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(54) **Title**: LONG-ACTING GLP-1/GLUCAGON RECEPTOR AGONISTS

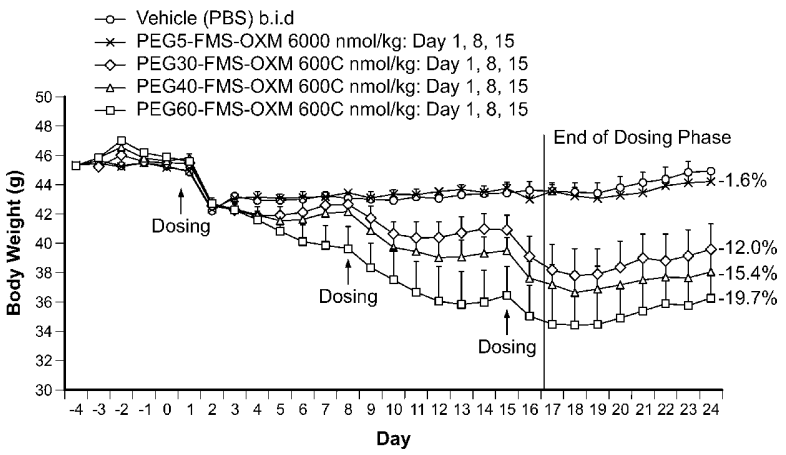

FIG. 13

(57) **Abstract**: Pegylated and reverse pegylated GLP-l/Glucaron receptor agonists including pharmaceutical compositions comprising the same and methods of using the same are disclosed.

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LONG-ACTING GLP-1/GLUCAGON RECEPTOR AGONISTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from United States Provisional Application Serial Number 61/492,448, filed June 2nd, 2011, and United States Provisional Application Serial Number 61/624,589, filed April 16th, 2012. These applications are hereby incorporated in their entirety by reference herein.

FIELD OF INVENTION

[0002] Pegylated and reverse pegylated oxyntomodulin including pharmaceutical compositions comprising the same and methods of using the same are disclosed.

BACKGROUND OF THE INVENTION

[0003] Proteins and especially short peptides are susceptible to denaturation or enzymatic degradation in the blood, liver or kidney. Accordingly, proteins typically have short circulatory half-lives of several hours. Because of their low stability, peptide drugs are usually delivered in a sustained frequency so as to maintain an effective plasma concentration of the active peptide. Moreover, since peptide drugs are usually administered by infusion, frequent injection of peptide drugs cause considerable discomfort to a subject. Thus, there is a need for technologies that will prolong the half-lives of therapeutic proteins and peptides while maintaining a high pharmacological efficacy thereof. Such desired peptide drugs should also meet the requirements of enhanced serum stability, high activity and a low probability of inducing an undesired immune response when injected into a subject.

[0004] Unfavorable pharmacokinetics, such as a short serum half-life, can prevent the pharmaceutical development of many otherwise promising drug candidates. Serum half-life is an empirical characteristic of a molecule, and must be determined experimentally for each new potential drug. For example, with lower molecular weight protein drugs, physiological clearance mechanisms such as renal filtration can make the maintenance of therapeutic levels of a drug unfeasible because of cost or frequency of the required dosing regimen.

[005] The gastrointestinal tract is responsible on synthesize and releasing of many peptide hormones that regulate eating behavior including pancreatic protein (PP), glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and Oxyntomodulin (OXM). OXM arises from a tissue-specific post-transitional processing of proglucagon in the intestine and the CNS. It contains 37 amino acids, including the complete glucagon sequence with a C-terminal basic

octapeptide extension that was shown to contribute to the properties of OXM both in-vitro and in-vivo but was not alone sufficient for the effects of the peptide. In response to food ingestion, OXM is secreted by intestinal L cells into the bloodstream proportionally to the meal caloric content.

[006] OXM enhances glucose clearance via stimulation of insulin secretion after both oral and intraperitoneal administration. It also regulates the control of food intake. Intracerebroventricular (ICV) and intranuclear injection of OXM into the paraventricular and arcuate nuclei (ARC) of the hypothalamus inhibits re-feeding in fasting rats (Dakin et al. 2001; Dakin et al. 2004). This inhibition has also been demonstrated in freely fed rats at the start of the dark phase. Moreover, peripheral administration of OXM dose-dependently inhibited both fast-induced and dark-phase food intake (Dakin et al. 2004).

[007] New conceptual approach termed reversible pegylation was previously described (PCT Publication No. WO 98/05361; Gershonov et al., 2000), for prolonging the half-life of proteins and peptides. According to this technology, prodrugs are prepared by derivatizing the drug with functional groups that are sensitive to bases and removable under mild basic conditions such as physiological conditions. The derivatization includes a substitution of at least one amino, hydroxyl mercapto and/or carboxyl groups of the drug molecule with a linker such as 9-fluorenylmethoxycarbonyl (Fmoc) and 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), to which a group of Polyethylene glycol (PEG) moiety is attached. The link between the PEG moiety and the drug is not direct but rather both residues are linked to different positions of the scaffold FMS or Fmoc structures that are highly sensitive to basic conditions. The present invention relates to OXM derivative in which the half-life of the peptide is prolonged utilizing the reversible pegylation technology.

SUMMARY OF THE INVENTION

[008] In one embodiment, the present invention provides a composition consisting of a dual GLP-1/Glucagon receptor agonist linked or bound to polyethylene glycol polymer (PEG polymer) via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[009] In another embodiment, the present invention further provides a method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering to the subject a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-

fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the linker is a cleavable linker.

[010] In another embodiment, the present invention further provides a method of inducing glucose tolerance, improving glycemic control, or both in a subject in need thereof, comprising the step of administering to the subject an effective amount of a composition consisting of a dual GLP-1/Glucagon receptor agonist linked to polyethylene glycol polymer (PEG polymer) via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) and a pharmaceutical acceptable carrier.

[011] In another embodiment, the present invention further provides a method for reducing insulin resistance in a subject, comprising the step of administering to the subject an effective amount of a composition comprising a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer).

[012] In another embodiment, the present invention further provides a method for extending the biological half life of a GLP-1/Glucagon receptor agonist consisting of the step of conjugating the agonist to polyethylene glycol polymer (PEG polymer) via a flexible linker comprising 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[013] In another embodiment, the present invention further provides a method for extending the biological half life of a dual GLP-1/Glucagon receptor agonist, consisting of the step of conjugating the agonist to polyethylene glycol polymer (PEG polymer) via a flexible linker comprising 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[014] In another embodiment, the present invention further provides a method for improving the area under the curve (AUC) of a GLP-1/Glucagon receptor agonist, consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of the agonist via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[015] In another embodiment, the present invention further provides a method for reducing a dosing frequency of a dual GLP-1/Glucagon receptor agonist, consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[016] In one embodiment, the present invention provides a method for increasing insulin sensitivity in a subject, comprising the step of administering to the subject an effective

amount of a composition comprising a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer). In another embodiment, the present invention provides a method for increasing insulin sensitivity in a subject following acute treatment or chronic treatment, comprising the step of administering to the subject an effective amount of a composition comprising a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer).

[017] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[018] **Figure 1** is a graph showing the pharmacokinetic profile of OXM, PEG_{10}-Fmoc-OXM and PEG_{20}-Fmoc-OXM in male rats. Rats received a single SC bolus injection of native OXM (62nmol/kg), PEG_{10}-Fmoc-OXM (containing 278μg/kg OXM peptide) or PEG_{20}-Fmoc-OXM (containing 62nmol/kg OXM peptide) in 0.5 ml PBS buffer. Serum samples were collected from the jugular vein at specified time points and OXM concentration was analyzed using OXM Elisa kit (Bachem, Switzerland).

[019] **Figure 2** are graphs showing the pharmacokinetic profile of OXM and PEG_{40}-Fmoc-OXM in male rats. Rats received a single IV bolus (A) or SC (B) injection of native OXM (62nmol/kg) or PEG_{40}-Fmoc-OXM (containing 62nmol/kg body weight OXM peptide) in 0.5 ml PBS buffer. Serum samples were collected from the jugular vein at specified time points and OXM concentration was analyzed using OXM Elisa kit (Bachem, Switzerland). The overlay insert highlight the OXM profile which is apparent in the first two hours after administration.

[020] **Figure 3** is a graph showing the in-vitro activity of native OXM, PEG_{40}-Fmoc-OXM and PEG_{40}-EMCS-OXM. CHO-K1 cells over-expressing GLP-1 receptor (Millipore HTS163C2) were seeded in 96 wells half-area white at a density of 200,000 cells/ml and incubated for 24 hours at 37°C. The cells were incubated with escalating concentrations of OXM (ALMAC), PEG40-EMCS-OXM and PEG40-Fmoc-OXM with or without Rat serum 1% (Bio reclamation). Cells cAMP concentrations were quantified by HTRF assay (Cisbio 62AM4PEB) and EC50 parameter was analyzed by PRISM software.

[021] **Figure 4** are graphs showing the induction of glucose tolerance in mice with native OXM and PEG_{40}-Fmoc-OXM and PEG_{40}-EMCS-OXM as measured by IP glucose tolerance test (IPGTT). C57BL/6 Mice were fasted overnight and then injected IP with PBS (vehicle), PEG_{40}-Osu as control (546nmol/kg), native OXM (333nmol/kg), PEG_{40}-Fmoc-OXM (202nmol/kg peptide content) and PEG_{40}-EMCS-OXM (333nmol/kg). Glucose (1.5gr/kg) was administrated IP either 15min after test article administration (vehicle, OXM and PEG_{40}-Osu) or 120 min after PEG_{40}-Fmoc-OXM administration. Blood glucose levels were measured by tail vein sampling prior to glucose administration and 10, 20, 30, 60 and 120 min after glucose administration using a handheld glucometer. Graph (A) provides the blood glucose profile and graph (B) shows the glucose AUC.

[022] **Figure 5** are graphs showing the effect of SC administration OXM (b.i.d) and PEG40-FMS-OXM (days 1, 3, 5, 7) on body weight (A) and cumulative food intake (B) in male C57BL/6J mice exhibiting diet induced obesity. Data are adjusted means (n = 10).

SEMs are calculated from the residuals of the statistical model. Mice were dosed for 7 days started on Day 1. Data analyzed by ANCOVA with body weight on Day 1 as covariate followed by Williams' test (OXM in PBS) or multiple t test (sibutramine and PEG40-FMS-OXM) vs appropriate vehicle. Significant differences vs. appropriate vehicle: *p<0.05, **p<0.01, ***p<0.001. Percentage values indicate difference from appropriate vehicle group on Day 8 (i.e. after 7 days dosing).

[023] **Figure 6** is a graph showing effect of SC administration OXM (b.i.d) and covalently bound pegylated OXM PEG40-EMCS-OXM (1000nmol/kg and 5000nmol/kg on Days 1, 4 and 7 or 8000nmol/kg on Days 1 and 7), PEG40-FMS-OXM (1000nmol/kg and 5000nmol/kg on Days 1, 4 and 7 or 8000nmol/kg on Days 1 and 7) and PEG30-FMS-OXM (5000nmol/kg on Days 1, 4 and 7) on body weight (A) and food intake (B) in male C57BL/6J mice exhibiting diet induced obesity. Data are adjusted means (n = 10). SEMs are calculated from the residuals of the statistical model. Mice were dosed for 7 days started on Day 1.

[024] **Figure** 7 shows the effects of reversible PEGylated OXM Administration on body weight in Diet Induced Obesity (DIO) Mice. During the first week of single housing (handling period), animals began a once-daily handling protocol and during the second week (baseline period), they were dosed with the appropriate vehicle b.i.d. or once a week as they were dosed during the treatment period) by a subcutaneous route. 7 groups (n=8) of DIO mice were dosed for 29 days as follows: A. PEG40-SH (662 mg/kg), B. PEG40-EMCS-OXM (6,000nmol/kg), C. PEG30-EMCS-OXM (6,000nmol/kg), D. PEG40-FMS-OXM (6,000nmol/kg), E. PEG30-FMS-OXM (6,000nmol/kg), F. Vehicle (PBS), and G. OXM (6,000nmol/kg; PBS). During the baseline and the treatment period food intake, water intake and body weight were recorded daily. Weekly administration of PEG40-FMS-OXM or PEG30-FMS-OXM significantly reduced body weight in Diet Induced Obesity (DIO) mice.

[025] **Figure 8** shows the acute effects of reversible PEGylated OXM administration on glucose tolerance in Diet Induced Obesity (DIO) Mice. On day 1 after the start of drug or vehicle administration, all the mice were overnight fasted. On day 2, the mice underwent an oral glucose tolerance test (OGTT). Each animal were dosed with vehicle or test compound and 60 minutes later were dosed with D-glucose (2 g/kg po). Baseline blood samples were taken immediately prior to dosing with vehicle or test compound (B1) and immediately before the glucose load (B2). Further blood samples were taken 10, 20, 30, 45, 60 and 120 minutes post glucose administration. All blood samples (approximately 20μl) were taken from the tail vein. Plasma samples were prepared and assayed for glucose (n = 2) and insulin

(n = 1) using the Thermoelectron Infinity glucose reagent (TR15421) and Alpco mouse ultrasensitive insulin ELISA (80-INSMSU-E10), respectively.

[026] **Figure 9** shows the effects of reversible PEGylated OXM administration on terminal glucose, glycerol, cholesterol and insulin in Diet Induced Obesity (DIO) Mice. Terminal plasma samples were collected (24 hours after the final dose of test or control compound on Day 29) by cardiac puncture and assayed for insulin, glucose and cholesterol using the mouse ultrasensitive insulin ELISA (80-INSMSU-E10), Thermoelectron Infinity glucose reagent (TR15421) and Thermoelectron Infinity cholesterol reagent (TR13421).

[027] **Figure 10** shows the effects of reversible PEGylated OXM administration on terminal body composition analysis of fat, water, protein and ash (bone) in Diet Induced Obesity (DIO) Mice. Body fat (A), water (B), protein (C), and ash levels (D) of DIO mouse carcasses were determined using standard chemical analysis techniques. The treatment groups were as follows: A. PEG40-SH (662 mg/kg), B. PEG40-EMCS-OXM (6,000nmol/kg), C. PEG30-EMCS-OXM (6,000nmol/kg), D. PEG40-FMS-OXM (6,000nmol/kg), E. PEG30-FMS-OXM (6,000nmol/kg), F. Vehicle (PBS), and G. OXM (6,000nmol/kg; PBS).

[028] **Figure 11** shows that administration of PEG-OXM variants PEG40-EMCS-OXM, PEG30-EMCS-OXM, PEG40-FMS-OXM, PEG30-FMS-OXM produced marked and significant reductions in fasting glucose and fasting plasma insulin when compared to controls.

[029] **Figure 12** shows that administration of PEG-OXM variants PEG30-FMS-OXM, PEG40-FMS-OXM and PEG60-FMS-OXM produced marked and significant reductions in fasting glucose and fasting plasma insulin when compared to controls.

[030] **Figure 13** shows that administration of PEG-OXM variants PEG5-FMS-OXM, PEG30-FMS-OXM, PEG40-FMS-OXM and PEG60-FMS-OXM produced marked and significant reductions in body weight when compared to controls.

DETAILED DESCRIPTION OF THE INVENTION

[031] In one embodiment, the present invention provides a long-acting dual GLP-1/ Glucagon receptor agonist and methods of producing and using the same. In another embodiment, the present invention provides a long-acting oxyntomodulin and methods of producing and using same. In one embodiment, a long-acting dual GLP-1/Glucagon receptor agonist is a composition comprising or consisting of oxyntomodulin, polyethylene glycol

polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, a long-acting oxyntomodulin is a composition comprising or consisting of oxyntomodulin, polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the present invention provides a modified oxyntomodulin peptide comprising an oxyntomodulin peptide, a polyethylene glycol (PEG) polymer, and a 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the present invention provides a modified oxyntomodulin peptide consisting of an oxyntomodulin peptide, a polyethylene glycol (PEG) polymer, and a 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In one embodiment, a long-acting oxyntomodulin is a composition comprising or consisting of oxyntomodulin and polyethylene glycol polymer (PEG polymer).

[032] In one embodiment, the terms dual "GLP-1/Glucagon receptor agonist" and "agonist" are used interchangeably herein. In another embodiment, the terms also include any GLP-1/Glucagon receptor agonist known in the art. In another embodiment, the preferred agonist is oxyntomodulin or OXM or a functional variant thereof.

[033] In one embodiment, the term "functional" refers to the ability of the agonist or OXM provided herein to have biological activity, which include but is not limited to, reducing weight, increasing insulin sensitivity, etc., as further provided herein.

[034] In another embodiment, a long-acting dual GLP-1/Glucagon receptor agonist is a pegylated oxyntomodulin. In another embodiment, a long-acting dual GLP-1/Glucagon receptor agonist is a reversed pegylated oxyntomodulin. In another embodiment, a long-acting oxyntomodulin is a pegylated oxyntomodulin. In another embodiment, a long-acting oxyntomodulin is a reversed pegylated oxyntomodulin. In another embodiment, the phrases "long-acting oxyntomodulin", "reversed pegylated oxyntomodulin", "reversible PEGylated OXM", and "a composition comprising or consisting of oxyntomodulin, polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS)" are used interchangeably. In another embodiment, a long-acting oxyntomodulin is OXM linked to PEG via Fmoc or FMS.

[035] In one embodiment, a long-acting dual GLP-1/Glucagon receptor agonist provided herein comprises a PEG polymer. In another embodiment, the agonist comprises a PEG polymer conjugated to the amino terminus of an oxyntomodulin peptide via Fmoc or FMS. In another embodiment, a long-acting oxyntomodulin of the invention comprises a PEG polymer. In another embodiment, a long-acting oxyntomodulin of the invention comprises a

PEG polymer conjugated to the amino terminus of an oxyntomodulin peptide via Fmoc or FMS.

[036] In another embodiment, a long-acting oxyntomodulin is a composition comprising or consisting of oxyntomodulin, polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of 1:0.2-10:0.2-10. In another embodiment, a long-acting oxyntomodulin is a composition comprising or consisting of oxyntomodulin, polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of 1:0.5-2:0.5-2. In another embodiment, a long-acting oxyntomodulin is a composition comprising or consisting of oxyntomodulin, polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of 1:1:1. In another embodiment, a long-acting oxyntomodulin includes a PEG polymer conjugated to the amino terminus of oxyntomodulin via Fmoc or FMS.

[037] In one embodiment, a long-acting dual GLP-1/Glucagon receptor agonist is linked to PEG via a reversible linker such as, but not limited to, Fmoc and FMS. In another embodiment, a long-acting oxyntomodulin is linked to PEG via a reversible linker such as, but not limited to, Fmoc and FMS. In another embodiment, Fmoc and FMS are sensitive to bases and are removable under physiological conditions. In another embodiment, a reversible linker is a linker that is sensitive to bases and is removable under physiological conditions. In another embodiment, a reversible linker is a linker that is sensitive to bases and is removable under physiological conditions in the blood, plasma, or lymph. In another embodiment, a reversible linker is a linker that is sensitive to bases and is removable under physiological conditions in a body fluid. In another embodiment, a reversible linker is a linker that is removable in a body fluid having a basic pH. In another embodiment, a linker that is sensitive to bases is cleaved upon exposure to a basic environment thus releasing OXM from the linker and PEG.

[038] In another embodiment, a reverse pegylated oxyntomodulin is a composition wherein OXM is linked to PEG via a reversible linker. In another embodiment, a reverse pegylated oxyntomodulin releases free OXM upon exposure to a basic environment. In another embodiment, a reverse pegylated oxyntomodulin releases free OXM upon exposure to blood or plasma. In another embodiment, a long-acting oxyntomodulin comprises PEG and oxyntomodulin that are not linked directly to each other, as in standard pegylation procedures, but rather both residues are linked to different positions of Fmoc or FMS which

are highly sensitive to bases and are removable under regular physiological conditions. In another embodiment, regular physiological conditions include a physiologic environment such as the blood or plasma.

[039] In one embodiment, a long-acting oxyntomodulin is non-reversibly conjugated to PEG using EMCS (see example 3).

[040] In another embodiment, the structures and the processes of making Fmoc and FMS are described in United States Patent No. 7585837. The disclosure of United States Patent No. 7585837 is hereby incorporated by reference in its entirety.

[041] In another embodiment, reverse pegylation renders OXM a long-acting OXM. In another embodiment, long-acting oxyntomodulin is an oxyntomodulin with an extended biological half-life.

[042] In one embodiment, reverse pegylation provides protection against degradation of a dual GLP-1/Glucagon receptor agonist. In another embodiment, reverse pegylation provides protection against degradation of OXM. In another embodiment, reverse pegylation affects the C_{max} of OXM to reduce harmful side effects. In another embodiment, reverse pegylation extends the T_{max} of OXM. In another embodiment, reverse pegylation extends the circulatory half-live of OXM. In another embodiment, reverse pegylated OXM has improved bioavailability compared to non-modified OXM. In another embodiment, reverse pegylated OXM has improved biological activity compared to non-modified OXM. In some embodiments, reverse pegylation enhances the potency of OXM.

[043] In other embodiments, a reverse pegylated OXM is at least equivalent to the non-modified OXM, in terms of biochemical measures. In other embodiments, a reverse pegylated OXM is at least equivalent to the non-modified OXM, in terms of pharmacological measures. In other embodiments, a reverse pegylated OXM is at least equivalent to the non-modified OXM, in terms of binding capacity (Kd). In other embodiments, a reverse pegylated OXM is at least equivalent to the non-modified OXM, in terms of absorption through the digestive system. In other embodiments, a reverse pegylated OXM is more stable during absorption through the digestive system than non-modified OXM.

[044] In another embodiment, a reverse pegylated dual GLP-1/Glucagon receptor agonist exhibits improved blood area under the curve (AUC) levels compared to free agonist. In another embodiment, a reverse pegylated OXM exhibits improved blood area under the curve (AUC) levels compared to free OXM. In another embodiment, a reverse pegylated OXM exhibits improved biological activity and blood area under the curve (AUC) levels compared to free OXM. In another embodiment, a reverse pegylated dual GLP-1/Glucagon receptor

agonist exhibits improved blood retention time ($t_{1/2}$) compared to free OXM. In another embodiment, a reverse pegylated OXM exhibits improved blood retention time ($t_{1/2}$) compared to free OXM. In another embodiment, a reverse pegylated OXM exhibits improved biological activity and blood retention time ($t_{1/2}$) compared to free OXM. In another embodiment, a reverse pegylated OXM exhibits improved blood C_{max} levels compared to free OXM, thereby reducing potentially harmful side effects. In another embodiment, a reverse pegylated OXM exhibits improved biological activity compared to free OXM. In another embodiment, provided herein a method of improving OXM's AUC, C_{max}, $t_{1/2}$, biological activity, or any combination thereof comprising or consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of free OXM via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). Hence, in one embodiment, the present invention further provides a method for improving the area under the curve (AUC) of oxyntomodulin, consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[045] In one embodiment, the GLP-1 or glucagon agonist activity of any given glucagon analogue peptide may be quantified by determining an EC_{50} value for that peptide in a selected assay for GLP-1 or glucagon activity. As the skilled person will be well aware, the EC50 value is a measure of the concentration at which half of that compound's maximal activity in the particular assay is achieved. In this specification, the EC_{50} value in an assay for GLP-1 or glucagon agonist activity will be referred to as EC_{50}[GLP-1] and EC_{50}[Glu] respectively. Where EC_{50} values for different compounds are compared, it will be understood that they describe the activity of the relevant compounds in the same assay under otherwise identical conditions.

[046] The ratio EC_{50}[Glu]/EC_{50}[GLP-1] for the glucagon analogue peptide may be greater than the ratio EC_{50}[Glu]/EC_{50}[GLP-1] for glucagon. This may be interpreted to mean that the glucagon analogue peptide has a greater selectivity for GLP-1 receptor than glucagon.

[047] In another embodiment, improvement of OXM's AUC, C_{max}, $t_{1/2}$, biological activity, or any combination thereof by conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of free OXM via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) enables the reduction in dosing frequency of OXM. In another embodiment, provided herein a method for reducing a dosing frequency of OXM, comprising or consisting of the step of conjugating a polyethylene glycol polymer (PEG

polymer) to the amino terminus or lysine residues of OXM via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, reverse pegylation of OXM is advantageous in permitting lower dosages to be used.

[048] In another embodiment, OXM comprises the amino acid sequence of SEQ ID NO: 1. In another embodiment, OXM consists of the amino acid sequence of SEQ ID NO: 1. In another embodiment, SEQ ID NO: 1 comprises or consists of the following amino acid (AA) sequence: HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA (SEQ ID NO: 1). In another embodiment, OXM comprises or consists of the amino acid sequence depicted in CAS No. 62340-29-8.

[049] In another embodiment, OXM is human OXM or any mammal OXM. In another embodiment, OXM is also referred to as glucagon-37 or bioactive enteroglucagon. In another embodiment, OXM is a dual GLP-1/Glucagon receptor agonist. In another embodiment, OXM is a biologically active fragment of OXM. In another embodiment, biologically active OXM extends from amino acid 30 to amino acid 37 of SEQ ID NO: 1. In another embodiment, biologically active OXM extends from amino acid 19 to amino acid 37 of SEQ ID NO: 1. In another embodiment, OXM of the invention corresponds to an octapeptide from which the two C-terminal amino acids are deleted. In another embodiment, OXM of the invention corresponds to any fragment of SEQ ID NO: 1 which retains OXM activity as described herein.

[050] In one embodiment, OXM refers to a peptide homologue of the peptide of SEQ ID NO: 1. In one embodiment, OXM amino acid sequence of the present invention is at least 40% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters. In one embodiment, OXM amino acid sequence of the present invention is at least 50% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters. In one embodiment, OXM amino acid sequence of the present invention is at least 60% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters. In one embodiment, OXM amino acid sequence of the present invention is at least 70% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters. In one embodiment, OXM amino acid sequence of the present invention is at least 80% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters.

[051] In one embodiment, OXM amino acid sequence of the present invention is at least 90% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters. In one embodiment, OXM amino acid sequence of the present invention is at least 95% homologous to the OXM sequence set forth in SEQ ID NO: 1 as determined using BlastP software of the NCBI using default parameters.

[052] In comparison to the wild-type OXM, the OXM derivatives or variants of the present invention contain several amino acid substitutions, and/or can be PEGylated or otherwise modified (e.g. recombinantly or chemically).

[053] The OXM provided herein also covers any analogue of the above OXM sequence. Any one or more amino acid residues in the sequence can be independently replaced with a conservative replacement as well known in the art i.e. replacing an amino acid with one of a similar chemical type such as replacing one hydrophobic amino acid with another. Alternatively, non-conservative amino acid mutations can be made that result in an enhanced effect or biological activity of OXM. In particular the OXM is modified to be resistant to cleavage and inactivation by dipeptidyl peptidase IV (DPP-IV)

[054] Derivatives, and variants of OXM and methods of generating the same are disclosed in US Patent Application Publication Nos. 2011/0152182, US Patent Application Publication Nos. 2011/0034374, US Patent Application Publication Nos.2010/0144617, all of which are incorporated by reference herein.

[055] In one embodiment, the dual GLP-1/Glucagon receptor agonist provided herein can be chemically modified. In another embodiment, the OXM provided herein can be chemically modified. In particular, the amino acid side chains, the amino terminus and/or the carboxy acid terminus of OXM can be modified. For example, the OXM can undergo one or more of alkylation, disulphide formation, metal complexation, acylation, esterification, amidation, nitration, treatment with acid, treatment with base, oxidation or reduction. Methods for carrying out these processes are well known in the art. In particular the OXM is provided as a lower alkyl ester, a lower alkyl amide, a lower dialkyl amide, an acid addition salt, a carboxylate salt or an alkali addition salt thereof. In particular, the amino or carboxylic termini of the OXM may be derivatised by for example, esterification, amidation, acylation, oxidation or reduction. In particular, the carboxylic terminus of the OXM can be derivatised to form an amide moiety.

[056] In one embodiment, the long-acting dual GLP-1/Glucagon receptor agonist of the invention maintains the biological activity of the unmodified agonist. In another embodiment, the OXM of the invention maintains the biological activity of unmodified

OXM. In one embodiment, the long-acting OXM of the invention maintains the biological activity of unmodified OXM. In another embodiment, the long-acting OXM of the invention comprising OXM biological activity. In another embodiment, the biological activity of a long-acting OXM of the invention comprises reducing digestive secretions. In another embodiment, the biological activity of a long-acting OXM of the invention comprises reducing and delaying gastric emptying. In another embodiment, the biological activity of a long-acting OXM of the invention comprises the inhibition of the fed motility pattern in the small intestine. In another embodiment, the biological activity of a long-acting OXM of the invention comprises the inhibition of acid secretion stimulated by pentagastrin. In another embodiment, the biological activity of a long-acting OXM of the invention comprises an increase of gastric somatostatin release. In another embodiment, the biological activity of a long-acting OXM of the invention comprises potentiating the effects of peptide YY. In another embodiment, the biological activity of a long-acting OXM of the invention comprises the inhibition of ghrelin release. In another embodiment, the biological activity of long-acting OXM of the invention comprises the up-regulation of adiponectin. In another embodiment, the biological activity of long-acting OXM of the invention comprises reducing free fatty acids. . In another embodiment, the biological activity of long-acting OXM of the invention comprises reducing triglycerides. In another embodiment, the biological activity of long-acting OXM of the invention comprises reducing cholesterol. In another embodiment, the biological activity of a long-acting OXM of the invention comprises the stimulation of aminopyrine accumulation and cAMP production. In another embodiment, the biological activity of a long-acting OXM of the invention comprises binding the GLP-1 receptor or the glucagon receptor. In another embodiment, the biological activity of a long-acting OXM of the invention comprises stimulating H+ production by activating the adenylate cyclase. In another embodiment, the biological activity of a long-acting OXM of the invention comprises inhibiting histamine-stimulated gastric acid secretion. In another embodiment, the biological activity of a long-acting OXM of the invention comprises inhibiting food intake. In another embodiment, the biological activity of a long-acting OXM of the invention comprises stimulating insulin release. In another embodiment, the biological activity of a long-acting OXM of the invention comprises inhibiting exocrine pancreatic secretion. In another embodiment, the biological activity of a long-acting OXM of the invention comprises increasing insulin sensitivity. In another embodiment, the biological activity of a long-acting OXM of the invention comprises reducing glucose levels.

