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(71) Applicant: UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; 1150 South Olive Street, Suite 2300, Los Angeles, CA 90015 (US).

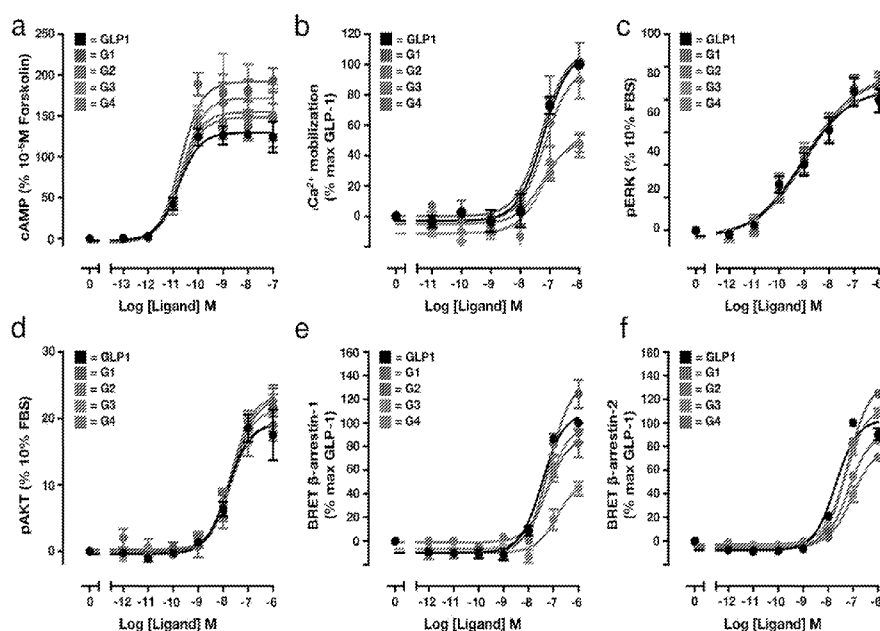
(72) Inventors: PRATT, Matthew; 1301 Clark Ln, Redondo Beach, CA 90278 (US). LEVINE, Paul; 967 N. 5091 Glen Iris Avenue, Apt. 201, Los Angeles, CA 90041 (US).

(74) Agent: GAMSON, Edward, P. et al.; Husch Blackwell, LLP, 120 South Riverside Plaza, Suite 2200, Chicago, IL 60606 (US).

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(54) Title: NON-NATIVE O-GLCNAc MODIFICATION OF PEPTIDE HORMONES YIELDS POTENT GPCR AGONISTS WITH IMPROVED SERUM STABILITY

Fig. 1



(57) Abstract: A synthetic peptide hormone comprising a sequence of about 15 to about 50 amino acid residues that are at least about 90% identical to the same length and aligned sequence of a native peptide hormone is disclosed. Each of the synthetic and native peptide sequences exhibits an amphipathic alpha-helical secondary structure. The synthetic peptide hormone includes a mono-O-glycosylated serine or threonine residue in its sequence that is not present in the native sequence; i.e., as a non-identical residue of the synthetic peptide hormone sequence. A contemplated synthetic peptide hormone has a half-life in human serum at 37°C of about 25 to about 800 percent longer than the native peptide hormone. A composition containing an agonistic effective amount of an above-described synthetic peptide hormone dissolved or dispersed in a pharmaceutically acceptable carrier and a method of treating a mammal having a GPCR-mediated condition are also disclosed.

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Non-native O-GlcNAc Modification of
Peptide Hormones Yields Potent GPCR
Agonists with Improved Serum Stability

Description

CROSS-REFERENCE TO RELATED APPLICATION

This international application claims priority to US application Serial No. 62/872,027, filed on July 9, 2019, whose disclosures are incorporated herein by reference.

**STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH OR DEVELOPMENT**

This invention was made with government support under R01 GM114537 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND ART

G protein-coupled receptors (GPCRs) are cell surface receptors that respond to a wide variety of stimuli, from light, odorants, hormones, and neurotransmitters to proteins and extracellular calcium. GPCRs represent the largest family of signaling proteins targeted by many clinically used drugs. Recent studies shed light on the conformational changes that accompany GPCR activation and continue through the cellular membrane to alter G protein and arrestin conformations, ultimately resulting signal transduction³⁷.

Glucagon-like peptide-1 (GLP-1) and the parathyroid hormone (PTH), which respectively help control glucose and calcium levels in the blood are

illustrative peptide hormone agonists that bind to GPCRs. Peptide hormone agonists of GPCRs and other receptors are powerful signaling molecules with high potential as biological tools and therapeutics, but they are typically plagued by instability and short half-lives *in vivo*.

According to the *Oxford Dictionary of Biochemistry and Molecular Biology*, A.D. Smith et al, eds., Oxford University Press, New York, page 308 (1997) a hormone is "any substance formed in very small amounts in one specialized organ or group of cells and carried (sometimes in the bloodstream) to another organ or group of cells, in the same organism, upon which it has a specific regulatory action; . . ." That same volume defines a peptide hormone as "any peptide with hormonal activity in animals, whether endocrine, neuroendocrine, or paracrine. Such substances form a very diverse group physiologically, and the boundary between peptide hormones and protein hormones is somewhat indistinct. (*Id.* at page 490.)

Peptide hormones can have amino acid residue sequences of about ten residues such as argipressin or oxytocin, each of which has a sequence of nine amino acid residues, or as many as nearly 250, such as erythropoietin with 165 residues and human chorionic gonadotropin that has a sequence of 237 amino acid residues. Those of interest here have a sequence length of about 15 residues to about 50 residues.

Glucagon-like peptide-1 (GLP-1) is an illustrative peptide hormone that contains a sequence of 30 amino acid residues deriving from the tissue-specific posttranslational processing of the

proglucagon gene product. GLP-1 is produced and secreted upon food consumption by intestinal enteroendocrine L-cells and certain neurons within the nucleus of the solitary tract in the brainstem.

The initial product GLP-1 (1-37) is susceptible to C-terminal proteolytic cleavage and amidation that gives rise to the two truncated and equipotent biologically active forms, GLP-1 (positions 7-36) amide and GLP-1 (positions 7-37). Aqueous, active GLP-1 contains two α -helices from amino acid residue positions 13-20 and 24-35 that are separated by a linker region.

Alongside glucose-dependent insulinotropic peptide (GIP), GLP-1 is an incretin; thus, it has the ability to decrease blood sugar levels in a glucose-dependent manner by enhancing the secretion of insulin. Beside the insulinotropic effects, GLP-1 has been associated with numerous regulatory and protective effects. Unlike GIP, the action of GLP-1 is preserved in patients with type 2 diabetes and substantial pharmaceutical research has therefore been directed towards the development of GLP-1-based treatment for type 2 diabetes.

Peptide therapeutics have recently garnered significant attention from the pharmaceutical industry due to their specificity, potency, and diminished off-target effects¹. This is particularly true in the case of type-2 diabetes mellitus, where agonists of both the insulin receptor and the glucagon-like peptide-1 receptor (GLP-1R) are well-established clinical targets^{2,3}. GLP-1R is a GPCR³³.

In fact, extensive efforts have focused on the development of small molecule modulators of GLP-1R with little to no success. However, peptide

agonists have proven to be effective as therapeutic agents.

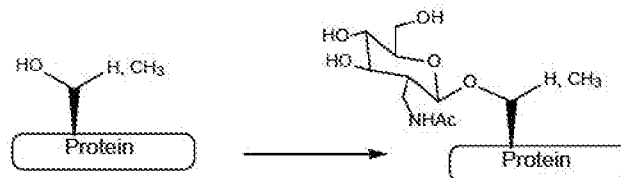
Parathyroid hormone (PTH) is a peptide hormone secreted by the parathyroid glands that regulates the serum calcium through its effects on bone, kidney, and intestine. In a similar manner, the PTH receptor (PTHr1)³³ has been clinically targeted by PTH(1-34), marketed by Eli Lilly as Forteo®, and a peptide analog of the parathyroid hormone-related protein, approved as Tymlos® from Radius Health. PTHr1 is also a GPCR³⁹.

One critical drawback to this strategy is the typically poor pharmacokinetic profiles of peptides *in vivo* due to proteolytic degradation by endogenous enzymes. For example, GLP-1(7-37) and PTH(1-34) have respective half-lives of only about 2 and 10 minutes in the bloodstream *in vivo*^{4,32}. This rapid degradation is carried out by endogenous proteases, mainly dipeptidyl peptidase-4 (DPP-4) cleavage at alanine 8 of GLP-1, which significantly compromises its potency *in vivo* and limits its ability to be used as an effective therapeutic agent⁵.

Given the increasing interest in preparing stable peptide and protein therapeutics, several strategies aimed toward engineering stable analogues of GLP-1 have emerged. Thus, unnatural amino acid modifications at the N-terminus of GLP-1, such as N-pyroglutamyl modification and thioamide substitution, can block its major degradation pathway through dipeptidyl peptidase (DPP-4) cleavage at alanine 8. However, these analogs can have reduced potency, as the N-terminal segment of GLP-1 is largely responsible for activation and signaling^{6,7}.

Additionally, PEG and lipid modifications introduced into peptides and proteins can often be immunogenic and must be tested on an individual basis. Nature uses protein oligoglycosylation to increase the serum stability of secreted proteins. However, these extracellular modifications are complex and heterogeneous in structure, making them an impractical solution. Therefore, new approaches to therapeutic peptide stabilization that circumvent some of these issues are still of great interest.

As is disclosed hereinafter, an alternative approach is taken through the unconventional application of the post-translational modification O-GlcNAc. O-GlcNAcylation is the addition of the monosaccharide N-acetylglucosamine through a β -linkage to proteins in the nucleus, cytosol, and mitochondria of metazoans as is shown below^{8,9}.



O-GlcNAc can have a variety of biological consequences on its protein substrates, including the modulation of protein-protein interactions and the inhibition of protein aggregation^{10,11}.

BRIEF SUMMARY OF THE INVENTION

The present invention contemplates a synthetic peptide hormone comprising a sequence of about 15 to about 50 amino acid residues that are at least about 90% identical to the same length and aligned sequence of a naturally-occurring peptide

hormone. Each of the synthetic and naturally-occurring (native) peptide sequences exhibiting an amphipathic alpha-helical secondary structure as measured by circular dichroism spectroscopy. Preferably, each of those peptide sequences is free of covalent intrachain linkage. The synthetic peptide hormone includes a mono-O-glycosylated serine or threonine residue in its sequence as a non-identical residue of the synthetic peptide hormone sequence; i.e., the naturally-occurring sequence does not include that mono-O-glycosylated residue. The synthetic peptide hormone containing the mono-O-glycosylated serine or threonine residue has a half-life in human serum at 37°C of about 25 to about 800 percent longer than the naturally-occurring peptide hormone.

A composition containing an agonistic effective amount of an above-described synthetic peptide hormone dissolved or dispersed in a pharmaceutically acceptable carrier is also contemplated.

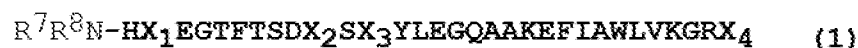
A method of treating a mammalian G protein-coupled receptor-mediated [GPCR-mediated] condition is also contemplated. Such conditions typically arise due to a lack or ineffectiveness of an appropriate endogenous peptide hormone.

That method comprises administering an agonistic effective amount of an above-described synthetic peptide hormone dissolved or dispersed in a pharmaceutically acceptable carrier to that mammal having that GPCR-mediated condition. This contemplated treatment can be repeated as needed to maintain the treated mammal.

One more specific aspect of the invention comprises a GLP-1 derivative peptide. The sequences of the two native GLP-1 peptide hormones are shown immediately below,

GLP-1(7-37) HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
(SEQ ID NO: 6); and
GPL-1(7-36)amide HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH₂
(SEQ ID NO: 7).

A contemplated synthetic GLP-1 peptide hormone sequence is shown below as Formula 1 and SEQ ID NO: 1, from left to right and in the direction from amino-terminus to carboxy-terminus,



where

$X_1 = -NH-(CH_2)_m-CR^1R^2-C(O)-$ or A (Ala);

$X_2 = -NH-(CH_2)_n-CR^3R^4-C(O)-$ or V (Val);

$X_3 = -NH-CH[(CHQ)-O-(Sac)]-C(O)-$;

$X_4 = G$ (Gly), $G-NR^5R^6$ or $-NR^5R^6$;

$m = 0$ or 1 ;

$n = 0$ or 1 ;

$Q = H$ or methyl;

R^1 and R^2 are the same or different substituents that are hydrogen (H) or C₁-C₃ hydrocarbyl, with the proviso that only one of R^1 and R^2 is hydrogen, or R^1 and R^2 together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R^3 and R^4 are the same or different substituents that are hydrogen (H) or C₁-C₃ hydrocarbyl, or R^3 and

R⁴ together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R⁵, R⁶, R⁷ and R⁸ are the same or different and are H, C₁-C₁₈ hydrocarbyl, C₁-C₁₈ acyl, C₁-C₁₈ hydrocarbylsulfonyl, or either or both of R⁵ and R⁶ and R⁷ and R⁸ together with their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R⁵ and R⁶ and R⁷ and R⁸ includes a hydrocarbyl, acyl or sulfonyl group having more than six carbon atoms; and

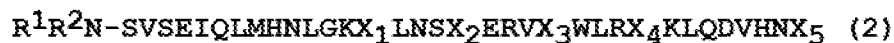
Sac is a glycosidically-linked C₅-C₆ saccharide, deoxysaccharide or 2-(N-acetylamino) C₅-C₆ saccharide or deoxysaccharide. Preferred saccharides are GlcNAc, GalNAc, ribose, deoxyribose, mannose, glucose and galactose.

A contemplated peptide of Formula 1 has been found to be particularly specific, potent, and exhibit diminished off-target effects as an agonist of the glucagon-like peptide-1 receptor (GLP-1R). GLP-1 is an agonist for each of GLP-1R and the insulin receptor (IR), both of which are well-established clinical targets^{2,3}. In addition, such a peptide also exhibits enhanced stability to hydrolysis by dipeptidyl peptidase-4 (DPP-4) and other endogenous proteases compared to GLP-1 itself.

A composition containing an IR- and/or GLP-1R- agonistic effective amount of a peptide of Formula 1 dissolved or dispersed in a pharmaceutically acceptable carrier is also contemplated.

A method of treating a mammalian G protein-coupled receptor-mediated [GPCR-mediated] such as an IR- or GLP-1R-mediated condition like type 2 diabetes is also contemplated. That method comprises administering an IR- or GLP-1R- agonistic effective amount of a peptide of Formula 1 dissolved or dispersed in a pharmaceutically acceptable carrier to that mammal having a GLP-1R-mediated condition such as type 2 diabetes. This contemplated treatment can be repeated as needed to maintain the treated mammal.

Another specific contemplated aspect of this invention is a parathyroid hormone (PTH) derivative peptide whose sequence is shown below, as Formula 2 and Seq. ID. No.: 7, from left to right and in the direction from amino-terminus to carboxy-terminus,



wherein

$-X_1-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or H (His);

$-X_2-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or M (Met);

$-X_3-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or E (Glu);

$-X_4-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or K (Lys);

$-X_5$ = F (Phe) or $F-NR^3R^4$, with the proviso that only one of $-X_1-$, $-X_2-$, $-X_3-$ and $-X_4-$ is other than the stated amino acid residue; i.e., His, Met, Glu and Lys, or only one O-glycosylated [O-(Sac)] serine or threonine residue is present in the peptide;

$n = 0$ or 1 and $p = 1$ or 2 , with the proviso that when $n = 1$, $p = 1$, and when $n = 0$, $p = 2$;

R^1 , R^2 , R^3 and R^4 are the same or different and are H, C_1 - C_{18} hydrocarbyl, C_1 - C_{18} acyl (hydrocarobyl), C_1 - C_{18} hydrocarbylsulfonyl, or either or both of R^1 and R^2 and R^3 and R^4 together with their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R^1 , R^2 , R^3 and R^4 includes a hydrocarbyl, acyl or sulfonyl group having a chain of carbon atoms that contains more than six carbon atoms; and

Sac is a glycosidically-linked C_5 - C_6 saccharide, deoxysaccharide or 2-(N-acetylamino) C_5 - C_6 saccharide. Preferred saccharides are GlcNAc, GalNAc, ribose, deoxyribose, mannose, glucose and galactose.

