lys was added to the C-terminus. The *in vitro* activity of the peptides was tested as essentially described in Example 14 and was additionally tested in a solution comprising 20% human plasma. The EC50 (nM) at each receptor for the peptides is shown in Table 28.

TABLE 28

Amino acid sequence	SEQ ID NO:	EC50 (nm) at Glucagon Receptor	EC50 (nm) at Glucagon Receptor (20% human plasma)	EC50 (nm) at GLP-1 Receptor	EC50 (nm) at GLP-1 Receptor (20% human plasma)
Glucagon	1	0.026	0.046		
GLP-1				0.022	0.028
AIB2, AIB16, K10(C16) Glucagon Amide	563	0.052	0.023	0.026	0.014
AIB2, AIB16, K10(C16), K30 Glucagon Amide	622	0.761	0.313	0.031	0.017

EXAMPLE 40

The discovery of leptin documented the existence of an endocrine system that regulates energy balance and body adiposity. It also recruited interest and investment in obesity research as a means to identify environmental and pharmacologic approaches to manage what has become a global epidemic of disease. Sufficiently efficacious and safe pharmacologic treatment for obesity has yet to emerge and surgery constitutes the only proven option to sustained weight loss. It is reported herein that the combinatorial efficacy of receptor agonism at two endocrine hormonal receptors to achieve potent satiety inducing and lipolytic effects in a single peptide of sustained duration of action. Two specific glucagon analogs with activity at the GLP1-R comparable to native GLP-1, but differing from each other in their level of glucagon receptor agonism were studied pharmacologically in rodent obesity models. Once weekly administration of these pegylated peptides selected from a series of high potency analogs with differential glucagon and GLP-1 activity normalized adiposity and glucose tolerance levels in diet induced obese mice (average body weight ca. 50g) within a month. Body weight loss was a consequence of body fat loss resulting from decreased food intake and increased energy expenditure, which increased with the level of glucagon receptor agonism. These co-agonist compounds also normalized glucose and lipid metabolism including liver steatosis. Effects were dose dependent and successfully repeated in diet induced obese rats. These preclinical studies indicate that when full GLP-1 agonism is enhanced with an appropriate degree of glucagon receptor activation, body fat reduction can be substantially and safely accelerated. The findings shown herein establish a basis for clinical testing and suggest an attractive novel treatment option for the metabolic syndrome.

EXAMPLE 41

The following materials and methods pertain to the experiments described in Examples 42 to 51.

Boc peptide synthesis and cleavage.

Peptide syntheses were performed using 0.2 mmol 4-methylbenzhydrylamine (MBHA) resin (Midwest Biotech, Fishers, Indiana) on a modified Applied Biosystems 430A peptide synthesizer. Solid-phase peptide syntheses utilized *in situ* neutralization for Boc-chemistry (Schnolzer, M. et al., *International Journal of*

Peptide Research and Therapeutics, 13:31-44 (2007)). Completed peptidyl-resins were treated with HF/*p*-cresol (10:0.5 v/v) at 0° C for 1 h. HF was removed *in vacuo* and the deprotected peptide was precipitated and washed in diethyl ether. The peptide was dissolved in 20% acetonitrile/1% acetic acid and lyophilized. Most peptides were prepared by Boc chemistry. The following side chain protecting groups were used for Boc-amino acids (Midwest Biotech): Arg(Tos), Asp(OcHex), Asn(Xan), Glu(OcHex), His(BOM), Lys(2-C1-Z), Ser(Bz1), Thr(Bz1), Trp(CH0), Tyr(Br-Z). Peptide molecular weights were confirmed by electrospray ionization or MALDI-TOF mass spectrometry and purified as described elsewhere.

Lactam Synthesis.

Cyclized peptides with i to i + 4 lactam formation were synthesized on resin. Glu(OFm)-OH gamma ester (Peptides International, Louisville, Kentucky) and Lys(Fmoc)-OH (Peptides International) were substituted for Glu(OcHex) and Lys(2-C1-Z) at positions involved in lactam formation. The fully protected peptidyl-resin was treated with 20% piperidine in DMF for 45 minutes to remove Fmoc and OFm protecting groups. On resin, lactam formation was achieved after treatment with 5 equivalents of benzotriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (Fluka) in DMF/DIEA for 5 h. Lactam formation was confirmed by ninhydrin analysis and mass reduction of 18 relative to the open form of the peptide.

Peptide Purification.

Following cleavage from the resin, crude peptide extracts were analyzed by analytical reverse-phase HPLC. Analytical separations were conducted in 0.1% TFA with an acetonitrile gradient on a Zorbax C8 column (0.46 X 5 cm). After analytical analysis, the crude extract was purified by semi-preparative chromatography in 0.1% TFA with an acetonitrile gradient on a Vydac C4 or C18 column (2.2 X 25 cm). Pegylated peptides were purifed using the same conditions. Preparative fractions were analyzed for purity (> 95%) by analytical reverse-phase HPLC utilizing the conditions listed for analytical separations. Peptide masses and purity were confirmed by electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Pegylated peptides showed a broad mass range spanning 43400 by MALDI-TOF. Purified peptides were lyophilized and stored at 4° C.

Pegylation of Peptides.

Purified peptides were mixed at a 1:1 molar ratio with methoxy poly(ethylene glycol) maleimido-propionamide-40K (Chirotech Technology Ltd, Cambridge) in 7M urea/50mM Tris, pH 8.0. Reaction progress was monitored by analytical reverse-phase HPLC and free peptide was consumed within 30 minutes. The reaction was quenched in 0.1% TFA, purified and characterized as described elsewhere.

Glucagon and GLP-I Receptor-Mediated cAMP Synthesis.

Each peptide analog was tested for its ability to stimulate cAMP production through the glucagon (Gcg) and GLP-1 receptors. HEK293 cells were co-transfected with the GcgR or GLP-1R cDNAs and a luciferase reporter gene-linked to a cAMP response element (CRE). Cells were serum deprived for 16 h by culturing in DMEM (Invitrogen, Carlsbad, CA) and supplemented with 0.25% Bovine Growth Serum (HyClone, Logan, UT). Serial dilutions of Glucagon and GLP-1 analogs were added to 96-well poly-D-Lysine-coated plates (BD Biosciences, San Jose, CA) containing co-transfected HEK293 cells, and plates were incubated for 5 h at 37° C, 5% CO_2. Following incubation, an equivalent volume (100 μL) of LucLite luminescence substrate reagent (Perkin-Elmer, Wellesley, MA) was added to each well and the plate was shaken for 3 min at 800 rpm. The plate was incubated for 10 min in the dark and light output was quantified on a MicroBeta1450 liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Effective 50% concentrations (EC50) were calculated by Origin software (OriginLab, Northampton, MA).

Circular dichroism measurements.

Peptides were dissolved in 10 mM phosphate buffer pH 5.9 with increasing concentrations of TFE, and peptide concentrations were quantified. Each sample was diluted to 10 μM for CD measurements. CD data were collected on a JASCO J-715 circular dichroism spectropolarimeter with constant nitrogen stream and temperature control of the 1 mm path length cell set at 25° C. Spectral data were accumulated for 5 scans from 270-190 nm with a scan speed of 100 nm/min and 1 nm wavelength step. Solvent signal was subtracted and data were smoothed (Savitzky and Golay, *Anal. Chem.* 36:1627 (1964)); in the JASCO Spectra Manager software. Millidegree values obtained were converted to mean residue ellipticity with units of deg·cm^{2}·dmol^{-1}. Calculated mean residue ellipticity values were input into DICHROWEB (Whitmore and Wallace, *Biopolymers* 89:392- 400 (2008); Whitmore and Wallace, *Nucleic Acids Research* 32:W668-W673 (2004) to obtain percent helicity values.

Animals.