[057] In one embodiment, the terms "reducing the level of" refers to a reduction of about 1-10% relative to an original, wild-type, normal or control level. In another embodiment, the reduction is of about 11-20%. In another embodiment, the reduction is of about 21-30%. In another embodiment, the reduction is of about 31-40%. In another embodiment, the reduction is of about 41-50%. In another embodiment, the reduction is of about 51-60%. In another embodiment, the reduction is of about 61-70%. In another embodiment, the reduction is of about 71-80%. In another embodiment, the reduction is of about 81-90%. In another embodiment, the reduction is of about 91-95%. In another embodiment, the reduction is of about 96-100%.

[058] In one embodiment, the terms "increasing the level of" or "extending" refers to a increase of about 1-10% relative to an original, wild-type, normal or control level. In another embodiment, the increase is of about 11-20%. In another embodiment, the increase is of about 21-30%. In another embodiment, the increase is of about 31-40%. In another embodiment, the increase is of about 41-50%. In another embodiment, the increase is of about 51-60%. In another embodiment, the increase is of about 61-70%. In another embodiment, the increase is of about 71-80%. In another embodiment, the increase is of about 81-90%. In another embodiment, the increase is of about 91-95%. In another embodiment, the increase is of about 96-100%.

[059] In another embodiment, the present invention further provides a method of inducing glucose tolerance, improving glycemic control, or both in a subject in need thereof, comprising the step of administering to the subject an effective amount of a composition consisting of a dual GLP-1/Glucagon receptor agonist linked to polyethylene glycol polymer (PEG polymer) via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) and a pharmaceutical acceptable carrier.

[060] In another embodiment, the present invention further provides a method of inducing glucose tolerance, improving glycemic control, or both in a subject in need thereof, comprising the step of administering to the subject an effective amount of a composition consisting of an oxyntomodulin linked to polyethylene glycol polymer (PEG polymer) via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) and a pharmaceutical acceptable carrier.

[061] In one embodiment, the present invention further provides a method for extending the biological half life of a dual GLP-1/Glucagon receptor agonist, consisting of the step of conjugating the agonist to polyethylene glycol polymer (PEG polymer) via a flexible linker

comprising 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

[062] In one embodiment, the present invention further provides a method for extending the biological half life of oxyntomodulin, consisting of the step of conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of about 1:1:0.5 to about 1:1:3.5. In another embodiment, the molar ratio is 1:1:10 OXM to PEG to linker. In another embodiment, the range is 1:1:5- 1:1:9, In another embodiment, the range is 1:1:3.7-1:1-4.9.

[063] In another embodiment, the present invention further provides a method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering to the subject a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the subject is afflicted with diabetes. In another embodiment, the subject is overweight. In another embodiment, the subject is afflicted with obesity.

[064] In another embodiment, the present invention further provides a method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering to the subject oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the subject is afflicted with diabetes. In another embodiment, the subject is overweight. In another embodiment, the subject is afflicted with obesity.

[065] In one embodiment, the PEG-OXM compounds provided herein induce significant reduction of glucose level without increasing insulin level. In another embodiment, the PEG-OXM compounds provided herein unexpectedly reduce glucose levels together with the reduction of fasted insulin levels following administration of a single dose of the PEG-OXM compounds (see Example 7, herein). Hence, in another embodiment, the present invention provides a method for increasing insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of a composition comprising a dual GLP-1/Glucagon receptor agonist conjugated to polyethylene glycol polymer (PEG polymer). In another embodiment, the present invention unexpectedly shows a marked increase in insulin sensitivity following acute treatment in a subject with the dual GLP-1/Glucagon receptor agonist composition provided herein (see Example 7). In another embodiment, the agonist is conjugated to said polyethylene glycol polymer (PEG polymer) via a linker. In another

embodiment, the agonist is OXM. In another embodiment, the linker is a flexible linker. In another embodiment, the linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS). In another embodiment, the linker is a non-cleavable linker. In another embodiment, the linker is *N*-(ε-Maleimidocaproyloxu) succinimide ester (EMCS).

[066] In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises inhibiting pancreatic secretion through a vagal neural indirect mechanism. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises reducing hydromineral transport through the small intestine. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises stimulating glucose uptake. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises controlling/stimulating somatostatin secretion. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises reduction in both food intake and body weight gain. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises reduction in adiposity. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises appetite suppression. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises induction of anorexia. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises reducing body weight in overweight and obese subjects. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises inducing changes in the levels of the adipose hormones leptin and adiponectin. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises increasing energy expenditure in addition to decreasing energy intake in overweight and obese subjects. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention comprises decreasing plasma triglycerides and increased ketone bodies.

[067] In one embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute treatment comprises decreasing plasma triglycerides and increased ketone bodies. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute

treatment comprises increasing expression of the gluconeogenic genes Pck1, Pgc1α, and Pdha1. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute treatment comprises decreasing liver pools of acetyl-CoA, the main product of pyruvate decarboxylation, and malonyl-CoA. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute treatment comprises upregulating genes that induce fatty acid oxidation (FAO) in the liver, including *Fgf21* and *Cpt1a*. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute treatment comprises downregulating lipogenic genes such as *ChREBP*. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following acute treatment comprises upregulating *Ldlr* gene.

[068] In one embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following chronic treatment comprises decreasing leptin levels. In another embodiment, the biological activity of a long-acting dual GLP-1/Glucagon receptor agonist of the invention, following chronic treatment comprises increasing b-hydroxybutyrate levels.

[069] In another embodiment, a PEG polymer is attached to the amino terminus or lysine residue of oxyntomodulin via Fmoc or FMS. In another embodiment, the terms "attached" and "linked" are use interchangeably. In another embodiment, the PEG polymer is linked to the α-amino side chain of OXM. In another embodiment, the PEG polymer is linked to the ε-amino side chain of OXM. In another embodiment, the PEG polymer is linked to one or more ε-amino side chain of OXM. In another embodiment, the PEG polymer comprises a sulfhydryl moiety.

[070] In another embodiment, PEG is linear. In another embodiment, PEG is branched. In another embodiment, PEG has a molecular weight in the range of 200 to 200,000 Da. In another embodiment, PEG has a molecular weight in the range of 5,000 to 80,000 Da. In another embodiment, PEG has a molecular weight in the range of 5,000 to 40,000 Da. In another embodiment, PEG has a molecular weight in the range of 20,000 Da to 40,000 Da.

[071] In another embodiment, a long-acting OXM is prepared using PEGylating agents, meaning any PEG derivative which is capable of reacting with a functional group such as, but not limited to, NH_2, OH, SH, COOH, CHO, --N=C=O, --N=C=S, --SO_2Cl, --$SO_2CH=CH_2$, --PO_2Cl, --(CH_2)xHal, present at the fluorene ring of the Fmoc or FMS moiety. In another embodiment, the PEGylating agent is usually used in its mono-methoxylated form where only one hydroxyl group at one terminus of the PEG molecule is

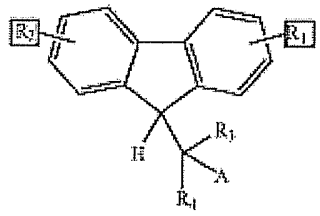
available for conjugation. In another embodiment, a bifunctional form of PEG where both termini are available for conjugation may be used if, for example, it is desired to obtain a conjugate with two peptide or protein residues covalently attached to a single PEG moiety.

[072] In another embodiment, branched PEGs are represented as $R(PEG\text{-}OH)_m$ in which R represents a central core moiety such as pentaerythritol or glycerol, and m represents the number of branching arms. The number of branching arms (m) can range from three to a hundred or more. In another embodiment, the hydroxyl groups are subject to chemical modification. In another embodiment, branched PEG molecules are described in U.S. Pat. No. 6,113,906, No. 5,919,455, No. 5,643,575, and No. 5,681,567, which are hereby incorporated by reference in their entirety.

[073] In one embodiment, the GLP-1/Glucagon receptor agonist is oxyntomodulin. In another embodiment, the GLP-1/Glucagon receptor agonist is an oxyntomodulin variant.

[074] In another embodiment, the present invention provides OXM with a PEG moiety which is not attached directly to the OXM, as in the standard pegylation procedure, but rather the PEG moiety is attached through a linker such as Fmoc or FMS. In another embodiment, the linker is highly sensitive to bases and is removable under mild basic conditions. In another embodiment, OXM connected to PEG via Fmoc or FMS is equivalently active to the free OXM. In another embodiment, OXM connected to PEG via Fmoc or FMS is more active than the free OXM. In another embodiment, OXM connected to PEG via Fmoc or FMS comprises different activity than the free OXM. In another embodiment, OXM connected to PEG via Fmoc or FMS unlike the free OXM, has central nervous system activity. In another embodiment, reversible Pegylated OXM crosses the blood-brain barrier and acts on the hypothalamus to exert the biological activities provided herein. In another embodiment, OXM connected to PEG via Fmoc or FMS unlike the free OXM, can not enter the brain through the blood brain barrier. In another embodiment, OXM connected to PEG via Fmoc or FMS comprises extended circulation half-life compared to the free OXM. In another embodiment, OXM connected to PEG via Fmoc or FMS loses its PEG moiety together with the Fmoc or FMS moiety thus recovering the free OXM.

[075] In another embodiment, the present invention provides a compound of the formula: (X)n—Y, wherein Y is a moiety of OXM bearing a free amino, carboxyl, or hydroxyl and X is a radical of formula (i):



[076] In another embodiment, R_1 is a radical containing a protein or polymer carrier moiety; polyethylene glycol (PEG) moiety; R_2 is selected from the group consisting of hydrogen, alkyl, alkoxy, alkoxyalkyl, aryl, alkaryl, aralkyl, halogen, nitro, --SO_3H, --SO_2NHR, amino, ammonium, carboxyl, PO_3H_2, and OPO_3H_2; R is selected from the group consisting of hydrogen, alkyl and aryl; R_3 and R_4, the same or different, are each selected from the group consisting of hydrogen, alkyl and aryl; A is a covalent bond when the radical is linked to an amino or hydroxyl group of the OXM-Y; n is an integer of at least one, and pharmaceutically acceptable salts thereof.

[077] In another embodiment, the terms "alkyl", "alkoxy", "alkoxyalkyl", "aryl", "alkaryl" and "aralkyl" are used to denote alkyl radicals of 1-8, preferably 1-4 carbon atoms, e.g. methyl, ethyl, propyl, isopropyl and butyl, and aryl radicals of 6-10 carbon atoms, e.g. phenyl and naphthyl. The term "halogen" includes bromo, fluoro, chloro and iodo.

[078] In another embodiment, R_2, R_3 and R_4 are each hydrogen and A is --OCO--, namely the 9-fluorenylmethoxycarbonyl radical (hereinafter "Fmoc"). In another embodiment, R is --SO_3H at position 2 of the fluorene ring, R_3 and R_4 are each hydrogen, and A is --OCO--, namely the 2-sulfo-9-fluorenylmethoxycarbonyl radical (hereinafter "FMS").

[079] In another embodiment, pegylation of OXM and preparation of the (PEG-Fmoc)n-OXM or (PEG-FMS)n- OXM conjugates includes attaching MAL-FMS-NHS or MAL-Fmoc-NHS to the amine component of OXM, thus obtaining a MAL-FMS- OXM or MAL-Fmoc- OXM conjugate, and then substituting PEG-SH for the maleimide moiety, producing the (PEG-FMS)n- OXM or (PEG-Fmoc)n- OXM conjugate, respectively.

[080] In another embodiment, pegylation of OXM includes reacting MAL-FMS-NHS or MAL-Fmoc-NHS with PEG-SH, thus forming a PEG-FMS-NHS or PEG-Fmoc-NHS conjugate, and then reacting it with the amine component of OXM resulting in the desired (PEG-FMS)n- OXM or (PEG-Fmoc)n- OXM conjugate, respectively. In another embodiment, pegylation of peptides/proteins such as OXM are described in United States Patent No. 7585837, which is incorporated herein by reference in its entirety. In another

embodiment, reverse-pegylation of peptides/proteins such as OXM with Fmoc or FMS are described in United States Patent No. 7585837.

[081] In another embodiment, the phrases "long acting OXM" and "reverse pegylated OXM" are used interchangeably. In another embodiment, reverse pegylated OXM is composed of PEG-FMS-OXM and PEG-Fmoc-OXM herein identified by the formulas: (PEG-FMS)n-OXM or (PEG-Fmoc)n-OXM, wherein n is an integer of at least one, and OXM is linked to the FMS or Fmoc radical through at least one amino group.

[082] In another embodiment, surprisingly, the long acting OXM described herein is both active in its pegylated form and in its peripheral form. In another embodiment, surprisingly, the construction of (PEG-FMS)n-OXM or (PEG-Fmoc)n-OXM does not render this conjugate inactive. In another embodiment, surprisingly, the construction of (PEG-FMS)n-OXM or (PEG-Fmoc)n-OXM does not render the OXM inactive.

Therapeutic Uses

[083] In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the prevention of hyperglycemia, for improving glycemic control, for treatment of diabetes mellitus selected from the group consisting of non-insulin dependent diabetes mellitus (in one embodiment, Type 2 diabetes), insulin-dependent diabetes mellitus (in one embodiment, Type 1 diabetes), and gestational diabetes mellitus, or any combination thereof. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for treating Type 2 Diabetes. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for increasing sensitivity to insulin. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for reducing insulin resistance.

[084] In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the suppression of appetite. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for inducing satiety. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the reduction of body weight. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the reduction of body fat. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the reduction of body mass index. In another

embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for the reduction of food consumption. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for treating obesity. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for treating diabetes mellitus associated with obesity. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for increasing heart rate. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for increasing the basal metabolic rate (BMR). In another embodiment, PEG-Fmoc-oxyntomodulin and PEG-FMS-oxyntomodulin and pharmaceutical compositions comprising them are utilized for increasing energy expenditure. In another embodiment, PEG-Fmoc-oxyntomodulin and PEG-FMS-oxyntomodulin and pharmaceutical compositions comprising them are utilized for inducing glucose tolerance. In another embodiment, PEG-Fmoc-oxyntomodulin and PEG-FMS-oxyntomodulin and pharmaceutical compositions comprising them are utilized for inducing glycemic control. In one embodiment, glycemic control refers to non-high and/or non-fluctuating blood glucose levels and/or non-high and/or non-fluctuating glycosylated hemoglobin levels.

[085] In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for inhibiting weight increase. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for reducing blood glucose levels (Figures 4A and 9). In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for decreasing caloric intake. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for decreasing appetite. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for weight control. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for inducing or promoting weight loss. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for maintaining any one or more of a desired body weight, a desired Body Mass Index, a desired appearance and good health. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for controlling a lipid profile. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for reducing

triglyceride levels. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for reducing glycerol levels (Figure 9D).

[086] In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for reducing cholesterol levels. In one embodiment, the reduction in cholesterol levels is greater than the reduction observed after administration of native OXM (Figure 9C). In one embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them lower cholesterol levels by 60-70%. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them lower cholesterol levels by 50-100%. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them lower cholesterol levels by 25-90%. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them lower cholesterol levels by 50-80%. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them lower cholesterol levels by 40-90%. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are utilized for increasing HDL cholesterol levels.

[087] In one embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them may be used for the purposes described herein without a significant decrease in effectiveness over the course of administration (Figures 5A, 6A, and 7). In one embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 1 day. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 2-6 days. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 1 week. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 2 weeks. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 3 weeks. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 4 weeks. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 6 weeks. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them

remains effective for 2 months. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 4 months. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 6 months. In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them remains effective for 1 year or more.

[088] In one embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them may be used for the purposes described herein and may be effective immediately upon administration of the first dose (Figure 8A). In another embodiment, PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them are effective after two or more doses have been administered.

[089] In another embodiment, methods of utilizing PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them as described hereinabove are applied to a human subject afflicted with a disease or condition that can be alleviated, inhibited, and/or treated by OXM. In another embodiment, methods of utilizing PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them as described hereinabove are veterinary methods. In another embodiment, methods of utilizing PEG-Fmoc-OXM and PEG-FMS-OXM and pharmaceutical compositions comprising them as described hereinabove are applied to animals such as farm animals, pets, and lab animals. Thus, in one embodiment, a subject of the present invention is feline, canine, bovine, porcine, murine, aquine, etc.

[090] In another embodiment, the present invention provides a method of treating or reducing a disease treatable or reducible by OXM or a pharmaceutical formulation comprising the same, in a subject, comprising the step of administering to a subject a therapeutically effective amount of PEG-Fmoc-OXM amd/or PEG-FMS-OXM as described herein, thereby treating or reducing a disease treatable or reducible by OXM in a subject.

[091] In another embodiment, OXM, "peptide" or "protein" as used herein encompasses native peptides (either degradation products, synthetically synthesized proteins or recombinant proteins) and peptidomimetics (typically, synthetically synthesized proteins), as well as peptoids and semipeptoids which are protein analogs, which have, in some embodiments, modifications rendering the proteins even more stable while in a body or more capable of penetrating into cells.

[092] In another embodiment, modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited

to, CH2-NH, CH2-S, CH2-S=O, O=C-NH, CH2-O, CH2-CH2, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

[093] In another embodiment, peptide bonds (-CO-NH-) within the peptide are substituted. In some embodiments, the peptide bonds are substituted by N-methylated bonds (-N(CH3)-CO-). In another embodiments, the peptide bonds are substituted by ester bonds (-C(R)H-C-O-O-C(R)-N-). In another embodiment, the peptide bonds are substituted by ketomethylen bonds (-CO-CH2-). In another embodiment, the peptide bonds are substituted by α-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH2-NH-). In another embodiments, the peptide bonds are substituted by hydroxyethylene bonds (-CH(OH)-CH2-). In another embodiment, the peptide bonds are substituted by thioamide bonds (-CS-NH-). In some embodiments, the peptide bonds are substituted by olefinic double bonds (-CH=CH-). In another embodiment, the peptide bonds are substituted by retro amide bonds (-NH-CO-). In another embodiment, the peptide bonds are substituted by peptide derivatives (-N(R)-CH2-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom. In some embodiments, these modifications occur at any of the bonds along the peptide chain and even at several (2-3 bonds) at the same time.

[094] In one embodiment, natural aromatic amino acids of the protein such as Trp, Tyr and Phe, are substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr. In another embodiment, the peptides of the present invention include one or more modified amino acid or one or more non-amino acid monomers (e.g. fatty acid, complex carbohydrates etc).

[095] In one embodiment, "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acid including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. In one embodiment, "amino acid" includes both D- and L-amino acids.

[096] In one embodiment, the OXM of the present invention are utilized in therapeutics which requires OXM to be in a soluble form. In another embodiment, OXM of the present invention includes one or more non-natural or natural polar amino acid, including, but not limited to, serine and threonine which are capable of increasing protein solubility due to their hydroxyl-containing side chain.

[097] In one embodiment, OXM of present invention is biochemically synthesized such as by using standard solid phase techniques. In another embodiment, these biochemical methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, or classical solution synthesis.

[098] In one embodiment, solid phase OXM synthesis procedures are well known to one skilled in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Protein Syntheses (2nd Ed., Pierce Chemical Company, 1984). In another embodiment, synthetic proteins are purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing by methods known to one skilled in the art.

[099] In another embodiment, recombinant protein techniques are used to generate the OXM of the present invention. In some embodiments, recombinant protein techniques are used for the generation of large amounts of the OXM of the present invention. In another embodiment, recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

[0100] In another embodiment, OXM of the present invention is synthesized using a polynucleotide encoding OXM of the present invention. In some embodiments, the polynucleotide encoding OXM of the present invention is ligated into an expression vector, comprising a transcriptional control of a cis-regulatory sequence (e.g., promoter sequence). In some embodiments, the cis-regulatory sequence is suitable for directing constitutive expression of the OXM of the present invention.

[0101] In one embodiment, the phrase "a polynucleotide" refers to a single or double stranded nucleic acid sequence which be isolated and provided in the form of an RNA

sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

[0102] In one embodiment, "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. In one embodiment, the sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA polymerase.

[0103] In one embodiment, "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

[0104] In one embodiment, "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. In one embodiment, a composite sequence can include some exonal sequences required to encode the peptide of the present invention, as well as some intronic sequences interposing there between. In one embodiment, the intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. In one embodiment, intronic sequences include cis acting expression regulatory elements.

[0105] In one embodiment, polynucleotides of the present invention are prepared using PCR techniques, or any other method or procedure known to one skilled in the art. In some embodiments, the procedure involves the ligation of two different DNA sequences (See, for example, "Current Protocols in Molecular Biology", eds. Ausubel et al., John Wiley & Sons, 1992).

[0106] In one embodiment, a variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the OXM of the present invention. In another embodiment, these include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the protein coding sequence; yeast transformed with recombinant yeast expression vectors containing the protein coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the protein coding sequence.

[0107] In one embodiment, non-bacterial expression systems are used (e.g. mammalian expression systems such as CHO cells) to express the OXM of the present invention. In one embodiment, the expression vector used to express polynucleotides of the present invention in mammalian cells is pCI-DHFR vector comprising a CMV promoter and a neomycin resistance gene.

[0108] In another embodiment, in bacterial systems of the present invention, a number of expression vectors can be advantageously selected depending upon the use intended for the protein expressed. In one embodiment, large quantities of OXM are desired. In one embodiment, vectors that direct the expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified are desired. In one embodiment, certain fusion protein engineered with a specific cleavage site to aid in recovery of the protein. In one embodiment, vectors adaptable to such manipulation include, but are not limited to, the pET series of *E. coli* expression vectors [Studier *et al.*, Methods in Enzymol. 185:60-89 (1990)].

[0109] In one embodiment, yeast expression systems are used. In one embodiment, a number of vectors containing constitutive or inducible promoters can be used in yeast as disclosed in U.S. Pat. Application. No: 5,932,447. In another embodiment, vectors which promote integration of foreign DNA sequences into the yeast chromosome are used.

[0110] In one embodiment, the expression vector of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric protein.

[0111] In one embodiment, mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

[0112] In another embodiment, expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses are used by the present invention. SV40 vectors include pSVT7 and pMT2. In another embodiment, vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0113] In one embodiment, plant expression vectors are used. In one embodiment, the expression of the dual GLP-1/Glucagon receptor agonist coding sequence (such as OXM) is driven by a number of promoters. In another embodiment, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV [Brisson *et al.*, Nature 310:511-514 (1984)], or the coat protein promoter to TMV [Takamatsu *et al.*, EMBO J. 6:307-311 (1987)] are used. In another embodiment, plant promoters are used such as, for example, the small subunit of RUBISCO [Coruzzi *et al.*, EMBO J. 3:1671-1680 (1984); and Brogli *et al.*, Science 224:838-843 (1984)] or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B [Gurley *et al.*, Mol. Cell. Biol. 6:559-565 (1986)]. In one embodiment, constructs are introduced into plant cells using Ti plasmid, Ri plasmid, plant viral vectors, direct DNA transformation, microinjection, electroporation and other techniques well known to the skilled artisan. See, for example, Weissbach & Weissbach [Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463 (1988)]. Other expression systems such as insects and mammalian host cell systems, which are well known in the art, can also be used by the present invention.

[0114] It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the protein), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed protein.

[0115] Various methods, in some embodiments, can be used to introduce the expression vector of the present invention into the host cell system. In some embodiments, such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[0116] In one embodiment, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant OXM. In another embodiment, effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. In one embodiment,

an effective medium refers to any medium in which a cell is cultured to produce the recombinant OXM of the present invention. In another embodiment, a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. In one embodiment, cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. In another embodiment, culturing is carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. In another embodiment, culturing conditions are within the expertise of one of ordinary skill in the art.

[0117] In one embodiment, depending on the vector and host system used for production, resultant OXM of the present invention either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

[0118] In one embodiment, following a predetermined time in culture, recovery of the recombinant OXM is effected.

[0119] In one embodiment, the phrase "recovering the recombinant OXM" used herein refers to collecting the whole fermentation medium containing the OXM and need not imply additional steps of separation or purification.

[0120] In one embodiment, OXM of the present invention is purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

[0121] In one embodiment, to facilitate recovery, the expressed coding sequence can be engineered to encode the protein of the present invention and fused cleavable moiety. In one embodiment, a fusion protein can be designed so that the protein can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. In one embodiment, a cleavage site is engineered between the protein and the cleavable moiety and the protein can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth *et al.*, Immunol. Lett. 19:65-70 (1988); and Gardella *et al.*, J. Biol. Chem. 265:15854-15859 (1990)]. In another embodiment, the OXM of the present invention is retrieved in "substantially pure" form. In another embodiment, the phrase "substantially

pure" refers to a purity that allows for the effective use of the OXM in the applications described herein.

[0122] In one embodiment, the dual GLP-1/Glucagon receptor agonist of the present invention can also be synthesized using *in vitro* expression systems. In one embodiment, *in vitro* synthesis methods are well known in the art and the components of the system are commercially available.

[0123] In another embodiment, *in vitro* binding activity is ascertained by measuring the ability of native, recombinant and/or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein as well as pharmaceutical compositions comprising the same to treat or ameliorate diseases or conditions such as but not limited to: diabetes mellitus, obesity, eating disorders, metabolic disorders, etc. In another embodiment, in vivo activity is deduced by known measures of the disease that is being treated.

[0124] In another embodiment, a dose of reverse pegylated OXM of the present invention comprises from 0.005 to 0.1 milligrams/kg OXM peptide. In another embodiment, a dose of reverse pegylated OXM of the present invention comprises from 0.005 to 0.5 milligrams/kg OXM peptide. In another embodiment, a dose of reverse pegylated OXM of the present invention comprises from 0.05 to 0.1 micrograms OXM peptide. In another embodiment, a dose of reverse pegylated OXM of the present invention comprises from 0.005 to 0.1 milligrams/kg OXM peptide in an injectable solution.

[0125] In another embodiment, a dose of reverse pegylated OXM is administered once a day. In another embodiment, a dose of reverse pegylated OXM is administered once every 36 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 48 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 60 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 72 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 84 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 96 hours. In another embodiment, a dose of reverse pegylated OXM is administered once every 5 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 6 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 7 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 8-10 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 10-12 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 12-15 days. In another embodiment, a dose of reverse pegylated OXM is administered once every 15-25 days.

[0126] In another embodiment, reverse pegylated OXM of the present invention is administered by an intramuscular (IM) injection, subcutaneous (SC) injection, or intravenous (IV) injection once a week.

[0127] In another embodiment, the reverse pegylated OXM of the present invention can be provided to the individual *per se*. In one embodiment, the reverse pegylated OXM of the present invention can be provided to the individual as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

[0128] In another embodiment, a "pharmaceutical composition" refers to a preparation of long-acting OXM as described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism. In another embodiment, a reverse pegylated OXM is accountable for the biological effect.

[0129] In another embodiment, any of the compositions of this invention will comprise at least a reverse pegylated OXM. In one embodiment, the present invention provides combined preparations. In one embodiment, "a combined preparation" defines especially a "kit of parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners i.e., simultaneously, concurrently, separately or sequentially. In some embodiments, the parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners, in some embodiments, can be administered in the combined preparation. In one embodiment, the combined preparation can be varied, e.g., in order to cope with the needs of a patient subpopulation to be treated or the needs of the single patient which different needs can be due to a particular disease, severity of a disease, age, sex, or body weight as can be readily made by a person skilled in the art.

[0130] In another embodiment, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. In one embodiment, one of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

[0131] In another embodiment, "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a long-acting OXN. In one embodiment, excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

[0132] Techniques for formulation and administration of drugs are found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

[0133] In another embodiment, suitable routes of administration of the peptide of the present invention, for example, include oral, rectal, transmucosal, transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0134] The present invention also includes reverse pegylated OXM for use in the manufacture of a medicament for administration by a route peripheral to the brain for any of the methods of treatment described above. Examples of peripheral routes include oral, rectal, parenteral e.g. intravenous, intramuscular, or intraperitoneal, mucosal e.g. buccal, sublingual, nasal, subcutaneous or transdermal administration, including administration by inhalation. Preferred dose amounts of OXM for the medicaments are given below.

[0135] The present invention provides a pharmaceutical composition comprising reverse pegylated OXM and a pharmaceutically suitable carrier, in a form suitable for oral, rectal, parenteral, e.g. intravenous, intramuscular, or intraperitoneal, mucosal e.g. buccal, sublingual, nasal, subcutaneous or transdermal administration, including administration by inhalation. If in unit dosage form, the dose per unit may be, for example, as described below or as calculated on the basis of the per kg doses given below.

[0136] In another embodiment, the preparation is administered in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body. In another embodiment, a reverse pegylated OXM is formulated in an intranasal dosage form. In another embodiment, a reverse pegylated OXM is formulated in an injectable dosage form.

[0137] Various embodiments of dosage ranges are contemplated by this invention: the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight per 3 days (only the weight of the OXM within the reverse pegylated OXM composition is provided as the size of PEG can differ substantially). In another embodiment, the OXM peptide component within of the reverse pegylated OXM

composition is administered in a range of 0.01-0.5 milligrams/kg body weight per 7 days. In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight per 10 days. In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight per 14 days. In another embodiment, unexpectedly, the effective amount of OXM in a reverse pegylated OXM composition is 1/4-1/10 of the effective amount of free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 50% compared with free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 70% compared with free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 75% compared with free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 80% compared with free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 85% compared with free OXM. In another embodiment, unexpectedly, reverse pegylation of OXM enables limiting the amount of OXM prescribed to a patient by at least 90% compared with free OXM.

[0138] In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight once every 3 days (only the weight of the OXM within the reverse pegylated OXM composition is provided as the size of PEG can differ substantially). In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight once every 7 days. In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight once every 10 days. In another embodiment, the OXM peptide component within of the reverse pegylated OXM composition is administered in a range of 0.01-0.5 milligrams/kg body weight once every 14 days.