A composition containing a PTHR1 agonistic effective amount of a peptide of Formula 2 dissolved or dispersed in a pharmaceutically acceptable carrier is also contemplated.

A method of treating a mammalian GPCR-mediated condition such as a PTHR1-mediated condition like osteoporosis is also contemplated. That method comprises administering a PTHRI agonistic effective amount of a peptide of Formula 2 dissolved or dispersed in a pharmaceutically acceptable carrier to that mammal having osteoporosis. This contemplated treatment can be repeated as needed to maintain the treated mammal.

Definitions

The word "hydrocarbyl" is used herein as a short hand term for a non-aromatic group that includes straight and branched chain aliphatic as well as alicyclic groups or radicals that contain only carbon and hydrogen. Thus, alkyl, alkenyl and alkynyl groups are contemplated, whereas aromatic hydrocarbons such as phenyl are not included for this term. Usual chemical suffix nomenclature is followed when using the word "hydrocarbyl" except that the usual practice of removing the terminal "yl" and adding an appropriate suffix is not always followed because of the possible similarity of a resulting name to one or more substituents.

Where a specific aliphatic hydrocarbyl substituent group is intended, that group is recited; i.e., C₁-C₃ alkyl, methyl or ethyl or iso-propyl. Exemplary hydrocarbyl groups contain a chain of 1 to 3 carbon atoms, and preferably 1 or 2 carbon atoms.

A particularly preferred hydrocarbyl group is an alkyl group. As a consequence, a generalized, but more preferred substituent can be recited by replacing the descriptor "hydrocarbyl" with "alkyl" in any of the substituent groups enumerated herein. A hydrocarbyl group can be straight chained, branched chain or cyclic. Hydrocarbyl groups with six or fewer carbon atoms are used below for illustrative purposes with the understanding that a skilled worker will be well aware of the substituents with larger numbers of carbon atoms, particularly the straight and branched chained substituents.

Examples of alkyl radicals include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl and the like. Examples of suitable alkenyl radicals

include ethenyl (vinyl), 2-propenyl, 3-propenyl, 1,4-pentadienyl, 1,4-butadienyl, 1-butenyl, 2-butenyl, 3-butenyl, hexenyl, hexadienyl and the like. Examples of alkynyl radicals include ethynyl, 2-propynyl, 3-propynyl, 1-butyne, 2-butyne, 3-butyne, and the like.

As a skilled worker will understand, a substituent that cannot exist such as a C₁ alkenyl or alkynyl group are not intended to be encompassed by the word "hydrocarbyl", although such substituents with two or more carbon atoms are intended, as is a C₁ alkyl or methyl group.

A contemplated hydrocarbyl group can also include a 5- to 7-membered hydrocarbyl ring. Illustrative of such rings are cyclopentyl, cyclohexyl and cycloheptyl, as well as 2-cyclopentenyl, 2- and 3-cyclohexenyl, and 2-, 3- and 4-cycloheptenyl groups.

A (C₁-C₁₈)hydrocarbonyl group is a straight, branched chain or cyclic acyl hydrocarbyl residue that can contain one to through seven carbon atoms. Illustrative (C₁-C₁₈)hydrocarbonyl groups include formyl, acetyl, propionyl, benzoyl, acryloyl, methacryloyl, cyclopentylcarbonyl, hexanoyl, lauroyl, stearoyl and the like.

C₅-C₆ monosaccharides, their 2-deoxy derivatives and the 2-N-acetyl (NAC) derivatives of both types are well known in the in the chemical and biochemical arts. Exemplary C₅-C₆ monosaccharides include allose, altrose, fructose, glucose, galactose, gulose, iodose, talose, mannose, xylose, ribose, fucose, arabinose, and lyxose. GlcNAC, GalNAC, ribose, deoxyribose, mannose, glucose and

galactose are preferred, whereas glucose (Glc) and galactose (Gal) and their 2-NAc derivatives are more preferred monosaccharides for use herein.

The terms "physiologically acceptable" and "pharmaceutically acceptable" in their various grammatical forms refer to any non-toxic cation or anion or combination thereof commonly used in the pharmaceutical industry, which can be prepared by methods known in the art. A contemplated cation and/or anion to provide ionic neutrality to an acid or basic amino acid residue side group, or can provide a water-soluble salt such as sodium chloride to raise osmolality above that of water, or a buffer such as dipotassium phosphate. Preferably, the cation salts are sodium, potassium, calcium and ammonium in either the mono or dibasic salt form. The preferred anions are halogens such as chloride and bromide, and C₁-C₆ hydrocarbyl carboxylates. The reader is directed to Berge, *J. Pharm. Sci.* 1977 **68(1)**:1-19 for lists of commonly used physiologically (or pharmaceutically) acceptable acids and bases that form physiologically acceptable salts with pharmaceutical compounds and buffers.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure,

Fig. 1A - Fig. 1F illustrate the cell-based characterization of the exemplary GLP-1 peptides prepared herein. Fig. 1A shows that neither O-GlcNAcylation at serine residue 18 alone nor O-GlcNAcylation plus 2-aminoisobutyric acid (AIB) substitution affects the EC₅₀ of cAMP production when compared to unmodified GLP-1. Fig. 1B shows that

O-GlcNAcylation of GLP-1 alone does not alter Ca^{2+} accumulation, but in combination with AIB substitution at residue 4 in Peptides **G3** and **G4** results in biased agonism. Fig. 1C and Fig. 1D illustrate that O-GlcNAcylation alone or in combination with AIB substitution does not affect kinase signaling. Fig. 1E and Fig. 1F illustrate that β -Arrestin-1 (1E) or β -Arrestin-2 (1F) recruitment to GLP-1R is unaffected by O-GlcNAcylation but is reduced by O-GlcNAcylation in combination with two AIB substitutions, resulting in biased agonism. All studies were performed in CHO cells expressing the human GLP-1R.

Fig. 2A and Fig. 2B illustrate that position 18 O-GlcNAcylation stabilizes GLP-1 from proteolysis and improves glucose clearance *in vivo* in human serum. Fig. 2A shows that O-GlcNAc modification improves the stability of GLP-1. The indicated peptides were incubated in triplicate with human serum for 24 hours. The stability of each peptide was measured using RP-HPLC after the indicated lengths of time in serum. Fig. 2B O-GlcNAcylation of GLP-1 improves glucose clearance in a glucose tolerance test. Lean mice ($n = 5$) were subjected to a single intraperitoneal co-injection of vehicle (saline) or the indicated concentrations of peptide with glucose challenge. Blood glucose levels were then measured after the indicated lengths of time. Error bars represent $\pm$ s.e.m., and statistical significance was calculated by one-way ANOVA followed by Dunnett's post-test.

Fig. 3 shows the circular dichroism (CD) spectrum of the GLP-1 peptide and also spectra of each of the synthesized peptides. Examination of the

five spectra shows that each is substantially identical to the others thereby indicating that the presence of the O-GlcNAc moiety O-bonded to the position 18 serine residue does not alter the GLP-1 secondary structure. The CD spectra were collected for freshly dissolved samples of the indicated peptides at 50 μ M concentration.

Fig. 4 shows a conformational model of the full length GLP-1R-**G1** complex and the **P2**-PTH1R complex. Fig. 4A shows an overview of the GLP-1R-**G1** molecular surface. Fig. 4B shows a close-up showing stabilizing interactions of the O-GlcNAc moiety. In both panels, GLP-1R is shown as grey, with certain regions additionally delineated. The **G1** peptide is represented as ribbon, with the O-GlcNAc moiety highlighted as transparent spheres. Intermolecular hydrogen bonds and salt bridges are shown by dotted lines. The **P2**-PTH1R complex is represented as molecular surface in Fig. 4C. Fig. 4D depicts a close-up showing predicted stabilizing interactions of the O-GlcNAc moiety. **P2** peptide is represented as ribbon, where the O-GlcNAc moiety is highlighted as transparent spheres.

Fig. 5A - Fig. 5E illustrate an analysis of O-GlcNAcylated PTH-analogs. Thus, Fig. 4A and Fig. 4B show cAMP production by O-GlcNAcylated PTH when compared to unmodified PTH in cells that either overexpress (Fig. 5A, GP-2.3) or endogenously express (Fig. 5B, SGS-72) PTH1R ($n = 4$ for each peptide). Fig. 5C shows that O-GlcNAc modification improves the stability of PTH. Unmodified PTH or **P2** ($n = 3$) were incubated with human serum for 48 hours at 37°C. The stability of each peptide was measured using RP-HPLC after the indicated lengths of time. Fig. 5D shows that O-GlcNAcylation of PTH exhibits similar Ca^{2+} mobilization compared to unmodified PTH *in vivo*.

Mice (n = 5) were subjected to a single subcutaneous injection of vehicle or the indicated concentrations of PTH or PTH peptide **P2**. Blood Ca^{2+} concentrations were then measured after the indicated lengths of time. Fig 5E shows that ionized Ca^{2+} area under the curve (AUC) calculated from the data in Fig. 5D over 8 hours with a baseline of 1.25 mM. Error bars represent $\pm$ s.e.m., and statistical significance of differences in AUC between vehicle and peptides was calculated using one-way ANOVA with a Dunnett post-test. Results with PTH and **P2** were not statistically-significantly different from each other.

Fig. 6A - Fig. 6F show the circular dichroism (CD) spectrum of the PTH peptide and also spectra of each of the synthesized, GlcNAcylated (**P1**, **P2**, **P3** and **P4**) peptides. Examination of the five spectra shows that the spectra for PTH and **P2** are similar and different from the other spectra, thereby indicating that the presence of the O-GlcNAc moiety O-bonded to the position synthetically-added serine residue does not alter the PTH secondary structure, whereas the other substitution do change the structure. The CD spectra were collected for freshly dissolved samples of the indicated peptides at 50 μM concentration.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention contemplates a synthetic peptide hormone comprising a sequence of about 15 to about 50 amino acid residues that are at least about 90% sequentially identical to the same length aligned sequence of a naturally-occurring peptide hormone. Each of the synthetic and

naturally-occurring peptide sequences exhibit an amphipathic alpha-helical secondary structure. Preferably, each of those peptide sequences is free of a covalent intrachain linkage. The synthetic peptide hormone includes a mono-O-glycosylated serine or threonine residue in its sequence as a non-identical residue compared to the naturally-occurring (native) peptide hormone sequence; i.e., the naturally-occurring sequence does not include that glycosylated residue. The synthetic peptide hormone containing the mono-O-glycosylated serine or threonine residue has a half-life in human serum at 37°C of about 25 to about 800 percent longer than the naturally-occurring peptide hormone.

The synthetic and naturally-occurring peptide hormones are agonists of a G protein-coupled receptor (GPCR). A GPCR for which a synthetic and naturally-occurring peptide hormone is an agonist mediates a mammal's bodily function via the G protein to which it is coupled and the protein(s) associated with that particular G protein as is well known. Exemplary particular naturally-occurring (native) peptide hormones agonists of G protein receptors are discussed hereinafter.

Turning to the synthetic peptide hormone and its corresponding native peptide hormone, it is seen that the two sequences have same number of amino acid residues in their linear sequences, and are therefore of the same length. The number of amino acid residues in such a sequence is about 15 to about 50. More preferably, that length is about 20 to about 45 residues, and most preferably about 25 to about 40 amino acid residues.

Not only do the two peptide sequences have the same sequence length, the synthetic peptide hormone is at least about 90% sequentially identical to the aligned sequence of a naturally-occurring peptide hormone. Thus, if the native peptide hormone contains a sequence of 50 residues, a contemplated synthetic peptide hormone can contain up a sequence of 50 residues including to about 5 residues, including the mono-O-glycosylated serine or threonine, that are at a different position in the sequence and/or of a different identity at a specified sequence position as compared to the naturally-occurring peptide hormone sequence. Because there are no partial amino acid residues, the "about 90%" utilized herein is to permit the "rounding up to a whole residue where the 90% calculation provides for a partial residue. Thus, for example, a contemplated synthetic peptide hormone whose corresponding native peptide hormone has a sequence of about 34 residues can contain three to four non-native residues including the mono-O-glycosylated serine or threonine.

It is to be understood that a mono-O-glycosylated serine or threonine residue utilizes the hydroxyl group of the serine or threonine to form a glycosidic bond with the saccharide portion of the newly-formed glycosylated amino acid residue. A C-O-C linkage joins the amino acid and saccharide portions of the residue. For written convenience, such residues will be referred to hereinafter as a "monoglycosylated serine or threonine".

Use of the word "aligned" herein means that when the two peptides are compared by their sequences, those sequences are arranged so that there

is the maximum identity of amino acid residues at each sequence position between them. Thus, if the native peptide hormone has a sequence of 35 residues and the synthetic peptide has 38 residues, the two sequences are first aligned to obtain the greatest sequence identity within the overlapping two lengths. Once the sequence alignment is considered, if the synthetic peptide hormone contains 35 residues that align with the native peptide hormone to provide at least an about 90% residue identity, the length and alignment criteria of the synthetic peptide hormone are fulfilled.

Each contemplated synthetic peptide hormone contains one monoglycosylated serine or threonine residue. In as much as there are no known naturally occurring monoglycosylated serine or threonine residues present in a naturally-occurring peptide hormone, such a glycosylated residue can constitute the required non-naturally-occurring (non-native) residue in a contemplated synthetic peptide hormone sequence. It is to be understood that a required non-native residue includes a monoglycosylated serine or threonine that is present at the same sequence position in the naturally-occurring form in a non-glycosylated form and/or a glycosylated serine or threonine that has replaced another residue at a given place in the sequence.

The native and synthetic peptide hormones also are preferably free of intrachain covalent linkages such as disulfides or amide bonds. Thus, a contemplated synthetic peptide hormone is preferably free of covalent cross-links such as disulfide or amide bonds.

The alpha-helical secondary structure exhibited by the native and synthetic peptide hormones is amphipathic in that opposing polar and nonpolar faces of the helix are oriented along the long axis of the helix. The monoglycosylated serine or threonine residue is present in the sequence that constitutes the polar face of the amphipathic alpha-helical peptide. Preferably, that residue is situated near the center of the polar face of the amphipathic alpha-helix.

Several means are well known for predicting amphipathic helical structures in peptides and proteins. The reader's attention on this point is invited to Schiffer et al., *Biophys J* **7**, 121-135 (1967); Jones et al., *J Lipid Res*, **33**, 287-296 (1992); Phoenix et al., *Curr Protein Pept Sci* **3**(2), 201-221 (April 2002); A. Drozdetskiy et al., *Nucl. Acids Res* **43**(W1):W389-W394 (2015); K. Lin et al. *Bioinformatics* **21**, 152-159 (2005); Buchan et al.; *Nucl Acids Res* **41**(W1): W340-W348 (2013); Gautier, et al, *Bioinformatics* **24**(18): 2101-2102 (2008); and Wadna et al., *JOSS* **3**(31): 1008 (2018).

The presence of an amphipathic alpha-helical secondary structure in a peptide is typically determined by circular dichroism (CD) spectroscopy. A CD spectroscopic determination is preferably carried out at a final peptide concentration of 50 μ M in a 10 mM aqueous phosphate buffer at pH 7.4.