C57BI/6 mice were obtained from Jackson Laboratories and fed a diabetogenic diet from Research Diets, a high sucrose diet with 58% kcal from fat. Mice were single or group-housed on a 12:12-h light-dark cycle at 22° C with free access to food and water. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

Body Composition Measurements.

Whole body composition (fat and lean mass) was measured using NMR technology (EchoMRI, Houston, TX).

Energy Balance Physiology Measurements.

Energy intake and expenditure, as well as home-cage activity, were assessed by using a combined indirect calorimetry system (TSE Systems, Bad Homburg, Germany). Oxygen consumption and CO_2 production were measured every 45 min for a total of 120 h (including 12 h of adaptation) to determine the respiratory quotient and energy expenditure. Food and water intake and meal patterns were determined continuously for 120 h at the same time as the indirect calorimetry assessments by integration of scales into the sealed cage environment. Meals were defined as food intake events with a minimum duration of 60 s, and a break of 300 s between food intake events. Home-cage locomotor activity was determined using a multidimensional infrared light beam system with beams scanning the bottom and top levels of the cage, and activity being expressed as beam breaks. Stationary motor activity (fidgeting) was defined as consecutive breaks of one single light beam at cage-bottom level, ambulatory movement as breaks of any two different light beams at cage-bottom level, and rearing as simultaneous breaks of light beams on both cage-bottom and the top level.

Blood Parameters.

Blood was collected after a 6-h fast from tail veins using EDTA-coated Microvette tubes (Sarstedt, Nuremberg, Germany) and immediately chilled on ice. After 15 min of centrifugation at 3,000 g and 4° C, plasma was stored at -80° C. Plasma insulin was quantified by a radioimmunoassay from Linco (Sensitive Rat Insulin RIA; Linco Research, St. Charles, MO). Plasma TGs and cholesterol levels were measured by enzymatic assay kits (Thermo Electron, Waltham, MA). Samples were analyzed individually with the exception that pooled samples (0.25 ml) from 5 animals/group were subjected to fast-performance liquid chromatography (FPLC) gel filtration on two Superose 6 columns connected in series for lipoprotein separation. All assays were performed according to the manufacturer's instructions.

Glucose tolerance test.

For the determination of glucose tolerance, mice were subjected to 6 h of fasting and injected intraperitoneally (i.p.) with 2 g glucose/kg body wt (50% D-glucose (Sigma) in 0.9% saline) for the glucose tolerance test (GTT). Tail blood glucose levels (mg/dl) were measured by using a hand-held glucometer (TheraSense Freestyle) before (0 min) and at 15, 30, 60, 90, and 120 min after injection.

Western blot of WAT HSl.

Adipose tissue was placed in a 1.5-ml microfuge tube and lysed in ice cold RIPA buffer (1X PBS, 1% Nonidet P40, 0.5% sodium doxycholate, 0.1% SDS with 50 mM NaF, 0.5 M phenylmethylsulfonyl fluoride, 0.1 mM Na Vanadate, 20 µg/ml Aprotinin, 10 µg/ml Leupeptin) using a tissue lyser (Retsch, Inc Newtown, PA Cat. # 85210) at 30 hz for 3 min. Samples were spun at 12,000 rpm for 15 min (4° C) at which time the internatant was removed to a new tube and sonicated for 15 sec on ice. Samples were spun at 14,000 rpm for 10 min (4° C) and the internatant was collected to a new tube. Samples were again spun at 19,000 rpm for 10 min (4° C) and the internatant collected to a new tube. An aliquot of sample was then taken for protein assay. Samples were then boiled in 4x SDS/DTT buffer for 2 min. 50 µg of protein from cell lysate were subjected to SDS/PAGE on 9% (w/v) acrylamide resolving gels and transferred to Hybond ECL nictrocellulose membranes. Membranes were blocked and probed with primary antibodies of interest (HSL (4107)) from Cell Signaling; Phospho-HSL (ser 660) (4126) from Cell Signaling). After washing, primary antibody detection was performed using either HRP-conjugated anti-(rabbit IgG) or anti-(mouse IgG) (HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Bio-Rad (170-6515 & 170-6516)) and detected using enhanced chemiluminescence (Amersham Biosciences) and exposed to CL-Xposure film (Pierce).

Immunohistochemistry.

Paraffin embedded sections of white epididymal adipose tissue (5 μm) were stained with hematoxylin/eosin as described (Ogden, C. L. et al. *JAMA* 295:1549-1555 (2006)). For each individual mouse tissue block, adipocyte size of 100 cells from each of three different high-power fields was quantified as areal measurement using Image Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA).

Oil Red Staining.

To visualize lipid accumulation in liver tissue, 4-8 mm cross-sections of the livers that were harvested at sacrifice were stained with Oil Red O dye. Images at both 20X and 40X magnification were acquired using a [compound-lens] microscope.

Quantitative RT-PCR procedure.

Animals were sacrificed by decapitation in the fed state (1-4 h after the morning feeding) and various tissues were sampled, freeze-clamped, and stored at -80° C for subsequent measurement of mRNA expression of PEPCK, G6P, and HPRT (housekeeping) by real-time quantitative PCR (icycler, BioRad).

Total RNA was extracted from frozen tissue samples using a RNeasy Lipid Tissue Kit (Qiagen, Ca# 74804) using the standard protocol. RNA concentrations and purity were determined by spectrophotometry using the Nanodrop. cDNA templates for RT-PCR were obtained using 2 μg of total RNA. Reverse transcription reaction was performed with 10X DNase I Reaction Buffer, DNase I, Amp Grade, 1 U/μl, depc-H_20, 25 mM EDTA, 10 mM dNTP Mix, oligo(dT)20 (50 μM), 5X First-Strand Buffer, 0.1 M DTT, RNaseOUT, and SuperScript III (Invitrogen).

The synthesized cDNAs were further amplified by PCR using the fluorescent dye SYBR green (BioRad, Ca# 1708882) containing a final concentration of 0.5 μM of forward and reverse primers. Product purity was confirmed by dissociation curves. No-template controls were included in all assays, yielding no consistent amplification. A standard curve was used to obtain the relative concentration of PEPCK or G6P, and the results were corrected according to the concentration of HPRT, used as housekeeping genes. The results are expressed as percent of vehicle, setting the mean of the vehicle group at 100% and then calculating each individual value of the 3 groups of animals studied.

Primer Sequences.

Primer sequences for PEPCK, G6P, and HPRT were taken from the NIH website and primers were generated by IDT DNA.

Reverse Transcription and Quantitative Real-Time RT-PCR.

CD68 mRNA expression was quantified by real-time RT-PCR as described (Nomiyama, T. et al. *Journal of Clinical Investigation* 117:2877-2888 (2007)). Briefly, upon sacrifice, 100 mg epididymal adipose tissue was homogenized in TRIZOL and total mRNA was reverse transcribed into cDNA. PCR reactions were performed using an iCycler (Bio-Rad) and SYBR Green I system (Bio-Rad). Each sample was analyzed in triplicate and normalized to values for TFIIB mRNA expression. Mouse primer sequences used were as follows:
CD68, 5'-CAAGGTCCAGGGAGGTTGTG-3' (forward) (SEQ ID NO: 638),
5'-CCAAAGGTAAGCTGTCCATAAGGA-3' (reverse) (SEQ ID NO: 639);
and TFIIB, 5'-CTCTCCCAAGAGTCACATGTCC (SEQ ID NO: 640),
5`-CAATAACTCGGTCCCCTACAAC-3' (reverse) (SEQ ID NO: 641).

Statistical Analyses.

Unless indicated otherwise, all statistical analyses were performed using GraphPad Prism one-way ANOVAs and column statistics. Stated P values are for one-way analysis of variance. All results are presented as means ± SE. (Receptor activation data is ± S.D.).