[0139] In another embodiment, reverse pegylated OXM compared to free OXM both reduces the effective dosing frequency by at least 2-fold and reduces the effective weekly dose by at least 2-fold, thus limiting the risk of adverse events and increasing compliance with the use of OXM therapy. In another embodiment, reverse pegylated OXM compared to free OXM both reduces the effective dosing frequency by at least 3-fold and reduces the effective weekly dose by at least 3-fold, thus limiting the risk of adverse events and increasing compliance with the use of OXM

therapy. In another embodiment, reverse pegylated OXM compared to free OXM both reduces the effective dosing frequency by at least 4-fold and reduces the effective weekly dose by at least 4-fold, thus limiting the risk of adverse events and increasing compliance with the use of OXM therapy. In another embodiment, reverse pegylated OXM compared to free OXM both reduces the effective dosing frequency by at least 5-fold and reduces the effective weekly dose by at least 5-fold, thus limiting the risk of adverse events and increasing compliance with the use of OXM therapy. In another embodiment, reverse pegylated OXM compared to free OXM both reduces the effective dosing frequency by at least 6-fold and reduces the effective weekly dose by at least 6-fold, thus limiting the risk of adverse events and increasing compliance with the use of OXM therapy. In another embodiment, effective dosing frequency and effective weekly dose are based on: (1) the weight of administered OXM component within the reverse pegylated OXM composition; and (2) the weight of administered OXM component within the free OXM (unmodified OXM) composition.

[0140] In another embodiment, the methods of the invention include increasing the compliance of patients afflicted with chronic illnesses that are in need of OXM therapy. In another embodiment, the methods of the invention enable reduction in the dosing frequency of OXM by reverse pegylating OXM as described hereinabove. In another embodiment, the term compliance comprises adherence. In another embodiment, the methods of the invention include increasing the compliance of patients in need of OXM therapy by reducing the frequency of administration of OXM. In another embodiment, reduction in the frequency of administration of the OXM is achieved thanks to reverse pegylation which render the OXM more stable and more potent. In another embodiment, reduction in the frequency of administration of the OXM is achieved as a result of increasing T1/2 of the OXM. In another embodiment, reduction in the frequency of administration of the OXM is achieved as a result of reducing blood clearance of OXM. In another embodiment, reduction in the frequency of administration of the OXM is achieved as a result of increasing T1/2 of the OXM. In another embodiment, reduction in the frequency of administration of the OXM is achieved as a result of increasing the AUC measure of the OXM.

[0141] In another embodiment, a reverse pegylated OXM is administered to a subject once a day. In another embodiment, a reverse pegylated OXM is administered to a subject once every two days. In another embodiment, a reverse pegylated OXM is administered to a subject once every three days. In another embodiment, a reverse pegylated OXM is administered to a subject once every four days. In another embodiment, a reverse pegylated OXM is administered to a subject once every five days. In another embodiment, a reverse

pegylated OXM is administered to a subject once every six days. In another embodiment, a reverse pegylated OXM is administered to a subject once every week. In another embodiment, a reverse pegylated OXM is administered to a subject once every 7-14 days. In another embodiment, a reverse pegylated OXM is administered to a subject once every 10-20 days. In another embodiment, a reverse pegylated OXM is administered to a subject once every 5-15 days. In another embodiment, a reverse pegylated OXM is administered to a subject once every 15-30 days.

[0142] In another embodiment, a pegylated OXM is administered to a subject once a day. In another embodiment, a pegylated OXM is administered to a subject once every two days. In another embodiment, a pegylated OXM is administered to a subject once every three days. In another embodiment, a pegylated OXM is administered to a subject once every four days. In another embodiment, a pegylated OXM is administered to a subject once every five days. In another embodiment, a pegylated OXM is administered to a subject once every six days. In another embodiment, a pegylated OXM is administered to a subject once every week. In another embodiment, a pegylated OXM is administered to a subject once every 7-14 days. In another embodiment, a pegylated OXM is administered to a subject once every 10-20 days. In another embodiment, a pegylated OXM is administered to a subject once every 5-15 days. In another embodiment, a pegylated OXM is administered to a subject once every 15-30 days.

[0143] In one embodiment, pegylated OXM variants provided herein unexpectedly reduce glucose together with reduction of fasted insulin levels following administration of a single dose of the PEG-OXM variant. In another embodiment, the pegylated OXM variants provided herein lead to increasing the sensitivity of a subject to insulin (see Example 6).

[0144] Oral administration, in one embodiment, comprises a unit dosage form comprising tablets, capsules, lozenges, chewable tablets, suspensions, emulsions and the like. Such unit dosage forms comprise a safe and effective amount of OXM of the invention, each of which is in one embodiment, from about 0.7 or 3.5 mg to about 280 mg/70 kg, or in another embodiment, about 0.5 or 10 mg to about 210 mg/70 kg. The pharmaceutically-acceptable carriers suitable for the preparation of unit dosage forms for peroral administration are well-known in the art. In some embodiments, tablets typically comprise conventional pharmaceutically-compatible adjuvants as inert diluents, such as calcium carbonate, sodium carbonate, mannitol, lactose and cellulose; binders such as starch, gelatin and sucrose; disintegrants such as starch, alginic acid and croscarmelose; lubricants such as magnesium stearate, stearic acid and talc. In one embodiment, glidants such as silicon dioxide can be used to improve flow characteristics of the

powder-mixture. In one embodiment, coloring agents, such as the FD&C dyes, can be added for appearance. Sweeteners and flavoring agents, such as aspartame, saccharin, menthol, peppermint, and fruit flavors, are useful adjuvants for chewable tablets. Capsules typically comprise one or more solid diluents disclosed above. In some embodiments, the selection of carrier components depends on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of this invention, and can be readily made by a person skilled in the art.

[0145] In one embodiment, the oral dosage form comprises predefined release profile. In one embodiment, the oral dosage form of the present invention comprises an extended release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises a slow release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises an immediate release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form is formulated according to the desired release profile of the long-acting OXM as known to one skilled in the art.

[0146] In another embodiment, compositions for use in the methods of this invention comprise solutions or emulsions, which in another embodiment are aqueous solutions or emulsions comprising a safe and effective amount of the compounds of the present invention and optionally, other compounds, intended for topical intranasal administration. In some embodiments, the compositions comprise from about 0.001% to about 10.0% w/v of a subject compound, more preferably from about 00.1% to about 2.0, which is used for systemic delivery of the compounds by the intranasal route.

[0147] In another embodiment, the pharmaceutical compositions are administered by intravenous, intra-arterial, subcutaneous or intramuscular injection of a liquid preparation. In another embodiment, liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously, and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially, and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions are administered intramuscularly, and are thus formulated in a form suitable for intramuscular administration.

[0148] Further, in another embodiment, the pharmaceutical compositions are administered topically to body surfaces, and are thus formulated in a form suitable for topical administration. Suitable topical formulations include gels, ointments, creams, lotions, drops and the like. For

topical administration, the compounds of the present invention are combined with an additional appropriate therapeutic agent or agents, prepared and applied as solutions, suspensions, or emulsions in a physiologically acceptable diluent with or without a pharmaceutical carrier.

[0149] In one embodiment, pharmaceutical compositions of the present invention are manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0150] In one embodiment, pharmaceutical compositions for use in accordance with the present invention is formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of OXM into preparations which, can be used pharmaceutically. In one embodiment, formulation is dependent upon the route of administration chosen.

[0151] In one embodiment, injectables, of the invention are formulated in aqueous solutions. In one embodiment, injectables, of the invention are formulated in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. In some embodiments, for transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0152] In one embodiment, the preparations described herein are formulated for parenteral administration, e.g., by bolus injection or continuous infusion. In another embodiment, formulations for injection are presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. In another embodiment, compositions are suspensions, solutions or emulsions in oily or aqueous vehicles, and contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0153] The compositions also comprise, in another embodiment, preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise, in some embodiments, local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0154] In one embodiment, pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally,

suspensions of long acting OXM, in some embodiments, are prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include, in some embodiments, fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions contain, in some embodiments, substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. In another embodiment, the suspension also contain suitable stabilizers or agents which increase the solubility of long acting OXM to allow for the preparation of highly concentrated solutions.

[0155] In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez- Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

[0156] In another embodiment, the pharmaceutical composition delivered in a controlled release system is formulated for intravenous infusion, implantable osmotic pump, transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump is used (see Langer, supra; Sefton, CRC Crit. Ref. *Biomed. Eng.* 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, supra, vol. 2, pp. 115-138 (1984). Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990).

[0157] In one embodiment, long acting OXM is in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use. Compositions are formulated, in some embodiments, for atomization and inhalation administration. In another embodiment, compositions are contained in a container with attached atomizing means.

[0158] In one embodiment, the preparation of the present invention is formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

[0159] In one embodiment, pharmaceutical compositions suitable for use in context of the present invention include compositions wherein long acting OXM is contained in an amount effective to achieve the intended purpose. In another embodiment, a therapeutically effective amount means an amount of long acting OXM effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

[0160] In one embodiment, determination of a therapeutically effective amount is well within the capability of those skilled in the art.

[0161] The compositions also comprise preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0162] Some examples of substances which can serve as pharmaceutically-acceptable carriers or components thereof are sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and methyl cellulose; powdered tragacanth; malt; gelatin; talc; solid lubricants, such as stearic acid and magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, mannitol, and polyethylene glycol; alginic acid; emulsifiers, such as the Tween™ brand emulsifiers; wetting agents, such sodium lauryl sulfate; coloring agents; flavoring agents; tableting agents, stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline; and phosphate buffer solutions. The choice of a pharmaceutically-acceptable carrier to be used in conjunction with the compound is basically determined by the way the compound is to be administered. If the subject compound is to be injected, in one embodiment, the pharmaceutically-acceptable carrier is sterile, physiological saline, with a blood-compatible suspending agent, the pH of which has been adjusted to about 7.4.

[0163] In addition, the compositions further comprise binders (e.g. acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g. cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (e.g., Tris-HCl., acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g. sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g. hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents(e.g. carbomer, colloidal silicon

dioxide, ethyl cellulose, guar gum), sweeteners (e.g. aspartame, citric acid), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), lubricants (e.g. stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g. colloidal silicon dioxide), plasticizers (e.g. diethyl phthalate, triethyl citrate), emulsifiers (e.g. carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g. ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[0164] Typical components of carriers for syrups, elixirs, emulsions and suspensions include ethanol, glycerol, propylene glycol, polyethylene glycol, liquid sucrose, sorbitol and water. For a suspension, typical suspending agents include methyl cellulose, sodium carboxymethyl cellulose, cellulose (e.g. Avicel™, RC-591), tragacanth and sodium alginate; typical wetting agents include lecithin and polyethylene oxide sorbitan (e.g. polysorbate 80). Typical preservatives include methyl paraben and sodium benzoate. In another embodiment, peroral liquid compositions also contain one or more components such as sweeteners, flavoring agents and colorants disclosed above.

[0165] The compositions also include incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance.

[0166] Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

[0167] In one embodiment, compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline. In another embodiment, the modified compounds exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds. In one embodiment, modifications also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. In another embodiment, the desired *in vivo* biological activity is achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

[0168] In another embodiment, preparation of effective amount or dose can be estimated initially from in vitro assays. In one embodiment, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

[0169] In one embodiment, toxicity and therapeutic efficacy of the long acting agonist (such as OXM) as described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. In one embodiment, the data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. In one embodiment, the dosages vary depending upon the dosage form employed and the route of administration utilized. In one embodiment, the exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

[0170] In one embodiment, depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

[0171] In one embodiment, the amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

[0172] In one embodiment, compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier are also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

[0173] In another embodiment, a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is administered via systemic administration. In another embodiment, a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is administered by intravenous, intramuscular or subcutaneous injection. In another embodiment, a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is lyophilized (i.e., freeze-dried) preparation in combination with complex organic excipients and stabilizers such as nonionic surface active agents (i.e., surfactants), various sugars, organic polyols and/or human serum albumin. In another embodiment, a pharmaceutical composition comprises a lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein in sterile water for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein in

sterile PBS for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein in sterile 0.9% NaCl for injection.

[0174] In another embodiment, the pharmaceutical composition comprises a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein and complex carriers such as human serum albumin, polyols, sugars, and anionic surface active stabilizing agents. See, for example, WO 89/10756 (Hara et al.- containing polyol and p-hydroxybenzoate). In another embodiment, the pharmaceutical composition comprises a reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein and lactobionic acid and an acetate/glycine buffer. In another embodiment, the pharmaceutical composition comprises a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein and amino acids, such as arginine or glutamate that increase the solubility of interferon compositions in water. In another embodiment, the pharmaceutical composition comprises a lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein and glycine or human serum albumin (HSA), a buffer (e g. acetate) and an isotonic agent (e.g NaCl). In another embodiment, the pharmaceutical composition comprises a lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein and phosphate buffer, glycine and HSA.

[0175] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is stabilized when placed in buffered solutions having a pH between about 4 and 7.2. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is stabilized with an amino acid as a stabilizing agent and in some cases a salt (if the amino acid does not contain a charged side chain).

[0176] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is a liquid composition comprising a stabilizing agent at between about 0.3% and 5% by weight which is an amino acid.

[0177] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein provides dosing accuracy and product safety. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein provides a biologically active, stable liquid formulation for use in injectable

applications. In another embodiment, the pharmaceutical composition comprises a non-lyophilized pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein.

[0178] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein provides a liquid formulation permitting storage for a long period of time in a liquid state facilitating storage and shipping prior to administration.

[0179] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises solid lipids as matrix material. In another embodiment, the injectable pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises solid lipids as matrix material. In another embodiment, the production of lipid microparticles by spray congealing was described by Speiser (Speiser and al., Pharm. Res. 8 (1991) 47-54) followed by lipid nanopellets for peroral administration (Speiser EP 0167825 (1990)). In another embodiment, lipids, which are used, are well tolerated by the body (e. g. glycerides composed of fatty acids which are present in the emulsions for parenteral nutrition).

[0180] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein is in the form of liposomes (J. E. Diederichs and al., Pharm./nd. 56 (1994) 267- 275).

[0181] In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises polymeric microparticles. In another embodiment, the injectable pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises polymeric microparticles. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises nanoparticles. In another embodiment, the pharmaceutical composition comprising a reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises liposomes. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated OXM as described herein comprises lipid emulsion. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises microspheres. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated

dual GLP-1/Glucagon receptor agonist as described herein comprises lipid nanoparticles. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises lipid nanoparticles comprising amphiphilic lipids. In another embodiment, the pharmaceutical composition comprising a pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist as described herein comprises lipid nanoparticles comprising a drug, a lipid matrix and a surfactant. In another embodiment, the lipid matrix has a monoglyceride content which is at least 50% w/w.

[0182] In one embodiment, compositions of the present invention are presented in a pack or dispenser device, such as an FDA approved kit, which contain one or more unit dosage forms containing the long acting dual GLP-1/Glucagon receptor agonist. In one embodiment, the pack, for example, comprise metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, in one embodiment, is labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

[0183] In one embodiment, it will be appreciated that the pegylated or reverse pegylated dual GLP-1/Glucagon receptor agonist of the present invention can be provided to the individual with additional active agents to achieve an improved therapeutic effect as compared to treatment with each agent by itself. In another embodiment, measures (e.g., dosing and selection of the complementary agent) are taken to adverse side effects which are associated with combination therapies.

[0184] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

[0185] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA

techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference. Other general references are provided throughout this document.

MATERIALS AND METHODS

PEG_{40}-Fmoc-OXM and PEG_{40}-FMS-OXM synthesis

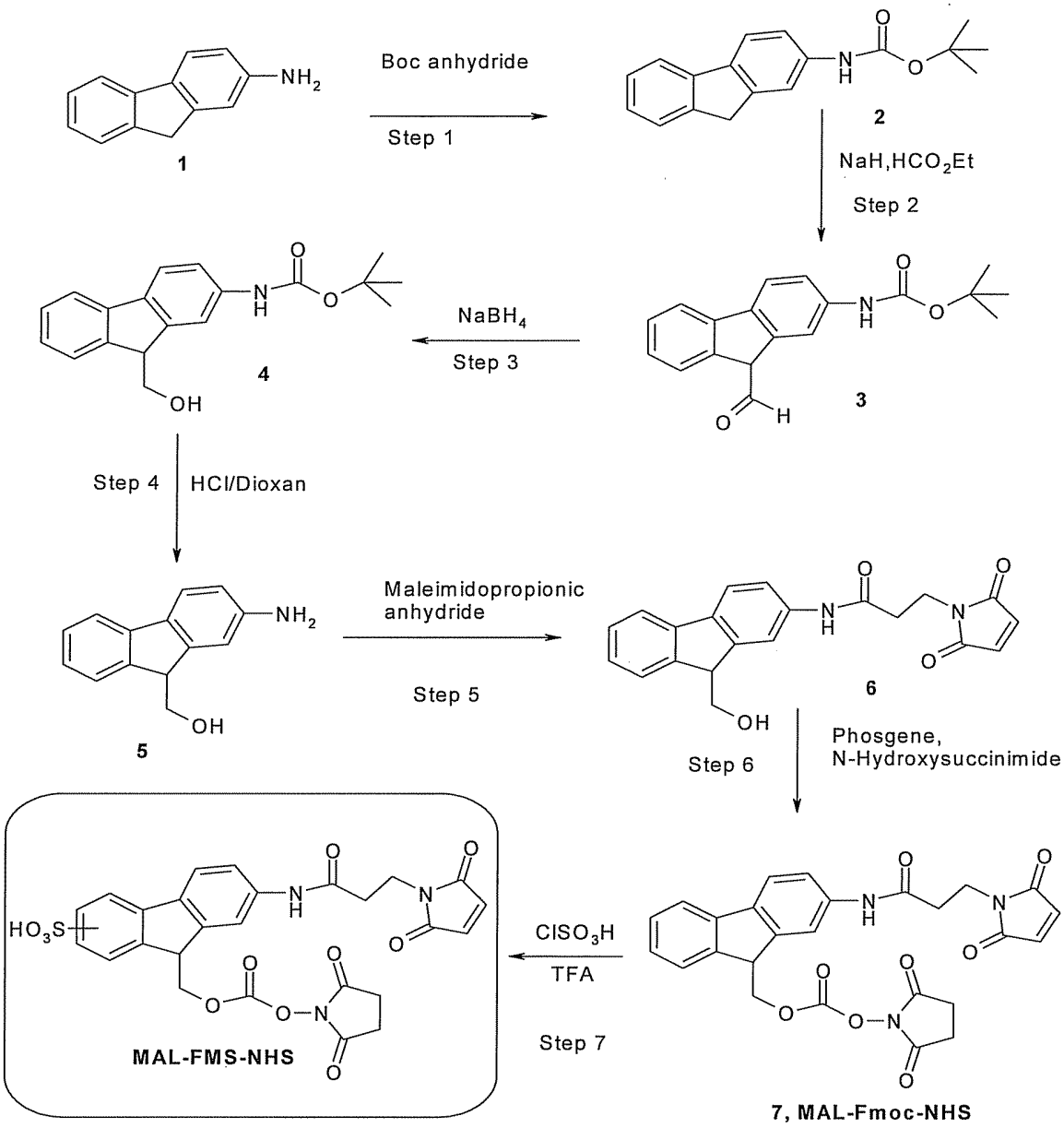
[0186] OXM synthesis: Oxyntomodulin of sequence: HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA (SEQ ID NO: 1) was synthesized by the solid phase method employing the Fmoc-strategy throughout the peptide chain assembly (Almac Sciences, Scotland). The peptide sequence was assembled using the following steps: (1) Capping: the resin was capped using 0.5M acetic anhydride (Fluka) solution in DMF (Rathburn); (2) De-protection: Fmoc-protecting group was removed from

the growing peptide chain using 20% v/v piperidine (Rathburn) solution in DMF (Rathburn); and (3) Amino Acid Coupling: 0.5 M Amino acid (Novabiochem) solution in DMF (Rathburn) was activated using 1M HOBt (Carbosynth) solution in DMF (Rathburn) and 1M DIC (Carbosynth) solution in DMF (Rathburn). Four equivalents of each amino acid were used per coupling.

[0187] The crude peptide was cleaved from the resin, and the protecting groups were removed by stirring in a cocktail of Triisopropylsilane (Fluka), water, dimethylsulphide (Aldrich), ammonium iodide (Aldrich) and TFA (Applied Biosystems) for 4 hours. The crude peptide was then collected by precipitation from cold diethyl ether.

[0188] Peptide Purification: Crude peptide was dissolved in acetonitrile (Rathburn)/water (MilliQ) (5:95) and loaded onto the preparative HPLC column. The chromatographic parameters are as follows: Column: Phenomenex Luna C18 250mm x 30mm, 15µm, 300A; Mobile Phase A: water + 0.1% v/v TFA (Applied Biosystems); Mobile Phase B: acetonitrile (Rathburn) + 0.1% v/v TFA (Applied Biosystems); UV Detection: 214 or 220 nm; Gradient: 25%B to 31%B over 4 column volumes; and flow rate 43mL/min.

Stage 2 – Linker Synthesis- Synthesis of MAL-FMS-NHS Linker:



[0189] The synthesis of compounds 2-5 was based on the procedures described by Albericio et al. in Synthetic Communication, 2001, 31(2), 225-232, which is incorporated herein by reference in its entirety.

[0190] 2-(Boc-amino)fluorene (2): 2- Aminofluorene (18g, 99mmol) was suspended in a mixture of dioxane:water (2:1) (200ml) and 2N NaOH (60ml) in an ice bath with magnetic stirring. Boc_2O (109mmol, 1.1 eq) was then added, and stirring continued at RT. The reaction was monitored by TLC (Rf= 0.5, Hexane/ Ethyl Acetate 2:1), and the pH was maintained between 9-10 by addition of 2N NaOH. Upon reaction completion, the suspension was

acidified with 1M KHSO4 to pH=3. The solid phase was filtered and washed with cold water (50ml), dioxane-water (2:1) and then azeotroped with toluene twice before using it in the next step.

[0191] 9-Formyl-2-(Boc-amino)fluorene (3): In a 3 necked RBF, NaH (60% in oil; 330mmol, 3.3eq) was suspended in dry THF (50ml), a solution of 2-(Boc-amino)fluorene from step 2 (28g; 100mmol) in dry THF (230ml) was added dropwise over 20 minutes. A thick, yellow slurry was observed, and the mixture stirred for 10 minutes at RT under nitrogen. Ethyl formate (20.1ml, 250mmol, 2.5eq) was added dropwise (caution: gas evolution). The slurry turned to a pale brown solution. The solution was stirred for 20 minutes. The reaction was monitored by TLC (Rf=0.5, Hexane/Ethyl acetate 1:1) and when only traces of starting material was observed, it was quenched with iced water (300ml). The mixture was evaporated under reduced pressure until most of the THF has been removed. The resulting mixture was treated with acetic acid to pH=5. The white precipitate obtained was dissolved in ethyl acetate and the organic layer separated. The aqueous layer was extracted with ethyl acetate and all the organic layer combined and washed with saturated sodium bicarbonate, brine and dried over $MgSO_4$. After filtration and solvent removal, a yellow solid was obtained. This material was used in the next step.

[0192] 9-Hydroxymethyl-2-(Boc-amino)fluorene (4): Compound 3 was suspended in MeOH (200ml) and sodium borohydride was added portion wise over 15 minutes. The mixture was stirred for 30 minutes (caution: exothermic reaction and gas evolution). The reaction was monitored by TLC (Rf=0.5, Hexane/EtOAc 1:1) and was completed. Water (500ml) was added and the pH adjusted to 5 with acetic acid. The work up involved extraction twice with ethyl acetate, washing the combined organic layers with sodium bicarbonate and brine, drying over $MgSO_4$, filtration and concentration to dryness. The crude obtained was purified by flask chromatography using Heptane/EtOAc (3:1) yielding a yellow foam (36g, 97.5% purity, traces of ethyl acetate and diethyl ether observed in the ^{1}H-NMR).

[0193] 9-Hydroxymethyl-2-aminofluorene (5): compound 4 was added to an ice cold solution of 4N HCl in dioxane. The reaction mixture was allowed to reach RT and stirred overnight. A pale yellow precipitate was obtained. The suspension was cold at 0°C and stirred further for 5 hours. After this time, the solid was filtered and washed thoroughly with DCM (5x30ml). After drying, a pale yellow solid was obtained (20g, 96.5% purity) with an overall yield of 80% over 3 steps.

[0194] 9-Hydroxymethyl-2-(amino-3-maleimidopropionate)fluorine (6): 9 Hydroxymethyl-2-aminofluorene (5, 5.5g, 26mmol) and maleimidopropionic anhydride

(6.93g, 26mmol) were placed in a 250ml RBF equipped with a stirrer, a reflux condenser and a nitrogen bubbler. Reaction mixture was refluxed at 85°C for 25 hours. TLC (Rf=0.25, Hexane/EtOAc 1:4) showed reaction completion after this time. The reaction mixture was concentrated under vacuum to afford a yellow solid. The product was purified by column chromatography.

[0195] MAL-Fmoc-NHS (7): A clean dry 500ml RBF with overhead agitation was charged triphosgene (1.58g, 0.35eq.) in dry THF (55ml) to form a solution at ambient. The solution was cooled to about 0°C with an ice/water bath, and a solution of NHS (0.67g, 0.38eq) in dry THF (19ml) was added dropwise over 10 minutes under nitrogen at 0°C. The resultant solution was stirred for 30 minutes. A further portion of NHS (1.34g, 0.77eq) in dry THF (36ml) was added dropwise at 0°C over 10 minutes and stirred for 15 minutes.

[0196] Compound 6 (5.5g, 1eq), dry THF (55ml) and pyridine (3.07ml, 2.5eq) were stirred together to form a suspension. This was added to the NHS solution in portions at 0-5°C and then allowed to go to RT by removing the ice bath. After 20 hours, the reaction was stopped (starting material still present, if the reaction is pushed to completion a dimmer impurity has been observed). The reaction mixture was filtered and to the filtrate, 4% brine (200ml) and EtOAc (200ml) were added. After separation, the organic layer was washed with 5% citric acid (220ml) and water (220ml). The organic layer was then concentrated to give 7.67g of crude MAL-Fmoc-NHS. The material was purified by column chromatography using a gradient cyclohexane/EtOAc 70:30 to 40:60. The fractions containing product were concentrated under vacuum to give 3.47g (45%) of MAL-Fmoc-NHS.

[0197] MAL-FMS-NHS (test reaction): to a solution of MAL-Fmoc-NHS (100mg, 0.2mmol) in trifluoroacetic acid (10ml), chlorosulfonic acid (0.5ml) was added. After 15 minutes, ice-cold diethyl ether (90ml) was added and the product precipitated. The material was collected by centrifugation, washed with diethyl ether and dried under vacuum. 41.3mg (35%) of beige solid was obtained.

Stage 3 – Conjugation

[0198] PEG-Fmoc-OXM conjugation: Conjugation with PEG, Fmoc and OXM were performed on a molar ratio of 1:1:1 e.g. PEG40-SH (44mg, in 4.4ml water equivalent to 1.0 μmol) added to peptide (4.5mg, equivalent to 1.0 μmol) and NaHCO3 (1M, 0.1ml) added. Fmoc (Almac, 10mg/ml in DMF, 50μl) added with stirring. Reaction stirred for 24h at RT.

[0199] PEG-FMS-OXM conjugation: All conjugations were performed at 1:1:1 molar ratio between the PEG the linker and OXM with the following reagents: PEG_{40}-SH and PEG_{30}-SH (NOF), FMS (Almac), EMCS (Termo Scientific), OXM (Almac). PEG_{40}-SH was

dissolved in 0.1M sodium phosphate buffer (Sigma) pH 7.2 to a concentration of 10mg/mL. The solution was added to one equivalent of purified OXM peptide (Almac). MAL-FMS-NHS (Almac) linker was dissolved in DMF to a concentration of 10mg/mL one equivalent added to the reaction. The mixture was stirred for 30 minutes. The solution was neutralised to pH 4 using glacial acetic acid (Fisher). The neutralised mixture was filtered (0.45μm) and separated using preparative chromatography. The reaction mixture was filtered and purified by preparative HPLC (Phenomenex Luna C18) lyophilized and stored frozen.

[0200] The chromatographic parameters were as follows: Column: Phenomenex Luna C18(2) 250mm x 30mm, 15μm prep, 100A; Mobile Phase A: water (MilliQ) + 0.1% v/v TFA (Applied Biosystems); Mobile Phase B: water/acetonitrile (Rathburn) (25:75) + 0.1% v/v TFA (Applied Biosystems); UV Detection: 214nm; Gradient: 10%B to 65%B over 41 minutes; and Flow: 43mL/min.

[0201] OXM content was determined using amino acid analysis (AAA) or basic hydrolysis. A defined quantity of lyophilized OXM conjugate was dissolved in water at a concentration of 20 mg/ml. The absorbance at 280nm was than determined, and the concentration according to the absorbance at 280nm was calculated using ε280=29,700. The concentration of the peptide was accurately quantitated by acid-hydrolyzing an aliquot followed by quantitative amino acid analysis; the ideal fraction is the one having close agreement between the calculated absorbance at 280nm and the peptide content.

Induction of cAMP cell based assay

[0202] CHO-K1 cells over-expressing GLP-1 receptor (Millipore HTS163C2) were seeded in 96 wells half-area white plate (Greiner) at a density of 200,000 cells/ml and incubated for 24 hours at 37°C. The cells were incubated with escalating concentrations of OXM (ALMAC), PEG40-EMCS-OXM and PEG40-Fmoc-OXM with or without rat serum 1% (Bio reclamation). Cells' cAMP concentrations were quantified by HTRF assay (Cisbio 62AM4PEB), and the EC50 parameter was analyzed by PRISM software.

Pharmacokinetic study

[0203] The pharmacokinetic profile of PEG_{40}-Fmoc-OXM was assessed as follows: Male Wistar rats were administrated intravenously (IV) or subcutaneously (SC) with a single dose of native OXM (n=9, 278μg/kg) or with PEG_{40}-Fmoc-OXM (n=6, 278μg/kg peptide equivalent). Cohorts of 3 animals per group were bled at alternating time points. OXM serum concentration was analyzed using a commercial ELISA kit (Cat# S-1393, Bachem).