However, some native peptide hormones and their contemplated corresponding synthetic peptides do exhibit their secondary structure in aqueous media. As noted by Kezdy et al., *Proc Natl Acad Sci USA*, **80**:1137-1143 (1983), some cross-link-free peptides having sequence lengths of 10 to 50 residues

that to not exhibit amphipathic alpha-helical structures in aqueous media do form amphipathic alpha-helical structures upon binding to cell surface proteins, lipids, phospholipids and the like. Kezdy et al. disclose methods to determine amphipathic alpha-helical secondary structures using egg lecithin vesicles and monolayer formation at an air-water interface. Separate X-ray crystallography analyses of the native peptide hormone and the synthetic peptide hormone bound to their receptor can also be useful. NMR techniques using as shown by NOSEY and chemical shift data can also be utilized to assay a GPCR-bound synthetic peptide hormone complex.

Still further, methanol, ethanol, acetonitrile, and trifluoroethanol alone or mixed with water can be used as solvents for circular dichroism determinations.

Exemplary GPCRs, their Native and Synthetic Agonists

The 178 residue proglucagon protein is a naturally occurring mammalian protein that is processed *in vivo* into shorter peptides (UniProtKB-1275). One of those peptides is known in the art as glucagon-like peptide-1 (GLP-1), and contains amino acid residues 92 through 128 of the proglucagon sequence. That peptide is relatively short-lived and is processed further into two peptides, both of which are also referred to in the art as glucagon-like peptide-1 (GLP-1).

As further identifiers, a first of those peptides contains 31 residues of the proglucagon molecule and is referred to as GLP-1(7-37). The second peptide contains 30 residues, is C-terminal amidated, and is further referred to as GLP-1(7-

36)amide (UniProtKB-1275). Any reference hereinafter to a GLP-1 peptide is directed to these latter two compounds, unless some other modifier is used to distinguish those peptides from the longer molecule. Both GLP-1(7-37) and GLP-1(7-36)amide bind to the GLP-1 receptor (GLP-1R).

The present invention contemplates a synthetic GLP-1 derivative peptide whose sequence is shown below, as Formula 1 (SEQ ID NO: 1), from left to right and in the direction from amino-terminus to carboxy-terminus in single letter amino acid code,



where

$X_1 = -NH-(CH_2)_m-CR^1R^2-C(O)-$ or A (Ala);

$X_2 = -NH-(CH_2)_n-CR^3R^4-C(O)-$ or V (Val);

$X_3 = -NH-CH[(CHQ)-O-(Sac)]-C(O)-$;

$X_4 = G$ (Gly), $G-NR^5R^6$ or $-NR^5R^6$;

$m = 0$ or 1 ;

$n = 0$ or 1 ;

$Q = H$ or methyl;

R^1 and R^2 are the same or different substituents that are hydrogen (H) or C_1 - C_3 hydrocarbyl, with the proviso that only one of R^1 and R^2 is hydrogen, or R^1 and R^2 together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R^3 and R^4 are the same or different substituents that are hydrogen (H) or C_1 - C_3 hydrocarbyl, or R^3 and R^4 together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R^5 , R^6 , R^7 and R^8 are the same or different and are H, C_1 - C_{18} hydrocarbyl, C_1 - C_{18} acyl, C_1 - C_{18} hydrocarbylsulfonyl, or either or both of R^5 and R^6 and R^7 and R^8 together with their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R^5 and R^6 and R^7 and R^8 includes a hydrocarbyl, acyl or sulfonyl group having more than six carbon atoms; and

Sac is a glycosidically-linked C_5 - C_6 saccharide, deoxysaccharide or 2-(N-acetylamino) C_5 - C_6 saccharide or deoxysaccharide.

In one preferred embodiment, the saccharide of **X₃** is O-GlcNAc. In another preference, R^1 , R^2 , R^3 and R^4 are each methyl. As a further preference, R^1 and R^2 are both methyl and **X₂** is a valyl residue (V). It is further preferred that **X₄** be G (Gly), and that n be 0 (zero) so that the $-(CH_2)_n$ group is absent. It is also preferred that R^7 and R^8 each be hydrogen (H). It is further preferred that Q be hydrogen.

Exemplary preferred peptide sequences include **G1**, **G2**, **G3** and **G4**, in which **X₁** = 2-amino-isobutyric acid (AIB), and **X₁** = **X₂** = AIB; **X₃** = -NH-CH[(CH₂)-O-(GlcNAc)]-C(O)-; and **X₄** is G (Gly), are set out below along with their SEQ ID NOs:

G1 HAEGTFTSDVSX₃YLEGQAAKEFIAWLVKGRX₄

(SEQ ID NO: 2);

G2 HAEGTFTSDX₂SX₃YLEGQAAKEFIAWLVKGRX₄

(SEQ ID NO: 3) R^3 and R^4 are both methyl;

G3 HX₁EGTFTSDVSX₃YLEGQAAKEFIAWLVKGRX₄

(SEQ ID NO: 4) R¹ and R² are both methyl;
and

G4 HX₁EGTFTSDX₂SX₃YLEGQAAKEFIAWLVKGRX₄

(SEQ ID NO: 5) R¹, R², R³ and R⁴ are each
methyl.

GLP-1(7-37) HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG
(Seq. ID. No.: 6) and GPL-1(7-36)amide
HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH₂ (SEQ ID NO: 7).

Another specific contemplated aspect of
this invention is a synthetic parathyroid hormone
(PTH) derivative peptide whose sequence is shown
below, as Formula 2 and SEQ ID NO: 8, from left to
right and in the direction from amino-terminus to
carboxy-terminus:

R¹R²N-SVSEIQLMHNLGKX₁LNSX₂ERVX₃WLRX₄KLQDVHNX₅ (2)

(SEQ ID NO: 8)

wherein

-X₁- = -NH-CH[(CH₃)_n(CH_p)O-(Sac)]-C(O)- or H
(His);

-X₂- = -NH-CH[(CH₃)_n(CH_p)O-(Sac)]-C(O)- or M
(Met);

-X₃- = -NH-CH[(CH₃)_n(CH_p)O-(Sac)]-C(O)- or E
(Glu);

-X₄- = -NH-CH[(CH₃)_n(CH_p)O-(Sac)]-C(O)- or K
(Lys);

-X₅ = F (Phe) or F-NR³R⁴, with the proviso that
only one of -X₁-, -X₂-, -X₃- and -X₄- is other than
the stated amino acid residue; i.e., His, Met, Glu
and Lys;

$n = 0$ or 1 and $p = 1$ or 2 , with the proviso that when $n = 1$, $p = 1$, and when $n = 0$, $p = 2$;

R^1 , R^2 , R^3 and R^4 are the same or different and are H, C_1 - C_{18} hydrocarbyl, C_1 - C_{18} acyl (hydrocarobyl), C_1 - C_{18} hydrocarbylsulfonyl, or either or both of R^1 and R^2 and R^3 and R^4 together with their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R^1 , R^2 , R^3 and R^4 includes a hydrocarbyl, acyl sulfonyl group contains more than six carbon atoms; and

Sac is a glycosidically-linked C_5 - C_6 saccharide, deoxysaccharide or 2-(N-acetylamino) C_5 - C_6 saccharide. Preferred saccharides are GlcNAc, GalNAc, ribose, deoxyribose, mannose, glucose and galactose.

Four illustrative synthetic peptides were prepared based upon the above PTH sequence. Although about 3 or 4 sequential differences are permitted within the 90% identity criterion, each sequence contained only one such difference an O-GlcNAcylated serine. Those four peptides are set out below, from left to right and in the direction from amino-terminus to carboxy-terminus, along with their SEQ ID NOS.

- P1** SVSEIQLMHNLGKX₁LNSMERVEWLRKKLQDVHNF
(SEQ ID NO: 9)
- P2** SVSEIQLMHNLGKHLNSX₂ERVEWLRKKLQDVHNF
(SEQ ID NO: 10)
- P3** SVSEIQLMHNLGKHLNSMERVX₃WLRKKLQDVHNF
(SEQ ID NO: 11)

P4 SVSEIQLMHNLGKHLNSMERVEWLRKX₄LQDVHNF

(SEQ ID NO: 12)

Listed below are five illustrative native peptide hormones that provide illustrative bases for contemplated synthetic peptide hormones:

Secretin⁴⁰: 27 amino acid residues, C-terminal amidated, forms an alpha-helix at residues 5-13 and also residues 17-26. Binds to family B GPCRs, including those that mediated diabetes mellitus, bone disease, pain, inflammation, migraine, anxiety, depression, short bowel syndrome and even some neoplastic diseases:

HSDGTFTSELSRLRDSARLQRLQLV-NH₂

(SEQ ID NO: 13);

Pancreatic polypeptide (PP)⁴¹: 36-amino acid residues whose C-terminus is amidated, forms an alpha-helix at residues 14-32. PP binds to the neuropeptide Y receptors that are class A GPCRs; increases gastric emptying and gut motility. Pancreatic polypeptide also relaxes the pyloric and ileoceccocolic sphincters, the colon, and gallbladder. PP levels increase after ingestion of food and remain elevated from four to eight hours:

APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH₂

(SEQ ID NO: 14);

β -Endorphin: 31-Amino acid residue peptide exhibits little alpha-helix content in water, but exhibit increasing amounts of alpha-helical conformation with increasing concentrations of methanol and also in solutions of 3 mM sodium dodecylsulfate (SDS) using CD spectroscopy⁴². There

are two alpha-helical regions; one is at residues 4-11 and the other is at residues 21-29. Beta-endorphin binds to the opiate pain GPCRs mu, delta and kappa⁴³:

YGGFMTSEKSQTPLVTLFKNALIKNAYKKGE
(SEQ ID NO: 15);

Human growth hormone-releasing fragment (hGRF): This hormone is a 44-amino acid residue C-terminal amidated peptide that is reported to exhibit two nested alpha-helical regions residues 22-29 when in low molar ratio aqueous SDS (e.g. 1.3:1-3.3:1) and residues 19-32 in SDS high molar ratios (e.g. 16:1 -72:1) as shown by NOSEY and chemical shift data⁴⁴. This peptide hormone binds to binding to the GPCR named growth hormone-releasing hormone receptor (GHRH-R). Residues 1-29 are reported to be important for biological activity, and residues 1-29 are preferred⁴⁵:

YADAIFTNSYRKVLGQLSARKLLQDIMSROQGESSNQERGARARL-NH₂
(SEQ ID NO: 16);

Glucagon: 29 Amino acid residue peptide hormone that binds to the GPCR glucagon receptor. The alpha-helical sequence is reported to be located at residues 19-27⁴⁶:

HSQGTFTSDYSKYLDSSRAQDFVQWLMNT
(SEQ ID NO: 17).

Pharmaceutical Compositions and Methods

A contemplated synthetic peptide hormone was discussed and defined previously. Briefly, such

a synthetic peptide hormone comprises a sequence of about 15 to about 50 amino acid residues that are at least about 90% sequentially identical to the same length aligned sequence of a naturally-occurring peptide hormone. A contemplated synthetic peptide hormone includes a monoglycosylated serine or threonine residue in its sequence as a non-identical amino acid residue relative to the naturally-occurring (native) peptide hormone sequence; i.e., the naturally-occurring sequence does not include that glycosylated residue. The synthetic peptide hormone containing the monoglycosylated serine or threonine residue has a half-life in human serum at 37°C of about 25 to about 800 percent longer than the naturally-occurring peptide hormone.

In one embodiment, a composition contains a contemplated synthetic peptide hormone is present in an agonistic-effective amount dissolved or dispersed in a pharmaceutically acceptable diluent or medium.

Illustratively, a contemplated synthetic GLP-1 derivative peptide hormone of Formula 1 can be used in the manufacture of a medicament (pharmaceutical composition) that is useful at for treating diabetes type-2. It is to be understood that a contemplated GLP-1 derivative peptide hormone exhibits activities in the standard assays illustrated herein.

A contemplated GLP-1 synthetic derivative peptide hormone, medicament or pharmaceutical composition containing the same lowers the blood glucose level of a recipient mammal as in a subject in need thereof, as does the parent GLP-1 peptide. However, contrary to the parent GLP-1 peptide that is quite unstable to proteolysis in a mammalian blood

stream, a contemplated GLP-1 derivative peptide is more stable in blood *in vivo*, and in plasma *in vitro*, while maintaining the parental potency. This maintained potency is also exhibited in *in vitro* cell-based assays as compared to parental GLP-1 itself.

Similarly, a contemplated PTH synthetic derivative peptide hormone, medicament or pharmaceutical composition containing the same can treat osteoporosis of a recipient mammal as in a subject in need thereof, as does the parent PTH peptide.

When used *in vitro* and *in vivo*, a contemplated synthetic peptide hormone is typically present along with pharmaceutically acceptable salts, buffers and the like that collectively are referred to as pharmaceutically acceptable diluents as compared to those salts and buffers that can be present in a composition that is not intended for pharmaceutical use, as in an *in vitro* assay or during synthesis.

A contemplated synthetic peptide hormone can be provided for use by itself, or as a pharmaceutically acceptable salt. A contemplated synthetic peptide hormone can contain basic and acidic side chains. As a result, one or both of cationic and anionic ions can be present as counterions.

Exemplary salts useful for a contemplated synthetic peptide hormone include but are not limited to the following: cations--sodium, potassium, magnesium, calcium, zinc, aluminum, choline, diethanol amine, ethylenediimine and procaine; anions--sulfate, hydrochloride, hydro bromides,

acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, cyclopentanepropionate, dodecylsulfate, ethanesulfonate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxy-ethanesulfonate, lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, palmoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, mesylate and undecanoate.

The reader is directed to Berge, *J. Pharm. Sci.* 1977 **68**(1):1-19 for lists of commonly used pharmaceutically acceptable acids and bases that form pharmaceutically acceptable salts with pharmaceutical compounds.

In some cases, a salt can also be used as an aid in the isolation, purification or resolution of the compounds of this invention. In such uses, the acid used and the salt prepared need not be pharmaceutically acceptable.

As is seen from the data herein, a contemplated synthetic peptide hormone is active in *in vitro* assay studies at picomolar (pM) to micromolar (μ M) amounts. When used in an assay such as an *in vitro* assay, an illustrative contemplated GLP-1 synthetic peptide hormone or a PTH synthetic peptide hormone is present in the composition in an amount that is sufficient to provide a concentration of about 0.001 nanomolar (nM) to about 1000 nM, preferably about 0.1 nM to about 50 nM, to contact cells to be assayed.

A composition is contemplated that contains an agonistic effective amount of a contemplated synthetic peptide hormone or a pharmaceutically acceptable salt thereof dissolved or dispersed in a physiologically (pharmaceutically) acceptable carrier. Such a composition can be administered to mammalian cells *in vitro* as in a cell culture to contact those cells, or the cells can be contacted *in vivo* as in a living, host mammal in need. As is seen from the results reported herein, illustrative effective amounts for laboratory mice were in the range of about 0.1 to about 10 mg/kg when provided by intraperitoneal administration.

Illustratively, those amounts along with the *in vivo* effective amounts for the GLP-1(7-37) derivative liraglutide (Victoza®) that contains an added palmitoyl side chain and one exchanged amino acid residue relative to native GLP-1(7-37) and the gila monster-derived 39 residue analog sold as Exendin-4 provide a skilled worker a more than adequate basis to determine an effective amount of a GLP-1 synthetic peptide hormone or salt appropriate to the intended recipient and desired effect.

A contemplated composition is typically administered *in vivo* to a subject in need thereof a plurality of times within one month, such as weekly, and can be administered over a period of several months to several years. More usually, a contemplated composition is administered a plurality of times over a course of treatment.