EXAMPLE 42

Two glucagon peptides, Peptides X and Y, comprising the amino acid sequence of SEQ ID NO: 1 with amino acid modifications were made as described herein. Both peptides comprised AIB at position 2, Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, and Cys at position 24. Site-specific 40-kd pegylation was achieved at Cys at position 24 through reaction with a maleimide-functionalized linear peg to yield Peptide X-PEG and Y-PEG. Peptides Y and Y-PEG differed from Peptides X and X-PEG, respectively, in that a single side-chain lactam bridge was introduced in the middle of Peptide Y or Peptide Y-PEG to stabilize the secondary structure and enhance glucagon agonism. The two side chains of Glu at position 16 and Lys at position 20 were covalently coupled in the course of peptide assembly as a side-chain amide. This macrocyclization of the peptide represents a 21-atom lactam. Peptides X-PEG and Y-PEG were tested for solubility and were found to be soluble in physiological buffers at concentrations that exceed 25 mg/ml, and Peptides X-PEG and Y-PEG proved completely resistant to *ex vivo* incubation with plasma for periods of one week.

EXAMPLE 43

The secondary conformation of peptides when solubilized in various concentrations of aqueous trifluoroethanol (TFE) was analyzed by circular dichroism (Figure 25). Glucagon was the least helical peptide tested, and had calculated helicity of 10, 15 and 33% in TFE solutions of 0, 10 and 20%, respectively (Table 29). Under the same experimental conditions, GLP-1 had enhanced helicity of 14, 29 and 55%, demonstrating that these two peptides differ in primary as well as secondary structure. There were no significant changes in the helicity of Peptides X and Y when pegylated, despite the fact that the pegylated portions represent more than 90% of the molecule by mass (Table 29). In contrast, the apparent helicity of Peptide Y in phosphate buffer in the absence of TFE was approximately double that of Peptide X, from 17% to 36%. Consequently, the pegylated forms of these two chimeric peptides (Peptide X-PEG and Peptide Y-PEG) differed appreciably in secondary structure (Figure 25) and the differences in biological properties are likely a function of these secondary structural differences.

TABLE 29

Peptide	Percent Helicity		
	0% TFE	10% TFE	20% TFE
Glucagon	10	15	33
GLP-1	14	29	55
Peptide X	17	34	60
Peptide X-PEG	12	31	-
Peptide Y	36	35	64
Peptide Y-PEG	37	51	-

EXAMPLE 44

The two peptides (Peptides X and Y) and their 40-kd pegylated derivatives (Peptides X-PEG and Y-PEG) were assessed for their ability to stimulate cAMP synthesis in cell-based CRE-luciferase reporter assays (Figure 26). As shown in

Table 30, native glucagon activated glucagon receptors half-maximally at an effective concentration (EC50) of 0.055 ± 0.014 nM and the GLP-1 receptor (GLP-1R) at a much higher concentration, EC50 of 3.29 ± 0.39 nM. In contrast, GLP-1 activated its receptor with an EC50 of 0.028 ± 0.009 nM and proved highly specific in that interaction at the glucagon receptor (GcgR) occurred at an EC50 exceeding 1 μM. The dynamic range in specificity exhibited for the native ligands at their receptors is in excess of a million. The potency of Peptide X-PEG at GLP-1R was twice that of native GLP-1 and even more enhanced at GcgR in a relative sense. However, the GcgR activity was only approximately 10% that of native glucagon. The introduction of the lactam restored full glucagon agonism without a change at GLP-I R. Consequently, Peptide Y-PEG is a fully potent, nearly balanced co-agonist relative to the native ligands at the two respective receptors. PEGylation of each peptide reduced potency by as much as ten-fold at GcgR and five-fold at GLP-1R. The slightly enhanced loss in activity at GcgR may be a function of the greater relative importance of the C-terminal sequence to glucagon receptor interaction. The pegylated peptides (Peptides X-PEG and Y-PEG) were slightly less potent at GLP-1R than native GLP-1 but still had a subnanomolar EC50. Peptide X-PEG is seven-fold more selective than the lactam version of this peptide, i.e., Peptide Y-PEG, at the GLP-1R. Therefore, these two DPP-4-resistant peptides are suitable for sustained in vivo time-action experiments and well-matched for GLP-1R agonism, but differ in glucagon agonism.

TABLE 30

Peptide	Glucagon Receptor			GLP-1 Receptor			Selectivity*
	EC50 (nM)	Standard Deviation	% activity of native glucagon	EC50 (nM)	Standard Deviation	% activity of native GLP-1	
Glucagon	0.055	0.014	100.00	3.293	0.389	0.86	0.009
GLP-1	>1000	-	<0.008	0.028	0.009	100.00	>12500
Peptide X	0.585	0.125	9.38	0.014	0.002	202.12	21.5

Peptide X-PEG	2.895	0.963	1.90	0.036	0.014	78.41	41.4
Peptide Y	0.055	0.011	99.51	0.013	0.005	219.78	2.21
Peptide Y-PEG	0.667	0.264	8.22	0.059	0.029	47.61	5.79

EXAMPLE 45

The 40-kd pegylated peptides Peptides X-PEG and Y-PEG were used as single weekly subcutaneous (s.c) injections in diet-induced obese (D10) C57B6 mice. A single injection of 325 nmol/kg of Peptide Y-PEG decreased body weight over one week by 25.8%, from 50.9 ± 1.4 g to 37.8 ± 0.8 g ($p < 0.0001$, n = 8/group). Comparable administration of Peptide X-PEG was effective but considerably less potent, as the decrease in body weight was 9% (49.1 ± 1.51 g to 44.68 ± 1.38 g). Saline-injected control mice did not change their body weight (before: 50.61 ± 1.32 g, after: 50.87 ± 1.46 g; Figure 27A). The body weight changes were a result of a decrease in fat mass (41.9% for the lactam peptide, 22.2% for open form, 2.3% for controls, $p < 0.001$; Figure 27B) and were paralleled by a significant decrease in average daily food intake (Peptide Y-PEG: 0.40 ± 0.29 g/day, Peptide X-PEG: 1.83 ± 0.81 g/day, saline: 2.70 ± 0.78 g/day, $p < 0.0001$, Figure 27C). Blood glucose was significantly decreased for both peptides when compared to control, and slightly more so in Peptide Y-PEG (Peptide Y-PEG: -90.1 mg/dL, Peptide X-PEG: -79.6 mg/dL, control: -23.9 g,/dL, p=0.0433; Figure 27D). The relative difference between the two peptides (Peptide X-PEG and Peptide Y-PEG) was not statistically significant.

EXAMPLE 46

In a separate experiment, single s.c. injections of six different doses (0, 7, 14, 35, 70, 140 and 350 nmol/kg) of Peptide Y-PEG and Peptide X-PEG demonstrated linearly responsive, dose-dependent decreases in body weight and blood glucose (Figure 28A, 28B, 28C and 28D). This suggests that the observed effects are pharmacologically relevant with no apparent toxicity, other than the indirect effects of rapid, excessive loss in body weight. The magnitude of the effect was more prominent with Peptide Y-PEG and indicates that the additional element of glucagon agonism improves the potency of the peptide.

EXAMPLE 47

In a separate experiment, weekly s.c. injections of 70 nmol/kg of Peptide Y-PEG or Peptide X-PEG decreased body weight of DIO mice by 28.1 % and 20.1 %, respectively ($p < 0.0001$, n= 7-8/group; Figure 29A). The body weight changes were associated with a decrease in fat mass (-62.9% for Peptide Y-PEG, -52.2% for Peptide X-PEG, and 5.1% for controls, $p < 0.0001$; Figure 29B). Long-term effects of these lower doses on food intake ($p = 0.95$; Figure 29C) were less impressive than short-term effects with a higher dose (Figure 27C). Energy expenditure was increased with Peptide Y-PEG (14.60 ± 0.69 kcal/[kg*h]) and Peptide X-PEG (17.19 ± 1.49 kcal/[kg*h]) compared to vehicle (12.71 ± 0.45 kcal/[kg*h]), $p = 0.0187$), whereas the respiratory quotient tended to be decreased (Figure 29D and 29E; 0.719 ± 0.01 for Peptide Y-PEG, 0.725 ± 0.01 for Peptide X-PEG, and 0.755 ± 0.01 for vehicle, $p = 0.1028$), indicating that increased thermogenesis and altered nutrient partitioning may explain the overall negative energy balance. Increased energy expenditure was not associated with a change in spontaneous physical activity induced thermogenesis (NEAT) since locomotor activity did not differ between treatment groups and controls ($p = 0.4281$; Figure 29F). Neither automated online monitoring of acute feeding nor chronic monitoring of food intake revealed any differences in caloric intake (automated $p = 0.667$, chronic $p = 0.9484$; Figure 30A).