IP glucose tolerance test

[0204] C57BL/6 male mice were fasted overnight and weighed, and blood glucose levels were measured by tail vein sampling using a handheld glucometer. Mice were IP injected with PBS (vehicle), OXM (333nmol/kg), PEG_{40}-EMCS-OXM (non–reversible pegylated OXM, 333nmol/kg body weight peptide content) and PEG40-Fmoc-OXM (202nmol/kg body weight peptide content) and PEG_{40}-Osu (546nmol/kg) as control. Glucose (1.5gr/kg) was administered IP either 15min after test article administration (vehicle, OXM and PEG_{40}-Osu) or 120 min after PEG_{40}-Fmoc-OXM administration. Blood glucose levels were measured by tail vein sampling prior to glucose administration and 10, 20, 30, 60 and 120 min after glucose administration using a handheld glucometer.

Diet-induced obesity mice model

[0205] Study 1: C57BL/6J mice (4-6 weeks of age, Harlan UK Limited, Bicester, Oxon, UK), were group housed upon arrival in polypropylene cages. All animals had free access to a high fat diet (D12451; 45% of kcal derived from fat; Research Diets, New Jersey, USA) and tap water at all times. Animals were maintained on a normal phase 12 h light-dark cycle (lights on 07:00). Animals were exposed to the appropriate diet for at least 6 months (until the average body weight was approximately 50g). Subsequently, animals were singly housed in polypropylene cages for a further two-week period and placed on reverse phase lighting (lights off for 8 h from 09:30 – 17:30 h). During the second week of single housing, animals began a once-daily handling protocol and a 7-day baseline period. Subsequently, mice were dosed with vehicle or test drug as given below in Table 1:

[0206] Table 1

Group	Treatment (sc)	Frequency	n
A	Vehicle (PBS)	b.i.d	10
B	OXM 5000nmol/kg body weight(PBS)	b.i.d	10
C	Sibutramine 20 mg/kg (PBS)	b.i.d	10
D	PEG40-FMS-OXM 5000nmol/kg body weight(citrate buffer)	Days 1, 3, 5, 7	10
E	556 mg/kg (27.8 mg/ml) PEG-SH (citrate buffer)	Days 1, 3, 5, 7	10

[0207] Measurements of body weight and food intake were performed daily until Day 8. The final measurement of body weight was carried out on Day 12. OXM and Sibutramine were formulated in PBS while PEG40-FMS-OXM and PEG-SH were formulated in 147mM

NaCl 10mM citrate buffer pH 6. OXM content in PEG40-FMS-OXM was determined by basic hydrolysis.

[0208] Study 2: Study 2 was carried out as described for Study 1. Following a baseline period, animals were dosed according to the following design described in Table 2:

Table 2Group	Treatment (SC)	Frequency	n
A	Vehicle (PBS)	b.i.d	8
B	OXM 5000nmol/kg body weight(PBS)	b.i.d	8
C	PEG40-FMS-OXM 1000nmol/kg body weight (citrate buffer)	Day 1,4,7	9
D	PEG40-FMS-OXM 5000nmol/kg body weight (citrate buffer)	Day 1,4,7	9
E	PEG40-FMS-OXM 8000nmol/kg body weight (citrate buffer)	Day 1,7	9
F	PEG40-EMCS-OXM 1000nmol/kg body weight (citrate buffer)	Day 1,4,7	9
G	PEG40-EMCS-OXM 5000nmol/kg body weight (citrate buffer)	Day 1,4,7	9
H	PEG40-EMCS-OXM 8000nmol/kg body weight (citrate buffer)	Day 1,7	9
I	PEG30-FMS-OXM 5000nmol/kg body weight (citrate buffer)	Day 1,4,7	9
J	PEG40-SH (citrate buffer)	Day 1,4,7	9
K	Sibutramine	b.i.d	8

[0209] Measurements of body weight and food intake were performed daily until Day 14.

[0210] Study 3: Study 3 was carried out as described for Study 1&2 with one difference, the mice at the beginning of the experiment were weight 45-46g. Following a baseline period, animals were dosed according to the following design described in Table 3:

[0211] Table 3

Group	Treatment (sc)	n
A	PEG5-FMS-OXM 6000 nmol/kg: Day 1, 8,15	7
B	PEG30-FMS-6000 nmol/kg: Day 1, 8, 15	7
C	PEG40—FMS-OXM 6000 nmol/kg: Day 1, 8, 15	7
D	PEG60-FMS-OXM 6000 nmol/kg: Day 1, 8,15	7
E	Vehicle (PBS sc)	7
F	Liraglutide (200 µg/kg bid) in PBS	7

Data and statistical analysis

[0212] OXM and Sibutramine were formulated in PBS while PEG40-EMCS-OXM, PEG40-FMS-OXM and PEG-SH were formulated in 147mM NaCl 10mM citrate buffer pH 6. OXM content in PEG40-FMS-OXM and PEG40-EMCS-OXM were determined by AAA.

[0213] Body weight and food intake are expressed as mean values ± SEM. Body weight, body weight gain, daily and average food intake data and cumulative food intake were analysed by ANCOVA with baseline as a covariate, followed by appropriate comparisons (two-tailed) to determine significant differences from the control group. $P<0.05$ is considered to be statistically significant. Baseline was Day 1 value for body weight or the average food or water consumption over the baseline period.

EXAMPLE 1

Synthesis and characterization of PEG-Fmoc-OXM

[0214] OXM peptide was synthesized by the solid phase method employing the Fmoc-strategy throughout the peptide chain assembly. The peptide was purified by preparative HPLC using Phenomenex Luna C18 (250 x 30mm) column by applying gradient between solution A (0.1% TFA+H_2O) and B (0.1% TFA + MeCN). Peptide purity was above 95%, the molecular weight was 4449 Da (measured by MALDI). Conjugation of OXM peptide to PEG_{40}-SH through Fmoc linker was performed in the presence of $NaHCO_3$. The reaction mixture was stirred for 24h at RT followed by filtration and purification by preparative HPLC (Jupiter C5). Conjugate molecular weight was analyzed by MALDI and OXM peptide content was analyzed by AAA. The average OXM peptide content was 189μg OXM per 1mg PEG_{10}-Fmoc-OXM conjugate 132.4μg OXM per 1mg PEG_{20}-Fmoc-OXM conjugate, 61.7μg OXM per 1mg PEG_{40}-Fmoc-OXM conjugate and 40μg OXM per 1mg PEG_{40}-FMS-OXM conjugate.

EXAMPLE 2

Pharmacokinetic profile of PEG10-Fmoc-OXM, PEG20-Fmoc-OXM and PEG40-Fmoc-OXM compared to native OXM

[0215] The pharmacokinetic profile of OXM compared to PEG_{10}-Fmoc-OXM and PEG_{20}-Fmoc-OXM was evaluated in male Wistar rats. Animals were administrated with a single SC injection of native OXM (278μg/kg peptide), PEG_{10}-Fmoc-OXM (278μg/kg peptide content) or PEG_{20}-Fmoc-OXM (278μg/kg peptide content). The serum concentration of the compound at indicated time intervals was measured (commercial ELISA, PK profile shown in Figure 1 and conventional noncompartmental PK parameters are summarized in Table 3). Reversible

pegylation of OXM conjugated to both PEG_{10} and PEG_{20} resulted in prolongation of the half-life of native OXM (0.15hr for native OXM; 16.16hr for PEG_{10}-Fmoc-OXM and 27.38hr for PEG_{20}-Fmoc-OXM). Exposure as, reflected by the AUC parameter, was increased by about ~450-fold for PEG_{10}-Fmoc-OXM and about ~2210 for PEG_{20}-Fmoc-OXM. Thus, reversible conjugation of OXM to PEG_{20} resulted in a more prolonged effect compared to PEG_{10}. In order to further characterize the PK profile of OXM reversibly conjugated to PEG_{40} through Fmoc linker, male Wistar rats were injected IV or SC with native OXM or PEG_{40}-Fmoc-OXM (278μg/kg peptide content) and serum concentration at indicated time points were analyzed (using commercial ELISA, PK profile shown in Figure 2 and conventional noncompartmental PK parameters are summarized in Table 4). The results indicated that reversible pegylation prolong the half life of OXM peptide by 100 fold, and increase the exposure significantly as reflected by AUC parameter, Moreover, the bioavailability of the native peptide was only 4.37% while administration of PEG_{40}-Fmoc-OXM resulted in 84% bioavailability.

[0216] Table 3: Non-compartmental PK parameters of OXM and PEG_{10}-Fmoc-OXM and PEG_{20}-Fmoc-OXM following SC administration in rats.

	AUC hr*ng/ml	**T1/2 term.** hr	**MRT** hr
OXM	3.2	0.15	0.3
PEG_{10}-Fmoc-OXM	1456	16.16	20.6
PEG_{20}-Fmoc-OXM	7079	27.38	37.2

[0217] Table 4: PK parameters of OXM and PEG_{40}-Fmoc-OXM following IV or SC administration in rats.

	Route of Administration	**AUC** hr*ng/ml	**T1/2 term.** hr	**MRT** hr	**F** %

OXM	IV	72.44	0.44	0.414	100
	SC	3.34	0.69	0.913	.374
PEG_{40}-Fmoc-OXM	IV	435,73	23.3	24.3	100
	SC	656,65	30.4	57.7	4.68

EXAMPLE 3

Induction of cAMP by OXM and reversible pegylated OXM

[0218] In order to assess the in vitro activity of the OXM compared to PEG40-Fmoc-OXM, and PEG40-EMCS-OXM (non –reversible pegylated OXM), CHO-K1 cells over-expressing GLP-1 receptor were incubated with escalating concentrations of the different compound followed by cAMP quantitation. Native OXM demonstrated improved activity compared to PEG_{40}-Fmoc-OXM and PEG_{40}-EMCS-OXM which had comparable in-vitro activity (EC50 of 2.53x10-9, 2.07x10-6 and 5.87x10-7 for OXM, PEG40-EMCS-OXM and PEG40-Fmoc-OXM respectively, Figure 3). Importantly, OXM pegylation didn't abrogate completely the GLP-1 receptor activation induced by OXM. In addition, while incubation of OXM in serum resulted in reduced activity, probably due partial proteolysis of the peptide, comparable activities in the present and absence of rat serum were obtained for PEG_{40}-Fmoc-OXM and PEG_{40}-EMCS-OXM, suggesting that pegylation masks potential proteolysis sites on OXM.

EXAMPLE 4

Reversible pegylated long acting OXM induced glucose tolerance

[0219] In order to evaluate the in vivo activity of the OXM or PEG_{40}-Fmoc-OXM, the IPGTT model was applied. Overnight fasted C57BL/6 mice were injected IP with OXM peptide or PEG_{40}-Fmoc-OXM followed by IP injection of glucose and measurement of blood glucose levels from the tail vein by glucometer. OXM (333nmol/kg), PEG_{40}-EMCS-OXM (non –reversible pegylated OXM, 333nmol/kg body weight peptide content) and PEG40-Fmoc-OXM (202nmol/kg body weight peptide content) were administrated IP 15 min (OXM and PEG_{40}-EMCS-OXM) or 2 hrs PEG_{40}-Fmoc-OXM, prior to glucose IP injection (1.5gr/kg). The induction of glucose tolerance was compared to vehicle group. As control to

the effect of PEG_{40}, a control group was administrated with PEG_{40}-Osu (546nmol/kg). While OXM peptide had a minor effect on the glucose tolerance compared to vehicle group, administration of PEG_{40}-Fmoc-OXM having even lower OXM molar content resulted in induced glucose tolerance (Figure 4). Surprisingly, administration of non-reversible pegylated resulted in induction of glucose tolerance suggesting that pegylated OXM is pharmacologically active in-vivo.

EXAMPLE 5

Reversible pegylated long acting OXM reduce body weight and inhibit food intake in DIO mice

[0220] The pharmacological activity of OXM was further evaluated in DIO mice following SC injection of native OXM, and reversibly-pegylated OXM. In study 1, male DIO mice (n=10 per group) were administered with either 5000nmol/kg body weight of OXM b.i.d or PEG_{40}-FMS-OXM containing 5000nmol/kg body weight OXM every other day for seven days of dosing. Body weight and food intake were measured daily for 8 days with a final measurement of body weight on day 12. Twice a day injection of OXM resulted in a moderate reduction in both body weight (6% weight loss on Day 8 compared to vehicle control group) and statistically significant inhibition of food intake. On the other hand, administration of PEG_{40}-FMS-OXM having the same OXM peptide content per dose but injected every other day resulted in a marked weight loss (24% weight loss on Day 8 compared to PEG-SH control group) and manifested a substantial inhibition of food intake (Figure 4). Sibutramine, neurotransmitter reuptake inhibitor, which was used as positive control reduced body weight by 15.6%. Of note, the reduction of body weight in the PEG_{40}-FMS-OXM group was consistent until the last measurement on Day 12 which is 5 days following the last dose, indicating a long lasting behavior of reversibly-pegylated OXM (Figure 5).

[0221] Since PEG_{40}-EMCS-OXM induced glucose tolerance in the IPGTT model it was important to compare the efficacy of non-reversible pegylated OXM to reversibly-pegylated OXM in the context of body weight and food intake. Consequently, a follow up study was designed to address this issue (study 2 in materials and methods). While administration of 5000nmol/kg body weight of PEG_{40}-FMS-OXM every 3 days (total of 3 injections) resulted in substantial reduction of body weight, injection of 5000nmol/kg body weight PEG_{40}-EMCS-OXM in the same frequency resulted in a negligible effect on body weight. Remarkably, single injection on Day 1 of 8000nmol/kg body weight of PEG_{40}-FMS-OXM

resulted in apparent weight reduction for 6 days. Surprisingly, administration of 5000nmol/kg body weight of PEG_{30}-FMS-OXM resulted in elevated reduction in body weight indicating an improved efficacy compared to PEG_{40}-FMS-OXM (Figure 6).

[0222] OXM is a potential peptide for the treatment of metabolic disorders such as diabetes and obesity, as demonstrated by the weight lost obtained by native OXM in over weight and obese healthy subject (Wynne et al, 2005). Yet, due to the short half-life of the peptide and its low stability in-vivo, repeated daily administrations of supra-physiological doses are required in order to achieve a pharmacological effect in humans. This patent provides effective means for stabilizing OXM in physiological conditions by reversibly pegylating the acting OXM thus rendering long-acting. Unexpectedly, the modified OXM-the pegylated version is active and is not a mere pro-drug.

[0223] Reversibly-pegylated OXM demonstrated superior pharmacokinetic profile in rats with a substantial increase in the exposure and elongated half-life compared to native OXM. When comparing the effect of PEGs with various molecular weights reversibly conjugated to OXM on OXM-PK profile, PEG_{40} -conjugate demonstrated a superior prolonging effect compared to PEG_{10} or PEG_{20}. Therefore, the PEG_{40} was further evaluated in pharmacological studies (Figures 1 and 2). Importantly, the bioavailability of OXM was significantly increased from 4.37% to 84.6% following SC administration of PEG40-Fmoc-OXM, contributing to the increased exposure of reversibly-pegylated peptide (Table 2). PEG_{40}-Fmoc-OXM improved glucose tolerance as compared to native OXM as assessed in overnight fasted C57BL/6 mice IPGTT model. In this model a non-reversible pegylated OXM conjugated to PEG_{40} (PEG_{40}-EMCS-OXM) demonstrated comparable glucose tolerance induction activity to the PEG40-Fmoc-OXM. This result further supported by the in-vitro activity observed for PEG_{40}-EMCS-OXM and PEG_{40}-Fmoc-OXM in which conventional pegylation of OXM does not completely abolish the binding of OXM to its receptor, a phenomenon observed for pegylated peptides due to steric interference, and consequently does not result in overall loss of biological activity (Figures 3 and 4).

[0224] Next, the effect of PEG_{40}-FMS-OXM on body weight and food intake was evaluated in DIO mice compared to native OXM. SC injection of 5000nmol/kg of native OXM administered twice daily resulted in a moderate reduction in body weight and food intake following 7 days of dosing. In contrast, injection of 5000nmol/kg PEG_{40}-FMS-OXM every other day resulted in a marked reduction in both body weight and food intake (6% and 24.9% reduction in body weight for OXM and PEG_{40}-FMS-OXM respectively, Figure 5) compared to control on Day 8. In conclusion, PEG_{40}-FMS-OXM exhibited a prolonged anti-obesity

effect and improved efficacy considering that the cumulative dose of OXM administered during the study for PEG_{40}-FMS-OXM was almost 4 times lower compared to the group administered with native OXM.

[0225] Non-reversible pegylated OXM, PEG_{40}-EMCS-OXM, was shown to improve glucose tolerance in IPGTT test. It was therefore imperative to evaluate the food regulation activity of conventional pegylation compared to reversibly pegylated OXM and native OXM in the DIO model. Administration of 5000nmol/kg of PEG_{40}-EMCS-OXM every three days resulted in a negligible reduction in body weight although inhibition of food intake was evident up to 3 days post dosing (Figure 6). The moderate inhibition of food intake probably results from the direct activity of OXM in the gastrointestinal tract and correlates with the peripheral activity observed in the IPGTT model. As OXM food regulation activity involves the crossing of the blood brain barrier and binding to receptors on neurons in the ARC, it is imperative that the ability of OXM to penetrate to the CNS will not be abolished. The observed peripheral bioactivity of PEG_{40}-EMCS-OXM as oppose to the lack of ability of this compound to reduce DIO mice body weight suggests that the covalent bond to the PEG moiety restrict the ability of PEG_{40}-EMCS-OXM to pass-through the BBB into the ARC which is the potential action site of OXM in the hypothalamus. In contrast, injection of 5000nmol/kg PEG_{40}-FMS-OXM in the same frequency markedly reduced body weight and inhibited food intake by 20% as measured on day 12. Remarkably, injection of 8000nmol/kg PEG_{40}-FMS-OXM once a week resulted in similar body weight reduction by 20%, indicating that in humans, significant weight lose can be achieved by once a week injection or even less frequent dosing of reversibly pegylated OXM.

[0226] The reversible pegylation strategy is aiming to overcome the loss of activity often observed in conventional pegylation while retaining the prolonging effect of the drug. In cases where the pegylated prodrug bioactivity is dramatically lost or even abolished the advantage in applying reversible pegylation was previously proven (United States Patent No. 7585837). Yet, it is unknown what will be the efficacy of a reversibly pegylated prodrug compared to covalently non-reversible pegylated drug that retain its biological activity. This is especially relevant as the PK profile of covalently pegylated peptide is expected to be superior compared to reversible pegylated peptide when assessing the conjugate blood concentrations, due to the slow release of the peptide from the conjugate. PEG_{40}-EMCS-OXM was shown to be active both in-vitro and in the IPGTT model in-vivo. Therefore, it was possible that this molecule will also induce satiety and reduce body weight following SC administration to mice. However, this was shown to be incorrect as PEG_{40}-EMCS-OXM fail

to reduce body weight in DIO mice while PEG_{40}-FMS-OXM had a marked effect. Previous publication presented conflicting data re the contribution of the peripheral activity of OXM and the CNS activity of OXM. On the one hand, the anorectic actions of ip OXM were blocked by prior intra-ARC administration of the GLP-1R, $exendin_{9-39}$ indicating the importance of the CNS-related activity of OXM (Dakin et al 2004). Yet, an oral delivery of Bifidobacterium expressing OXM to overweight mice resulted in reduction in body weight while OXM was not detected in the plasma of this mice, suggesting that the direct activation of gastrointestinal cells is important for the weight loss activity of OXM (Long et al, 2010). As mentioned above the lack of information on mode of action of OXM and the impact of pegylation, it was impossible to predict what will be the efficacy of reversibly pegylated OXM compared to native OXM and covalently bound pegylated OXM.

[0227] The superior efficacy of PEG_{30}-FMS-OXM compared to PEG_{40}-FMS-OXM as shown in study 2 was surprising. Although the PK profile of PEG_{30}-FMS-OXM was not evaluated, PEG_{40}-FMS-OXM PK profile was clearly superior to PEG_{10}-FMS-OXM and PEG_{20}-FMS-OXM. It is possible that the use of PEG_{30} present the favorable PEG size that on the one hand reduce significantly the renal clearance of the conjugated PEG_{30}-FMS-OXM, while facilitate OXM rate of hydrolysis from the conjugate that enable a sustained presence of OXM required for eliciting its pharmacological activities.

[0228] **Study 3**

[0229] In this study the conjugation of OXM to PEG of various sizes was evaluated. As was shown in the previous studies, administration of PEG30-FMS-OXM and PEG40-FMS-OXM once weekly had a marked effect on body weight. Surprisingly, PEG5-FMS-OXM completely lost the ability to induce weight loss as compared to the vehicle group, while PEG60-FMS-OXM induced an even more pronounced reduction than PEG30-FMS-OXM. The difference in weight loss between PEG30-FMS-OXM and PEG40-FMS-OXM in this study was in the experiment variability range and it was not significant.

EXAMPLE 6

Improved Glycemic and Lipidemic Profiles in Obese Mice Treated with Reversible PEGylated OXM

Materials and Methods

Experimental Procedures for Diet Induced Obesity (DIO) Mouse Model:

[0230] The DIO model was carried out at RenaSci Ltd Company (Nottingham, UK). C57BL/6J mice (4-6 weeks of age, Harlan UK Limited, Bicester, Oxon, UK), were exposed

to a high fat diet (D12451; 45% of kcal derived from fat; Research Diets, New Jersey, USA) for at least 6 months (until the average body weight is approximately 50g). Two weeks prior to drug administration, animals were singly housed and placed on reverse phase lighting (lights off for 8 h from 09:30 – 17:30 h). During the first week of single housing (handling period), animals began a once-daily handling protocol and during the second week (baseline period), they were dosed with the appropriate vehicle b.i.d. or once a week as they were dosed during the treatment period) by a subcutaneous route. 7 groups (n=8) of DIO mice were dosed for 29 days as follows:

Group	Treatment (SC)	Frequency
A	PEG40-SH (662 mg/kg)	Once a week (1, 8, 15, 22, 29)
B	PEG40-EMCS-OXM (6,000nmol/kg)	Once a week (1, 8, 15, 22, 29)
C	PEG30-EMCS-OXM (6,000nmol/kg)	Once a week (1, 8, 15, 22, 29)
D	PEG40-FMS-OXM (6,000nmol/kg)	Once a week (1, 8, 15, 22, 29)
E	PEG30-FMS-OXM (6,000nmol/kg)	Once a week (1, 8, 15, 22, 29)
F	Vehicle (PBS)	b.i.d
G	OXM (6,000nmol/kg; PBS)	b.i.d

[0231] During the baseline and the treatment period food intake, water intake and body weight were recorded daily. On days 1 and 22 after a two-week baseline, all the mice were overnight fasted. On days 2 and 23, the mice underwent an oral glucose tolerance test (OGTT). Each animal were dosed with vehicle or test compound and 60 minutes later were dosed with D-glucose (2 g/kg po). Baseline blood samples were taken immediately prior to dosing with vehicle or test compound (B1) and immediately before the glucose load (B2). Further blood samples were taken 10, 20, 30, 45, 60 and 120 minutes post glucose administration. All blood samples (approximately 20µl) were taken from the tail vein. Plasma samples were prepared and assayed for glucose (n = 2) and insulin (n = 1) using the Thermoelectron Infinity glucose reagent (TR15421) and Alpco mouse ultrasensitive insulin ELISA (80-INSMSU-E10), respectively. On Day 30, terminal plasma samples were collected (24 hours after the final dose on Day 29) by cardiac puncture and assayed for insulin,

glucose, cholesterol and triglycerides using the mouse ultrasensitive insulin ELISA (80-INSMSU-E10), Thermoelectron Infinity glucose reagent (TR15421), Thermoelectron Infinity cholesterol reagent (TR13421) and the Sigma Triglyceride kit (TR0100). Final carcass weights were recorded after terminal blood sampling and carcasses frozen at -20°C.

Experimental procedures for body composition studies:

[0232] Body fat, protein, water and ash levels of the carcasses were determined using standard chemical analysis techniques. Only fat, protein, water and ash content were measured, since other components (mainly carbohydrate) form less than 2% of total body composition. Carcass water was determined by freeze-drying the mouse carcasses to constant weight. Dried carcasses were then ground in a laboratory grinder ready for subsequent analyses. Carcass fat was determined on the freeze-dried samples using a modified Soxhlet extraction protocol (petroleum ether at 40-60°C) with a Tecator Soxtec 2050 system (Foss UK Ltd, Wheldrake, UK) according to the manufacturer's recommended protocol. Carcass protein was determined using a micro-Kjeldahl procedure on the freeze-dried samples using a Tecator 2012 digestion block and 2200 distilling unit (Foss UK Ltd). Residual carcass ash was determined by firing the freeze-dried samples at high temperatures using a muffle ashing furnace.

Data and statistical analysis:

[0233] Body weights, food intake and water intake expressed as mean values ± SEM. Body weight, body weight gain, daily and average food and water intake data and cumulative food intake were analysed by ANCOVA with baseline as a covariate, followed by appropriate comparisons (two-tailed) to determine significant differences from the control group. $P<0.05$ is considered to be statistically significant. Baseline was Day 1 value for body weight or the average food or water consumption over the baseline period.

[0234] Terminal plasma insulin, cholesterol and triglycerides were analysed by general linear model with treatment as a factor and bleeding order and baseline body weight as covariates followed by appropriate comparisons (two-tailed) to determine significant differences from the relevant vehicle group. A log transformation and/or robust regression techniques were used if appropriate.

[0235] Data for each body composition parameter (fat, protein, water and ash) were presented as g/carcass and % total. Final carcass weights were also analysed as a direct comparison. The analysis was done by robust regression with treatment as a factor and body weight at baseline as a covariate, followed by appropriate multiple comparisons tests (two-tailed) to compare the effects of each treatment group with the relevant vehicle group.

Results

[0236] A weekly injection of reversible PEG30 (PEG30-FMS-OXM (6,000 nmol/kg; citrate buffer)) or reversible PEG40 (PEG40-FMS-OXM (6,000 nmol/kg; citrate buffer)), during a 30-day period, provided 28% and 23% weight loss, respectively, compared to 17% weight loss for the group injected twice per day with native oxyntomodulin (Figure 7) – while the cumulative dosing of net oxyntomodulin injected with reversible PEG30 was only 8.6% for the 30-day period. Non-reversibly PEGylated OXM (PEG40-EMCS-OXM and PEG30-EMCS-OXM) were even less effective in reducing body weight.

[0237] Glucose tolerance in DIO mice after weekly injections with reversible PEGylated OXM (PEG30-FMS-OXM or PEG40-FMS-OXM) was comparable to the glucose tolerance elicited by a twice per day injection of native oxyntomodulin at Day 2 (Figure 8A) and at Day 23 (Figure 8B).

[0238] In addition, a once weekly administration of reversible PEGylated OXM improved the glycemic and lipidic profiles in DIO mice, demonstrated by a reduction in terminal glucose (Figure 9A), a reduction in terminal insulin (Figure 9B), a reduction in terminal cholesterol (Figure 9C), and a reduction in terminal glycerol (Figure 9D).

[0239] Finally, a body composition analysis of the DIO mice demonstrated that the weight loss demonstrated by mice treated with reversible PEGylated OXM resulted from a specific reduction in fat (Figure 10).

[0240] Taken together, reverse PEGylation was shown to be safe and tolerable in different toxicological rodent animal models. Reverse PEGylation also enables elongation of OXM half-life, while maintaining its potential to penetrate target tissues (e.g. penetrate the BBB).

[0241] Reversibly PEGylated OXM demonstrated superior long acting properties, supporting once weekly injection in humans. Reversibly PEGylated OXM reduced the body weight by a specific reduction in fat (Body Composition assessment). Reversibly PEGylated OXM improved the glycemic and lipidemic profiles. Reversibly PEGylated OXM is expected to provide long-term therapy for obesity and Type II Diabetes patients via its impressive effects on glycemic activity and fat loss.

EXAMPLE 7

Effect of reversible pegylated OXM on glucose level and insulin secretion

Experimental procedures for Diet induced Obesity (DIO) mice model:

[0242] The DIO model was carried out at RenaSci Ltd Company (Nottingham, UK). 57BL/6J mice (4 - 6 weeks of age, Harlan UK Limited, Bicester, Oxon, UK), were exposed to a high fat diet (D12451; 45% of kcal derived from fat; Research Diets, New Jersey, USA) for at least 6 months (until the average body weight was approximately 50g). Two weeks prior to drug administration, animals were singly housed, where they began an acclimation period. On the first week, the handling period, animals began a once-daily handling protocol and during the second week, the baseline period, they were dosed with the appropriate vehicle; (b.i.d or once a week as they were dose during the treatment period) by a subcutaneous route. During the baseline and the treatment period food intake, water intake and body weight were recorded daily. On the morning of day 1 the mice were dosed followed by an overnight fasting. On days 2, 24h following administration (groups A-E) or prior to the morning dosing (groups F-H), the mice were sampled for fasting glucose and fasting insulin. All blood samples (approximately 20µl) were taken from the tail vein. Plasma samples were prepared and assayed for glucose (n = 2) and insulin (n = 1) using the Thermoelectron Infinity glucose reagent (TR15421) and Alpco mouse ultrasensitive insulin ELISA (80-INSMSU-E10), respectively.

Results

[0243] In this set of experiments two independent *in vivo* studies were carried out. The first experiment included 8 groups (n=8) of DIO mice that were dosed for 2 days as follows:

Group	Treatment (SC)	Frequency
A	PEG40-SH (662 mg/kg)	Single injection on day 1
B	PEG40-EMCS-OXM (6,000nmol/kg)	Single injection on day 1
C	PEG30-EMCS-OXM (6,000nmol/kg)	Single injection on day 1
D	PEG40-FMS-OXM (6,000nmol/kg)	Single injection on day 1
E	PEG30-FMS-OXM (6,000nmol/kg)	Single injection on day 1
F	Vehicle (PBS)	b.i.d
G	OXM (6,000nmol/kg; PBS)	b.i.d
H	Liraglutide (200µg/kg)	b.i.d

[0244] The second experiment included 7 groups (n=7) of DIO mice that were dosed for 2 days as follows:

Group	Treatment (SC)	Frequency
A	PEG60-SH (947.4 mg/kg)	Single injection on day 1
B	PEG5-FMS-OXM 6000 nmol/kg	Single injection on day 1

C	PEG30-FMS-6000 nmol/kg	Single injection on day 1
D	PEG40—FMS-OXM 6000 nmol/kg	Single injection on day 1
E	PEG60-FMS-OXM 6000 nmol/kg	Single injection on day 1
F	Vehicle (PBS)	b.i.d
G	Liraglutide (200 μg/kg bid) in PBS	b.i.d

[0245] In both studies administration of a single dose of all PEG-OXM variants: PEG40-EMCS-OXM, PEG30-EMCS-OXM, PEG40-FMS-OXM, PEG30-FMS-OXM (Exp. #1) or PEG30-FMS-OXM, PEG40-FMS-OXM and PEG60-FMS-OXM (Exp. #2) produced marked and significant reductions in fasting glucose when compared to vehicle (figures 11 and 12). In experiment #1 the vehicle group (PEG40-SH) exhibits glucose level of 9.5 mM while the PEG-OXM treated groups exhibit glucose level of 5.18 to 5.8 mM. The same reduction of glucose level was obtained also for PEG-OXM treated group in experiment #2 (except PEG5-OXM group) that showed reduction of glucose from 11.9 mM of vehicle group to 5-5.7mM of PEG-OXM treated groups. This effect was associated with reduction in fasted plasma insulin levels in experiment #1 from 2.8 ng/ml of vehicle group to 1.4-1.9 ng/ml of PEG-OXM treated groups as shown in Figure 11. In Experiment #2 fasted insulin level was 0.99 ng/ml while fasted insulin level of PEG-OXM (except PEG5-OXM) was 0.78 to 0.91 ng/ml.