A contemplated pharmaceutical composition can be administered orally (perorally) or parenterally, which is preferred, in a formulation containing conventional nontoxic pharmaceutically

acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous (which is most preferred), intramuscular, intrasternal injection, or infusion techniques. Formulation of drugs is discussed in, for example, Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania; 1975 and Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980.

Solid dosage forms for oral administration can include capsules, tablets, pills, powders, and granules. The amount of a contemplated compound in a solid dosage form is as discussed previously, an amount sufficient to provide a concentration of about about 0.001 nanomolar (nM) to about 1000 nM, preferably about 0.1 nM to about 50 nM in the serum or blood plasma. A solid dosage form can also be administered a plurality of times during a one week time period.

In such solid dosage forms, a compound of this invention is ordinarily combined with one or more adjuvants appropriate to the indicated route of administration. If administered per os, the compounds can be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets can contain a controlled-release formulation as can be provided in a dispersion of active compound

in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms can also comprise buffering agents such as sodium citrate, magnesium or calcium carbonate or bicarbonate. Tablets and pills can additionally be prepared with enteric coatings.

A contemplated pharmaceutical composition is preferably adapted for parenteral administration. Thus, a pharmaceutical composition is preferably in liquid form when administered, and most preferably, the liquid is an aqueous liquid, although other liquids are contemplated as discussed below, and a presently most preferred composition is an injectable preparation.

Thus, injectable preparations, for example, sterile injectable aqueous or oleaginous solutions or suspensions can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution, phosphate-buffered saline.

Other liquid pharmaceutical compositions include, for example, solutions suitable for parenteral administration. Sterile aqueous saline solutions of a contemplated synthetic peptide hormone or sterile solution of a contemplated synthetic peptide hormone in solvents comprising water, ethanol, or propylene glycol are examples of liquid compositions suitable for parenteral administration.

In some aspects, a contemplated synthetic peptide hormone is provided as a dry powder that is to be dissolved in an appropriate liquid medium such as aqueous sodium chloride for injection prior to use.

In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of an injectable composition. Dimethyl acetamide, surfactants including ionic and non-ionic detergents, polyethylene glycols can be used. Mixtures of solvents and wetting agents such as those discussed above are also useful. Sterile solutions can be prepared by dissolving the active component in the desired solvent system, and then passing the resulting solution through a membrane filter to sterilize it or, alternatively, by dissolving the sterile compound in a previously sterilized solvent under sterile conditions.

A mammal in need of treatment (a subject) and to which a pharmaceutical composition containing a contemplated synthetic peptide hormone is administered can be a primate such as a human, an ape such as a chimpanzee or gorilla, a monkey such as a cynomolgus monkey or a macaque, a laboratory animal such as a rat, mouse or rabbit, a companion animal such as a dog, cat, horse, or a food animal such as a cow or steer, sheep, lamb, pig, goat, llama or the like.

Where an *in vitro* assay is contemplated, a sample to be assayed such as cells and tissue can be used. These *in vitro* compositions typically contain the water, sodium or potassium chloride, and one or

more buffer salts such as acetate and phosphate salts, Hepes or the like, a metal ion chelator such as EDTA that are buffered to a desired pH value such as pH 4.0 -8.5, preferably about pH 7.2-7.4, depending on the assay to be performed, as is well known.

Preferably, the pharmaceutical composition is in unit dosage form. In such form, the composition is divided into unit doses containing appropriate quantities of the active compound. The unit dosage form can be a packaged preparation, the package containing discrete quantities of the preparation, for example, in vials or ampules.

Results

During the course of studies on O-GlcNAcylation of the Parkinson's disease associated protein α -synuclein, which is modified by O-GlcNAc *in vivo*, it was found that single modifications of this protein dramatically inhibited the proteolysis of α -synuclein by the protease calpain¹². Notably, this inhibition occurred despite the fact that the O-GlcNAc modifications were very distant in primary sequence (10-15 amino acids) from certain cleavage sites.

Therefore, it was queried whether this property of "remote stabilization," by O-GlcNAcylation could be repurposed for therapeutic peptides. Accordingly, we first synthesized four analogs of GLP-1 that contained a single O-GlcNAc at serine 18 or in combination with one or two 2-aminoisobutyric acid (AIB) residues at sequence positions 8 and/or 16, which have been shown to stabilize GLP-1 by the Gellman laboratory^{13,14}. We

then used a range of cell-based, *in vitro*, and *in vivo* assays to demonstrate that O-GlcNAcylation does not compromise the agonism of GLP-1R by the peptide, but does indeed stabilize GLP-1 against cleavage by serum proteases, including DPP-4, resulting in improved glucose clearance in mice.

Surprisingly, we found that O-GlcNAc in combination with AIB substitutions not only further stabilized the peptide to cleavage by serum proteases [inhibited peptide cleavage by serum proteases], but resulted in significantly increased biased agonism towards certain G-protein signaling pathways and reduced recruitment of β -arrestins compared the to AIB substitutions alone^{13,14}, which could result in improved therapeutic properties. Finally, we used molecular modeling to show that a combination of O-GlcNAc in S18, and the AIB mutations in positions V16 and A8, can create new key interactions on the peptide-receptor interface that can facilitate the conformational changes leading to agonism bias.

Design and synthesis of O-GlcNAcylated variants of GLP-1

As mentioned above, biochemical and structural studies have demonstrated that the N-terminal residues of GLP-1 are critical for making the appropriate interactions with GLP-1R that result in receptor activation. Additionally, the receptor makes additional interactions with the C-terminus of GLP-1 starting around residue 24.¹⁴ With these considerations in mind, we chose to place O-GlcNAc at serine 18 of GLP-1, as we hypothesized that this position would be on the polar face of the amphipathic helix and solvent-exposed upon receptor

binding. Accordingly, we prepared the suitably protected O-GlcNAcylated serine using a published procedure¹⁵ and prepared the peptides (**G1-G4**, sometimes also referred to herein as **C1-C4**) using standard Fmoc-based solid phase peptide synthesis. Each peptide was purified.

Unmodified GLP-1 has significant α -helical character in solution. Therefore, we next used circular dichroism (CD) spectroscopy to examine the secondary structure of all of our peptides and found that neither O-GlcNAc nor the AIB substitutions had any noticeable consequences (Fig. 3).

O-GlcNAcylation does not inhibit GLP-1R agonism alone but causes biased agonism in combination with AIB. Endogenous binding of GLP-1 to the GLP-1R results in recruitment of the $G\alpha_s$ G-protein, subsequently stimulating the production of cyclic AMP (cAMP) from ATP and leading to glucose-stimulated insulin secretion¹⁶. To assess the ability of the O-GlcNAc-modified GLP-1 peptides **G1-G4** to agonize human GLP-1R, cAMP accumulation was measured in FlpIn CHO cells stably expressing human GLP-1R¹⁴.

As a positive control for full human GLP-1R activation, cells were treated with native GLP-1, which exhibited an EC_{50} = 4.1 pM (Fig. 1A and Table 1A). O-GlcNAc peptide analogues **G1-G4** showed very similar potencies (5.1-6.0 pM) that were not significantly different from unmodified GLP-1. Notably peptide **G1** also resulted in a small but significant higher maximum efficacy compared to unmodified GLP-1.

Again, using the CHO-cell system, the ability of peptides **G1-G4** to promote intracellular calcium mobilization was next measured, an

established assay that reports on $G\alpha_q$ activation by GLP-1R^{14,17}. In this assay, recruitment of $G\alpha_q$ activates phospholipase C β (PLC β), catalyzing the formation of inositol-1,4,5-trisphosphate (IP $_3$) that can bind and open the IP $_3$ -gated calcium channel, increasing calcium concentration in the cytoplasm¹⁶.

We found GLP-1 to exhibit an EC_{50} = 58.5 nM (Fig. 1B and Table 1A) in this assay. The O-GlcNAc peptide analogues **G1** and **G2** showed comparable potencies and maximum response relative to GLP-1. In contrast, O-GlcNAc peptide analogues **G3** and **G4** displayed reduced potencies relative to GLP-1 and their maximal response was reduced significantly, indicating that **G3** and **G4** could be biased $G\alpha_s$ agonists.

In addition to these G-protein coupled pathways, GLP-1 has been shown to activate kinases through non-canonical pathways; therefore, we tested whether peptide analogues **G1-G4** were assessed for their ability to promote ERK1/2 and AKT phosphorylation^{18,19}. As expected, unmodified GLP-1 resulted in dose-dependent phosphorylation/activation of both ERK and AKT (Fig. 1C, Fig. 1D and Tables 1A & 1B). Likewise, all of the O-GlcNAcylated analogs resulted in phosphorylation of both kinases that were essentially indistinguishable from the unmodified peptide (Fig. 2C, Fig. 2D and **Tables 1A** and **1B**).

Like most a G protein-coupled receptors (GPCRs), GLP-1R activation can also result in recruitment of β -arrestin proteins that play a central role in receptor desensitization²⁰. An established bioluminescence resonance energy transfer (BRET) assay in FlpInCHO cells transiently expressing GLP-1R was used to evaluate this pathway²¹.

In this assay, GLP-1 exhibited an EC_{50} = 34.8 nM for β -arrestin-1 and an EC_{50} = 20.0 nM for β -arrestin-2 (Fig. 1E, Fig. 1F and Table 1B). O-GlcNAc modified peptides **G1-G3** displayed comparable EC_{50} values and curves to GLP-1 (Table 1B).

In contrast, we observed both a shift in EC_{50} and a substantial decline in the recruitment of the β -arrestins, relative to GLP-1, upon treatment with peptide **G4**. These results further support peptide **G4** as a biased agonist towards the $G\alpha_s$ /cAMP signaling pathway. We previously showed that AIB substitution at both residues 8 and 16 can cause a modest bias in the same direction¹⁴. However, those magnitudes (about 15-25%) are considerably less than those observed for **G4**, indicating that the O-GlcNAc modification is playing a direct role in increasing bias.

Table 1A

Activity data for GLP and O-GlcNAcylated peptides G1-G4 in cAMP accumulation, Ca^{2+} mobilization, ERK1/2 phosphorylation*

Peptide	cAMP		iCa ²⁺		pERK1/2	
	pEC ₅₀ (M)	E _{max}	pEC ₅₀ (M)	E _{max}	pEC ₅₀ (M)	E _{max}
GLP-1	11.4 ± 0.1	100 ± 2	7.2 ± 0.1	100 ± 6	9.1 ± 0.2	100 ± 6
G1	11.2 ± 0.1	112 ± 2 ⁺	7.3 ± 0.2	100 ± 8	9.0 ± 0.2	105 ± 6
G2	11.2 ± 0.1	107 ± 3	7.2 ± 0.2	90 ± 10	9.0 ± 0.2	107 ± 7
G3	11.3 ± 0.1	108 ± 2	7.2 ± 0.3	49 ± 8 ⁺	9.1 ± 0.2	106 ± 7
G4	11.3 ± 0.1	105 ± 2	7.3 ± 0.2	46 ± 5 ⁺	9.0 ± 0.2	107 ± 6

* Notes follow Table 1B.

Table 1B

Activity data for GLP and O-GlcNAcylated peptides G1-G4 in AKT phosphorylation, and β -arrestin 1 or 2 recruitment*

Peptide	pAKT		β -Arrestin-1		β -Arrestin-2	
	pEC ₅₀ (M)	E _{max}	pEC ₅₀ (M)	E _{max}	pEC ₅₀ (M)	E _{max}
GLP-1	7.8 ± 0.2	100 ± 7	7.5 ± 0.1	100 ± 4	7.7 ± 0.1	100 ± 4
G1	7.6 ± 0.2	120 ± 8	7.2 ± 0.1	125 ± 7	7.2 ± 0.1 ⁺	130 ± 7 ⁺
G2	7.7 ± 0.1	122 ± 6	7.2 ± 0.1	82 ± 6	7.2 ± 0.1 ⁺	89 ± 3
G3	7.7 ± 0.1	111 ± 7	7.2 ± 0.1	91 ± 5	7.3 ± 0.1 ⁺	112 ± 3
G4	7.7 ± 0.1	101 ± 6	6.9 ± 0.1 [#]	48 ± 8 [*]	7.0 ± 0.1 ⁺	76 ± 4 ⁺

*All studies were performed in FlpIn CHO cells either stably or transiently expressing human GLP-1R. pEC₅₀ and E_{max} values were calculated by logarithmic fitting in GraphPad. Values are the mean ± s.e.m. of at least four biological replicates. Statistical significant difference from GLP-1 was calculated using either one way ANOVA followed by Tukey's post-hoc analysis (+) or students t-test (#).

O-GlcNAc modification stabilizes GLP-1 from degradation and improves its function *in vivo*. To complement our *ex vivo* characterization of the GLP-1 analogs **G1-G4**, we next tested our original hypothesis that O-GlcNAcylation would improve the stability of GLP-1 in the presence of serum proteases.

Accordingly, unmodified GLP-1, **G1**, or **G4** were incubated in triplicate in human serum for 24 hours.

After different lengths of time, aliquots were removed and analyzed by RP-HPLC to measure the remaining full-length peptide²². The resulting data were then fit to an exponential decay curve to calculate the peptide half-lives (Fig. 2A). Gratifyingly, the half-life of peptide **G1** was twice that of unmodified GLP-1, confirming our O-GlcNAc hypothesis, and the incorporation of AIB units in **G4** further increased this stability.

Interestingly, we could identify the major cleavage product of GLP-1 and **G1** by dipeptidyl peptidase-4 (DDP-4) by mass spectrometry and found that O-GlcNAcylation appears to inhibit this proteolysis event despite the fact that this modification is 10 amino acid residues away from the cleavage site in the primary sequence. Given this promising *in vitro* data, we next tested our peptides *in vivo* using a glucose clearance assay, or glucose tolerance test (GTT)²³.

More specifically, after fasting, mice (n = 5 per group) were injected intraperitoneally (IP) with 1 mg/kg of glucose and either vehicle, or vehicle plus GLP-1, or one of our O-GlcNAcylated analogs. After different lengths of time, tail blood-glucose levels were measured. In an acute GTT, all of the modified peptides (**G1-G4**) displayed significantly improved glucose disposal efficiency compared to unmodified GLP-1 (Fig. 2B).

Notably, contemplated peptides **G1-G4** lowered fasting blood glucose levels with a faster onset than GLP-1 as indicated by a greater correction of glucose levels at the 15 minute time point. These data fully support our cell-based assays that show near identical levels of cAMP accumulation after GLP-1R activation by peptides **G1-G4**, and demonstrate that O-GlcNAc modification results in improved *in vivo* activity.

Structure-based modeling of the modified peptides to GLP-1R suggests the molecular determinants of binding and signaling bias. In order to gain molecular insight into the effect of O-GlcNAc on GLP-1R binding by peptide **G1**, and potentially more interestingly the biased agonism of peptide **G4**, we

used molecular modeling based on a Cryo-EM structure of unmodified GLP-1 bound to GLP-1R 9 as discussed hereinafter²⁴.

First, we generated a model of peptide **G1** in complex with GLP-1R complex and found that the O-GlcNAc moiety introduced at S18 is accommodated in the shallow cleft located between the extracellular loops ECL2 and ECL3 of the 7 transmembrane (7TM) domain and the extracellular domain (ECD) of GLP-1R (Fig. 4). The energy-optimized conformation of the sugar makes hydrogen bond interactions with N300^{ECL2} of the receptor and an internal hydrogen bond with D15 of GLP-1, which is further stabilized by a salt bridge with R376^{ECL3}, while maintaining the crucial salt bridge with the R380⁷.³⁴ residue, both of GLP-1R²⁵.