Blood glucose levels were markedly decreased over the treatment period starting at Day 3 after the first injection (mean decrease: Peptide Y-PEG -32%, Peptide X-PEG -24.5%, controls: -2.7%, $p < 0.0001$; Figure 29G). In response to an intraperitoneally (i.p.) glucose challenge on Day 3, blood glucose peaks (Figure 29H) and profiles (AUC) (Figure 30F) were markedly lower in the two treated groups (Peptide Y-PEG 14183 ± 1072, Peptide X-PEG 13794 ± 824.1) compared to the vehicle-treated controls (34125 ± 3142, $p < 0.0001$). After one month of treatment with Peptide Y-PEG or Peptide X-PEG, plasma insulin was lower in the treatment groups (1194 pg/ml, 1034 pg/ml , p=0.0244) compared to controls (2675 pg/ml), suggesting improved insulin sensitivity (Figure 29I). Plasma C- peptide levels tended to be decreased after one month of treatment with Peptide Y-PEG or Peptide X-PEG (738.8 pg/ml, 624.7 pg/ml) versus vehicle (1077 pg/ ml) ($p = 0.108$) (Figure 30G).

To determine if the principal phenomenon generalizes across species, both compounds were administered to diet-induced obese rats (mean weight 777.4 +/- 2.1 g, dose 70 nmol/kg/week, once-a-week injection, 3-week treatment). Peptide Y-PEG and Peptide X-PEG each decreased body weight (Peptide X-PEG: -11.15 +/- 0.88%; Peptide Y-PEG: -20.58 +/- 2.26%, vehicle: 1.09 +/- 0.56%) ($p < 0.0001$) and fat mass of the DIO rats (Peptide X-PEG: -19.17 +/- 2.03%; Peptide Y-PEG: - 33.76 +/- 4.76%, vehicle: 0.65 +/- 1.20%; $p < 0.0001$), confirming a species-independent applicability of this anti-obesity treatment approach.

EXAMPLE 48

Chronic s.c. treatment over 27 days with Peptide X-PEG and Peptide Y-PEG decreased total cholesterol in DIO mice (106.9 ± 6.3 mg/dL and 200.8 ± 29.58 mg/dL, respectively) relative to vehicle (254.0 25.33 mg/dL, $p = 0.0441$; Figure 31A). In a separate experiment, DIO mice received 70 nmol/kg s.c. of Peptide X-PEG, Peptide Y-PEG or vehicle on Days 0 and 7 and were evaluated on Day 9. Peptide Y-PEG decreased plasma triglycerides, LDL cholesterol and total cholesterol (total cholesterol 63.0 2.49 mg/dL compared to vehicle 177.7 ± 11.8 mg/dL) ($p < 0.0001$), while potentially causing a switch from LDL to HDL cholesterol (Figure 31B). Peptide X-PEG decreased both LDL and HDL cholesterol but had no significant effect on triglycerides (Figure 31C). There was a significant decrease in leptin (3343 ± 723.3 pg/ml for Peptide Y-PEG; 7308 ± 2927 for Peptide X-PEG, and 18,642 ± 6124 for vehicle; p=0.0426; Figure 31D, 31E, 31F). Chronic treatment for 27 days also normalized liver lipid content while control DIO mice maintained significant liver steatosis (data not shown).

EXAMPLE 49

One month treatment with Peptide X-PEG or Peptide Y-PEG resulted in increased phosphorylation of hormone sensitive lipase (HSL) in white adipose tissue (WAT) of DIO mice (Peptide X-PEG: 1.135 ± 0.315; Peptide Y-PEG: 1.625 ± 0.149; vehicle: 0.597 ± 0.204; $p = 0.0369$; Figure 32B), implying a glucagon-specific direct effect on WAT lipolysis. Concomitant with the decrease in fat mass of mice treated for two weeks at a dose of 35 nmol/kg/week with the Peptide Y-PEG and Peptide X-PEG, there was a significant reduction of adipocyte size in epididymal adipose tissues when compared to control mice (data not shown). However, despite having decreased fat mass and smaller adipocytes, this short term treatment of two weeks with the Peptide Y-PEG and Peptide X-PEG was not associated with a significant reduction of adipose tissue macrophage content as quantified by real-time RT-PCR for CD68 (Figure 33C). Uncoupling protein I (UCP1) levels in brown adipose tissue (BAT) were increased by Peptide X-PEG, but not by Peptide Y-PEG treatment (Peptide X-PEG 2.167 ± 0.429, Peptide Y-PEG 1.287 ± 0.1558, and vehicle 1.0 ± 0.118; $p = 0.0264$; Figure 32A), consistent with a GLP-1-specific action on BAT resting thermogenesis. Hepatic gene expression reflective of hepatic gluconeogenesis was not affected by either Peptide X-PEG or Peptide Y-PEG (Figure

30H and 30I). Histology indicated that pancreatic islets tended to be smaller following Peptide X-PEG treatment (data not shown).

EXAMPLE 50

In order to dissect the contributions of the GLP-1R and the GcgR agonist components of Peptides X-PEG and Y-PEG, each was administered for one month to GLP-1 receptor knock out (GLP-1R -/-) mice maintained on high-fat diet. Peptide X-PEG caused a reduction of body weight ($p > 0.05$; Figures 34A and 34B) and fat mass ($p > 0.05$; Figure 34C) compared to saline. Peptide Y-PEG caused a significant decrease in body weight ($p = 0.0025$) and fat mass ($p = 0.0025$) in the GLP-1R -/- mice (Figures 34A-34C). Peptide X-PEG had no effect on food intake in GLP-1R -/- mice, while Peptide Y-PEG suppressed food intake significantly ($p = 0.017$) (Figure 34D). Peptide Y-PEG (but not Peptide X-PEG) had a tendency to increase blood glucose in a glucose tolerance test in the absence of a functional GLP-1R ($p = 0.03$) (Figures 34E and 34F), implying that the GLP-1 component of the co-agonist is needed to protect against glucagon-induced hyperglycemia.

EXAMPLE 51

As an independent assessment of the effect of Peptides X-PEG and Y-PEG that can be attributable to glucagon agonism, two additional peptide agonists with comparable GLP-1R potency but markedly different GcgR activity were studied. The two peptides (Peptides U and V) are related to the Peptides X-PEG and Y-PEG. Peptides U and V comprised the amino acid sequence of SEQ ID NO: 1 with the following modifications: Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, and Cys at position 24, but comprised a 20-kd pegylation at the Cys at position 24 and did not comprise AIB at position 2. Peptide V additionally comprised a substitution of Gln3 with Glu which selectively reduced glucagon agonism by more than ten-fold. Neither Peptide U nor Peptide V comprised a lactam bridge. Treatment of DIO mice each day for one week at 50 nmol/kg s.c. with Peptide V revealed a reduced effect on body weight lowering relative to the Peptide U (-9.09 ± 0.80 vs. -13.71 ± 0.92 g, respectively ($p < 0.0001$; Figure 35A).

EXAMPLE 52

Glucagon peptides comprising a C16 fatty acyl group attached to a Lys residue via a γ-Glu spacer or a γ-Glu-γ-Glu dipeptide spacer, wherein the Lys residue is located at position 10 or at the C-terminus (at position 29), were made as essentially described herein. The peptides were tested for in vitro activities at the glucagon and GLP-1 receptors as described herein. The results are shown in Table 31.