[0246] Liraglutide in both of the experiments significantly reduced fasting glucose when compared to vehicle (PBS); 9.3 mM of vehicle was decreased to 6.06 mM in experiment.#1 and 11.5 mM of vehicle was decreased to 6.7 mM in experiment. #2. Together with this reduction of glucose this treated group exhibited significant increase in plasma insulin from 2.5 ng/ml of vehicle to 4.4 ng/ml in experiment. #1 and from 1.98 ng/ml to 3 ng/ml in experiment. #2. OXM native peptide was analyzed in experiment. #1 and did not show any significant difference in glucose and insulin levels as compared to the vehicle, probably due to its short serum half-life and very rapid clearance from the body.

[0247] The results from these two independent experiments in DIO mice reveal that PEG-OXM compounds induce significant reduction of glucose level but without increasing insulin level as was observed following Liraglutide administration, and as expected from previously data that had been shown for OXM native peptide. This unexpected reduction of glucose level together with reduction of fasted insulin levels indicates that a single dose of PEG-OXM lead to increasing the sensitivity of the animals to insulin already following acute exposure and not due to chronic treatment.

[0248] While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

CLAIMS

What is claimed is:

1. A composition consisting of an oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

2. The composition of claim 1, wherein said PEG polymer is attached to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

3. The composition of claim 1, wherein said oxyntomodulin consists of the amino acid sequence set forth in SEQ ID NO: 1.

4. The composition of claim 1, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

5. The composition of claim 1, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

6. A pharmaceutical composition comprising the composition of claim 1 and a pharmaceutical acceptable carrier.

7. A method for extending the biological half life of oxyntomodulin, consisting of the step of conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of about 1:1:0.5 to about 1:1:3.5.

8. The method of claim 7, wherein said oxyntomodulin consists of an amino acid sequence of SEQ ID NO: 1.

9. The method of claim 7, wherein said PEG polymer is conjugated to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

10. The method of claim 7, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

11. The method of claim 7, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

12. A method of inducing glucose tolerance, glycemic control, or both in a subject in need thereof, comprising the step of administering to said subject an effective amount of the composition of claim 1 and a pharmaceutical acceptable carrier.

13. The method of claim 12, wherein said oxyntomodulin consists of an amino acid sequence of SEQ ID NO: 1.

14. The method of claim 12, wherein said PEG polymer is conjugated to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

15. The method of claim 12, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

16. The method of claim 12, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

17. A method of improving the area under the curve (AUC) of oxyntomodulin, consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

18. The method of claim 17, wherein said oxyntomodulin consists of an amino acid sequence of SEQ ID NO: 1.

19. The method of claim 17, wherein said PEG polymer is conjugated to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

20. The method of claim 17, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

21. The method of claim 17, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

22. A method of reducing the dosing frequency of oxyntomodulin, consisting of the step of conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

23. The method of claim 22, wherein said oxyntomodulin consists of an amino acid sequence of SEQ ID NO: 1.

24. The method of claim 22, wherein said PEG polymer is conjugated to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

25. The method of claim 22, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

26. The method of claim 22, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

27. A method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker to said subject, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

28. The method of claim 27, wherein said oxyntomodulin consists of an amino acid sequence of SEQ ID NO: 1.

29. The method of claim 27, wherein said PEG polymer is conjugated to the amino terminus or lysine residue of said oxyntomodulin via Fmoc or FMS.

30. The method of claim 27, wherein said PEG polymer is a PEG polymer with a sulfhydryl moiety.

31. The method of claim 27, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

32. A method for increasing insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of a composition comprising oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer).

33. The method of claim 32, wherein said PEG polymer is PEG_{30}, PEG_{40} or PEG_{60}.

34. The method of claim 32, wherein said oxyntomodulin is conjugated to said polyethylene glycol polymer (PEG polymer) via a linker.

35. The method of claim 33, wherein said linker is a cleavable flexible linker.

36. The method of claim 33, wherein the linker is a non-cleavable linker.

37. The method of claim 35, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

38. The method of claim 36, wherein said linker is *N*-(ε-Maleimidocaproyloxu) succinimide ester (EMCS).

39. The method of claim 32, wherein administering said composition results in an acute increase in insulin sensitivity in said subject.

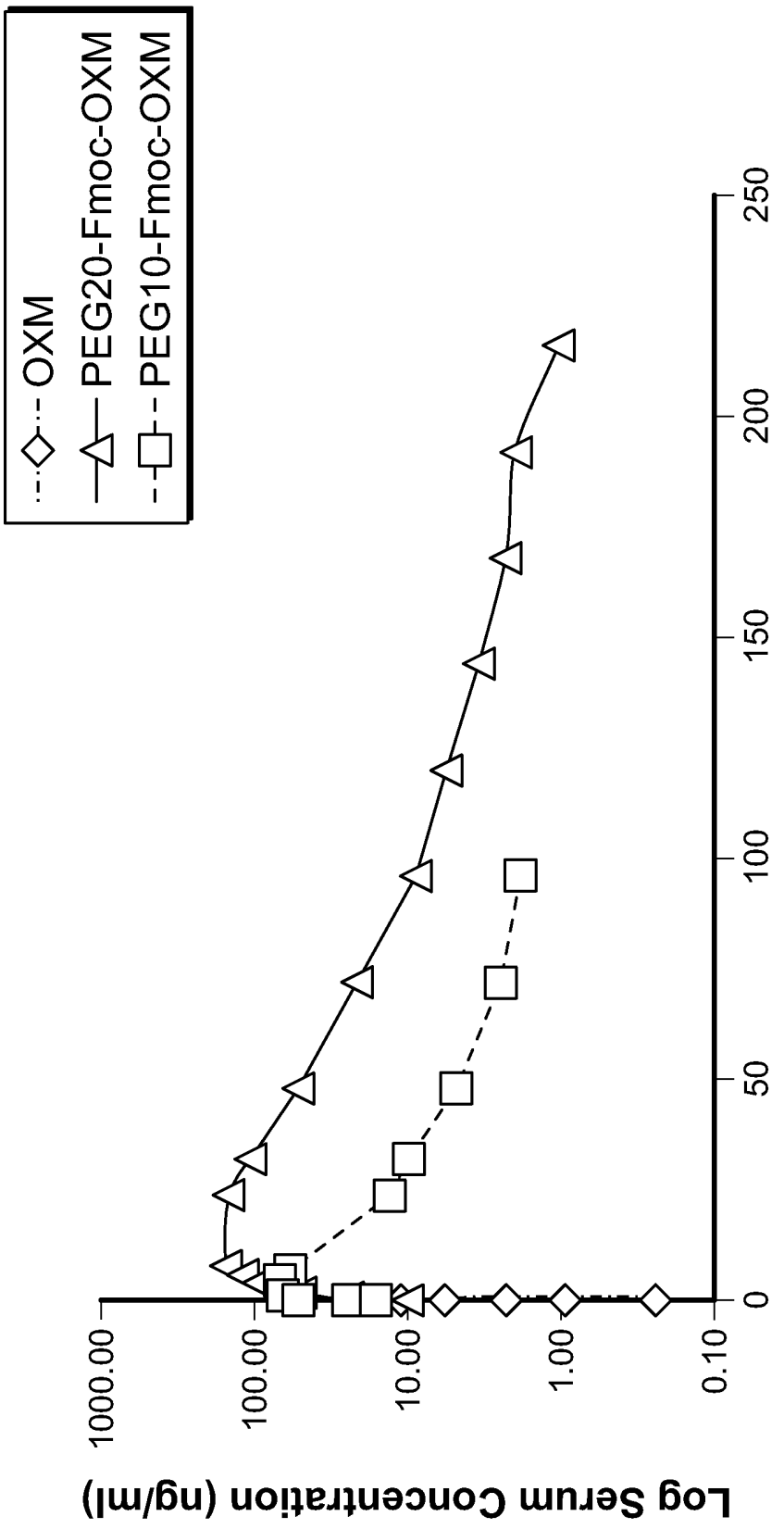

OXM
PEG20-Fmoc-OXM
PEG10-Fmoc-OXM
Log Serum Concentration (ng/ml)
1000.00
100.00
10.00
1.00
0.10
0
50
100
150
200
250
Time Post Injection (hr)

FIG. 1

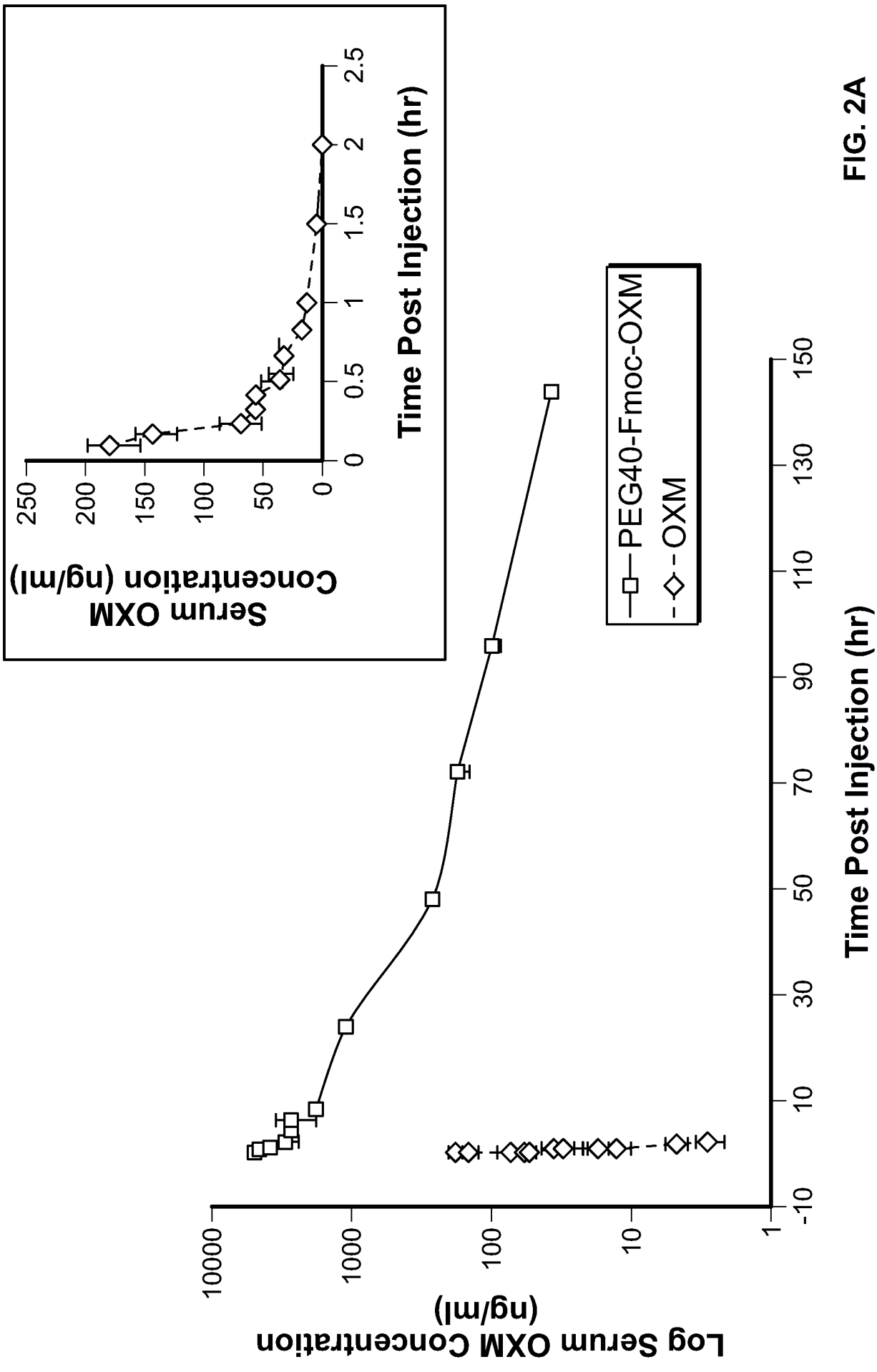

Serum OXM Concentration (ng/ml)
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
Time Post Injection (hr)
Log Serum OXM Concentration (ng/ml)
10000
1000
100
10
1
-10
10
30
50
70
90
110
130
150
Time Post Injection (hr)
PEG40-Fmoc-OXM
OXM

FIG. 2A

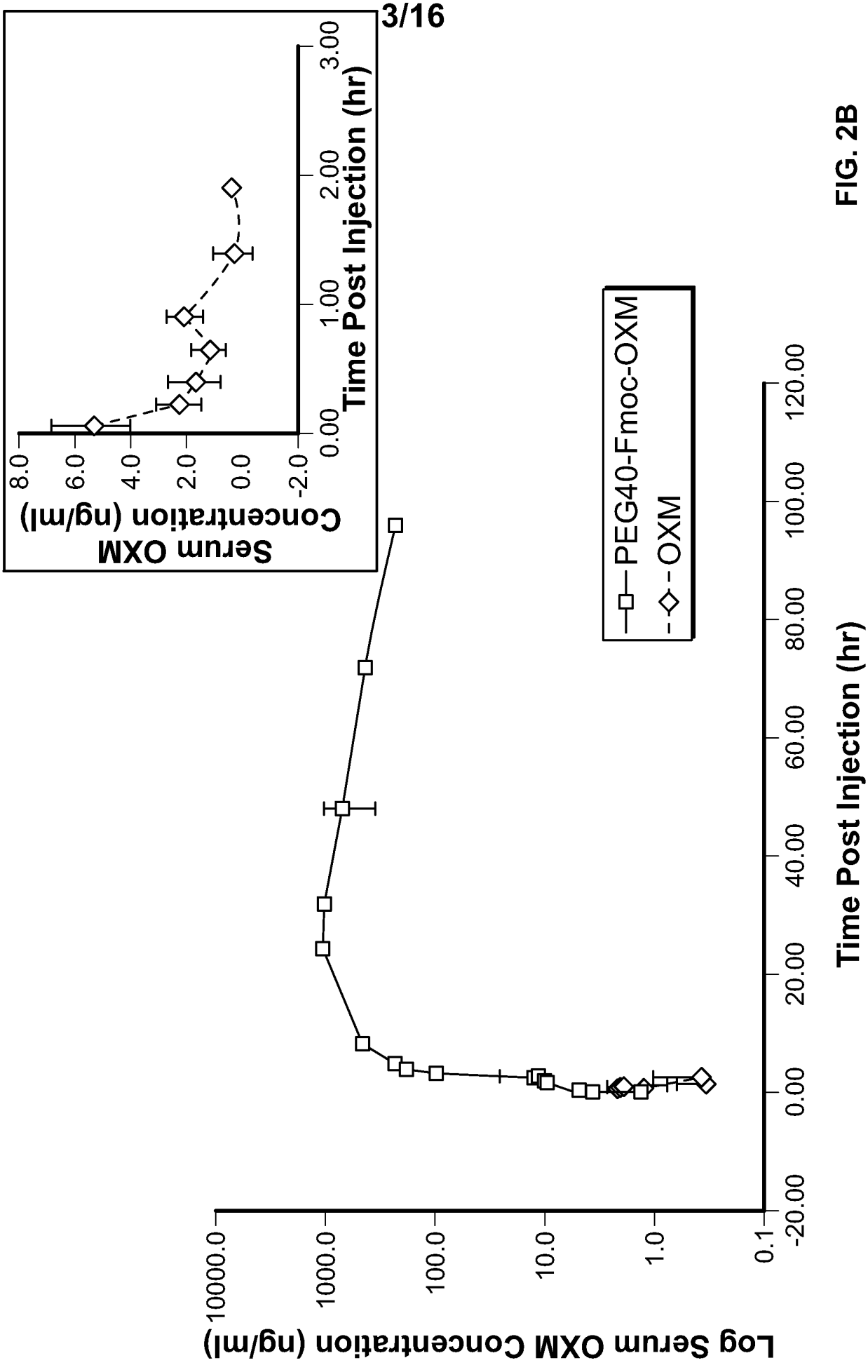

Log Serum OXM Concentration (ng/ml)
10000.0
1000.0
100.0
10.0
1.0
0.1
-20.00
0.00
20.00
40.00
60.00
80.00
100.00
120.00
Time Post Injection (hr)
PEG40-Fmoc-OXM
OXM
Serum OXM Concentration (ng/ml)
8.0
6.0
4.0
2.0
0.0
-2.0
0.00
1.00
2.00
3.00
Time Post Injection (hr)

FIG. 2B

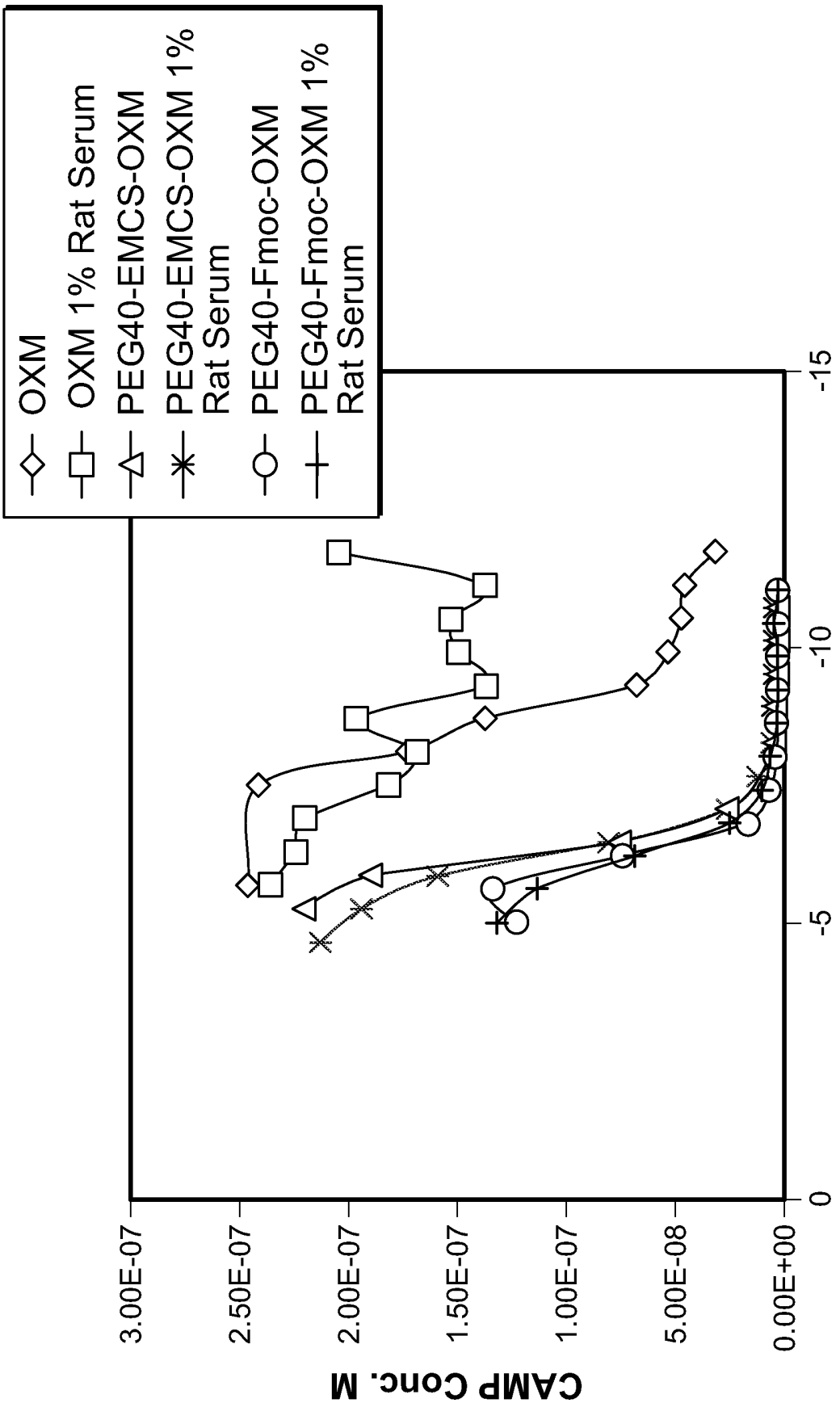

OXM
OXM 1% Rat Serum
PEG40-EMCS-OXM
PEG40-EMCS-OXM 1% Rat Serum
PEG40-Fmoc-OXM
PEG40-Fmoc-OXM 1% Rat Serum
cAMP Conc. M
3.00E-07
2.50E-07
2.00E-07
1.50E-07
1.00E-07
5.00E-08
0.00E+00
0
-5
-10
-15
Ligand Conc. (LogM)

FIG. 3

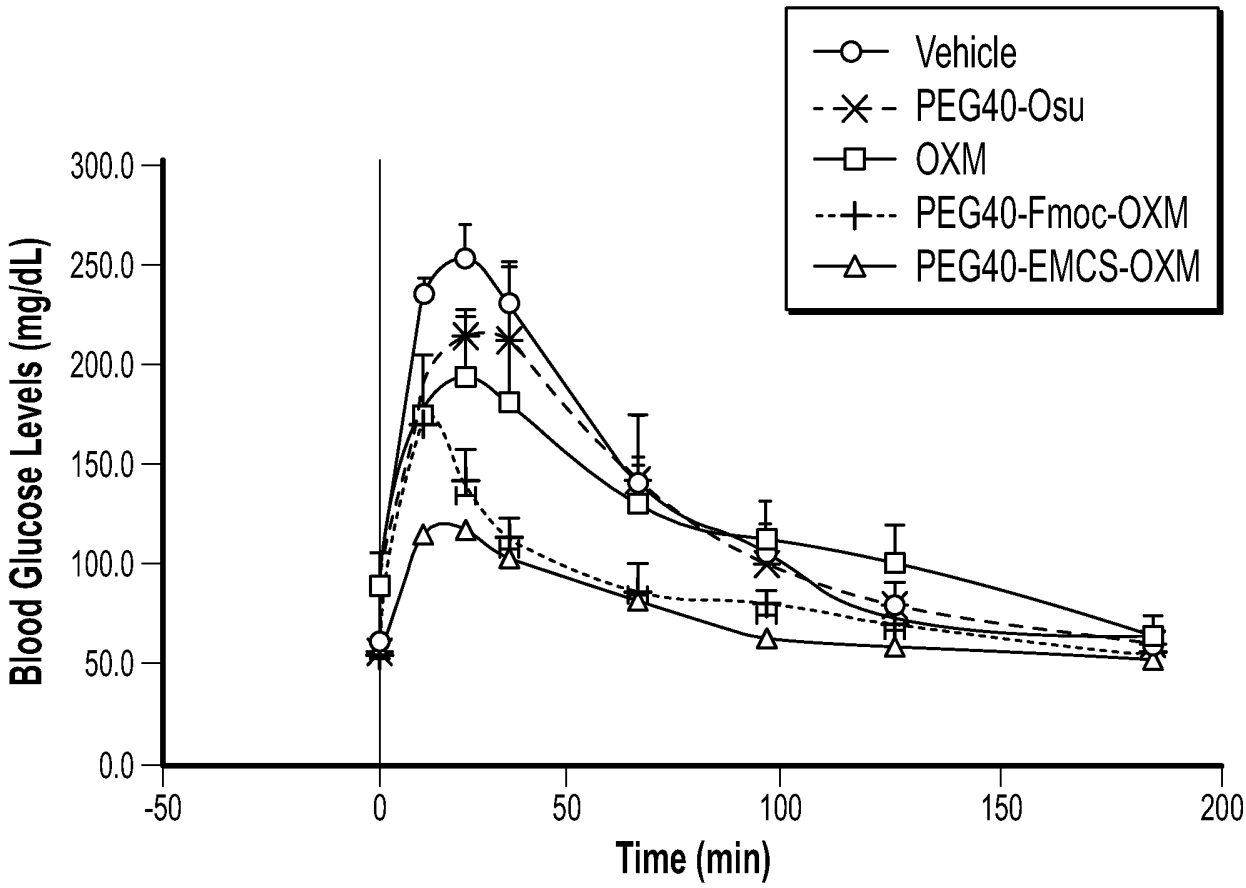

Vehicle
PEG40-Osu
OXM
PEG40-Fmoc-OXM
PEG40-EMCS-OXM
Blood Glucose Levels (mg/dL)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50
0
50
100
150
200
Time (min)

FIG. 4A

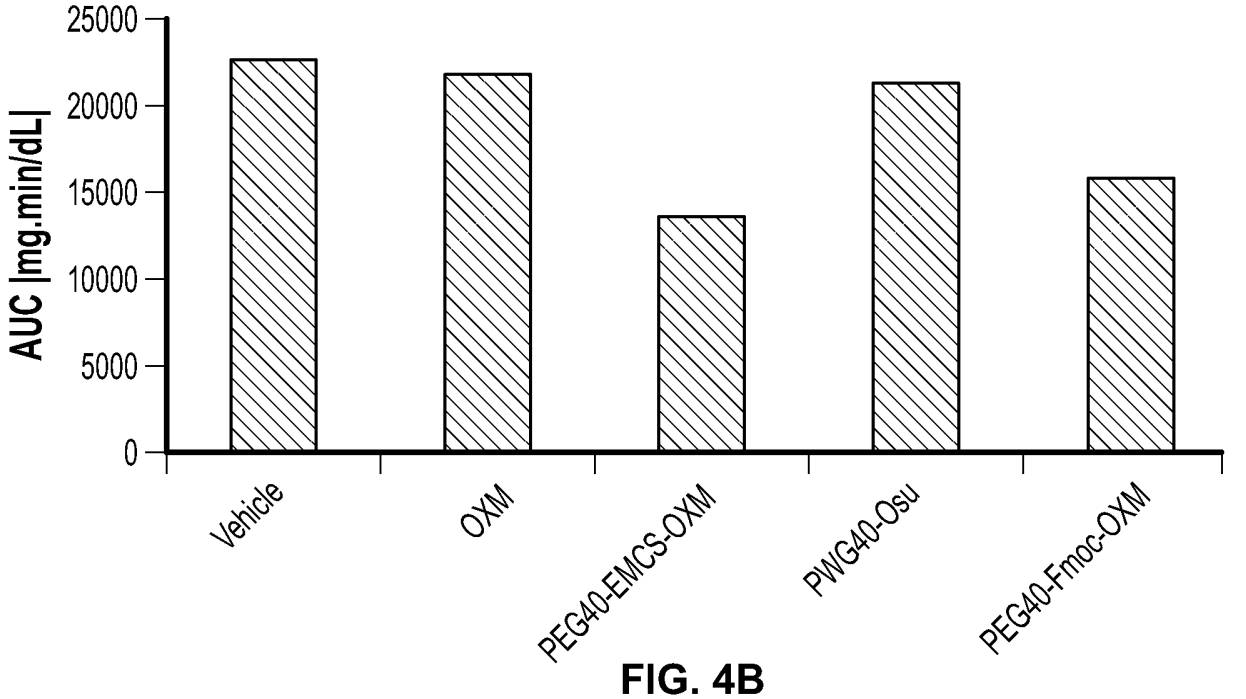

AUC |mg.min/dL|
25000
20000
15000
10000
5000
0
Vehicle
OXM
PEG40-EMCS-OXM
PWG40-Osu
PEG40-Fmoc-OXM

FIG. 4B

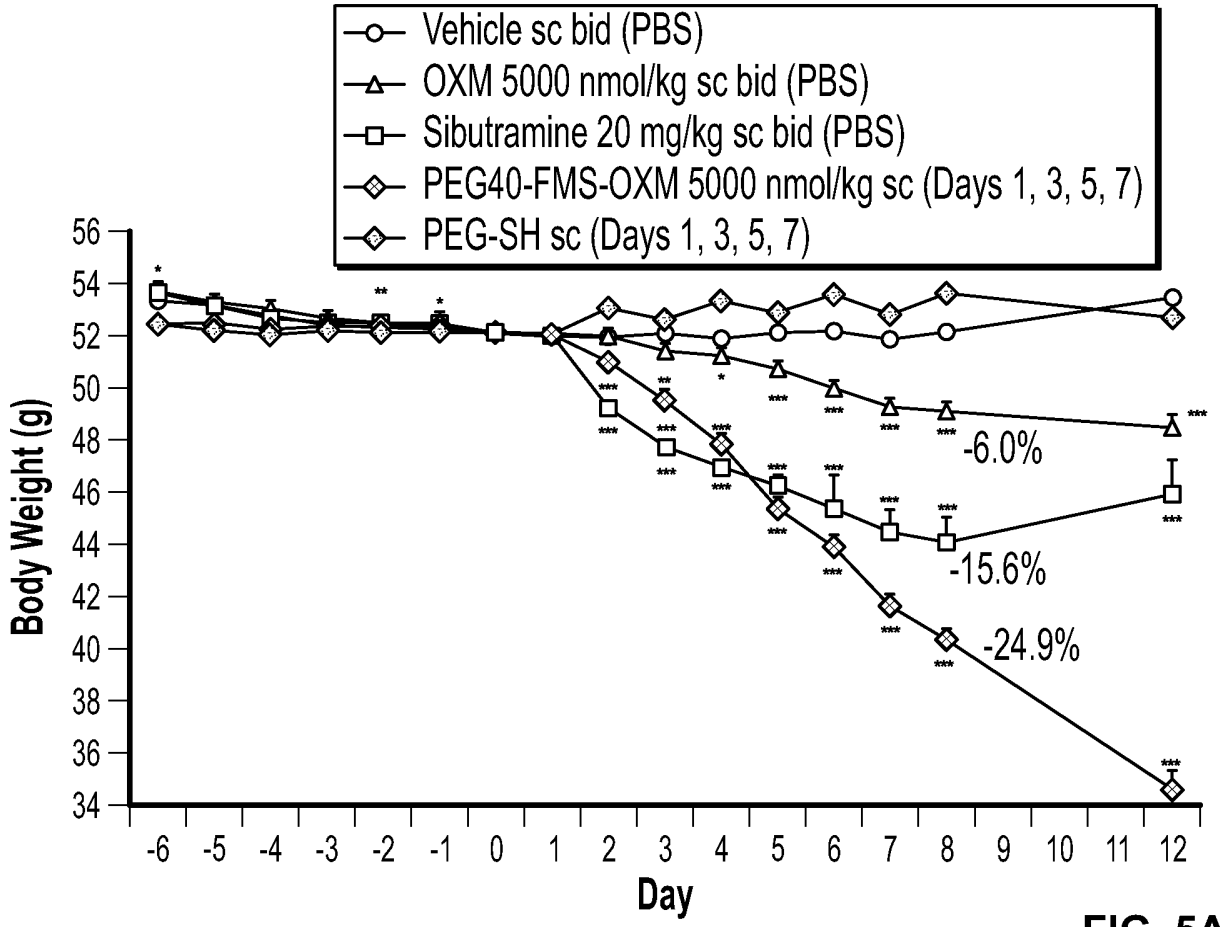

Vehicle sc bid (PBS)
OXM 5000 nmol/kg sc bid (PBS)
Sibutramine 20 mg/kg sc bid (PBS)
PEG40-FMS-OXM 5000 nmol/kg sc (Days 1, 3, 5, 7)
PEG-SH sc (Days 1, 3, 5, 7)
Body Weight (g)
56
54
52
50
48
46
44
42
40
38
36
34
-6.0%
-15.6%
-24.9%
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12
Day