Additionally, introduction of the O-GlcNAc moiety leads to rearrangement of the Y19 and Q23 side chains in GLP-1, where Y19 forms anion- π interaction with receptor residue E139¹.³⁴ and an intramolecular hydrogen bond with Q23. This extended interaction network resulting from introduction of O-GlcNAc on S18 is likely to contribute to increased rigidity in the GLP-1R-G1 complex.

Likewise, introduction of AIB in position V16 of **G2** can impose additional conformational rigidity in the peptide. This model matches the experimental data, where both of these modifications of GLP-1 are well tolerated but do not have notable effects on signaling potency or bias.

The additional modification that confers signaling bias introduces AIB at position A8 at the very N-terminus of GLP-1, peptide **G4**. In this structural model, the additional methyl unit of AIB8 impinges on the receptor F367⁶.⁵⁶ phenyl ring,

resulting in a dramatic shift of this side-chain. This shift is likely to result in a dynamic change in the helix VI backbone itself, which is known to couple to downstream conformational changes, facilitating G-protein signaling, while potentially compromising other signaling pathways (e.g. β -arrestin). Corroborating this hypothesis, our recent Cryo-EM structure with another biased agonist, Exendin-P5²⁶, shows a similar outward shift and rotation of residue F367^{6.56} and the tip of helix VI (PDB ID 6B3J), and Exendin-P5 has a bulky residue (V8) in the same position as AIB8 in **G4**.

This analysis suggests that the distinct interaction of N-terminal residues of the biased ligand with helix VI, and particular the F367^{6.56} side chain, might serve as a major determinant of G-protein bias in GLP-1R. As noted above, AIB modifications without the O-GlcNAcylation induce much less bias^{13,14}. We propose that this may be due to flexibility in the ligand, which can absorb the introduced strain of the AIB8 - F367^{6.56} interaction.

In contrast, when all 3 changes are combined in the **G4** ligand, the increased overall rigidity of ligand-GLP-1 interactions by O-GlcNAc on S18 and AIB16 results in very pronounced bias effect from the AIB8 mutation. In other words, whereas AIB8 creates a lever for inducing the conformational change in helix VI, the other two modifications in the peptide enhance the foothold for this lever and make it more rigid.

Thus, all three mutations contribute to the dramatic changes in signaling profile and introduce ligand bias. Although the model effectively describes our cellular data, direct structural

information may be needed to further validate the mechanism of biased mode of action of the **G4** ligand.

This model is validated by our experimental data, where the O-GlcNAcylation of Ser18 is accommodated by GLP-1R and does not inhibit potency of our GLP-1 peptide analogs in the cell-based signaling assays. Moreover, mutation of key residues within this pocket alter the potency and/or maximum response of GLP-1, **G1** and **G4** peptides when assessed in both cAMP and pERK1/2, albeit there are distinctions in the extent of these effects when comparing between cAMP and pERK1/2 and between ligands, as shown in **Tables 3A** and **3B**, respectively, below.

Table 3A¹

	cAMP		
	GLP-1	G1	G4
WT	9.93 ± 0.14	9.94 ± 0.13	9.57 ± 0.15
L142A	9.18 ± 0.16* p = 0.02	9.06 ± 0.18 p = 0.13	8.28 ± 0.17* p = 0.001
N300A	7.09 ± 0.14* p = <0.0001	7.46 ± 0.29* p = <0.0001	6.98 ± 0.24* p = <0.0001
R376A	9.44 ± 0.18 p = 0.14	9.49 ± 0.29 p = 0.63	9.07 ± 0.18 p = 0.22
R380A	6.70 ± 0.18* p = <0.0001	7.34 ± 0.42* p = <0.0001	7.17 ± 0.18* p = <0.0001
	E_{max} (%WT)	E_{max} (%WT)	E_{max} (%WT)
WT	100 ± 4	100 ± 4	100 ± 4
L142A	72 ± 4* p = 0.03	86 ± 6 p > 0.99	79 ± 5* p = 0.02
N300A	54 ± 6* p = 0.001	53 ± 9* p = 0.001	25 ± 5* p = <0.0001
R376A	99 ± 6 p < 0.01	82 ± 5 p < 0.001	88 ± 6 p = 0.23
R380A	66 ± 11* p = 0.01	34 ± 9* p = <0.0001	9 ± 1* p = <0.0001

¹ Notes follow Table 3B.

Table 3B¹

pERK1/2			
	GLP-1	G1	G4
WT	9.09 ± 0.15	9.24 ± 0.25	8.73 ± 0.27
L142A	9.96 ± 0.19* p = 0.02	9.93 ± 0.29 p = 0.39	9.85 ± 0.22* p = 0.007
N300A	6.98 ± 0.11* p = <0.0001	7.69 ± 0.29* p = 0.01	7.98 ± 0.24 p = 0.076
R376A	8.07 ± 0.15* p = 0.003	7.69 ± 0.43* p = 0.01	7.18 ± 0.14* p = 0.0004
R380A	7.67 ± 0.24* p = 0.0001	7.81 ± 0.33* p = 0.02	7.30 ± 0.16* p = 0.001
	E_{max} (%WT)	E_{max} (%WT)	E_{max} (%WT)
WT	100 ± 5	100 ± 5	100 ± 9
L142A	71 ± 4 p = 0.09	98 ± 8 p = 1.00	77 ± 4 p = 0.15
N300A	105 ± 14 p = 0.98	52 ± 7* p = 0.04	43 ± 4* p = 0.0004
R376A	92 ± 10 p = 0.90	107 ± 24 p = 0.98	93 ± 13 p = 0.91
R380A	36 ± 4* p = 0.0003	24 ± 4* p = 0.002	31 ± 3* p < 0.001

¹ All studies were performed in FlpIn CHO cells either stably or transiently expressing wildtype or mutant human GLP-1R. pEC50, E_{max}, and error (s.e.m.) were calculated by nonlinear fitting (three parameter logistic fit for all cAMP data, and four parameter fit for pERK) in GraphPad. Values are from at least four replicates. P values were calculated for each ligand using one-way ANOVA followed by Dunnett's post-test with wildtype as the control. Statistically significant differences (P<0.05) are denoted by *.

For example, L142A reduces potency for all ligands in cAMP but has more limited effect in pERK1/2 whereas R376A/ECL3 had a greater effect of pERK1/2 potency relative to cAMP. Ala mutation of N300ECL2 and R380ECL3 heavily impaired cAMP and pERK1/2 signaling mediated by all three peptides, but G4 was more sensitive to these mutations in cAMP.

For pERK1/2, potency was impacted for all three peptides, but maximal response was only reduced for G1 and G4. These results are in excellent

agreement with the additional interactions predicted by the modelling between the sugar of the O-GlcNAc peptides and receptor residues N300, R376, and R380.

Design and synthesis of O-GlcNAcylated variants of PTH

In the case of PTH, the O-GlcNAcylation sites on the crystal structure of PTH(15-34) bound to the extracellular domain of PTHr1²¹. More specifically, the individual O-GlcNAc moieties were mapped along the α -helical face of PTH that points away from the extracellular domain of PTHr1, yielding peptides **P1-P4**. As with GLP-1, Fmoc-based solid phase peptide synthesis was employed, the peptides were purified by RP-HPLC, and characterized them by ESI-MS.

PTH also tends to form an α -helix in aqueous solution, CD spectroscopy was used to examine any effects of O-GlcNAcylation (Fig. 6). Similar to GLP-1, modification on peptides **P1** and **P2** had little-to-no effect on the structure of PTH. However, peptides **P3** and **P4** were more unstructured in solution with **P4** having the largest degree of disorder.

O-GlcNAcylation analogs of PTH maintain canonical cAMP signaling

Much like GLP-1R, activation of PTHr1 can result in the recruitment of G α_s that promotes cAMP production and associated downstream signaling events in bone and the kidneys to regulate blood calcium levels. To test the ability of our PTH analogs (n = 4) to stimulate cAMP production, we used a HEK-293 cell line, GP-2.3, which stably overexpresses the human PTHr1 and a cAMP sensor **Table 2**, as well as a human osteoblastic cell line (SGS-72) that endogenously expresses PTHr1 **Table 2**, below.

Table 2

EC ₅₀ and maximal activity (E _{max}) data for PTH and O-GlcNAcylated peptides P1-P4 in cAMP accumulation ¹				
	GP-2.3 cAMP		SGS-72 cAMP	
	pEC ₅₀ (M)	E _{max}	pEC ₅₀ (M)	E _{max}
PTH	9.7 ± 0.1	100 ± 1	10.0 ± 0.1	102 ± 4
P1	9.6 ± 0.1 p = 0.63	131 ± 3* p < 0.0001	9.4 ± 0.2* p < 0.05	123 ± 6* p < 0.05
P2	9.4 ± 0.1 p = 0.06	140 ± 2* p < 0.0001	9.7 ± 0.1 p = 0.28	115 ± 4 p = 0.19
P3	9.3 ± 0.1* p < 0.01	127 ± 4* p < 0.0001	9.6 ± 0.1 p = 0.11	115 ± 5 p = 0.19
P4	9.8 ± 0.1 p = 0.41	119 ± 3* p < 0.001	9.4 ± 0.1* p < 0.05	113 ± 4 p = 0.31

¹ All studies were performed in the indicated cell lines that either overexpress (GP-2.3) or endogenously produce (SGS-72) PTHR1. pEC₅₀, E_{max}, and error (±s.e.m.) were calculated by nonlinear fitting (three parameters logistic fit) in GraphPad. Values are from at least four biological replicates. P values were calculated using one-way ANOVA followed by a Dunnett's post-test. Statistically significant differences are denoted by *.

Overall, there was limited impact of the O-GlcNAcylation modification on PTH-mediated cAMP responses, with only small reductions in potency observed, in a peptide- and cell-specific manner. However, similar to what we observed with GLP-1 modifications, the most robust effect was an enhancement of E_{max}, particularly in the case of the GP-2.3 model.

O-GlcNAc modification inhibits PTH serum degradation while maintaining its in vivo function

To determine if the O-GlcNAc modified PTH analogs could also enhance serum stability, we selected one of the analogs, P2 for further characterization. P2 was selected as it displayed

the best potency compared to unmodified PTH in the endogenous PTHR1 expression-model (SGS-72, **Table 2**).

Thus, either **P2** or PTH was incubated with human serum for 48 hours (n = 3 for each peptide) *ex vivo*. As with the GLP-1 peptides, aliquots of the reaction mixture were removed after different lengths of time and analyzed by RP-HPLC and peptide half-lives were calculated. Strikingly, we found that **P2** was approximately twice as stable as unmodified PTH, an increase in stability very similar to that of **G1** compared to GLP-1.

We next tested these two peptides *in vivo* measuring blood Ca^{2+} levels in mice, which increase upon PTH administration through release of calcium from bone. Specifically, either PTH or **P2** was injected subcutaneously into the tail vein at a dose of 20 nmol/kg. We then analyzed the ionizable Ca^{2+} levels from blood that was withdrawn immediately before injection and at various time points after injection.

As expected, we observed an increase in blood Ca^{2+} levels upon administration of PTH. However, despite the increased serum stability of **P2**, we did not observe an additional increase in blood Ca^{2+} over PTH. Nevertheless, these data indicate that O-GlcNAcylation can function as a general stabilizer of peptides in serum.

Structure-based modeling of the modified peptides to PTHR1 suggests the molecular determinants of binding and signaling bias. Analogous to the work described above for the effect of O-GlcNAc on GLP-1R binding by peptide **G1**, we used molecular modeling based on the high resolution crystal structure of

ePTH, a peptide agonist with 79% sequence homology to PTH, in complex with the full length PTH1R (2.5 Å).⁴⁸

First, we generated a model of **P2** in complex with PTH1R and we found that the O-GlcNAc moiety introduced at Met18 can be accommodated in the proximity of the stalk region spanning between the 7TM domain and the ECD (170-179 residues). The optimized conformation of the O-GlcNAc moiety is accommodated in the receptor model even without conformational changes in the protein or peptide backbone. Moreover, there is a crystallographic water molecule located in the proximity of residue Met18, which is coordinated by Glu22 and Agr25 of ePTH, and might also form an additional hydrogen bond to the O-GlcNAc moiety.

Taken together, the model suggests that any observed increase in the maximal signaling from **P2** can be explained by the shape complementarity and polar contacts with the PTH1R residues N176 and E180 in the stalk region as well as overall increased rigidity of the complex (Figs. 4C and 4D).

DISCUSSION

Inspired by our studies showing that O-GlcNAcylation of the much larger α -synuclein (140 amino acid residues) inhibits its proteolytic degradation¹², we report here that this property is portable to the much shorter peptide hormones GLP-1, PTH and others having up to about 50 amino acid residues. More specifically, we first found that modification of GLP-1 at S18 (peptide **G1**) had no detrimental effects on the ability of GLP-1 to engage its receptor in living cells and initiate the

physiologically-relevant signaling pathways (Fig. 1A-1F).

However, this monosaccharide does indeed slow the degradation of GLP-1 in human serum *in vitro* by 2-fold compared to the unmodified peptide (Fig. 2A). Although this level of stabilization is at first glance modest, O-GlcNAcylation significantly improves glucose disposal *in vivo* (Fig. 2B), a property that we attribute to its increased circulation time. Remarkably, the increased stability of GLP-1 extends to the major cleavage of the peptide at residue 8 by the protease DPP4 that is 10 amino acid residues away from the site of O-GlcNAc modification, mirroring our α -synuclein results.

We believe that this "remote stabilization" property of O-GlcNAc will make it significantly more flexible in its application than other strategies that target the specific site of proteolysis. For example, the N-terminus of GLP-1 is essential for signaling, and previous strategies that directly stabilize the amide bond at residue 8^{7,27} or use larger hydrophilic groups²⁸ can result in reduced signaling potency.

This flexibility of remote stabilization by O-GlcNAc renders it applicable to other peptide hormones or even proteins, as the modification would likely be placed in a solvent-exposed position distant from the proteolytically-labile residues that are also key for receptor binding. This is being currently explored.

In addition to this single modification of GLP-1 by O-GlcNAc, we also explored the potential for additional effects when glycosylation was combined with backbone modifications. Serendipitously, two

AIB substitutions at residues 8 and 16 of **G1**, producing **G4**, resulted in fairly large biased agonism towards cAMP accumulation and away from Ca^{2+} mobilization and arrestin recruitment (Figs. 1B, 1E and 1F). This result demonstrates that O-GlcNAc can be combined with other strategies, including other natural and unnatural amino acids, to further improve the clinical properties of peptides. Furthermore, because O-GlcNAc is a native modification of either intracellular proteins it has a low potential to elicit immune responses.

Given these interesting biological results and potential clinical applications, we next used molecular modeling to examine how O-GlcNAcylation in **G1** affected binding to the GLP-1 receptor and found that this modification was mostly solvent exposed, as hypothesized. In this model, the N-terminus of **G1** can adopt a conformation similar to the unmodified peptide consistent with our cell-based results that show very little changes in signaling between GLP-1 and C1 (Figs. 1A-1F).

We also wanted to explore the basis of **G4**'s signaling profile in our cell assays and again used molecular modeling. Strikingly, we found that an AIB substitution at residue 8 in GLP-1 clashed with F367^{5.56} of the receptor in a manner similar to that of our previous Cryo-EM structure of GLP-1R in complex with the biased agonist Exendin-P5²⁶, which is consistent with the cellular signaling profile of **G4** (Figs. 1A-1F). We hypothesize that the positioning of O-GlcNAc in a cleft of GLP-1R, and potentially the specific interactions around the 2-N-acetate, rigidify GLP-1 with respect to the receptor and allow

it to "push harder" against helix VI than either G1 or the AIB substitutions alone.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's Modified Eagles Medium (DMEM), Fluo-4 acetoxymethyl ester and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA). Polyethylenimine (PEI) was purchased from Polysciences (Warrington, PA). AlphaScreen® reagents, LANCE® cAMP reagents and 384-well ProxiPlates™ were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). SureFire® extracellular signal-regulated kinases 1 and 2 (ERK1/2) and protein kinase B (Akt) reagents were obtained from TGR Biosciences (Adelaide, SA, Australia). Coelenterazine H was purchased from Nanolight Technology (Pinetop, AZ). GLP-1(7-37) was purchased from American Peptide (Sunnyvale, CA).