TABLE 31

Peptide			SEQ ID NO:	EC50 (nM) at Glucagon Receptor	EC50 at GLP-1 Receptor
	Acylated AA	Spacer			
Chi-2, d-Ser2	Lys10	γE	643	0.011	0.0014
Chi-2, d-Ser2	Lys10	γE-γE	644	0.008	0.003
Chi-2, AIB2	Lys10	γE	645	0.025	0.0014
Chi-2, AIB2	Lys10	γE-γE	646	0.014	0.0018
Chi-2, AIB2, E3	Lys10	None	647	46.084	0.005
Chi-2, AIB2, E3	Lys10	γE-γE	648	2.922	0.004
Chi-2, AIB2, I7	Lys10	γE	649	0.014	0.024 (0.044*)
Chi-2, AIB2, I7	Lys10	γE-γE	650	0.007	0.010
DMIA1, E16/K20 lactam	Lys10	γE	651	0.019	0.006
DMIA1, E16/K20 lactam	Lys10	γE-γE	652	0.014	0.004
DMIA1, E16/K20 lactam	Lys29	γE	653	0.107	0.075

DMIA1, E16/K20 lactam	Lys29	γE-γE	654	0.025	0.070
AIB2, AIB16, A18, D28	Lys10	γE-γE	655	0.003	0.004
AIB2, AIB16, A18, D28	Lys10	γE	656	0.006	0.004

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EXAMPLE 53

The peptides shown in Table 32 were made as essentially described herein:

TABLE 32

Peptide Name	SEQ ID NO:	Sequence
Chimera-2 Aib2C24Mal40KPEG	624	H(Aib)QGTFTSDYSKYLDEQAAKEFICWLMNT-amide
Chimera-2 Aib2E16K20lactamC24Mal40KPEG	625	H(Aib)QGTFTSDYSKYLDEQAAKEFICWLMNT-amide
Glucagon Aib2E16K20lactamC24amideMal40KPEG	626	H(Aib)QGTFTSDYSKYLDERRAKDFVCWLMNT-amide lactam
Glucagon Dmia1E16K20lactamC24Mal40KPEG	628	(Dmia)SQGTFTSDYSKYLDERRAKDFVCWLMNT-amide lactam
Glucagon Dmia1E16K20lactamC24Mal40KPEG	629	(Dmia)SQGTFTSDYSKYLDERRAKDFVCWLMNT-OH lactam
Glucagon Dmia1E16K20lactamC24thioether40KPEG	630	(Dmia)SQGTFTSDYSKYLDERRAKDFVCWLMNT-amide lactam
Chimera2 Aib2E3C24-Thioether40K PEG	631	H(Aib)EGTFTSDYSKYLDEQAAKEFICWLMNT-amide
Glucagon DMIA1, E3, E15, E16, K20, C24-Peg	632	(Dmia)SEGTFTSDYSKYLEERRAKDFVC(PEG40K)WLMNT-amide
Glucagon Aib2Aib16C24K10(rErE-C14)C24PEG40K TE)amide	633	H(Aib)QGTFTSDK(rErE-C14)SKYLDAibRRAQDFVC(PEG40K TE)WLMNT-amide
Glucagon Aib2Aib16K10(AA-C14)C24PEG40K TE amide	634	H(Aib)QGTFTSDK(AA-C14)SKYLDAibRRAQDFVC(PEG40K TE)WLMNT-amide
Glucagon Aib2Aib16K10(AA-C16) amide	635	H(Aib)QGTFTSD K(AA-C16)SKYLDAibRRAQDFVQWLMNT amide

Glucagon Aib2Aib16K10(rErE-C16) amide	636	H(Aib)QGTFTSD K(rErE-C16)SKYLDAibRRAQDFVQWLMNT amide

All peptides of Table 32 demonstrated potent *in vitro* activities at both the glucagon and GLP-1 receptors, except for the peptides of SEQ ID NOs: 624, 631, and 632

Peptides of Set A comprising the amino acid sequence of native glucagon (SEQ ID NO: 1) except for the changes outlined in Table 33 are made as essentially described herein.

TABLE 33

DPP-IV Protection	Alpha Helix Stabilization	Position 3	Backbone*	C-Terminal Amide?
DMIA at position 1	AIB at position 16	Gln (wild-type)	Wild-type	yes
AIB at position 2	AIB at position 16	Gln (wild-type)	Wild-type	yes
d-Ser at position 2	AIB at position 16	Gln (wild-type)	Wild-type	yes
DMIA at position 1	AIB at position 16	Glu	Wild-type	yes
AIB at position 2	AIB at position 16	Glu	Wild-type	yes
d-Ser at position 2	AIB at position 16	Glu	Wild-type	yes
DMIA at position 1	AIB at positions 16 and 20	Gln (wild-type)	Wild-type	yes
AIB at position 2	AIB at positions 16 and 20	Gln (wild-type)	Wild-type	yes
d-Ser at position 2	AIB at positions 16 and 20	Gln (wild-type)	Wild-type	yes

DPP-IV Protection	Alpha Helix Stabilization	Position 3	Backbone*	C-Terminal Amide?
DMIA at position 1	AIB at positions 16 and 20	Glu	Wild-type	yes
AIB at position 2	AIB at positions 16 and 20	Glu	Wild-type	yes
d-Ser at position 2	AIB at positions 16 and 20	Glu	Wild-type	yes
DMIA at position 1	Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
AIB at position 2	Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
d-Ser at position 2	Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
DMIA at position 1	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
AIB at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
d-Ser at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Wild-type	yes
DMIA at position 1	Glu at position 16 and Lys at position 20	Glu	Wild-type	yes
AIB at position 2	Glu at position 16 and Lys at position 20	Glu	Wild-type	yes
d-Ser at position 2	Glu at position 16 and Lys at position 20	Glu	Wild-type	yes

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DPP-IV Protection	Alpha Helix Stabilization	Position 3	Backbone*	C-Terminal Amide?
DMIA at position 1	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Wild-type	yes
AIB at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Wild-type	yes
d-Ser at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Wild-type	yes
DMIA at position 1	Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
AIB at position 2	Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
d-Ser at position 2	Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
DMIA at position 1	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
AIB at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
d-Ser at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Gln (wild-type)	Chimera 2	yes
DMIA at position 1	Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes
AIB at position 2	Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes

DPP-IV Protection	Alpha Helix Stabilization	Position 3	Backbone*	C-Terminal Amide?
d-Ser at position 2	Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes
DMIA at position 1	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes
AIB at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes
d-Ser at position 2	Lactam bridge between side chains of Glu at position 16 and Lys at position 20	Glu	Chimera 2	yes

* indicates amino acids at positions 17, 28, 21, and 23 as wild-type or as Chimera 2 (Gln at position 17, Ala at position 18, Glu at position 21, and Ile at position 23).

Peptides having the same structure as the peptides of Set A, except that the Met at position 27 is replaced with a Norleucine, are made as essentially described herein. These modified peptides are the peptides of Set B.

Peptides having the same structure as the peptides of Sets A and B, except that the Gln at position 24 is replaced with a Cys covalently attached to a 40 kDa PEG, are made as essentially described herein. These pegylated peptides form the peptides of Set C.

Peptides having the same structure as the peptides of Set A, B, or C, except that the Tyr at position 10 is replaced with a Lys covalently attached to a C8, C12, C14, C16, or C18 fatty acyl group, are made as essentially described herein. The peptides acylated with a C8 fatty acyl group form the peptides of Set D. The peptides acylated with a C12 fatty acyl group form the peptides of Set E. The peptides acylated with a C14 fatty acyl group form the peptides of Set F. The peptides acylated with a C16 fatty acyl group form the peptides of Set G. The peptides acylated with a C18 fatty acyl group form the peptides of Set H.