FIG. 5A

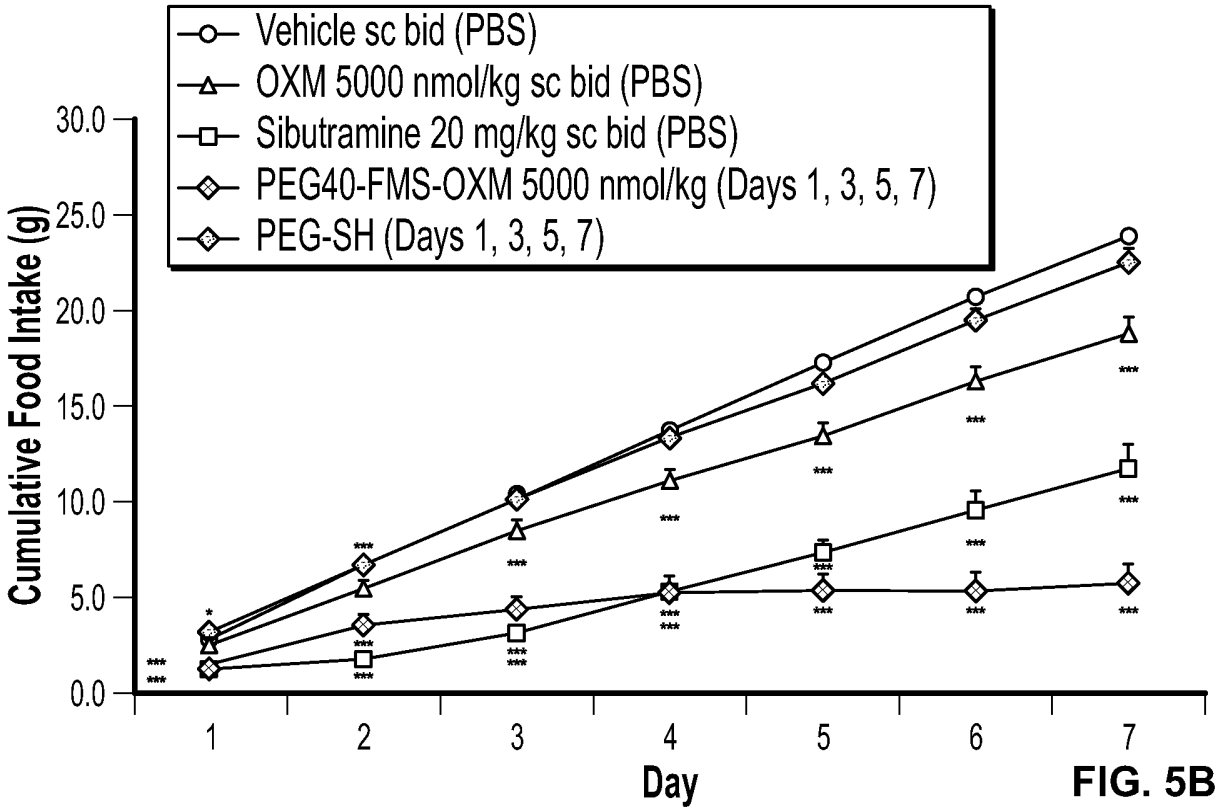

Vehicle sc bid (PBS)
OXM 5000 nmol/kg sc bid (PBS)
Sibutramine 20 mg/kg sc bid (PBS)
PEG40-FMS-OXM 5000 nmol/kg (Days 1, 3, 5, 7)
PEG-SH (Days 1, 3, 5, 7)
Cumulative Food Intake (g)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1 2 3 4 5 6 7
Day

FIG. 5B

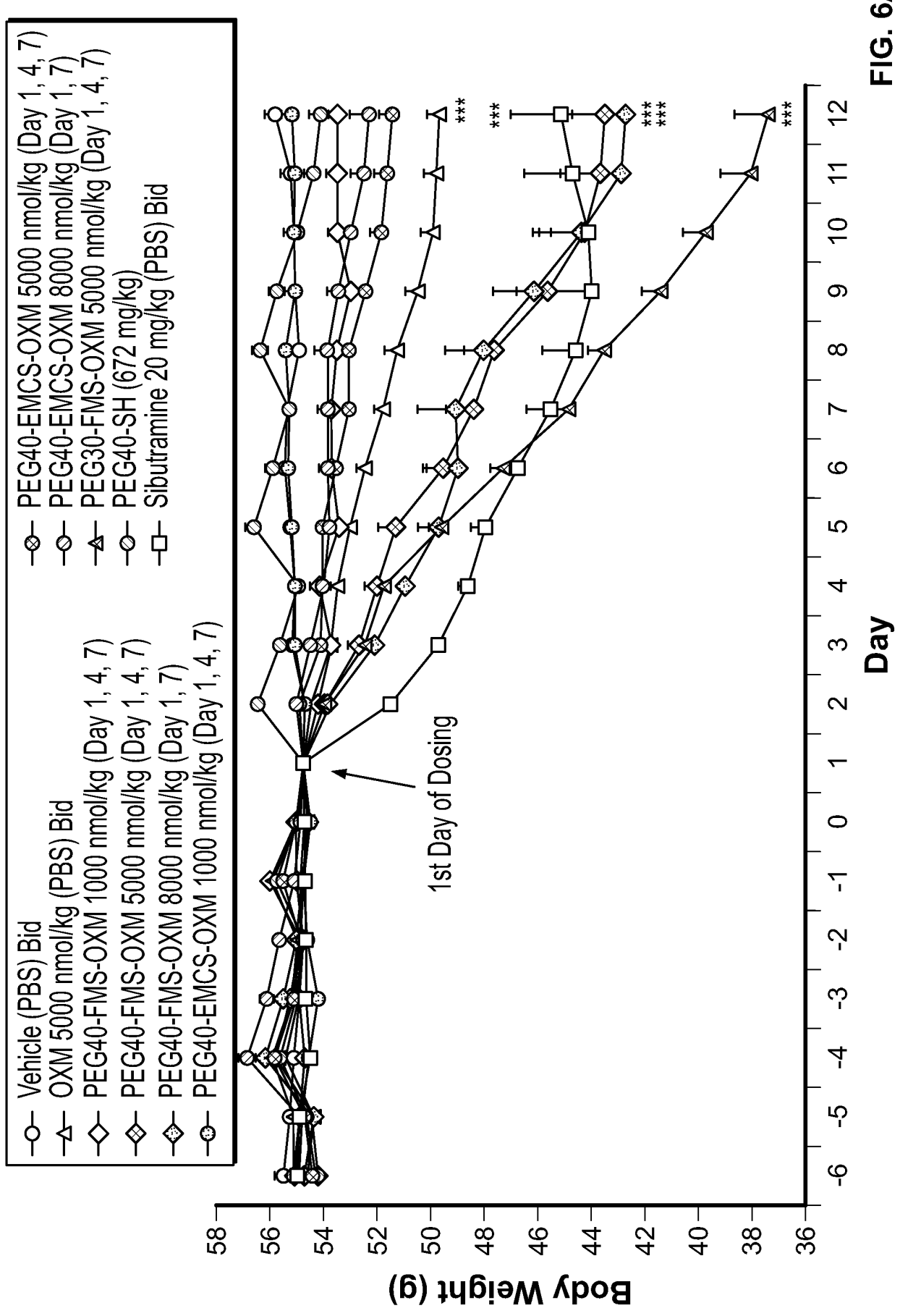

Vehicle (PBS) Bid
OXM 5000 nmol/kg (PBS) Bid
PEG40-FMS-OXM 1000 nmol/kg (Day 1, 4, 7)
PEG40-FMS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-FMS-OXM 8000 nmol/kg (Day 1, 7)
PEG40-EMCS-OXM 1000 nmol/kg (Day 1, 4, 7)
PEG40-EMCS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-EMCS-OXM 8000 nmol/kg (Day 1, 7)
PEG30-FMS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-SH (672 mg/kg)
Sibutramine 20 mg/kg (PBS) Bid
1st Day of Dosing
***
Body Weight (g)
Day
58
56
54
52
50
48
46
44
42
40
38
36
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12

FIG. 6A

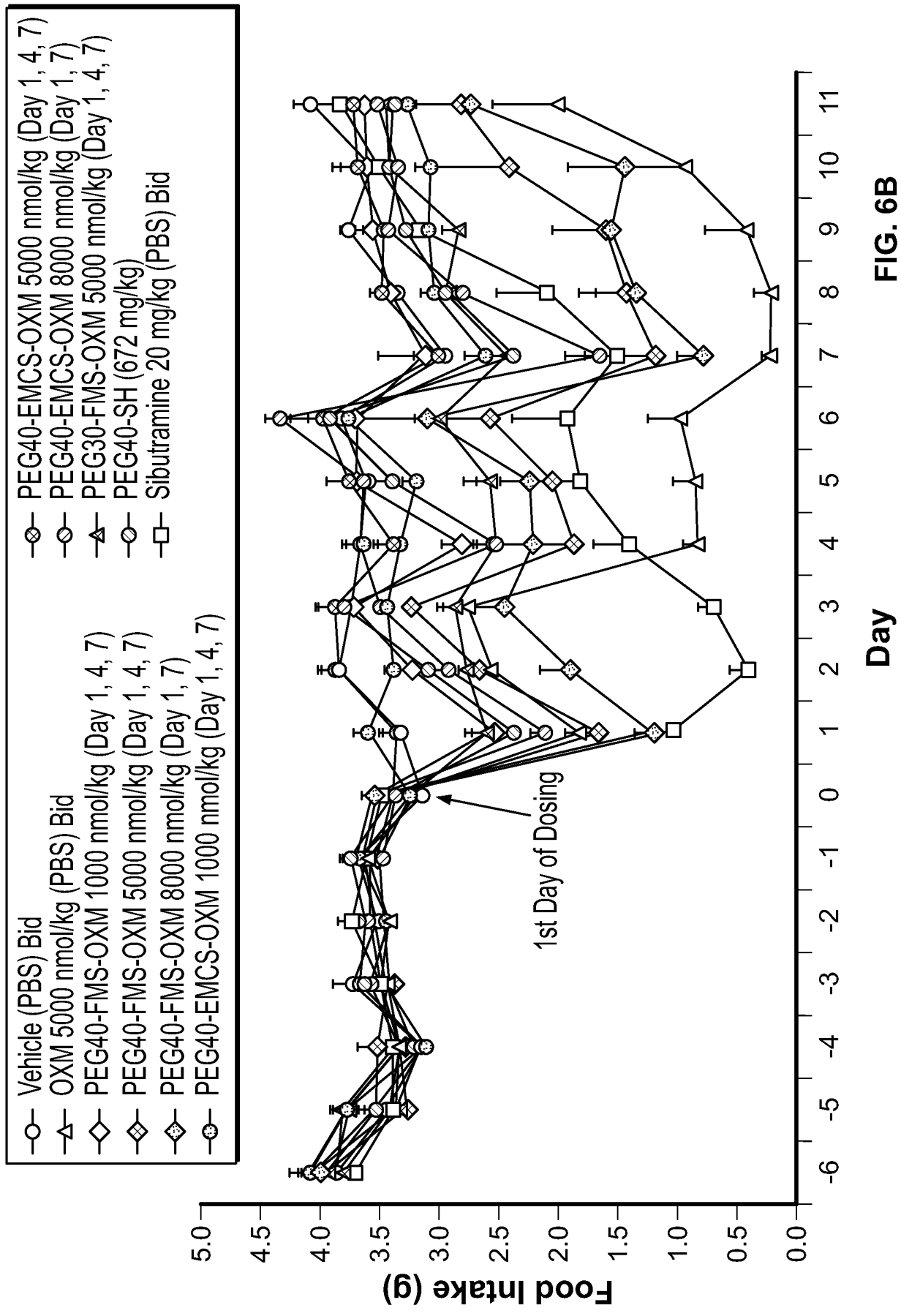

Vehicle (PBS) Bid
OXM 5000 nmol/kg (PBS) Bid
PEG40-FMS-OXM 1000 nmol/kg (Day 1, 4, 7)
PEG40-FMS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-FMS-OXM 8000 nmol/kg (Day 1, 7)
PEG40-EMCS-OXM 1000 nmol/kg (Day 1, 4, 7)
PEG40-EMCS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-EMCS-OXM 8000 nmol/kg (Day 1, 7)
PEG30-FMS-OXM 5000 nmol/kg (Day 1, 4, 7)
PEG40-SH (672 mg/kg)
Sibutramine 20 mg/kg (PBS) Bid
1st Day of Dosing
Food Intake (g)
Day
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11

FIG. 6B

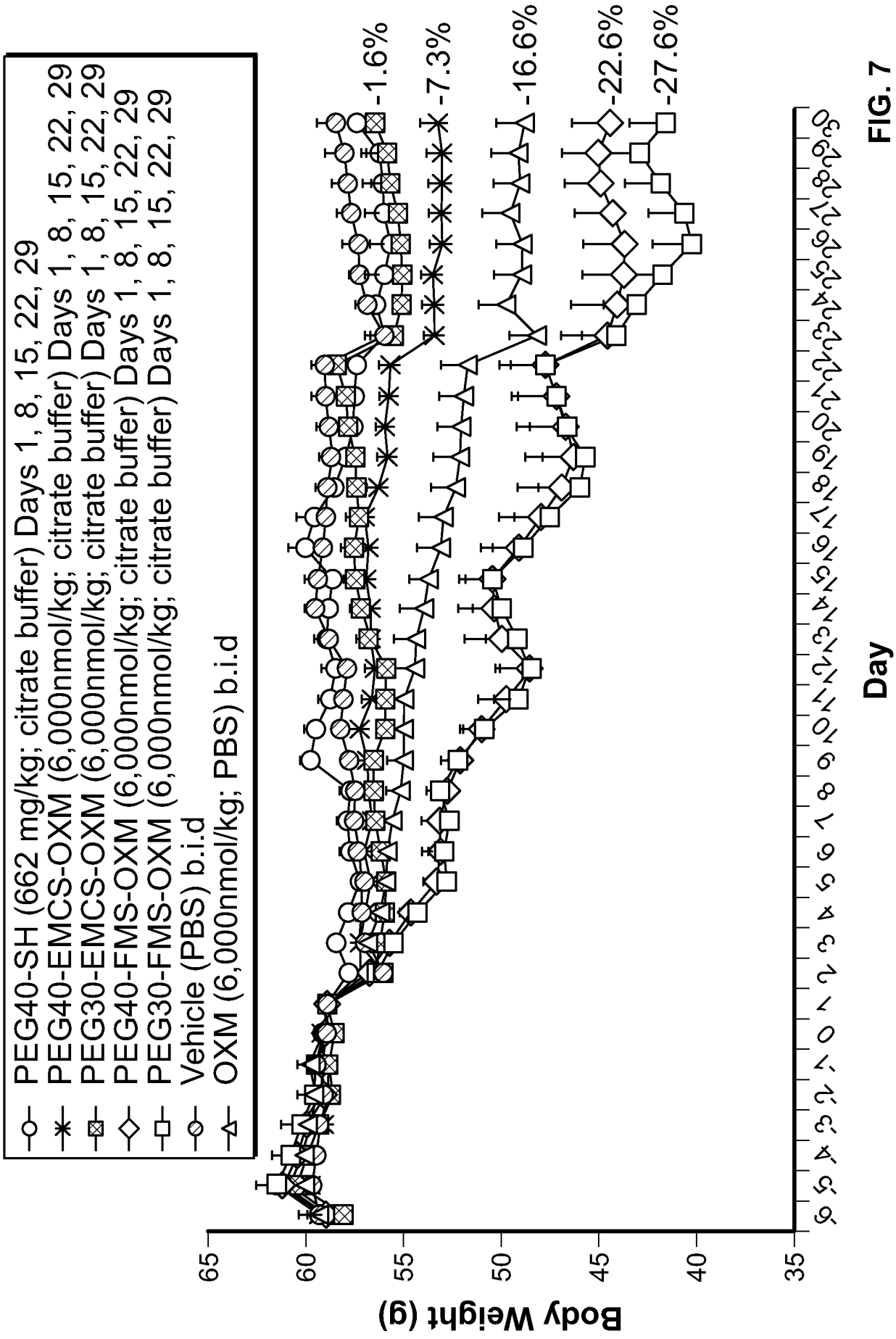

PEG40-SH (662 mg/kg; citrate buffer) Days 1, 8, 15, 22, 29
PEG40-EMCS-OXM (6,000nmol/kg; citrate buffer) Days 1, 8, 15, 22, 29
PEG30-EMCS-OXM (6,000nmol/kg; citrate buffer) Days 1, 8, 15, 22, 29
PEG40-FMS-OXM (6,000nmol/kg; citrate buffer) Days 1, 8, 15, 22, 29
PEG30-FMS-OXM (6,000nmol/kg; citrate buffer) Days 1, 8, 15, 22, 29
Vehicle (PBS) b.i.d
OXM (6,000nmol/kg; PBS) b.i.d
-1.6%
-7.3%
-16.6%
-22.6%
-27.6%
Body Weight (g)
65
60
55
50
45
40
35
Day

FIG. 7

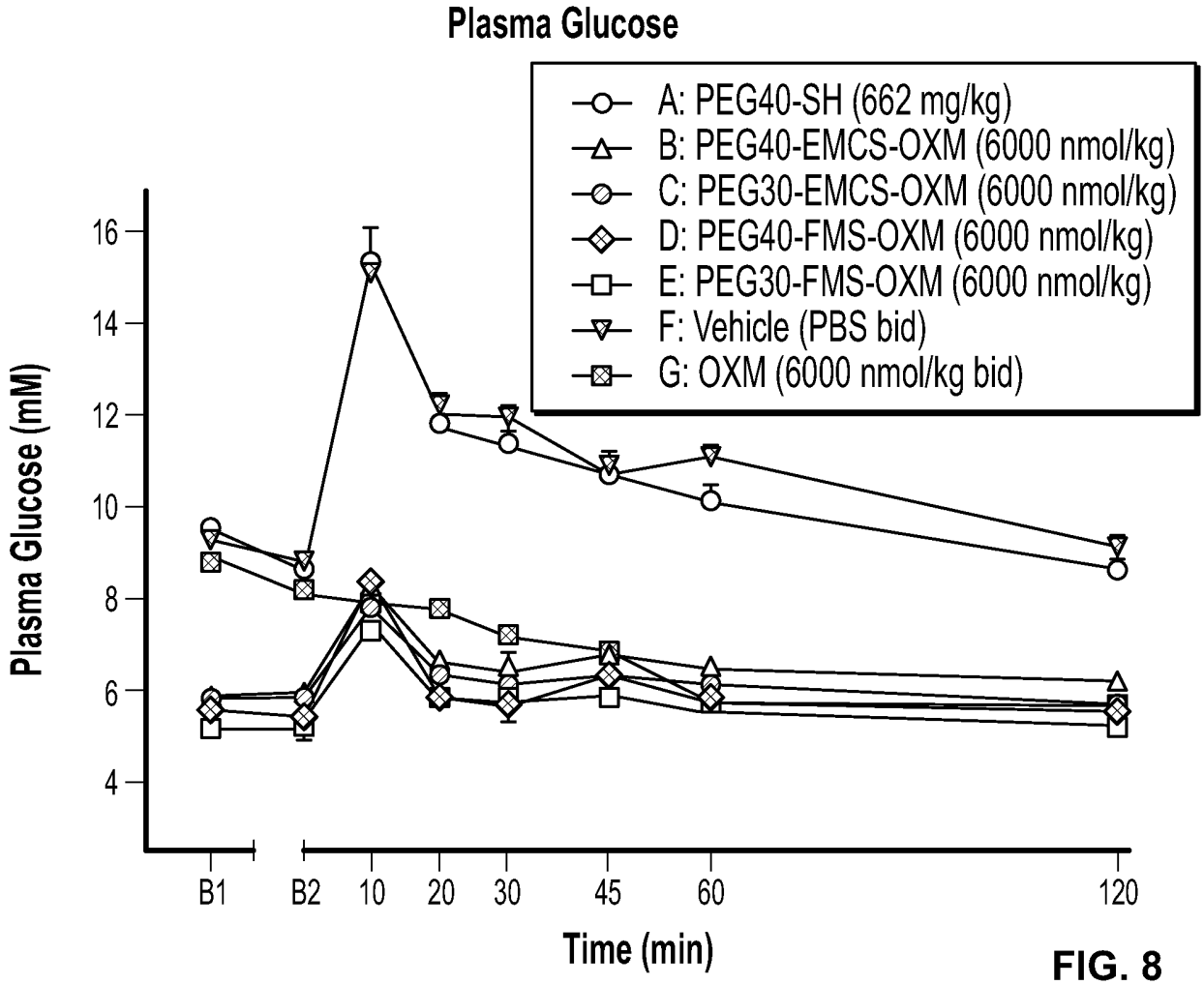

Plasma Glucose
A: PEG40-SH (662 mg/kg)
B: PEG40-EMCS-OXM (6000 nmol/kg)
C: PEG30-EMCS-OXM (6000 nmol/kg)
D: PEG40-FMS-OXM (6000 nmol/kg)
E: PEG30-FMS-OXM (6000 nmol/kg)
F: Vehicle (PBS bid)
G: OXM (6000 nmol/kg bid)
Plasma Glucose (mM)
16
14
12
10
8
6
4
B1
B2
10
20
30
45
60
120
Time (min)

FIG. 8

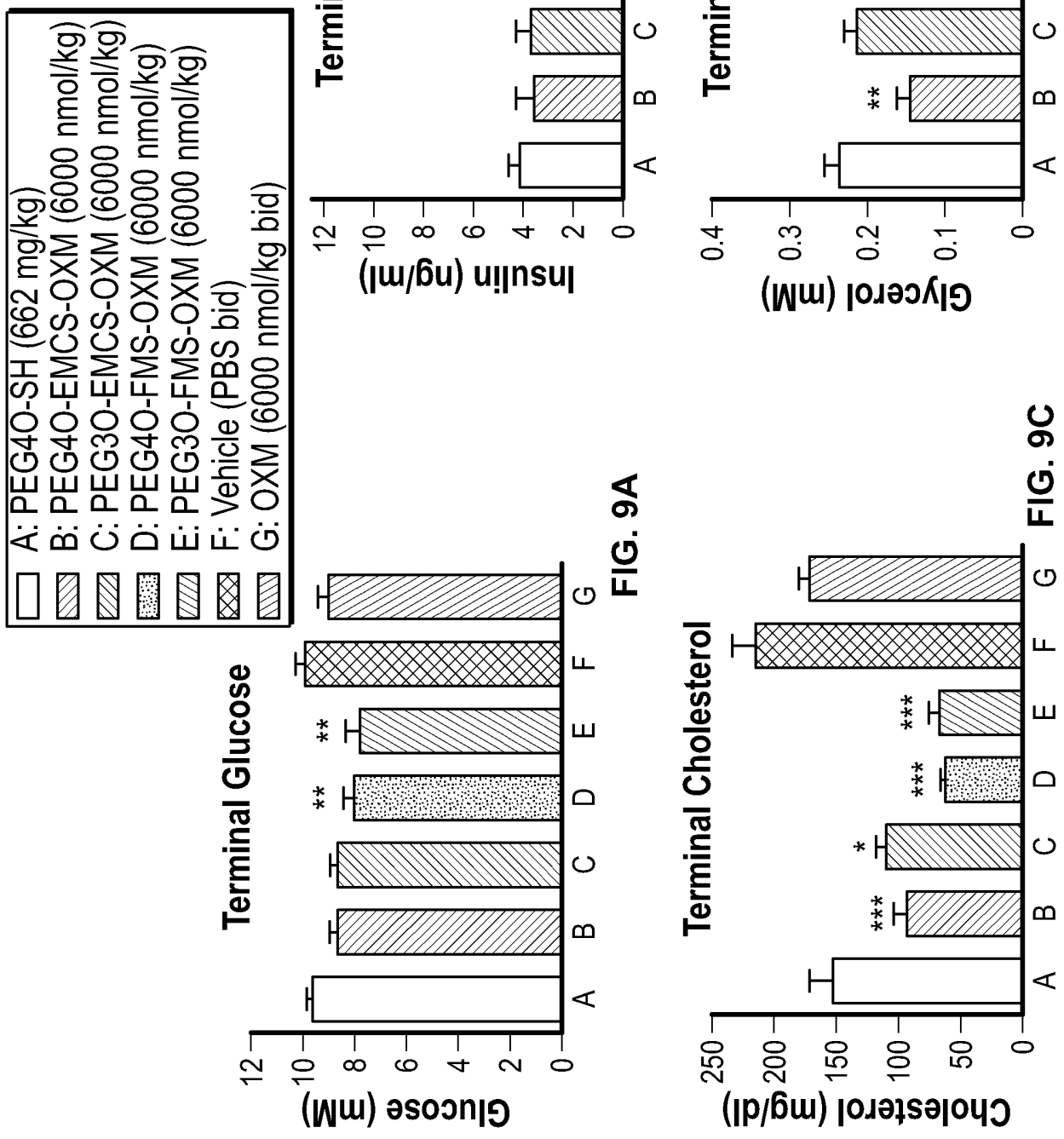
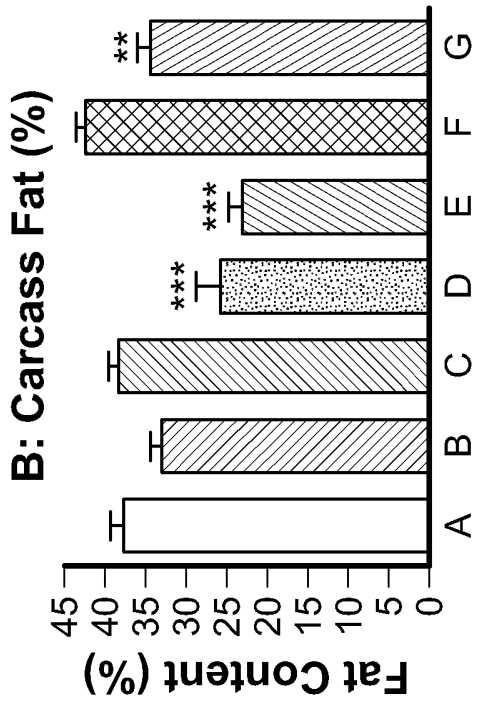


FIG. 9A

FIG. 9B

FIG. 9C

FIG. 9D



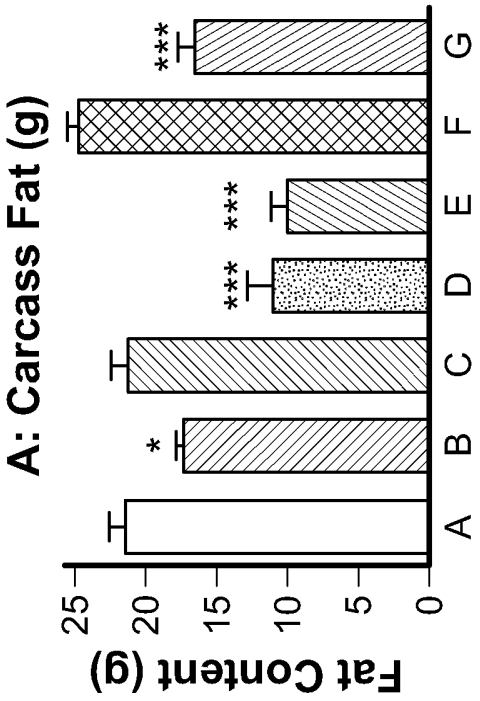
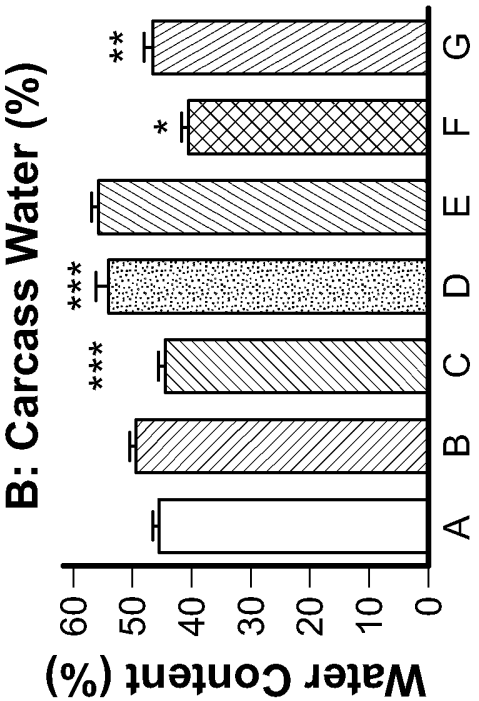