All solvents and reagents were purchased from commercial sources (Sigma-Aldrich, Fluka, EMD, Novagen, etc.) and used without any further purification. All aqueous solutions were prepared using ultrapure laboratory grade water (deionized, filtered, and sterilized) obtained from an in-house ELGA® water purification system and filter sterilized with 0.45 µm syringe filters (VWR) before use.

Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed using an Agilent Technologies 1200 Series HPLC instrument with a diode array detector. Unless otherwise stated, the following HPLC buffers were used: buffer A, 0.1% trifluoroacetic acid (TFA) in H₂O; buffer B, 0.1% TFA

and 90% acetonitrile (ACN) in H₂O. Mass spectra were acquired on an API 3000 LC/MS-MS system (Applied Biosystems/MDS SCIEX).

Peptide Syntheses

All peptides were synthesized using standard Fmoc solid-phase chemistry on Rink amide ChemMatrix® (PCAS BioMatrix, 0.45 mmol/g) resin using HBTU (5 equiv, Novabiochem) and DIEA (10 equiv, Sigma) in DMF for 1 hour. For activated glycosylated amino acids, 2 equivalents of monomer in 3 mL of DMF was coupled overnight (about 18 hours). When peptides were completed, acetyl groups were deprotected with hydrazine monohydrate [80% (v/v) in MeOH] twice for 30 minutes with mixing. All peptides were then cleaved using (95:2.5:2.5 TFA/H₂O/triisopropylsilane) for 3.5 hours at room temperature, precipitated out of cold ether, and purified by reverse-phase HPLC using preparative chromatography. All peptides were characterized by mass analysis using ESI-MS, and the sequence purity was assessed by analytical HPLC.

Circular Dichroism Spectroscopy

Circular dichroism spectra were recorded on a Jasco J-815 CD spectrometer. Peptides were freshly diluted to a final concentration of 50 µM in 10 mM phosphate buffer (pH 7.4) prior to sample measurement. Spectra were recorded from 250 to 190 nm with a 0.1 nm data pitch, a 50 nm min⁻¹ scanning speed, a 4 second data integration time, a 1 nm bandwidth, and a 1 mm path length with 3 accumulations, at 25°C. All data were background-subtracted from a sample containing only phosphate buffer.

Cell Culture

Chinese Hamster Ovary (CHO) FlpIn cells stably transfected with an N-terminally double myc labeled human GLP-1R (CHO FlpIn huGLP-1R) were generated using Gateway technology (Invitrogen) as previously described²⁹. These cells were used for all studies, with the exception of β -arrestin recruitment assays, for which CHO FlpIn parental cells were transiently cotransfected with C-terminally Rluc8 labeled huGLP-1R and C-terminally YFP labeled β -arrestin-1 or β -arrestin-2. For transient transfections, cells were seeded at 3×10^6 cells per 10 cm dish or prepared in a 10 mL FBS-enriched DMEM suspension at a density of 3×10^6 cells, and transfected using a 1:6 ratio of DNA:PEI, prepared in 150 mM NaCl for 15 minutes room temperature (RT) and then added to cells. Cells transfected in suspension were then immediately seeded at a density of 30,000 cells/well into 96-well white culture plates. In all cases, total DNA transfected did not exceed 5 μ g, with receptor: β -arrestin transfected at a ratio of 1:4. All cells were maintained in DMEM supplemented with 5% FBS in a humidified environment at 37°C in 5% CO₂.

GLP-1 cAMP Accumulation Assay

CHO FlpIn huGLP-1R cells were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO₂, and peptide-mediated cAMP generation was carried out using the LANCE[®] cAMP protocol (PerkinElmer) as described previously¹⁴. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data subsequently normalized to the response elicited by 10 μ M forskolin.

Intracellular Ca^{2+} Mobilization Assay

CHO FlpIn huGLP-1R cells were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated overnight (about 18 hours) at 37°C in 5% CO_2 , and peptide-mediated intracellular Ca^{2+} mobilization was determined as described previously¹⁷. The peak value following peptide addition was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP.

GLP-1 ERK1/2 and Akt Phosphorylation Assays

CHO FlpIn huGLP-1R cells were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated overnight (about 18 hours) at 37°C in 5% CO_2 , and peptide-mediated ERK1/2 and Akt phosphorylation was determined using the AlphaScreen® SureFire® protocol (PerkinElmer) as described previously²⁹. Initial phosphorylation studies were performed over a 1 hour time course to determine the peak phosphorylation time point. Cells were stimulated with peptide at the time required to cause a maximal response (pERK1/2, 6 minutes; pAkt, 5 minutes). Data were normalized to the response of 10% FBS at 6 minutes (pERK1/2) or 4 minutes (pAkt) (peak FBS response).

GLP-1 β -Arrestin Recruitment Assay

Twenty-four hours following transient transfection, CHO FlpIn cells expressing the huGLP-1R and β -arrestin-1 or β -arrestin-2 were seeded from 10 cm dishes into 96-well white culture plates at a density of 30,000 cells/well, and incubated for a further 24 hours at 37°C in 5% CO_2 prior to assay. CHO FlpIn cells transiently expressing the huGLP-1R and β -arrestin-1 or β -arrestin-2 using the suspension

method were incubated for 48 hours at 37°C in 5% CO₂ prior to assay.

Peptide-mediated recruitment of β -arrestin-1 or β -arrestin-2 was conducted using BRET, as previously described²¹. Initial recruitment studies were performed over a 15 minute time course to determine the peak BRET signal. Subsequent studies were performed at the time required to cause a maximal BRET response (β -arrestin-1, 3 minutes; β -arrestin-2, 2.5 minutes). Data were corrected for vehicle-treated cells (Hank's buffered salt solution).

Data Analysis

All data were analyzed using Prism 7.0 (GraphPad Software Inc., San Diego, CA). For all analyses, the data were unweighted and each y value (mean of replicates for each individual study) was considered an individual point. Concentration response signaling data were analyzed with a three-parameter logistic equation as described previously²³:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log EC_{50} - \log[A])}}$$

(Equation 1)

where "Bottom" represents the y value in the absence of ligand(s), "Top" represents maximal stimulation in the presence of ligand(s), "[A]" is the molar concentration of agonist and "EC₅₀" is the molar concentration of ligand required to generate a response halfway between Top and Bottom. pERK1/2 concentration response signaling data were also analyzed with a biphasic dose response model:

$$Y = Bottom + \frac{(Top - Bottom)(Frac)}{1 + 10^{(LogEC_{50(I)} - \log[A])nH_I}} + \frac{(Top - Bottom)(1 - Frac)}{1 + 10^{(LogEC_{50(II)} - \log[A])nH_{II}}}$$

(Equation 2)

where the parameters are as listed above and $LogEC_{50(I)}$ and $LogEC_{50(II)}$ are the midpoint potencies for the phases 1 and 2, respectively, " nH_I " and " nH_{II} " are their respective Hill slopes, and "Frac" is the more potent fraction of the curve.

Glucose Tolerance Test

Male C57BL/6J mice (Jackson Laboratory) were tested between 10 and 12 weeks of age. Mice were group-housed on a 12:12 light-dark cycle in a temperature-controlled environment with free access to standard chow diet and water. Mice were divided into groups (n= 5) and fasted 18 hours (overnight). Mice were injected intraperitoneally with compound 20 minutes prior to intraperitoneal injection of glucose bolus at 1g per kilogram body weight. Blood glucose level was monitored at the indicated time points using an AphaTRAK2 glucose meter Zoetis Services LLC (Parsippany, New Jersey). All mouse studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Human Serum Stability Assay

Stock solutions of the peptides (500 μ M in PBS, pH 7.4) and reconstituted normal human serum (Thermo Scientific) were pre-equilibrated to 37°C for 15 minutes in separate tubes. For each replicate, peptides and serum were combined to a final concentration of 50 μ M and 50% (v/v) respectively, final volume of 150 μ L. The reaction tubes were then

incubated at 37°C with 300 rpm shaking in an Eppendorf® ThermoMixer®.

At each time point, 20 µL of the reaction was taken from each replicate and diluted with four-fold excess of cold acetone. The serum proteins were allowed to precipitate for 15 minutes at -20°C and were removed by centrifugation at 14,000 g, 4°C for 10 minutes. The supernatant was transferred to a new tube, diluted with the same volume of water, and were stored at -20°C until HPLC analysis.

Chromatographic separations were performed on a C18 reversed phase analytical column (Higgins Analytical, 150x4.6 mm, 5 µM pore size) using a 30-50%B linear gradient over 30 minutes. Solvent A consisted of 0.1% (v/v) TFA in water, and solvent B contained 90% acetonitrile, 0.1% TFA in water. Peak areas corresponding to the intact peptide (as confirmed by MALDI-TOF) were obtained by manual integration using Agilent OpenLab CDS ChemStation.

PTH cAMP Accumulation Assay

Agonist activity at the human PTHR1 was assessed using HEK-293-derived cell lines that stably express the Glosensor cAMP reporter (Promega Corp.) along with wild-type human PTHR1 (GP-2.3 cells). The cells were cultured in 96-well plates and treated 24-48 hours after reaching confluency. Cells were pre-incubated with CO₂ independent media (Life Sciences) containing d-luciferin (0.5 mM) in 96-well plates at room temperature until a stable baseline level of luminescence was established (30 minutes). Then, varying concentrations of test ligands were added, and the time course of luminescence response was recorded using a Perkin Elmer plate reader following peptide addition.

The maximal luminescence response (observed about 10-30 minutes after ligand addition) was expressed as per-cent of the maximum response observed for used for generating dose response curves. Reported EC₅₀ values are the average of 4 independent studies, each in duplicate. Data were fitted to a sigmoidal dose-response model with variable slope.

In Vivo Calcemic Response

Mice (CD1, female, age 11 weeks) were treated in accordance with the ethical guidelines adopted by Massachusetts General Hospital. Mice (n = 5 per compound) were injected subcutaneously with vehicle (10 mM citric acid/150 mM NaCl/0.05% Tween®-80, pH 5.0) containing PTH(1-34) or modified analog each at a dose of 20 nmol/kg body weight. Tail vein blood was withdrawn just prior to injection (t = 0) and at times thereafter and immediately measured for Ca²⁺ concentration using a Siemens RapidLab 348 Ca²⁺/pH analyzer.

Molecular Modeling

The GLP-1R-G1 and GLP-1R-G4 models were created based on Cryo-EM structure of GLP-1R-GLP-1 complex (PDB ID: 5VAI)²⁴. The N-terminal 1-28 residues of the GLP-1R are not resolved in the structural template, and likely highly flexible and were not modeled. The residue R380 side chain, unresolved in the structure was modeled and additional minimization of the model at the tip of helix VII, where R380 is located was performed to restore the known key interaction between D15 of GLP-1 and R380 of GLP-1R²⁵.

The G1 and G4 peptides were prepared by adding O-GlcNAc modification, and two 2-amino-

isobutyric acid (AIB) residues to the GLP1, followed by extensive energy-based conformational optimization of the GLP-1R-**G1** and GLP-1R-**G4** complexes in ICM3.8-Pro molecular modeling software (Molsoft LLC, San Diego). Optimization runs were performed for all peptide side chains and receptor side chains located 5 Å from the peptide. The GLP-1R-**G4** model was compared to the Cryo-EM structure of GLP-1R complex with a bias agonist Exendin-P5 (PDB ID 6B3J)²⁶.

The PTH1R-**P2** model was generated based on high resolution structure of PTH1R-ePTH complex (PDB: 6FJ3)²⁶. The **P2** peptide was generated by mutating the stabilizing point mutations in PTH and mutating residue 18 into serine and adding O-GlcNAc modification, respectively. The PTH1R-**P2** complex was submitted to energy based conformational optimization including O-GlcNAc modification and side chains located 5 Å from the peptide. Optimization was performed in ICM3.8-Pro molecular modeling software (Molsoft LLC, San Diego).

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Each of the patents, patent applications and articles cited herein is incorporated by reference.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and

will readily present themselves to those skilled in the art.

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CLAIMS:

1. A synthetic peptide hormone comprising a sequence of about 15 to about 50 amino acid residues that are at least about 90 percent identical to the same length and aligned sequence of a naturally-occurring peptide hormone; each of the synthetic and naturally-occurring peptide sequences exhibiting an amphipathic alpha-helical secondary structure; said synthetic peptide hormone including a monoglycosylated serine or threonine residue in its sequence as a non-identical residue of the synthetic peptide hormone sequence that is present in the sequence of polar face of the helical structure; said synthetic peptide hormone containing said monoglycosylated serine or threonine residue has a half-life in human serum at 37°C of about 25 to about 800 percent longer than the naturally-occurring peptide hormone.

2. The synthetic peptide hormone according to claim 1, wherein each of said synthetic and naturally-occurring peptide hormone peptides is free of a covalent intrachain linkage.

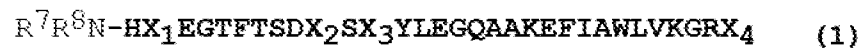
3. The synthetic peptide hormone according to claim 1, wherein each of said synthetic and naturally-occurring peptide hormone is an agonists of a G protein-coupled receptor (GPCR).

4. The synthetic peptide hormone according to claim 1 that comprises a sequence length of about 20 to about 45 residues.

5. The synthetic peptide hormone according to claim 1, wherein said alpha-helical secondary structure is determined by circular dichroism (CD) spectroscopy.

6. The synthetic peptide hormone according to claim 1, wherein said naturally-occurring peptide hormone has the sequence of the glucagon-like peptide-1 GLP-1(7-37) (SEQ ID NO: 6) or GLP-1(7-3)amide .

7. The synthetic peptide hormone according to claim 6, wherein said GLP-1 has the amino acid residue sequence of GLP-1(7-37) or GLP-1(7-36)amide whose amino acid residue sequence, from left to right and from amino-terminus to carboxy-terminus, corresponds to Formula 1 (SEQ ID NO: 1):



where

$X_1 = -NH-(CH_2)_m-CR^1R^2-C(O)-$ or A (Ala);

$X_2 = -NH-(CH_2)_n-CR^3R^4-C(O)-$ or V (Val);

$X_3 = -NH-CH[(CHQ)-O-(Sac)]-C(O)-$;

$X_4 = G$ (Gly), $G-NR^5R^6$ or $-NR^5R^6$;

$m = 0$ or 1 ;

$n = 0$ or 1 ;

$Q = H$ or methyl;

R^1 and R^2 are the same or different substituents that are hydrogen (H) or C_1 - C_3 hydrocarbyl, with the proviso that only one of R^1 and R^2 is hydrogen, or R^1 and R^2 together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R^3 and R^4 are the same or different substituents that are hydrogen (H) or C_1 - C_3 hydrocarbyl, or R^3 and R^4 together with the depicted carbon to which they are bonded form a 5-7-membered ring;

R^5 , R^6 , R^7 and R^8 are the same or different and are H, C_1 - C_{18} hydrocarbyl, C_1 - C_{18} acyl, C_1 - C_{18} hydrocarbylsulfonyl, or either or both of R^5 and R^6 and R^7 and R^8 together with their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R^5 and R^6 and R^7 and R^8 includes a hydrocarbyl, acyl or sulfonyl group that contains more than six carbon atoms; and

Sac is a glycosidically-linked C_5 - C_6 saccharide, deoxysaccharide or 2-(N-acetylamino) C_5 - C_6 saccharide or deoxysaccharide.