Peptides having the same structure as the peptides of Sets D through H, except that the fatty acyl group is attached to the Lys at position 10 via a spacer, are made as essentially described herein. The peptides comprising a γ-Glu- γ-Glu spacer form the peptides of Set I. The peptides comprising a γ-Glu spacer form the peptides of Set J. The peptides comprising an Ala-Ala spacer form the peptides of Set K. The peptides comprising a β-Ala- β-Ala spacer form the peptides of Set L.

EXAMPLE 54

Glucagon peptides comprising SEQ ID NO: 1 with up to 15 amino acid modifications, a C-terminal extension of 1-21 amino acids, wherein (a) the extension is acylated or alkylated, and/or (b) the extension comprises 1-6 positive-charged amino acids, were made as essentially described herein. The peptides were tested for *in vitro* activity at the glucagon, GLP-1, and GIP receptors as essentially described in Example 14. The structures of the peptides and their *in vitro* activities are summarized in Table 34.

TABLE 34

Peptide	SEQ ID NO:	Glucagon Receptor			GLP-1 Receptor			GIP Receptor		
		EC50 (nM)	Standard deviation	% relative activity*	EC50 (nM)	Standard deviation	% relative activity*	EC50 (nM)	Standard deviation	% relative activity*
mt-345	657	0.0183	0.0353	192.90	0.0036	0.0055	152.78	0.0541	0.0027	4.99
mt-346	658	0.9535	0.0353	3.70	0.0015	0.0055	366.67	0.7961	0.0027	0.34
mt-347	659	0.0317	0.0353	111.36	0.0082	0.0055	67.07	0.3830	0.0027	0.70
mt-348	660	0.4327	0.0353	8.16	0.0037	0.0055	148.65	1.7605	0.0027	0.15
mt-349	661	0.5494	0.0210	3.82	0.0022	0.0063	286.36	1.9906	0.0036	0.18
mt-350	662	nd	nd	Nd	0.0107	0.0063	58.88	77.7229	0.0036	0.00
mt-351	663	0.1963	0.0210	10.70	0.0042	0.0063	150.00	4.1217	0.0036	0.09
mt-352	664	nd	nd	nd	0.0273	0.0063	23.08	51.1125	0.0036	0.01
mt-361	666	1.7701	0.0639	3.61	0.0109	0.0247	226.35	1.0488	0.0031	0.29
mt-362	667	0.0768	0.0639	83.17	0.102	0.0247	240.96	0.0782	0.0031	3.95
mt-363	668	0.8230	0.0639	7.76	0.0037	0.0247	671.66	0.4250	0.0031	0.73
mt-364	669	0.0183	0.0639	349.97	0.0070	0.0247	354.68	0.0197	0.0031	15.66

* Activity relative to the native hormone at the indicated receptor

As shown in Table 34, all unpegylated peptides demonstrated agonist activity at the GIP receptor that was greater than 0.1%, Peptides mt-345, mt-362, and mt-364 demonstrated exceptionally potent activity at the GIP receptor. Peptides mt-350, mt-351, and mt-352 demonstrated activities less than 0.1% likely due to the fact that these peptides were pegylated.

EXAMPLE 55

The *in vivo* effects of mt-345 (having the structure of SEQ ID NO: 657) were compared to the effects of other acylated peptides (Liraglutide, mt-261 (SEQ ID NO: 670), and mt-347 (SEQ ID NO: 659)) and a non-acylated peptide (mt-348; SEQ ID NO: 660). Peptides mt-345, mt-261, and mt-347 comprised the amino acid sequence of glucagon (SEQ ID NO: 1) with amino acid modifications and further comprised an extension of GPSSGAPPPS (SEQ ID NO: 26) C-terminal to the amino acid at position 29 followed by an acylated Lys as position 40. Peptide mt-345 comprised AIB at position 2, Glu at position 16, Gln at position 17, Ala at position 18, Lys at position 20, Glu at position 21, Ile at position 23, Cys at position 24, Gly at position 29, SEQ ID NO: 26 c-terminal to the Gly at position 29, and an acylated Lys as position 40. The acyl group of mt-345 was a C14 fatty acyl group. Peptide mt-261 had a similar structure to that of mt-345, except that mt-261 comprised a Tyr at position 1, Glu at position 3, Ile at position 12, Lys at position 16, AIB at position 20, Val at position 23, Asn at position 24, Leu at position 27, Ala at position 28, and a C16 fatty acyl group at position 40. The structures of mt-347 and mt-348 were identical to the structure of mt-345, except that mt-347 and mt-348 comprised an AIB at position 16. The structure of mt-347 was the same as that of mt-348 except that mt-347 comprised a C14 fatty acyl group at position 40, whereas mt-348 lacked an acyl group.

The *in vitro* activities at the glucagon, GLP-1, and GIP receptors of the peptides were tested as essentially described in Example 14. The relative activities are shown in Table 35.

TABLE 35

Peptide	% Relative activity at the Receptor for		
	Glucagon	GLP-1	GIP
Liraglutide	0.04	138	n/a
mt-261	13.4	372	700
mt-345	193	153	5
mt-347	111	67	0.7
mt-348	8.2	149	0.2

% relative activity is relative to the native hormone of the indicated receptor

The peptides were administered daily for two weeks to 11 groups of 8 C57Bl/6 mice by subcutaneous injection at a dose of 10 or 30 nmol/kg. The initial average body weight of the mice was 51 g. The mice were 6 months old and had been on a diabetogenic diet for 4 months. One group of mice received a vehicle control and another received a glucagon amide control peptide which comprised the amino acid sequence of SEQ ID NO: 1 with a C-terminal amidation in place of the C-terminal alpha carboxylate.

The body weight of each group of mice receiving a peptide steadily declined over the course of the study, as compared to the vehicle control. However, as shown in Figure 36, the total change in body weight of mice receiving 10 nmol/kg of mt-261 or mt-345 was the most significant (around 20%). Mice that were administered the glucagon amide peptide also demonstrated a similar total decrease in body weight as those of mt-261 and mt-345, although the dose of the glucagon amide was three times as much as the dose of mt-261 and mt-345. The effects on body weight were clearly more significant than the effects of liraglutide.

Food intake by the groups of mice were also evaluated during the study. Mice that received the mt-261 or mt-345 peptide demonstrated the lowest food intake.

Blood glucose levels of the mice were also studied. Mice that were administered liraglutide, mt-261, or mt-345 demonstrated a total decrease in blood glucose levels on Day 7 (7 days after the first administration of peptide). The extent of the decrease was less however when measured on Day 14.

EXAMPLE 56

Glucagon peptides comprising an acylated C-terminal extension were made as essentially described herein. Peptides mt-278, mt-261, mt-297, and mt-358 comprised the amino acid sequence of SEQ ID NO: 1 with the following amino acid modifications: Tyr at position 1, AIB at positions 2 and 20, Lys at position 16, Ile at position 12, Gln at position 17, Ala at position 18, Glu at position 21, Gly at position 29, the amino acid sequence of SEQ ID NO: 26 C-terminal to the amino acid at position 29, and an acylated Lys at position 40. Peptide mt-364 varied in structure from the other peptides by comprising a His at position 1, Glu at position 16 and a Lys at position 20. The full descriptions of the structures of these peptides are described in the sequence listing attached hereto: mt-364 (SEQ ID NO: 669), mt-261 (SEQ ID NO: 270), mt-278 (SEQ ID NO: 271), mt-297 (SEQ ID NO: 272), and mt-358 (SEQ ID NO: 673).

The peptides were tested for *in vitro* activities at the glucagon, GLP-1, and GIP receptors as essentially described in Example 14. The activities of the peptides in comparison to the native hormones at the indicated receptor are shown in Table 36.