FIG. 10A



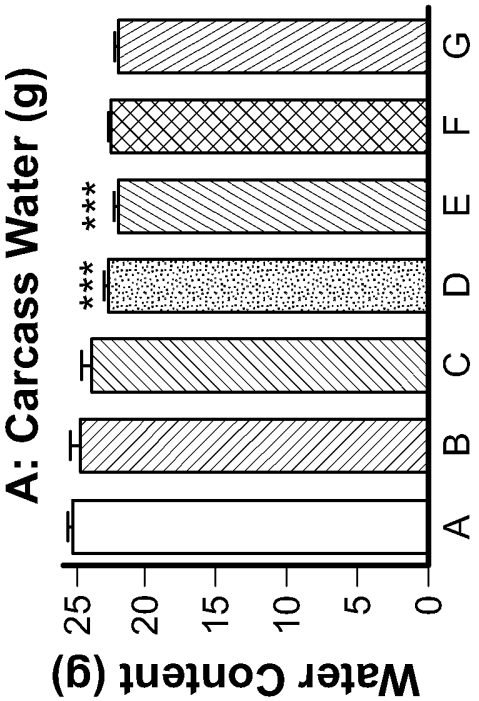


FIG. 10B

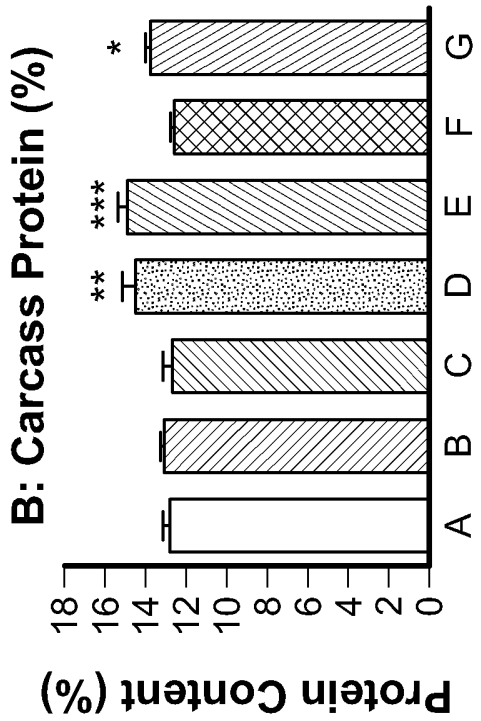


FIG. 10C

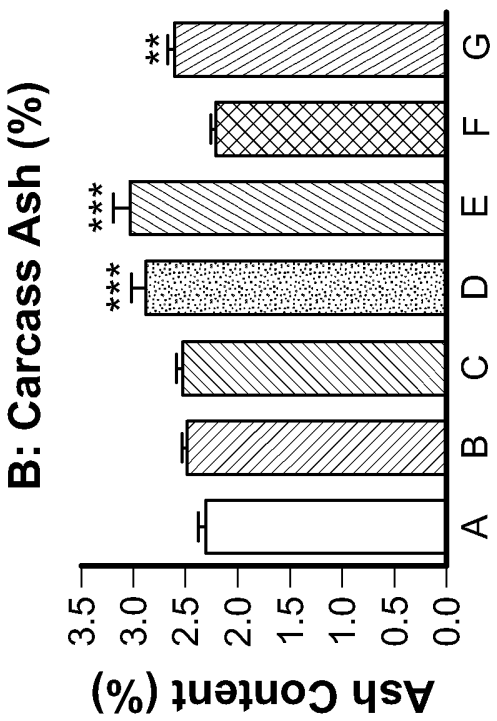
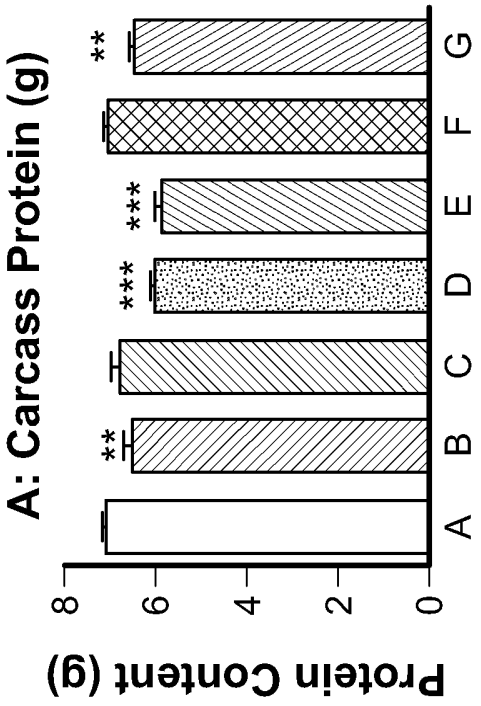
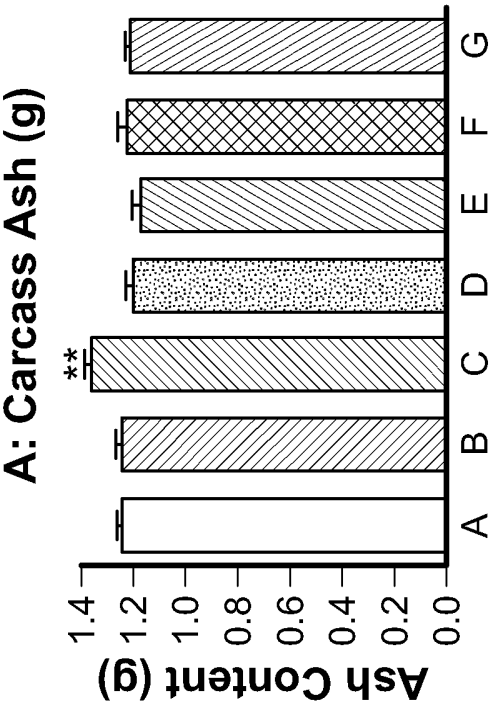


FIG. 10D





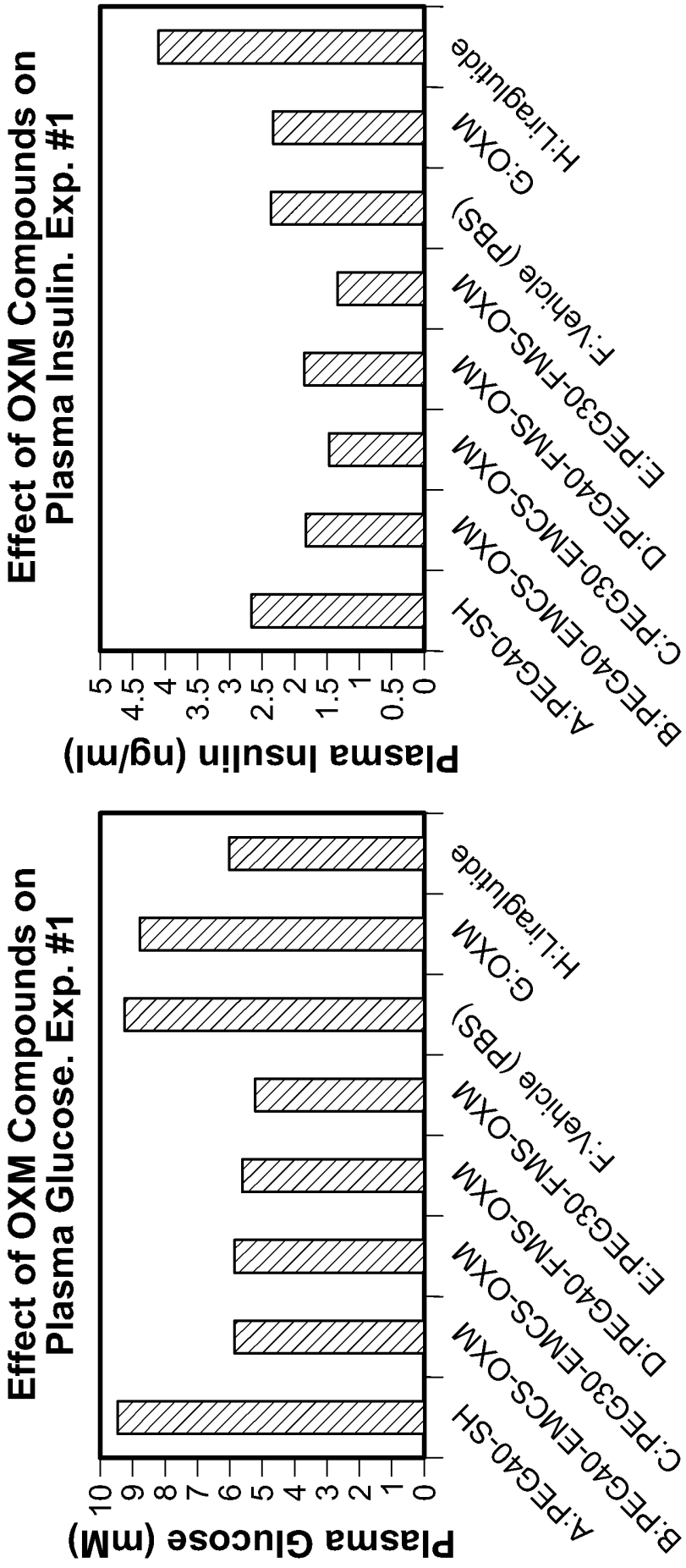

Effect of OXM Compounds on Plasma Insulin. Exp. #1
Plasma Insulin (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
A:PEG40-SH
B:PEG40-EMCS-OXM
C:PEG30-EMCS-OXM
D:PEG40-FMS-OXM
E:PEG30-FMS-OXM
F:Vehicle (PBS)
G:OXM
H:Liraglutide
Effect of OXM Compounds on Plasma Glucose. Exp. #1
Plasma Glucose (mM)
0
1
2
3
4
5
6
7
8
9
10
A:PEG40-SH
B:PEG40-EMCS-OXM
C:PEG30-EMCS-OXM
D:PEG40-FMS-OXM
E:PEG30-FMS-OXM
F:Vehicle (PBS)
G:OXM
H:Liraglutide

FIG. 11

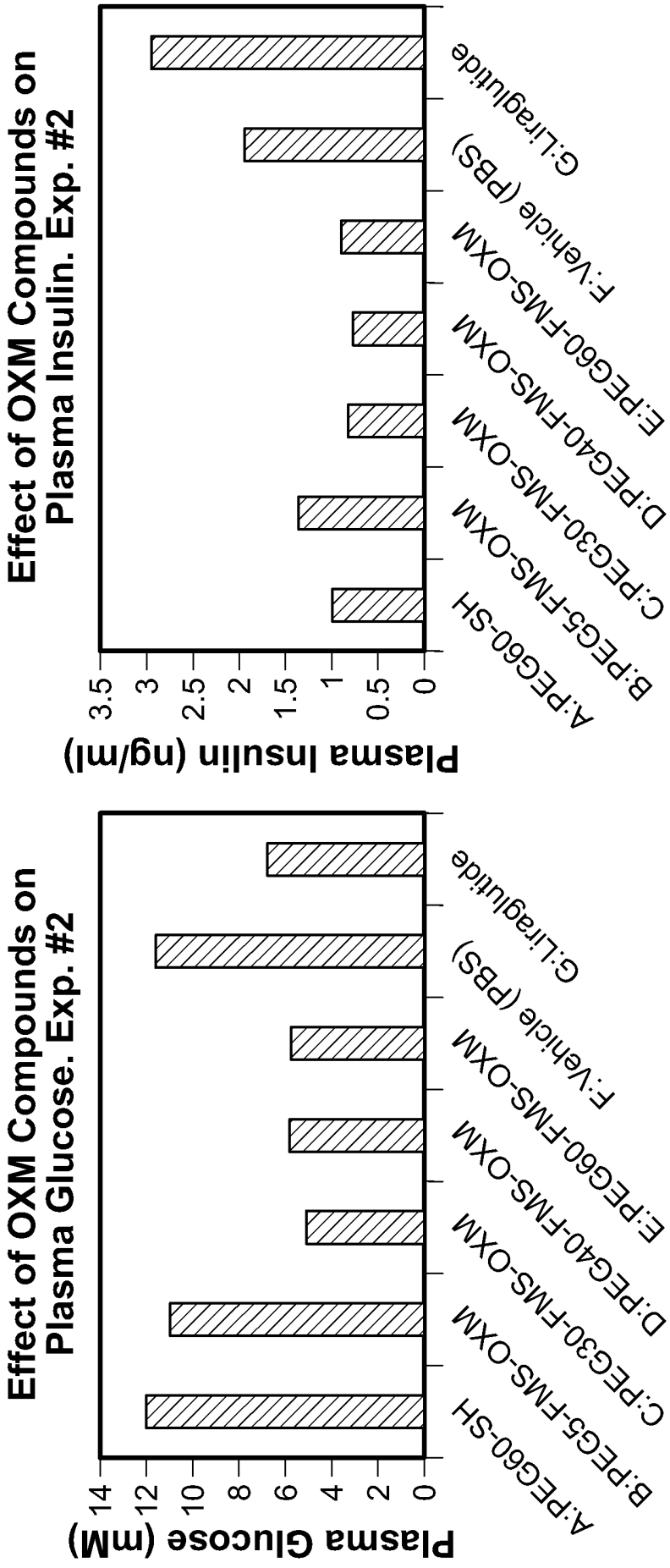

Effect of OXM Compounds on Plasma Insulin. Exp. #2
Plasma Insulin (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
A:PEG60-SH
B:PEG5-FMS-OXM
C:PEG30-FMS-OXM
D:PEG40-FMS-OXM
E:PEG60-FMS-OXM
F:Vehicle (PBS)
G:Liraglutide
Effect of OXM Compounds on Plasma Glucose. Exp. #2
Plasma Glucose (mM)
0
2
4
6
8
10
12
14
A:PEG60-SH
B:PEG5-FMS-OXM
C:PEG30-FMS-OXM
D:PEG40-FMS-OXM
E:PEG60-FMS-OXM
F:Vehicle (PBS)
G:Liraglutide

FIG. 12

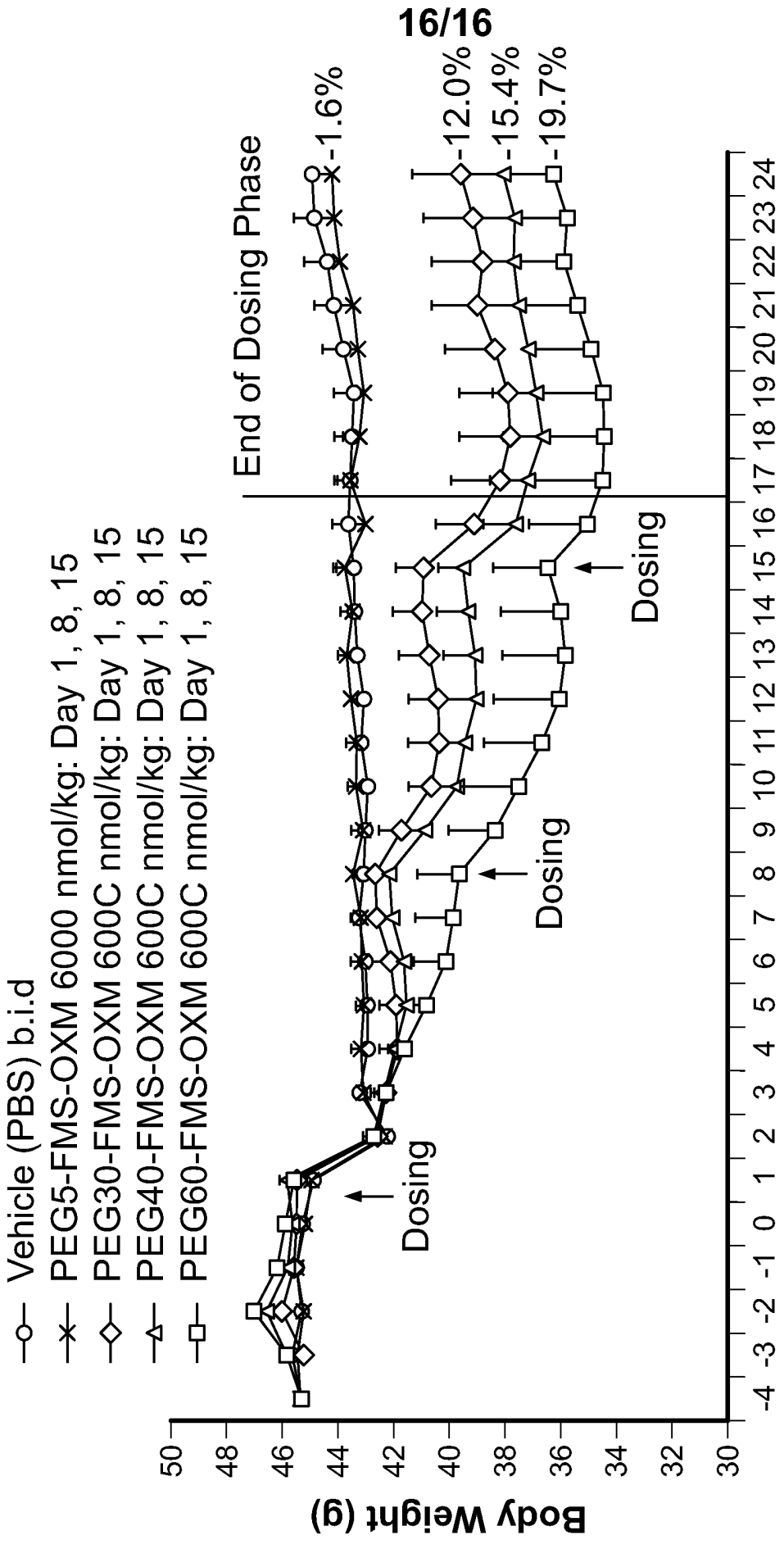

16/16
Vehicle (PBS) b.i.d
PEG5-FMS-OXM 6000 nmol/kg: Day 1, 8, 15
PEG30-FMS-OXM 600C nmol/kg: Day 1, 8, 15
PEG40-FMS-OXM 600C nmol/kg: Day 1, 8, 15
PEG60-FMS-OXM 600C nmol/kg: Day 1, 8, 15
End of Dosing Phase
-1.6%
-12.0%
-15.4%
-19.7%
Dosing
Dosing
Dosing
Body Weight (g)
Day
50
48
46
44
42
40
38
36
34
32
30
-4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/40744

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/26; C07K 14/605; A61P 3/10, 7/12 (2012.01)
USPC - 514/7.2, 11.7; 530/308
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)
IPC(8) - A61K 38/26; C07K 14/605; A61P 3/10, 7/12 (2012.01)
USPC - 514/7.2, 11.7; 530/308

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/4.8, 6.7, 6.8, 6.9
(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase; PubWest (PGPB,USPT,USOC,EPAB,JPAB); PubMed (MEDLINE) and Google Scholar.
Search Terms: oxyntomodulin, polyethylene glycol, fluorenylmethoxycarbonyl, Fmoc, fluorenylmethoxycarbonyl, FMS, PEG.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0144617 A1 (SINHA ROY et al.) 10 June 2010 (10.06.2010) abstract; para [0001], [0071], [0127], [0128], [0142]; Table 1; SEQ ID NO: 4	1-6
Y	US 2006/0171920 A1 (SHECHTER et al.) 03 August 2006 (03.08.2006) abstract; para [0003], [0015], [0066], [0164], [0257], [0339], [0359]-[0360].	1-6

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"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	Date of mailing of the international search report
19 October 2012 (19.10.2012)	26 OCT 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/40744

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-6, drawn to a composition consisting of oxyntomodulin, a polyethylene glycol polymer (PEG polymer), and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

Group II: Claims 7-11, drawn to a method for extending the biological half life of oxyntomodulin consisting of the step of conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a molar ratio of about 1:1:0.5 to about 1:1:3.5.
Group III: Claims 12-16, drawn to a method of inducing glucose tolerance, glycemic control, or both in a subject in need thereof, comprising the step of administering to said subject an effective amount of the composition of claim 1 and a pharmaceutical acceptable carrier.
**********Continued in Supplemental Box**********

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 additional fees, this Authority did not invite payment of 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.:
Claims 1-6

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

International application No.
PCT/US 12/40744

Continuation of Box III. Observations where unity of invention is lacking (Continuation of item 3 of first sheet):

Group IV: Claims 17-26, drawn to a method of improving the area under the curve (AUC) of oxyntomodulin / a method of reducing dosing frequency of oxyntomodulin, consisting of the step of conjugated a polyethylene glycol polymer (PEG polymer) to the amino terminus of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).
Groups V: Claims 27-31, drawn to a method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).
Group VI: Claims 32-39, drawn to a method for increasing insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of a composition comprising oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer).
The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Groups I-II and IV do not include the inventive concept of a method for increasing insulin sensitivity in a subject, comprising the step of administering to the subject an effective amount of a composition comprising oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer), as required by Group VI.
Group III does not include the inventive concept of oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer), as required by Group VI.
Groups I-II and IV do not include the inventive concept of a method for reducing food intake, reducing body weight, or both in a subject, comprising the step of administering oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), as required by Group V.
Groups III and VI do not include the inventive concept of oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) via a flexible linker, wherein said flexible linker is 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), as required by Group V.
Groups I-III and V-VI do not include the inventive concept of conjugated a polyethylene glycol polymer (PEG polymer) to the AMINO TERMINUS of said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), as required by Group IV.
Groups I-II and IV do not include the inventive concept of a method of inducing glucose tolerance, glycemic control, or both in a subject in need thereof, comprising the step of administering to said subject an effective amount of the composition of claim 1 and a pharmaceutical acceptable carrier, as required by Group III.
Group V does not include the inventive concept of a pharmaceutical acceptable carrier, as required by Group III.
Group VI does not include the inventive concept of a composition comprising 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) (of claim 1), as required by Group III.
Groups I and III-VI do not include the inventive concept of conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) in a MOLAR RATIO of about 1:1:0.5 to about 1:1:3.5, as required by Group II.
Group VI does not include the inventive concept of 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), as required by Group I.

Group I-VI share the technical feature of a composition comprising oxyntomodulin and a polyethylene glycol polymer (PEG polymer).
Groups I-V further share the technical feature of a composition consisting of oxyntomodulin, a polyethylene glycol polymer (PEG polymer), and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), namely, Claim 1.
Groups II and IV further share the technical feature of a method for extending the biological half life of oxyntomodulin / a method of improving the area under the curve (AUC) of oxyntomodulin / a method of reducing dosing frequency of oxyntomodulin comprising conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).
Groups II and IV-V share the technical feature of conjugating oxyntomodulin, a polyethylene glycol polymer (PEG polymer) and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).
Groups III and V-VI further share the technical feature of a method of inducing glucose tolerance, glycemic control, or both in a subject in need thereof / a method for reducing food intake, reducing body weight, or both in a subject / a method for increasing insulin sensitivity in
a subject, comprising the step of administering to said subject an effective amount of a composition comprising oxyntomodulin and PEG.
Groups IV-V further share the technical feature of conjugating a polyethylene glycol polymer (PEG polymer) to said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).
However, these shared technical features do not represent a contribution over the prior arts as being obvious over US 2010/0144617 A1 to Sinha Roy et al. (hereinafter 'Sinha Roy') in view of US 2006/0171920 A1 to Shechter et al. (hereinafter 'Shechter') as follows:
Sinha Roy teaches a composition comprising oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) (table 1- SEQ ID No: 4; para [0001], [0127]-[0128]-'The invention contemplates the use of multi-functional polymer derivatives, as exemplified by bifunctional and multi-arm N-maleimidyl PEG derivatives. A wide variety of polyethylene glycol (PEG) species may be used for PEGylation of the novel OXM derivatives of the present invention').

**********Continued on Next Page**********

Continuation of Previous page:

Sinha Roy further teaches a method of inducing glucose tolerance, glycemic control, or both in a subject in need thereof / a method for reducing food intake, reducing body weight, or both in a subject / a method for increasing insulin sensitivity in a subject comprising the step of administering to the subject an effective amount of a composition comprising oxyntomodulin conjugated to polyethylene glycol polymer (PEG polymer) (table 1- SEQ ID No: 4; para [0001], [0127]-[0128]-'The invention contemplates the use of multi-functional polymer derivatives, as exemplified by bifunctional and multi-arm N-maleimidyl PEG derivatives. A wide variety of polyethylene glycol (PEG) species may be used for PEGylation of the novel OXM derivatives of the present invention'; [0051]-'a method for the treatment of a metabolic disease in a subject comprising administering to the subject a polypeptide as described above. The metabolic disease may be selected from the group consisting of diabetes, metabolic syndrome, hyperglycemia, and obesity', [0142]-'at a dosage range of 0.001 mg/kg to 10 mg/kg, more preferably from 1 .mu.g/kg to 200 mg/kg with a dosing frequency ranging from twice daily to once per week or longer', [0140]-'the glucose lowering activity of OXM is comparable to that of GLP-1 in a mouse intraperitoneal glucose tolerance test (IPGTT)', [0224]-[0225]-'Animals were then injected intraperitoneally (i.p.) with vehicle (saline) or a polypeptide of the invention (0.01-10 mg/kg)', 'Incretin activity of a polypeptide of the invention in IPGTT is manifested as a dose-dependent increase in percent inhibition of glucose excursion, reaching at least 30% at the 10 mg/kg dosage', [0122]-'The OXM derivatives of the present invention may be useful in the reduction of food intake and body weight and may mediate glucose-stimulated insulin secretion (GSIS) from pancreatic islets', [0226]-'Reductions in food intake at any time point and/or in overnight body weight gain are considered to be statistically significant for P values .ltoreq.0.05 and denotes efficacy of the corresponding OXM polypeptide (OXM2 or OXM3) in this model. (FIG. 3)', [0227]-[0232]-'glucosestimulated insulin secretion (GSIS)').

Sinha Roy does not expressly disclose that said composition further comprises 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), wherein said composition is achieve by conjugating a polyethylene glycol polymer (PEG polymer) to said oxyntomodulin via 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS).

However, Shechter teaches a composition consisting of a protein, a polyethylene glycol polymer (PEG polymer), and 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), wherein said composition is achieved by conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of the protein via a flexible linker such as 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) (abstract-'In these pegylated drugs, the PEG moiety and the drug residue are not linked directly to each other, but rather both residues are linked to different positions of the scaffold Fmoc or FMS structure that is highly sensitive to bases and is removable under physiological conditions. The drugs are preferably drugs containing an amino group, most preferably peptides and proteins of low or medium molecular weight', para [0066]-'whereby the PEG moiety and the drug residue are not linked directly to each other, as in standard pegylation procedures, but rather both residues are linked to different positions of a scaffold structure that is highly sensitive to bases and is removable under physiological conditions', [0359]-[0360]-'PEG.sub.40-FMS-PYY.sub.3-36', 'Furthermore, based on the sequencing yields, the PEG.sub.40-FMS group is primarily linked to the N-terminal .alpha.-amino group of PYY.sub.3-36').

Shechter further teaches a a method for extending the biological half life of oxyntomodulin / a method of improving the area under the curve (AUC) of oxyntomodulin / a method of reducing dosing frequency of a protein drug comprising conjugating a polyethylene glycol polymer (PEG polymer) to the amino terminus of the protein via a flexible linker such as 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) (abstract-'In these pegylated drugs, the PEG moiety and the drug residue are not linked directly to each other, but rather both residues are linked to different positions of the scaffold Fmoc or FMS structure that is highly sensitive to bases and is removable under physiological conditions. The drugs are preferably drugs containing an amino group, most preferably peptides and proteins of low or medium molecular weight'; para [0013]-[0015]-'drugs with a prolonged circulating half-life can be obtained by combining the technology of derivatization of the drug with Fmoc or FMS or similar moieties removable under mild basic conditions with the technology of attaching a suitable natural or synthetic carrier to the thus derivatized drug molecule, such carrier serving for delivery of the drug and providing further benefits', 'In one preferred embodiment, the polymeric carrier is PEG', [0177]-'the pegylation technique has been extensively used for modifying molecules, in particular peptide and protein drugs, in an attempt to improve some of its characteristics such as improved stability and solubility, reduced immunogenicity, reduced proteolysis, reduced toxicity, reduced clearance by the kidneys, improved bioavailability, and extended circulating life thus less frequent dosing being required', [0066]-'whereby the PEG moiety and the drug residue are not linked directly to each other, as in standard pegylation procedures, but rather both residues are linked to different positions of a scaffold structure that is highly sensitive to bases and is removable under physiological conditions', [0359]-[0360]-'PEG.sub.40-FMS-PYY.sub.3-36', 'Furthermore, based on the sequencing yields, the PEG.sub.40-FMS group is primarily linked to the N-terminal .alpha.-amino group of PYY.sub.3-36').

Thus one of ordinary skill in the art would have found it obvious use said flexible linker FMS or Fmoc to modify the PEG-oxyntomodulin conjugate of Sinha Roy so that 'major deficiencies of the protein-pegylation technology, mainly the loss of biological and pharmacological potencies in the PEG conjugates in vivo, may be overcome', as disclosed by Shechter (para [0015]).

As said shared technical features would have been obvious to one of ordinary skill in the art at the time of the invention, these cannot be considered special technical feature that would otherwise unify the groups.