8. The synthetic peptide hormone according to claim 7, wherein the saccharide (Sac) of **X₃** is O-GlcNAc, **X₄** is a glycyl residue (G), and n and m are both zero (SEQ ID NO: 2).

9. The synthetic peptide hormone according to claim 8, wherein R^1 , R^2 , R^3 and R^4 are each methyl (SEQ ID NO: 5).

10. The synthetic peptide hormone according to claim 8, wherein the R^1 and R^2 of **X₁** are both methyl, and **X₂** is a valyl residue (V) (SEQ ID NO: 4).

11. The synthetic peptide hormone according to claim 8, wherein X_1 is an alanyl residue (A) and R^3 and R^4 are both methyl (SEQ ID NO: 3).

12. The synthetic peptide hormone according to claim 1, wherein said naturally-occurring peptide hormone has the sequence of the parathyroid hormone (PTH) peptide whose sequence is shown below, as Formula 2 and SEQ ID NO: 7, from left to right and in the direction from amino-terminus to carboxy-terminus:



(SEQ ID NO: 7)

wherein

$-X_1-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or H (His);

$-X_2-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or M (Met);

$-X_3-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or E (Glu);

$-X_4-$ = $-NH-CH[(CH_3)_n(CH_p)O-(Sac)]-C(O)-$ or K (Lys);

$-X_5$ = F (Phe) or $F-NR^3R^4$, with the proviso that only one of $-X_1-$, $-X_2-$, $-X_3-$ and $-X_4-$ is other than the stated amino acid residue; i.e., His, Met, Glu and Lys;

$n = 0$ or 1 and $p = 1$ or 2 , with the proviso that when $n = 1$, $p = 1$, and when $n = 0$, $p = 2$;

R^1 , R^2 , R^3 and R^4 are the same or different and are H, C_1 - C_{18} hydrocarbyl, C_1 - C_{18} acyl (hydrocarobyl), C_1 - C_{18} hydrocarbylsulfonyl, or either or both of R^1 and R^2 and R^3 and R^4 together with

their depicted nitrogen atoms forms a 5-7-membered ring that optionally contains 1 or 2 additional hetero atoms that independently are nitrogen, oxygen or sulfur, with the proviso that only one of R¹, R², R³ and R⁴ includes a hydrocarbyl, acyl or sulfonyl group that contains more than six carbon atoms; and

Sac is a glycosidically-linked C₅-C₆ saccharide, deoxysaccharide or 2-(N-acetylamino) C₅-C₆ saccharide.

13. The synthetic peptide hormone according to claim 6, wherein said glycosidically-linked C₅-C₆ saccharide, deoxysaccharide or 2-(N-acetylamino) C₅-C₆ saccharide is selected from the group consisting of GlcNAc, GalNAc, ribose, deoxyribose, mannose, glucose and galactose.

14. The synthetic peptide hormone according to claim 13, wherein said glycosidically-linked 2-(N-acetylamino) C₅-C₆ saccharide is GlcNAc.

15. The synthetic peptide hormone according to claim 12 that is one or more of:

P1 SVSEIQLMHNLGKX₁LNSMERVEWLRKKLQDVHNF

(SEQ ID NO: 8);

P2 SVSEIQLMHNLGKHLNSX₂ERVEWLRKKLQDVHNF

(SEQ ID NO: 9);

P3 SVSEIQLMHNLGKHLNSMERVX₃WLRKKLQDVHNF

(SEQ ID NO: 10); and

P4 SVSEIQLMHNLGKHLNSMERVEWLRKX₄LQDVHNF

(SEQ ID NO: 11).

16. A composition containing an agonistic effective amount of a synthetic peptide hormone according to claim 1 dissolved or dispersed in a pharmaceutically acceptable carrier.

17. The pharmaceutical composition according to claim 16, wherein said synthetic peptide hormone is a GLP-1 derivative peptide of Formula 1 (SEQ ID NO: 1) that is present in an IR- and/or GLP-1R- agonist effective amount dissolved or dispersed in a pharmaceutically acceptable carrier.

18. The pharmaceutical composition according claim 17, wherein said GLP-1 derivative peptide of Formula 1 is a peptide of SEQ ID NO: 2.

19. The pharmaceutical composition according claim 17, wherein said GLP-1 derivative peptide of Formula 1 is a peptide of SEQ ID NO: 5.

20. The pharmaceutical composition according to claim 16, wherein said synthetic peptide hormone is a PTH derivative peptide of Formula 2 (SEQ ID NO: 8) dissolved or dispersed in a pharmaceutically acceptable carrier.

21. The pharmaceutical composition according claim 20, wherein said PTH derivative peptide of Formula 2 is a peptide of SEQ ID NO: 10.

22. A method of treating a mammalian G protein-coupled receptor-mediated [GPCR-mediated] condition of a mammal that comprises administering an agonistic effective amount of a synthetic peptide

hormone according to claim 1 dissolved or dispersed in a pharmaceutically acceptable carrier to that mammal having that GPCR-mediated condition.

23. The method of treating according to claim 22, wherein said administration is repeated as needed to maintain said mammal.

24. The method of treating according to claim 22, wherein said GPCR-mediated condition is type-2 diabetes that comprises administering said pharmaceutical composition contains an IR- or GLP-1R- agonist effective amount of a peptide of Formula 1 (SEQ ID NO: 1).

25. The method of treating according to claim 24, wherein said administration is repeated as needed to maintain said mammal.

26. The method of treating according to claim 22, wherein said synthetic peptide hormone is a peptide of SEQ ID NO: 8.

27. The method of treating according to claim 26, wherein said administration is repeated as needed to maintain said mammal.