TABLE 36

Peptide	% Relative activity at the Receptor for		
	Glucagon	GLP-1	GIP
mt-261	31.62	299.24	297.6
mt-278	761.36	319.58	266.40
mt-297	276.50	144.16	68.63
mt-358	710.29	11.89	313.61
mt-364	349.97	354.68	15.66

% relative activity is relative to the native hormone of the indicated receptor

The peptides were then tested *in vivo* for their effects on body weight, food intake, blood glucose levels, and fat mass in eight-month old C57Bl/6 mice that had been on a diabetogenic diet for 6 months. Eleven groups of eight mice were subcutaneously injected with 10 nmol/kg of peptide daily for one week. The mice had an initial body weight of 53 g.

The body weight of mice that were injected with mt-278, mt-261, or mt-364 steadily decreased over the course of the study, as compared to mice that were injected with vehicle only. As shown in Figure 37, mice injected with mt-364 demonstrated the greatest decrease in body weight by the end of the week-long study, achieving a weight loss of greater than 15%. Mice injected with mt-278 or mt-261 also demonstrated substantial (greater than 10%) decreases in body weight.

Total food intake by the mice was monitored and by the end of the study, total food intake by mice injected with mt-364 or mt-261 demonstrated a total food intake which was less than 50% of the total food intake by mice injected with vehicle only.

The fat mass of mice was measured throughout the study. Mice injected with mt-364, mt-278, or mt-261 exhibited a fat mass which was less than the fat mass of mice injected with vehicle only.

The effects on blood glucose levels were additionally evaluated. On the last day of the study (Day 7), mice injected with mt-364 or mt-261 exhibited a decrease in blood glucose levels that was greater than 60% as compared to the blood glucose levels measured on Day 0. Mice injected with mt-278 additionally demonstrated a substantial decrease in blood glucose (about 40% decrease).

EXAMPLE 57

Analogs of glucagon having a C-terminal amide in place of the C-terminal alpha carboxylate were made as essentially described herein:

Peptide 83 comprised the structure of SEQ ID NO: 1 with AIB at position 2, AIB at position 16, and Lys at position 10, which Lys comprised a C16 fatty acyl group via a γ-Glu-γ-Glu dipeptide spacer.

Peptide 900 comprised the structure of SEQ ID NO: 1 with AIB at position 2, AIB at position 16, a C-terminal extension comprising SEQ ID NO: 26, and Lys at position 40, which Lys comprised a C16 fatty acyl group via a γ-Glu-γ-Glu dipeptide spacer.

Peptide 901 comprised the structure of SEQ ID NO: 1 with AIB at position 2, AIB at position 16, a C-terminal extension comprising SEQ ID NO: 26, and Lys at position 10, which Lys comprised a C16 fatty acyl group via a γ-Glu-γ-Glu dipeptide spacer.

Peptide mt-364 comprised the structure of SEQ ID NO: 669.

The analogs were tested for in vitro activity at each of the glucagon, GLP-1, and GIP receptors as essentially described herein. The results are shown in Table 37.

TABLE 37

Peptide	% Relative activity at the Receptor for		
	Glucagon	GLP-1	GIP
83	211	84	nd
900	560	383	2.89
901	560	460	8.65
mt-364	349.97	354.68	15.66

nd = not determined

Nine groups of 8 DIO mice (strain: C57Bl6 WT) were subcutaneously injected daily for 7 days with 10 nmol/kg of one of the peptides of Table 37. The average initial body weight of the mice was 57.6 g. The mice were approximately 10 months old and had been on a high fat diet for about 8 months.

The total change in body weight was measured on Day 7. As shown in Figure 38, all mice injected with a peptide of Table 37 demonstrated a decrease in body weight as compared to vehicle control. Among Peptides 83, 900 and 901, all of which have a similar peptide structure, Peptides 900 and 901 demonstrated the greatest decreases in body weight. Interestingly, each of Peptides 900 and 901 comprised a C-terminal extension and was acylated, in contrast to Peptide 83, which was acylated but did not comprise the C-terminal extension. Peptide 901 which was acylated at position 10 demonstrated a greater decrease (~ 15% decrease) than Peptide 900 (~ 10% decrease) which was acylated at position 40. The *in vivo* effects on body weight of these peptides, however, were still not as significant as those of MT-364. Mice that were injected with MT-364 demonstrated about a 20% decrease.

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.

WHAT IS CLAIMED IS:

1. An analog of a glucagon peptide, the analog comprising:

(i) SEQ ID NO: 1 with at least one and up to 10 amino acid modifications, wherein at least one of the amino acid modifications confers a stabilized alpha helix structure in the C-terminal portion of the analog;

(ii) an extension of 1 to 21 amino acids C-terminal to the amino acid at position 29, and

(iii) at least one of the following:

(a) at least one of the amino acids of the extension located at any of positions 37-43 (according to the numbering of SEQ ID NO: 1) comprises an acyl or alkyl group which is non-native to a naturally-occurring amino acid,

(b) 1-6 amino acids of the extension are positive-charged amino acids,

(c) the analog comprises an amino acid comprising an acyl or alkyl group, which is non-native to a naturally-occurring amino acid, at position 10 of the analog, or

(d) a combination of (a), (b), and (c);

wherein, when the analog lacks a hydrophilic moiety, the analog exhibits at least 0.1% activity of native GIP at the GIP receptor.

2. The analog of claim 1, wherein (i) the analog comprises an intramolecular bridge between the side chains of an amino acid at position i and an amino acid at position i+4 or between the side chains of amino acids at positions j and j+3, wherein i is 12, 13, 16, 17, 20 or 24 and j is 17, (ii) one, two, three or more of positions 16, 20, 21 or 24 of the analog are substituted with an α, α-disubstituted amino acid, or (iii) both (i) and (ii).

3. The analog of claim 1 or 2, comprising Glu at position 16 and Lys at position 20, wherein optionally a lactam bridge links the Glu and the Lys.

4. The analog of claim 3, further comprising one or more of: Gln at position 17, Ala at position 18, Glu at position 21, Ile at position 23, and Ala or Cys at position 24, or one or more conservative amino acid substitutions thereof.

5. The analog of any of claims 1 to 4, comprising a C-terminal amide.

6. The analog of any of claims 1 to 5 comprising an amino acid substitution at position 1, position 2, or positions 1 and 2, wherein the amino acid substitution(s) achieve DPP-IV protease resistance.

7. The analog of claim 6, wherein the His at position 1 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.

8. The analog of claim 6 or 7, wherein the Ser at position 2 is substituted with an amino acid selected from the group consisting of: D-serine, alanine, D-alanine, valine, glycine, N-methyl serine, N-methyl alanine, and amino isobutyric acid (AIB).

9. The analog of any of claims 1 to 8, wherein the amino acid at position 1 comprises an imidazole ring.

10. The analog of claim 9, wherein the amino acid at position 1 is His.

11. The analog of any of claims 1 to 6, wherein the amino acid at position 1 of the analog is not a large, aromatic amino acid.

12. The analog of claim 11, wherein the amino acid at position 1 of the analog is not Tyr.

13. The analog of any of claims 1 to 12, comprising amino acid modifications at one, two or all of positions 27, 28 and 29.

14. The analog of claim 13, wherein (a) the Met at position 27 is substituted with a large aliphatic amino acid, optionally Leu, (b) the Asn at position 28 is substituted with a small aliphatic amino acid, optionally Ala, (c) the Thr at position 29 is substituted with a small aliphatic amino acid, optionally Gly, or (d) a combination of two or all of (a), (b), and (c).

15. The analog of claim 14, comprising Leu at position 27, Ala at position 28, and Gly or Thr at position 29.

16. The analog of any of claims 1 to 15, comprising one or more of the following modifications:

a. Ser at position 2 substituted with Ala;

b. Gln at position 3 substituted with Glu or a glutamine analog;

c. Thr at position 7 substituted with a Ile;

d. Tyr at position 10 substituted with Trp or an amino acid comprising an acyl or alkyl group which is non-native to a naturally-occurring amino acid;

e. Lys at position 12 substituted with Ile;

f. Asp at position 15 substituted with Glu;

g. Ser at position 16 substituted with Glu;

h. Gln at position 20 substituted with Ser, Thr, Ala, AIB;

i. Gln at position 24 substituted with Ser, Thr, Ala, AIB;

j. Met at position 27 substituted with Leu or Nle;

k. Asn at position 29 substituted with a charged amino acid, optionally, Asp or Glu; and

l. Thr at position 29 substituted with Gly or a charged amino acid, optionally, Asp or Glu.

17. The analog of any of claims 1 to 16, comprising (i) the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 26) or XGPSSGAPPPS (SEQ ID NO: 674), wherein X is any amino acid, (ii) an amino acid sequence which has at least 80% sequence identity to SEQ ID NO: 26 or SEQ ID NO: 674, or (iii) the amino acid sequence of (i) or (ii) with one or more conservative amino acid substitutions, wherein the amino acid sequence is C-terminal to the amino acid at position 29 of the analog.

18. The analog of claim 17, comprising the amino acid sequence of GPSSGAPPPS (SEQ ID NO: 26) or XGPSSGAPPPS (SEQ ID NO: 674), wherein X is any amino acid, C-terminal to the amino acid at position 29 of the analog.

19. The analog of any of claims 1 to 18, wherein the amino acid comprising an acyl or alkyl group is an amino acid of Formula I, II, or III.

20. The analog of claim 19, wherein the amino acid comprising an acyl or alkyl group is Lys.

21. The analog of any of claims 1 to 20, wherein the amino acid comprising an acyl or alkyl group is located at any of positions 37, 38, 39, 40, 41, 42 or 43 of the analog.

22. The analog of claim 21, wherein the amino acid comprising an acyl or alkyl group is located at position 40 of the analog.

23. The analog of any of claims 1 to 22, wherein the acyl group is a C4 to C30 fatty acyl group.

24. The analog of any of claims 1 to 23, wherein the acyl or alkyl group is covalently attached to the side chain of the amino acid via a spacer.

25. The analog of claim 24, wherein the spacer is 3 to 10 atoms in length.

26. The analog of claim 25, wherein the spacer is an amino acid or dipeptide.

27. The analog of claim 26, wherein the spacer is 6-amino hexanoic acid.

28. The analog of claim 26, wherein the spacer is a dipeptide selected from the group consisting of: Ala-Ala, β-Ala- β-Ala, Leu-Leu, and Pro-Pro.

29. The analog of any of claims 24 to 28, wherein the total length of the spacer and the acyl group is about 14 to about 28 atoms in length.

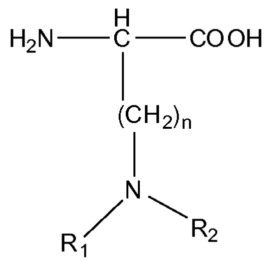
30. The analog of any of claims 1 to 29, wherein the acyl group is a C12 to C18 fatty acyl group.

31. The analog of claim 30, wherein the acyl group is a C14 or C16 fatty acyl group.

32. The analog of any of claims 1 to 31, wherein the 1-6 positive-charged amino acids are located at any of located at any of positions 37, 38, 39, 40, 41, 42, and 43 (according to the numbering of SEQ ID NO: 1) of the analog.

33. The analog of claim 32, comprising a positive-charged amino acid at position 40.

34. The analog of any of claims 1 to 33, wherein the positive-charged amino acid comprises the structure of Formula IV:



[Formula IV],

wherein n is 1 to 16, or 1 to 10, or 1 to 7, or 1 to 6, or 2 to 6, or 2 or 3 or 4 or 5, each of R_1 and R_2 is independently selected from the group consisting of H, C_1-C_{18} alkyl, (C_1-C_{18} alkyl)OH, (C_1-C_{18} alkyl)NH_2, (C_1-C_{18} alkyl)SH, (C_0-C_4 alkyl)(C_3-C_6)cycloalkyl, (C_0-C_4 alkyl)(C_2-C_5 heterocyclic), (C_0-C_4 alkyl)(C_6-C_{10} aryl)R_7, and (C_1-C_4 alkyl)(C_3-C_9 heteroaryl), wherein R_7 is H or OH, and the side chain of the amino acid of Formula IV comprises a free amino group.

35. The analog of claim 34, wherein the amino acid of Formula IV is Orn, Dab, Lys, or homoLys.

36. The analog of any of the preceding claims, wherein the analog has at least about 4% of the activity of GLP-1 (SEQ ID NO: 2) at the GLP-1 receptor, when the analog does not comprise a hydrophilic moiety.

37. The analog of any of the preceding claims, wherein the analog has at least about 20% of the activity of glucagon at the glucagon receptor, when the analog does not comprise a hydrophilic moiety.

38. The analog of any of the preceding claims excluding claim 37, wherein the analog comprises an amino acid modification at position 3 and has less than 1% of the activity of glucagon at the glucagon receptor, when the analog does not comprise a hydrophilic moiety.

39. The analog of any of the preceding claims excluding claim 36, wherein the analog comprises an amino acid modification at position 7 and has less than about 10% of the activity of GLP-1 at the GLP-1 receptor when the analog does not comprise a hydrophilic moiety.

40. The analog of any of the preceding claims, wherein the glucagon peptide is covalently linked to a hydrophilic moiety at any of amino acid positions 19, 20, 23, 24, 27, 32, 43 or the C-terminus.

41. The analog of claim 40, wherein the glucagon peptide is covalently linked to a hydrophilic moiety at amino acid position 24 or 40.

42. The analog of claim 40 or 41, wherein the hydrophilic moiety is covalently linked to Lys, Cys, Orn, homocysteine, or acetyl-phenylalanine.

43. The analog of any of claims 40 to 42, wherein the hydrophilic moiety is a polyethylene glycol (PEG).

44. The analog of claim 43, wherein the PEG has a molecular weight of about 1,000 Daltons to about 40,000 Daltons.

45. The analog of claim 43, wherein the PEG has a molecular weight of about 20,000 Daltons to about 40,000 Daltons.

46. The analog of any of claims 40 to 45 wherein the EC50 of the analog for GIP receptor activation is about 10 nM or less.

47. The analog of any of claims 40 to 46, wherein the analog has at least about 0.01% of the activity of wild-type GIP (SEQ ID NO: 4) at the GIP receptor.

48. The analog of any of claims 40 to 47, wherein the analog has at least about 0.4% of the activity of GLP-1 (SEQ ID NO: 2) at the GLP-1 receptor.

49. The analog of any of claims 40 to 48, wherein the analog has at least 2% of the activity of glucagon at the glucagon receptor.

50. The analog of any of claims 1 to 47 excluding claim 39, wherein the ratio of the EC50 of the analog at the GLP-1 receptor to the EC50 of the analog at the GIP receptor is less than about 0.01.

51. The analog of any of the preceding claims, wherein the GIP potency of the analog is within about 500-fold of GLP-1 potency of the analog.

52. The analog of any of the preceding claims, wherein the GIP potency of the analog is within about 500-fold of the glucagon potency of the analog.

53. A dimer comprising two glucagon peptides, wherein at least one of the glucagon peptides is an analog of any of claims 1 to 52, bound to one another through a linker.

54. A pharmaceutical composition comprising an analog of any of claims 1 to 52, a dimer of claim 53, and a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

55. A method of treating hypoglycemia using a pre-formulated aqueous composition, said method comprising the steps of administering an effective amount of a pharmaceutical composition of claim 54.

56. A method of treating diabetes, said method comprising administering an effective amount of a pharmaceutical composition of claim 54.

57. A method of causing temporary paralysis of the intestinal tract, said method comprising administering an effective amount of a pharmaceutical composition of claim 54.

58. A method of reducing weight gain or inducing weight loss, said method comprising administering an effective amount of a pharmaceutical composition of claim 54.