Fig. 1

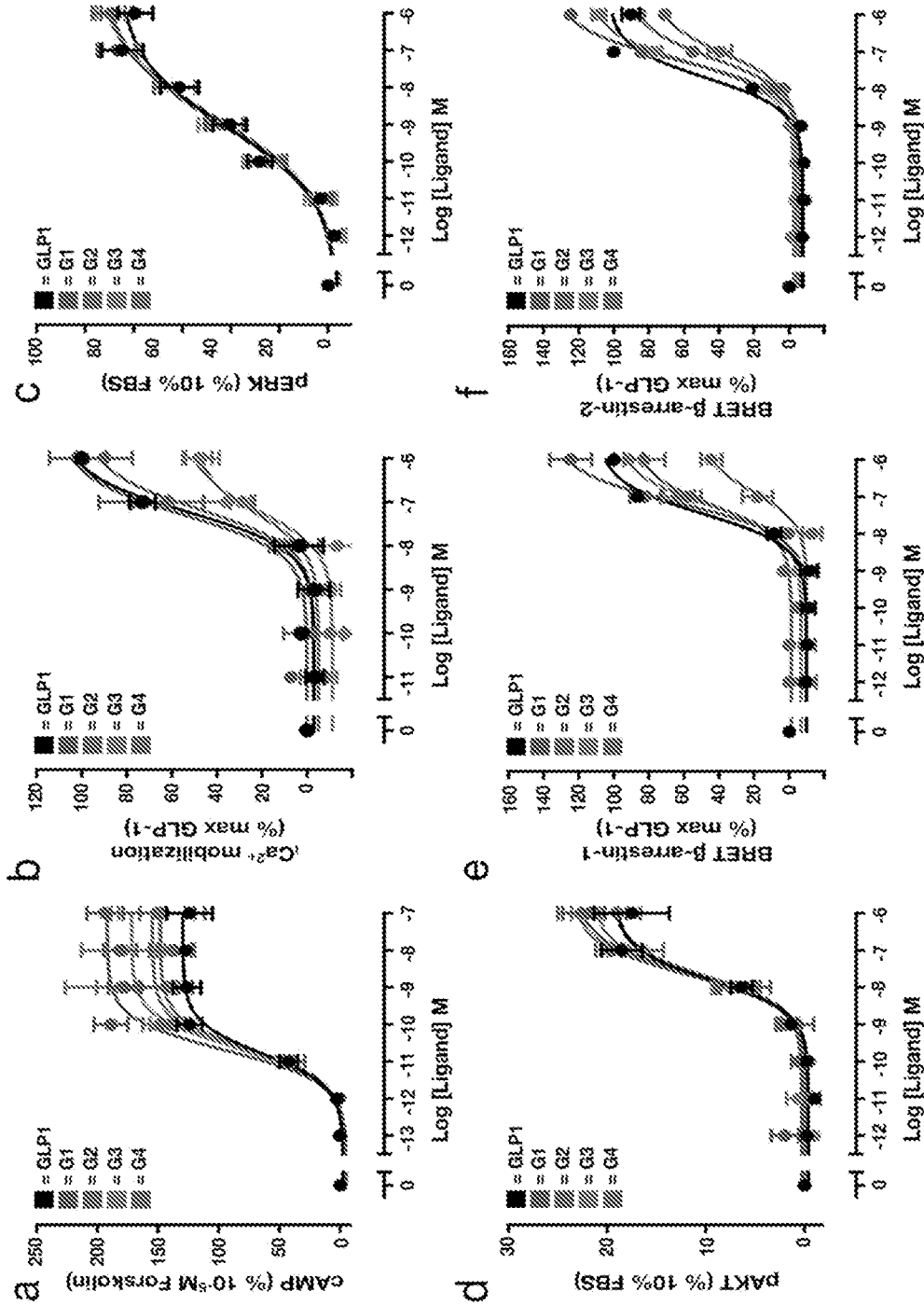


Fig. 2

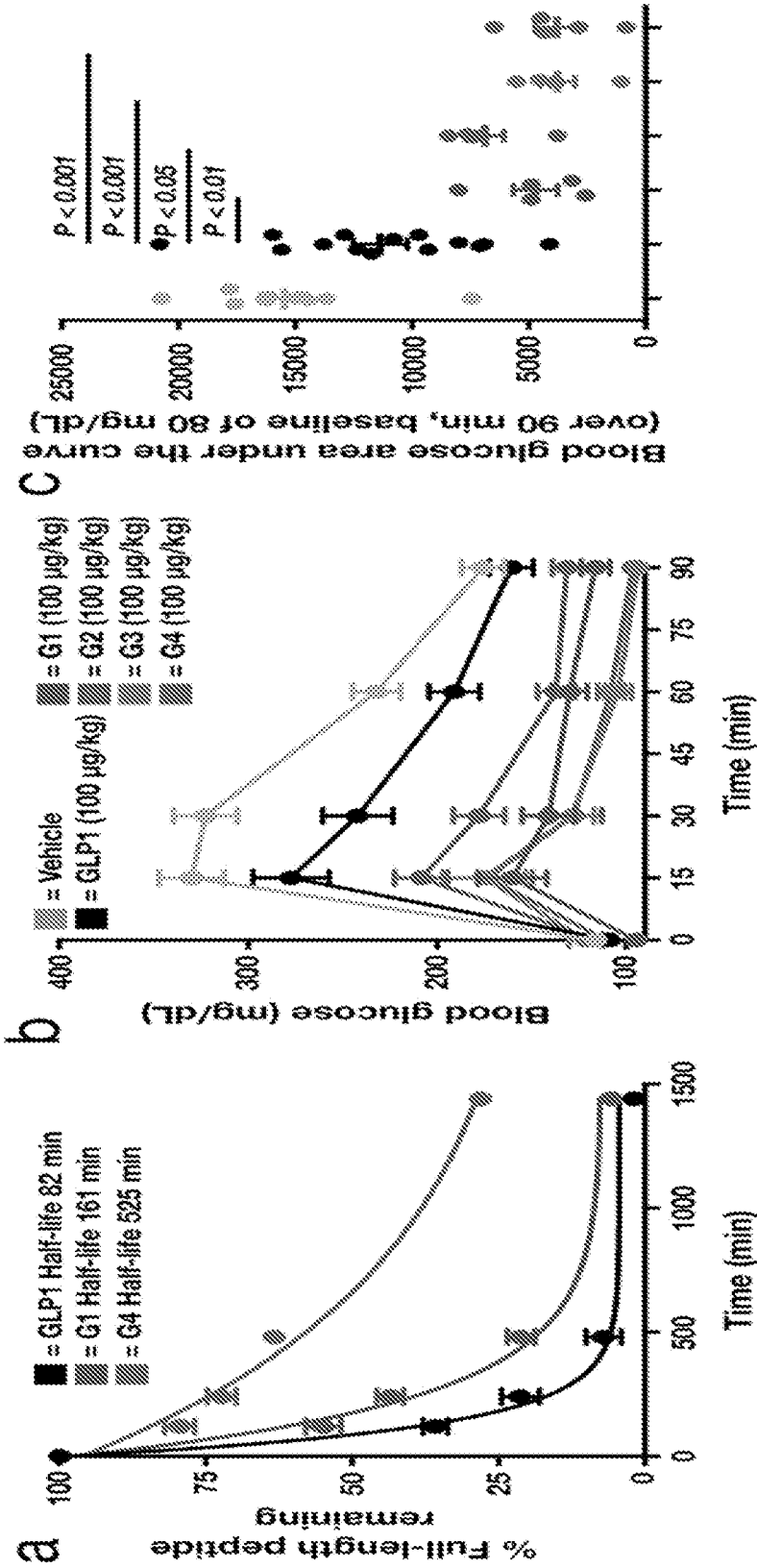


Fig. 3

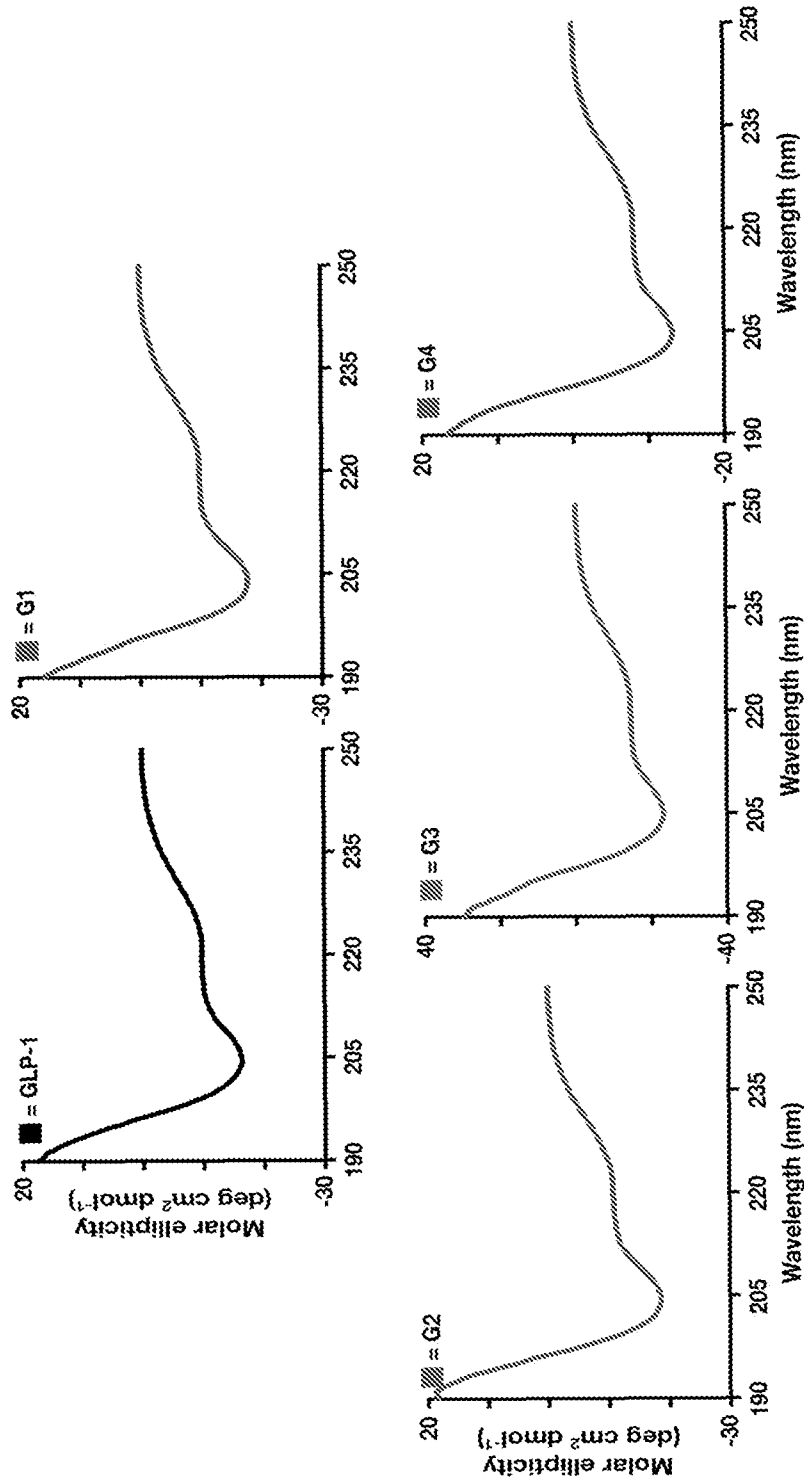


Fig. 4

Fig. 4A

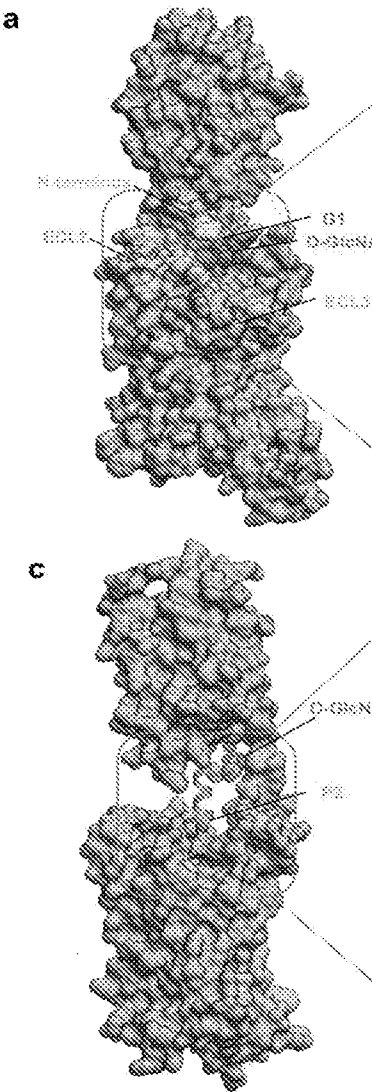


Fig. 4 B

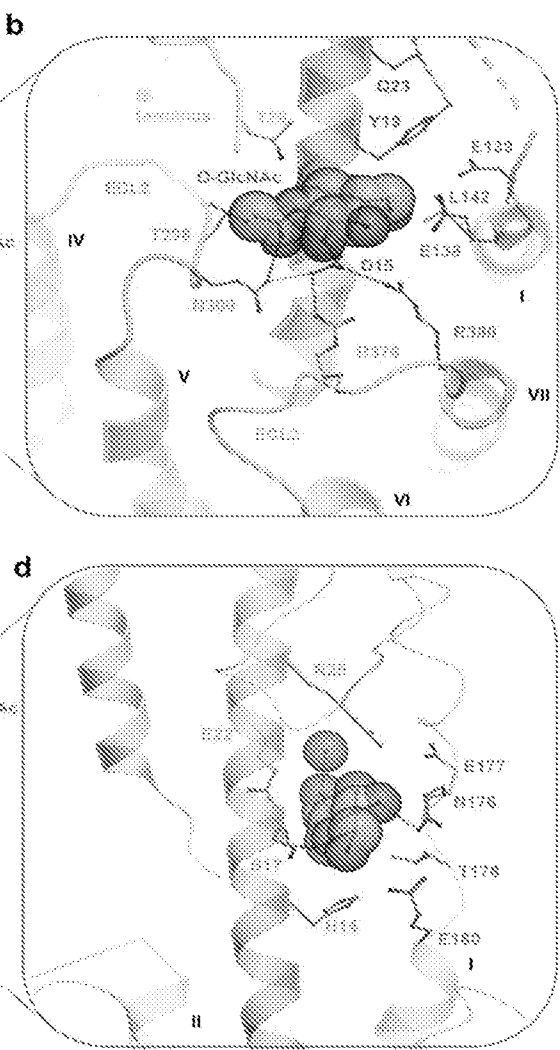


Fig. 4C

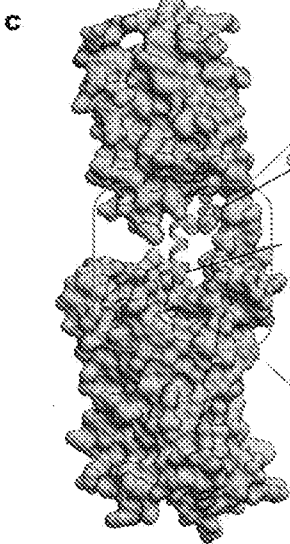


Fig. 4

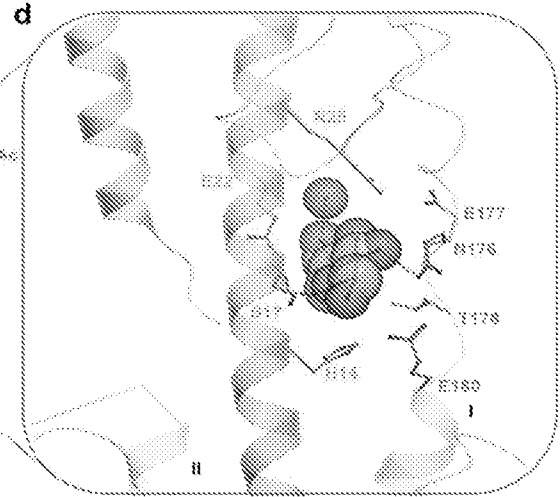


Fig. 5

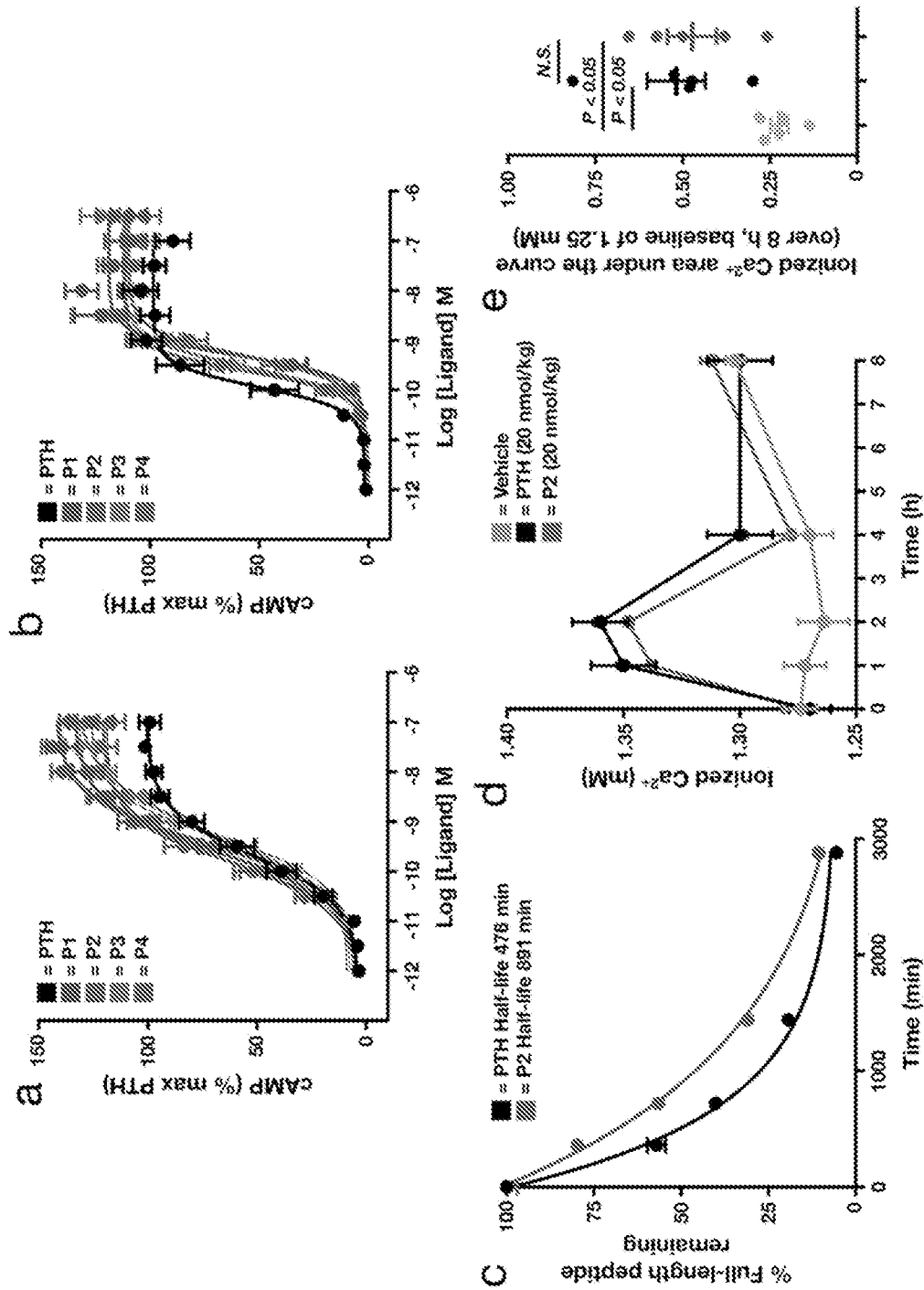
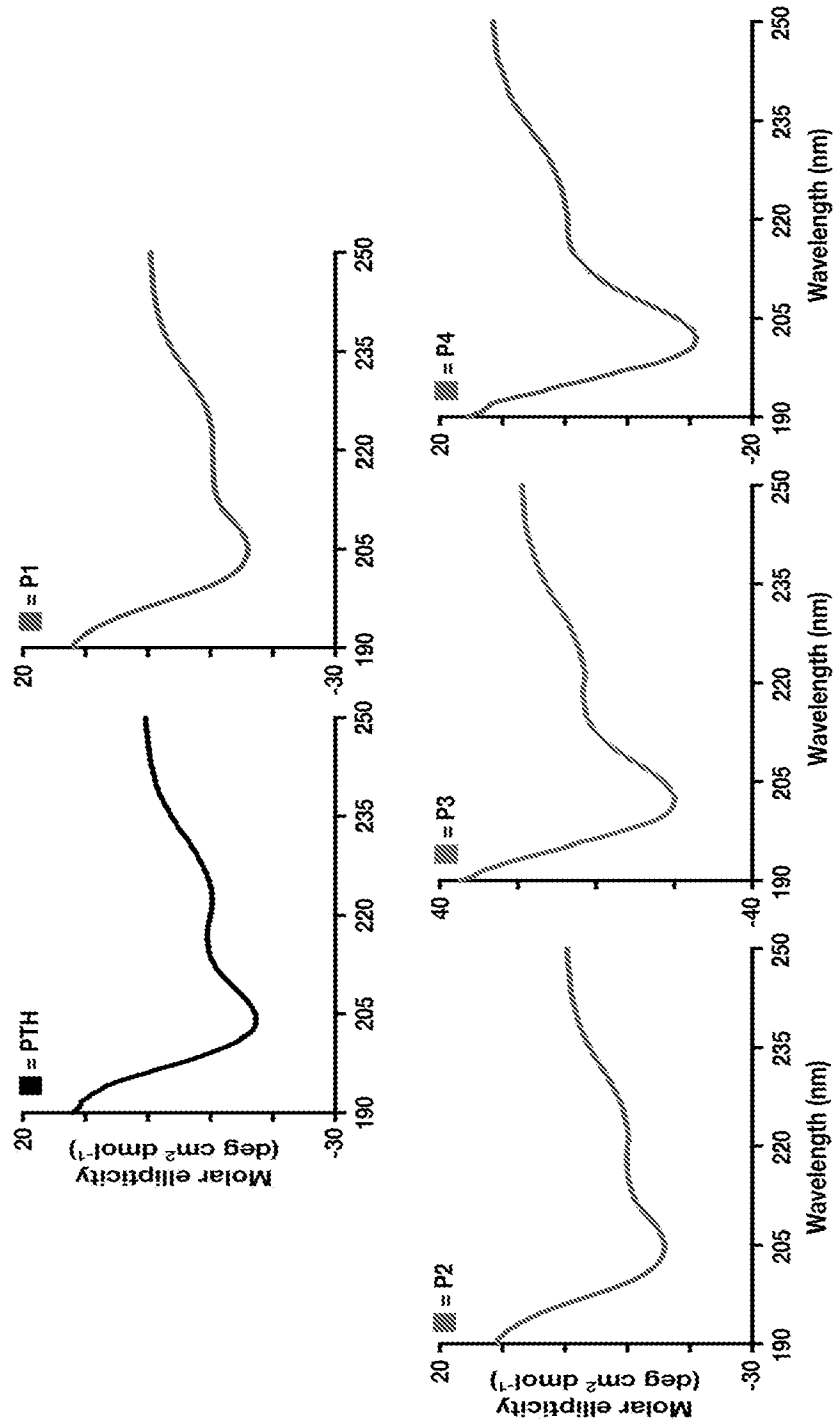


Fig. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/041049**A. CLASSIFICATION OF SUBJECT MATTER****C07K 14/605(2006.01)i, C07K 14/635(2006.01)i, A61K 38/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/605; C07K 14/635; C07K 7/08; A61K 38/00

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: glucagon-like peptide-1(GLP-1), parathyroid hormone(PTH), O-GlcNAc, glycosylation, GPCR, polar face, alpha-helix, serine, threonine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANANDHAR, B. et al., 'Glucagon-like Peptide-1 (GLP-1) Analogs: Recent Advances, New Possibilities, and Therapeutic Implications', J. Med. Chem., 2015, vol. 58, pp. 1020-1037 abstract; pages 1020-1024; table 2; figures 1-4	1-21
Y	US 2010-0016547 A1 (ITO, T. et al.) 21 January 2010 abstract; paragraphs [0018], [0032]-[0039]; claims 1-12	1-21
Y	US 2014-0228293 A1 (DANISHEFSKY, S. J. et al.) 14 August 2014 abstract; paragraphs [0016], [0026], [0080]-[0082], [0098], [0116]; claims 1-31; figure 1	12-15, 20-21
A	DE LEON, C. A. et al., 'The Sulfur-Linked Analogue of O-GlcNAc (S-GlcNAc) Is an Enzymatically Stable and Reasonable Structural Surrogate for O-GlcNAc at the Peptide and Protein Levels', Biochemistry, 11 July 2017, vol. 56, no. 27, pp. 3507-3517, HHS Public Access Author manuscript version internal pages 1-23 the whole document	1-21

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

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

19 October 2020 (19.10.2020)

Date of mailing of the international search report

19 October 2020 (19.10.2020)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

Facsimile No. +82-42-481-8578

Authorized officer

Jung, Da Won

Telephone No. +82-42-481-5373



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/041049

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	LEVINE, P. M. et al., 'O-GlcNAc Engineering of GPCR Peptide-Agonists Improves Their Stability and in Vivo Activity', J. Am. Chem. Soc., 16 August 2019, vol. 141, pp. 14210-14219 the whole document	1-21

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2020/041049**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☒ Claims Nos.: 22-27
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 22-27 pertain to a method for treatment of the human body by therapy or surgery, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2020/041049

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