Groups I-VI therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

(19)中华人民共和国国家知识产权局

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(51)Int.Cl.
A61K 38/26(2006.01)
C07K 14/605(2006.01)
A61P 3/10(2006.01)
A61P 7/12(2006.01)

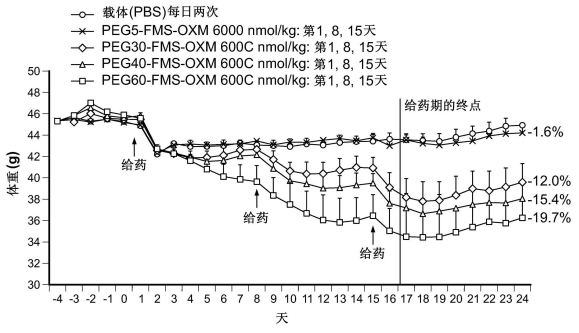
权利要求书2页 说明书41页
序列表1页 附图17页

(54)发明名称

长效GLP-1/胰高血糖素受体激动剂

(57)摘要

本发明公开了聚乙二醇化的和反向聚乙二醇化的GLP-1/胰高血糖素受体激动剂，包括包含其的药物组合物，及其使用方法。



CN 104023739 A

1. 一种组合物，由胃泌酸调节素、聚乙二醇聚合物（PEG 聚合物）和 9- 芴基甲氧基羰基（Fmoc）或 2- 磺基 -9- 芴基甲氧基羰基（FMS）组成。

2. 根据权利要求 1 所述的组合物，其中，通过 Fmoc 或 FMS，将所述 PEG 聚合物连接于所述胃泌酸调节素的氨基末端或赖氨酸残基。

3. 根据权利要求 1 所述的组合物，其中，所述胃泌酸调节素由在 SEQ ID NO:1 中给出的氨基酸序列组成。

4. 根据权利要求 1 所述的组合物，其中，所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

5. 根据权利要求 1 所述的组合物，其中，所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

6. 一种药物组合物，包含根据权利要求 1 所述的组合物和药用载体。

7. 一种用于延长胃泌酸调节素的生物半衰期的方法，由以下步骤组成：以约 1:1:0.5 至约 1:1:3.5 的摩尔比率结合胃泌酸调节素、聚乙二醇聚合物（PEG 聚合物）和 9- 芴基甲氧基羰基（Fmoc）或 2- 磺基 -9- 芴基甲氧基羰基（FMS）。

8. 根据权利要求 7 所述的方法，其中，所述胃泌酸调节素由 SEQ ID NO:1 的氨基酸序列组成。

9. 根据权利要求 7 所述的方法，其中，通过 Fmoc 或 FMS，将所述 PEG 聚合物结合于所述胃泌酸调节素的氨基末端或赖氨酸残基。

10. 根据权利要求 7 所述的方法，其中，所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

11. 根据权利要求 7 所述的方法，其中，所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

12. 一种用于在需要其的受试者中诱导葡萄糖耐受、血糖控制、或两者的方法，包括以下步骤：给予所述受试者有效量的根据权利要求 1 所述的组合物和药用载体。

13. 根据权利要求 12 所述的方法，其中，所述胃泌酸调节素由 SEQ ID NO:1 的氨基酸序列组成。

14. 根据权利要求 12 所述的方法，其中，通过 Fmoc 或 FMS，将所述 PEG 聚合物结合于所述胃泌酸调节素的氨基末端或赖氨酸残基。

15. 根据权利要求所 12 述的方法，其中，所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

16. 根据权利要求 12 所述的方法，其中，所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

17. 一种用于改善胃泌酸调节素的曲线下面积（AUC）的方法，由以下步骤组成：通过 9- 芴基甲氧基羰基（Fmoc）或 2- 磺基 -9- 芴基甲氧基羰基（FMS），将聚乙二醇聚合物（PEG 聚合物）结合于所述胃泌酸调节素的氨基末端。

18. 根据权利要求 17 所述的方法，其中，所述胃泌酸调节素由 SEQ ID NO:1 的氨基酸序列组成。

19. 根据权利要求 17 所述的方法，其中，通过 Fmoc 或 FMS，将所述 PEG 聚合物结合于所述胃泌酸调节素的氨基末端或赖氨酸残基。

20. 根据权利要求 17 所述的方法，其中，所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

21. 根据权利要求 17 所述的方法，其中，所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

22. 一种用于降低胃泌酸调节素的给药频率的方法，由以下步骤组成：通过 9- 芴基甲

氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 将聚乙二醇聚合物 (PEG 聚合物) 结合于所述胃泌酸调节素的氨基末端。

23. 根据权利要求 22 所述的方法,其中,所述胃泌酸调节素由 SEQ ID NO:1 的氨基酸序列组成。

24. 根据权利要求 22 所述的方法,其中,通过 Fmoc 或 FMS,将所述 PEG 聚合物结合于所述胃泌酸调节素的氨基末端或赖氨酸残基。

25. 根据权利要求 22 所述的方法,其中,所述所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

26. 根据权利要求 22 所述的方法,其中,所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

27. 一种用于在受试者中减少食物摄入量、减少体重、或两者的方法,包括以下步骤 :将通过柔性连接基团结合于聚乙二醇聚合物 (PEG 聚合物) 的胃泌酸调节素给予所述受试者,其中所述柔性连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。

28. 根据权利要求 27 所述的方法,其中,所述胃泌酸调节素由 SEQ ID NO:1 的氨基酸序列组成。

29. 根据权利要求 27 所述的方法,其中,通过 Fmoc 或 FMS,将所述 PEG 聚合物结合于所述胃泌酸调节素的氨基末端或赖氨酸残基。

30. 根据权利要求 27 所述的方法,其中,所述 PEG 聚合物是具有巯基部分的 PEG 聚合物。

31. 根据权利要求 27 所述的方法,其中,所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

32. 一种用于在受试者中增加胰岛素敏感度的方法,包括以下步骤 :给予所述受试者有效量的包含结合于聚乙二醇聚合物 (PEG 聚合物) 的胃泌酸调节素的组合物。

33. 根据权利要求 32 所述的方法,其中,所述 PEG 聚合物是 PEG_{30}、PEG_{40} 或 PEG_{60}。

34. 根据权利要求 32 所述的方法,其中,通过连接基团,将所述胃泌酸调节素结合于所述聚乙二醇聚合物 (PEG 聚合物)。

35. 根据权利要求 33 所述的方法,其中,所述连接基团是可断链的柔性连接基团。

36. 根据权利要求 33 所述的方法,其中,所述连接基团是不可断链的连接基团。

37. 根据权利要求 35 所述的方法,其中,所述柔性连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。

38. 根据权利要求 36 所述的方法,其中,所述连接基团是 N-(ε - 马来酰亚胺己酸) 琥珀酰亚胺酯 (EMCS)。

39. 根据权利要求 32 所述的方法,其中,给予所述组合物导致在所述受试者中胰岛素敏感度的急剧增加。

长效 GLP-1/ 胰高血糖素受体激动剂

[0001] 相关申请的引用

[0002] 本申请要求于 2011 年 6 月 2 日提交的美国临时申请序列号 61/492,448 和于 2012 年 4 月 16 日提交的美国临时申请序列号 61/624,589 的优先权。这些申请的全部内容以引用方式结合于本文。

技术领域

[0003] 本发明公开了聚乙二醇化和反向聚乙二醇化胃泌酸调节素，包括包含其的药物组合物，及其使用方法。

背景技术

[0004] 蛋白质以及尤其是短肽在血液、肝脏或肾脏中易变性或酶解。因此，蛋白质通常具有数小时的较短的循环半衰期。由于它们的低稳定性，通常以持续的频率来递送肽类药物以维持活性肽的有效的血浆浓度。此外，因为通常通过输注来给予肽类药物，肽类药物的频繁注射会引起受试者相当不舒服。因此，需要这样的技术来延长治疗性蛋白和肽类的半衰期，同时维持其高药理功效。这样的所期望的肽类药物还应满足以下要求 :当注入受试者时，增强的血清稳定性、高活性和诱导不希望的免疫反应的低概率。

[0005] 不利的药代动力学，如较短的血清半衰期，可能妨碍许多另外地有前途的候选药物的药物开发。血清半衰期是分子的经验性特征，并且必须针对每种新型潜在药物以实验方式确定。例如，对于较低分子量蛋白质类药物，由于所需要的给药方案的成本或频率，生理清除机制如肾脏过滤可能使得维持药物的治疗水平不可行。

[0006] 胃肠道负责调节饮食行为的多种肽激素的合成和释放，包括胰蛋白 (PP)、高血糖素样多肽 1(GLP-1)、肽 YY(PYY) 和胃泌酸调节素 (OXM)。OXM 来自在肠和 CNS 中胰高血糖素原的组织特异性翻译后加工。它包含 37 个氨基酸，包括具有 C 端碱性八肽延伸的完全胰高血糖素序列，其显示有助于体内和体外的 OXM 性能，但对于肽的效应而言，并不是单独足够的。响应于食品摄取，正比于膳食的含热量，OXM 被肠 L 细胞分泌进入血流。

[0007] 在口服和腹膜内给予以后，通过刺激胰岛素分泌，OXM 会增强葡萄糖清除率。它还调节食物摄入量的控制。在空腹大鼠中，将 OXM 脑室内 (ICV) 和核内注射进入下丘脑的室旁和弓状核 (ARC) 会抑制再喂养 (Dakin et al.2001;Dakin et al.2004)。在黑暗阶段的开始时，在自由喂养大鼠中也已证明了这种抑制。此外，OXM 的外周给予剂量依赖性地抑制禁食诱导和黑暗阶段的食物摄入量 (Dakin et al.2004)。

[0008] 先前描述了 (PCT 公开号 WO98/05361;Gershonov et al.,2000) 称作可逆聚乙二醇化的新概念方法，用于延长蛋白质和肽的半衰期。根据此技术，通过用对碱敏感并在温和的碱性条件如生理条件下可除去的官能团来衍化药物，从而制备前药。衍生化包括用一组聚乙二醇 (PEG) 部分连接到其上的连接基团如 9- 芴基甲氧基羰基 (Fmoc) 和 2- 磺基 -9- 芴基甲氧基羰基 (FMS)，来代替药物分子的至少一个氨基、羟基、巯基和 / 或羧基。在 PEG 部分和药物之间的连接不是直接的，而是两个残基连接于支架 FMS 或 Fmoc 结构(其对于碱性条

件是高度敏感的)的不同位置。本发明涉及 OXM 衍生物,其中利用可逆聚乙二醇化技术来延长肽的半衰期。

发明内容

[0009] 在一种实施方式中,本发明提供了一组组合物,该组合物由通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 连接或结合至聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂组成。

[0010] 在另一种实施方式中,本发明进一步提供了用于在受试者中减少食物摄入量、减少体重、或两者的方法,包括以下步骤 :给予受试者通过柔性连接基团结合至聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂,其中所述柔性连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中,连接基团是可断链连接基团。

[0011] 在另一种实施方式中,本发明进一步提供了用于在需要其的受试者中诱导葡萄糖耐受、改善血糖控制、或两者的方法,包括以下步骤 :给予受试者有效量的组合物,该组合物由通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 连接至聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂以及药用载体组成。

[0012] 在另一种实施方式中,本发明进一步提供了用于在受试者中降低胰岛素耐受性的方法,包括以下步骤 :给予受试者有效量的组合物,该组合物包含结合至聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂。

[0013] 在另一种实施方式中,本发明进一步提供了用于延长 GLP-1/ 胰高血糖素受体激动剂的生物半衰期的方法,包括以下步骤 :通过柔性连接基团(其包含 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)),将激动剂结合于聚乙二醇聚合物 (PEG 聚合物)。

[0014] 在另一种实施方式中,本发明进一步提供了用于延长双重 GLP-1/ 胰高血糖素受体激动剂的生物半衰期的方法,包括以下步骤 :通过柔性连接基团(其包含 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)),将激动剂结合于聚乙二醇聚合物 (PEG 聚合物)。

[0015] 在另一种实施方式中,本发明进一步提供了用于改善 GLP-1/ 胰高血糖素受体激动剂的曲线下面积 (AUC) 的方法,该方法由以下步骤组成 :通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS),将聚乙二醇聚合物 (PEG 聚合物) 结合于激动剂的氨基末端。

[0016] 在另一种实施方式中,本发明进一步提供了用于降低双重 GLP-1/ 胰高血糖素受体激动剂的给药频率的方法,该方法由以下步骤组成 :通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS),将聚乙二醇聚合物 (PEG 聚合物) 结合于所述胃泌酸调节素的氨基末端。

[0017] 在一种实施方式中,本发明提供了用于在受试者中增加胰岛素敏感度的方法,该方法包括以下步骤 :给予受试者有效量的组合物,该组合物包含结合于聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂。在另一种实施方式中,本发明提供了用于在受试者中在急性治疗或慢性治疗以后增加胰岛素敏感度的方法,该方法包括以下步

骤：给予受试者有效量的组合物，该组合物包含结合于聚乙二醇聚合物（PEG 聚合物）的双重 GLP-1/ 胰高血糖素受体激动剂。

[0018] 依据以下详细描述的实施例和附图，本发明的其他特点和优点将变得清楚。然而，应当明了，具体实施方式和具体实施例（虽然表示本发明的优选实施方式）仅通过举例说明的方式给出，因为依据本发明的具体实施方式本领域技术人员可以清楚在本发明的精神和范围内的各种改变和变更。

附图说明

[0019] 图 1 是曲线图，其示出在雄性大鼠中 OXM、PEG_{10}-Fmoc-OXM 和 PEG_{20}-Fmoc-OXM 的药代动力学曲线。大鼠接收单次 SC 推注的处于 0.5ml PBS 缓冲液中的天然 OXM(62nmol/kg)、PEG_{10}-Fmoc-OXM（包含 278μg/kg OXM 肽）或 PEG_{20}-Fmoc-OXM（包含 62nmol/kg OXM 肽）。在指定的时间点，从颈静脉收集血清样品并利用 OXM Elisa 试剂盒 (Bachem，瑞士）来分析 OXM 浓度。

[0020] 图 2 是曲线图，其示出在雄性大鼠中 OXM 和 PEG_{40}-Fmoc-OXM 的药代动力学曲线。大鼠接收单次 IV 推注 (A) 或 SC(B) 注射的处于 0.5ml PBS 缓冲液中的天然 OXM(62mnol/kg) 或 PEG_{40}-Fmoc-OXM（包含 62nmol/kg 体重的 OXM 肽）。在指定的时间点，从颈静脉收集血清样品并利用 OXM Elisa 试剂盒 (Bachem，瑞士）来分析 OXM 浓度。叠加插图突出显示 OXM 曲线，其在给予以后的最初两个小时内是明显的。

[0021] 图 3 是曲线图，其示出天然 OXM、PEG_{40}-Fmoc-OXM 和 PEG_{40}-EMCS-OXM 的体外活性。在 96 孔半区白板中以 200,000 个细胞 /ml 的密度接种过表达 GLP-1 受体的 CHO-K1 细胞 (Millipore HTS163C2) 并在 37℃下温育 24 小时。使用具有或不具有大鼠血清 1%(Bioreclamation) 的逐步升高浓度的 OXM(ALMAC)、PEG_{40}-EMCS-OXM 和 PEG_{40}-Fmoc-OXM 来温育细胞。通过 HTRF 测定 (Cisbio62AM4PEB) 来定量细胞 cAMP 浓度并通过 PRISM 软件来分析 EC50 参数。

[0022] 图 4 是曲线图，其示出在小鼠中用天然 OXM 和 PEG_{40}-Fmoc-OXM 和 PEG_{40}-EMCS-OXM 诱导葡萄糖耐受，如通过 IP 葡萄糖耐受试验 (IPGTT) 所测得的。禁食 C57BL/6 小鼠过夜，然后 IP 注射 PBS（载体），PEG_{40}-Osu 作为对照 (546nmol/kg)，天然 OXM(333nmol/kg)，PEG_{40}-Fmoc-OXM(202nmol/kg 肽含量）和 PEG_{40}-EMCS-OXM(333nmol/kg)。在给予试验物质（载体、OXM 和 PEG_{40}-Osu) 以后 15 分钟或在给予 PEG_{40}-Fmoc-OXM 以后 120 分钟，IP 给予葡萄糖 (1.5gr/kg)。在给予葡萄糖以前以及在给予葡萄糖以后 10、20、30、60 和 120 分钟，利用便携式血糖仪，通过尾静脉采样，来测量血糖水平。曲线图 (A) 提供血糖曲线以及曲线图 (B) 示出葡萄糖 AUC。

[0023] 图 5 是曲线图，其示出在显示饮食诱导肥胖症的雄性 C57BL/6J 小鼠中，SC 给予 OXM（每日两次）和 PEG_{40}-FMS-OXM（第 1、3、5、7 天）对体重 (A) 和累积食物摄入量 (B) 的影响。数据是调整的平均值 (n=10)。SEM 由统计模型的残差计算。在第 1 天开始，小鼠给药持续 7 天。通过 ANCOVA 来分析数据，其中在第 1 天的体重作为协变量，接着 Williams 检验（处于 PBS 中的 OXM) 或多重 t 检验（西布曲明和 PEG_{40}-FMS-OXM) 相对于适当的载体。显著差异相对于适当的载体：*$p<0.05$，**$p<0.01$，***$p<0.001$。百分比值表示在第 8 天（即，在给予 7 天以后）与适当的载体组的差异。

[0024] 图 6 是曲线图，其示出在显示饮食诱导肥胖症的雄性 C57BL/6J 小鼠中，SC 给予 OXM（每日两次）和共价结合的聚乙二醇化 OXM PEG_{40}-EMCS-OXM(1000nmol/kg 和 5000nmol/kg，在第 1、4 和 7 天，或 8000mnol/kg，在第 1 和 7 天）、PEG_{40}-FMS-OXM(1000nmol/kg 和 5000nmol/kg，在第 1、4 和 7 天，或 8000nmol/kg，在第 1 和 7 天）以及 PEG_{30}-FMS-OXM(5000nmol/kg，在第 1、4 和 7 天）对体重 (A) 和食物摄入量 (B) 的影响。数据是调整的平均值 (n=10)。SEM 由统计模型的残差计算。在第 1 天开始，小鼠给药持续 7 天。

[0025] 图 7 示出，在饮食诱导肥胖症 (DIO) 小鼠中，可逆聚乙二醇化 OXM 给予对体重的影响。在单独圈养的第一周（处理期）期间，动物开始每日一次执行方案并在第二周（基线期）期间，通过皮下途径，给予它们适当的载体，每日两次、或每周一次，如在治疗期期间给予它们的）。7 组 (n=8)DIO 小鼠给药 29 天，具体如下 :A. PEG_{40}-SH(662mg/kg)，B. PEG40-EMCS-OXM(6,000nmol/kg)，C. PEG30-EMCS-OXM(6,000nmol/kg)，D. PEG40-FMS-OXM(6,000nmol/kg)，E. PEG30-FMS-OXM(6,000nmol/kg)，F. 载体 (PBS)，以及 G. OXM(6,000nmol/kg ;PBS)。在基线和治疗期期间，每日记录食物摄入量、水摄入量和体重。在饮食诱导肥胖症 (DIO) 小鼠中，每周给予 PEG40-FMS-OXM 或 PEG30-FMS-OXM 显著降低了体重。

[0026] 图 8 示出，在饮食诱导肥胖症 (DIO) 小鼠中，可逆聚乙二醇化 OXM 给予对葡萄糖耐受的急性效应。在开始给予药物或载体以后第 1 天，所有小鼠禁食过夜。在第 2 天，小鼠经受口服葡萄糖耐受试验 (OGTT)。每只动物给予载体或测试化合物，并在 60 分钟以后给予 D- 葡萄糖 (2g/kg po)。在给予载体或测试化合物 (B1) 以前立即地以及在葡萄糖负荷 (B2) 以前立即地获取基线血液样品。另外，在给予葡萄糖以后 10、20、30、45、60 和 120 分钟获取血液样品。所有血液样品（大约 20μl）获自尾静脉。制备血浆样品并分别利用 Thermoelectron Infinity 葡萄糖试剂 (TR15421) 和 Alpco 小鼠超敏感胰岛素 ELISA(80-INSMSU-E10) 来测定葡萄糖 (n=2) 和胰岛素 (n=1)。

[0027] 图 9 示出，在饮食诱导肥胖症 (DIO) 小鼠中，可逆聚乙二醇化 OXM 给予对末端葡萄糖、甘油、胆固醇和胰岛素的影响。通过心脏穿刺来收集末端血浆样品（在第 29 天，测试或对照化合物的最后剂量以后 24 小时）并利用小鼠超敏感胰岛素 ELISA(80-INSMSU-E10)、Thermoelectron Infinity 葡萄糖试剂 (TR15421) 和 Thermoelectron Infinity 胆固醇试剂 (TR13421) 来测定胰岛素、葡萄糖和胆固醇。

[0028] 图 10 示出，在饮食诱导肥胖症 (DIO) 小鼠中，可逆聚乙二醇化 OXM 给予对脂肪、水分、蛋白质和灰分（骨）的末端身体成分分析的影响。利用标准化学分析技术，确定了 DIO 小鼠尸体的身体脂肪 (A)、水 (B)、蛋白质 (C)、和灰分水平 (D)。治疗组如下 :A. PEG40-SH(662mg/kg)，B. PEG40-EMCS-OXM(6,000nmol/kg)，C. PEG30-EMCS-OXM(6,000nmol/kg)，D. PEG40-FMS-OXM(6,000nmol/kg)，E. PEG30-FMS-OXM(6,000nmol/kg)，F. 载体 (PBS)，以及 G. OXM(6,000nmol/kg ;PBS)。

[0029] 图 11 示出，当和对照相比时，给予 PEG-OXM 变体 PEG40-EMCS-OXM、PEG30-EMCS-OXM、PEG40-FMS-OXM、PEG30-FMS-OXM 产生明显和显著减少的空腹血糖和空腹血浆胰岛素。

[0030] 图 12 示出，当和对照相比时，给予 PEG-OXM 变体 PEG30-FMS-OXM、PEG40-FMS-OXM

和 PEG60-FMS-OXM 产生明显和显著减少的空腹血糖和空腹血浆胰岛素。

[0031] 图 13 示出，当和对照相比时，给予 PEG-OXM 变体 PEG5-FMS-OXM、PEG30-FMS-OXM、PEG40-FMS-OXM 和 PEG60-FMS-OXM 产生明显和显著减少的体重。

具体实施方式

[0032] 在一种实施方式中，本发明提供了长效双重 GLP-1/ 胰高血糖素受体激动剂及其产生和使用方法。在另一种实施方式中，本发明提供了长效胃泌酸调节素及其产生和使用方法。在一种实施方式中，长效双重 GLP-1/ 胰高血糖素受体激动剂是包括或由以下各项组成的组合物 ：胃泌酸调节素、聚乙二醇聚合物 (PEG 聚合物) 和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，长效胃泌酸调节素是包括或由以下各项组成的组合物 ：胃泌酸调节素、聚乙二醇聚合物 (PEG 聚合物) 和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，本发明提供了包括以下各项的修饰的胃泌酸调节素肽 ：胃泌酸调节素肽、聚乙二醇 (PEG) 聚合物、和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，本发明提供了由以下各项组成的修饰的胃泌酸调节素肽 ：胃泌酸调节素肽、聚乙二醇 (PEG) 聚合物、和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在一种实施方式中，长效胃泌酸调节素是包括或由以下各项组成的组合物 ：胃泌酸调节素和聚乙二醇聚合物 (PEG 聚合物)。

[0033] 在一种实施方式中，术语双重 "GLP-1/ 胰高血糖素受体激动剂 " 和 " 激动剂 " 在本文中可互换使用。在另一种实施方式中，此术语还包括本领域中已知的任何 GLP-1/ 胰高血糖素受体激动剂。在另一种实施方式中，优选的激动剂是胃泌酸调节素或 OXM 或其功能变体。

[0034] 在一种实施方式中，术语 " 功能的 " 是指本文提供的激动剂或 OXM 具有生物活性的能力，其包括但不限于减少体重、增加胰岛素敏感度等，如本文中进一步提供的。

[0035] 在另一种实施方式中，长效双重 GLP-1/ 胰高血糖素受体激动剂是聚乙二醇化胃泌酸调节素。在另一种实施方式中，长效双重 GLP-1/ 胰高血糖素受体激动剂是反向聚乙二醇化胃泌酸调节素。在另一种实施方式中，长效胃泌酸调节素是聚乙二醇化胃泌酸调节素。在另一种实施方式中，长效胃泌酸调节素是反向聚乙二醇化胃泌酸调节素。在另一种实施方式中，短语 " 长效胃泌酸调节素 "、" 反向聚乙二醇化胃泌酸调节素 "、" 可逆聚乙二醇化 OXM"、和 " 包含或由胃泌酸调节素、聚乙二醇聚合物 (PEG 聚合物) 和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 组成的组合物 " 可互换使用。在另一种实施方式中，长效胃泌酸调节素是通过 Fmoc 或 FMS 连接于 PEG 的 OXM。

[0036] 在一种实施方式中，本文提供的长效双重 GLP-1/ 胰高血糖素受体激动剂包含 PEG 聚合物。在另一种实施方式中，激动剂包含通过 Fmoc 或 FMS 结合于胃泌酸调节素肽的氨基末端的 PEG 聚合物。在另一种实施方式中，本发明的长效胃泌酸调节素包含 PEG 聚合物。在另一种实施方式中，本发明的长效胃泌酸调节素包含通过 Fmoc 或 FMS 结合于胃泌酸调节素肽的氨基末端的 PEG 聚合物。

[0037] 在另一种实施方式中，长效胃泌酸调节素是包括或由以下各项组成的组合物 ：摩尔比率为 1:0.2-10:0.2-10 的胃泌酸调节素、聚乙二醇聚合物 (PEG 聚合物) 和 9- 芴基甲

氧基羰基(Fmoc)或2-磺基-9-芴基甲氧基羰基(FMS)。在另一种实施方式中,长效胃泌酸调节素是包括或由以下各项组成的组合物:摩尔比率为1:0.5-2:0.5-2的胃泌酸调节素、聚乙二醇聚合物(PEG聚合物)和9-芴基甲氧基羰基(Fmoc)或2-磺基-9-芴基甲氧基羰基(FMS)。在另一种实施方式中,长效胃泌酸调节素是包括或由以下各项组成的组合物:摩尔比率为1:1:1的胃泌酸调节素、聚乙二醇聚合物(PEG聚合物)和9-芴基甲氧基羰基(Fmoc)或2-磺基-9-芴基甲氧基羰基(FMS)。在另一种实施方式中,长效胃泌酸调节素包括通过Fmoc或FMS结合于胃泌酸调节素的氨基末端的PEG聚合物。

[0038] 在一种实施方式中,长效双重GLP-1/胰高血糖素受体激动剂,通过可逆连接基团例如但不限于Fmoc和FMS,连接于PEG。在另一种实施方式中,长效胃泌酸调节素,通过可逆连接基团例如但不限于Fmoc和FMS,连接于PEG。在另一种实施方式中,Fmoc和FMS对碱敏感并在生理条件下可除去。在另一种实施方式中,可逆连接基团是这样的连接基团,其对碱敏感并且在生理条件下可除去。在另一种实施方式中,可逆连接基团是这样的连接基团,其对碱敏感,并且在血液、血浆、或淋巴中的生理条件下可除去。在另一种实施方式中,可逆连接基团是这样的连接基团,其对碱敏感,并且在体液中的生理条件下可除去。在另一种实施方式中,可逆连接基团是这样的连接基团,其在具有碱性pH的体液中可除去。在另一种实施方式中,在暴露于碱性环境后,对碱敏感的连接基团被切割,因而由连接基团和PEG释放OXM。

[0039] 在另一种实施方式中,反向聚乙二醇化胃泌酸调节素是组合物,其中,通过可逆连接基团,OXM连接于PEG。在另一种实施方式中,在暴露于碱性环境以后,反向聚乙二醇化胃泌酸调节素释放自由OXM。在另一种实施方式中,在暴露于血液或血浆以后,反向聚乙二醇化胃泌酸调节素释放自由OXM。在另一种实施方式中,长效胃泌酸调节素包含PEG和胃泌酸调节素,其并不彼此直接连接,如在标准聚乙二醇化步骤中,而是两种残基连接于Fmoc或FMS的不同位置,其对碱高度敏感并在常规生理条件下可除去。在另一种实施方式中,常规生理条件包括生理环境如血液或血浆。

[0040] 在一种实施方式中,利用EMCS,长效胃泌酸调节素非可逆地结合于PEG(参见实施例3)。

[0041] 在另一种实施方式中,Fmoc和FMS的结构和制备方法描述于美国专利号7585837。美国专利号7585837所公开的全部内容以引用方式结合于本文。

[0042] 在另一种实施方式中,反向聚乙二醇化使OXM成为长效OXM。在另一种实施方式中,长效胃泌酸调节素是具有延长的生物半衰期的胃泌酸调节素。

[0043] 在一种实施方式中,反向聚乙二醇化提供保护以防止双重GLP-1/胰高血糖素受体激动剂降解。在另一种实施方式中,反向聚乙二醇化提供保护以防止OXM降解。在另一种实施方式中,反向聚乙二醇化影响OXM的C_{max}以减少有害副作用。在另一种实施方式中,反向聚乙二醇化延长OXM的T_{max}。在另一种实施方式中,反向聚乙二醇化延长OXM的循环半衰期。在另一种实施方式中,相比于未修饰的OXM,反向聚乙二醇化OXM具有改善的生物利用度。在另一种实施方式中,相比于未修饰的OXM,反向聚乙二醇化OXM具有改善的生物活性。在一些实施方式中,反向聚乙二醇化增强OXM的效力。

[0044] 在其他实施方式中,就生化措施而言,反向聚乙二醇化OXM至少相当于未修饰的OXM。在其他实施方式中,就药理度量而言,反向聚乙二醇化OXM至少相当于未修饰的OXM。

在其他实施方式中，就结合能力 (Kd) 而言，反向聚乙二醇化 OXM 至少相当于未修饰的 OXM。在其他实施方式中，就通过消化系统吸收而言，反向聚乙二醇化 OXM 至少相当于未修饰的 OXM。在其他实施方式中，和未修饰的 OXM 相比，在通过消化系统吸收期间，反向聚乙二醇化 OXM 是更加稳定的。

[0045] 在另一种实施方式中，相比于自由激动剂，反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂显示出改善的血液曲线下面积 (AUC) 水平。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 显示出改善的血液曲线下面积 (AUC) 水平。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 表现出改善的生物活性和血液曲线下面积 (AUC) 水平。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂表现出改善的血液滞留时间 ($t_{1/2}$)。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 表现出改善的血液滞留时间 ($t_{1/2}$)。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 表现出改善的生物活性和血液滞留时间 ($t_{1/2}$)。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 表现出改善的血液 C_{max} 水平，从而减少潜在有害的副作用。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 表现出改善的生物活性。在另一种实施方式中，本文提供了用于包括或由以下步骤组成的改善 OXM 的 AUC、C_{max}、$t_{1/2}$、生物活性、或它们的任何组合的方法 ：通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)，将聚乙二醇聚合物 (PEG 聚合物) 结合于自由 OXM 的氨基末端。因此，在一种实施方式中，本发明进一步提供了由以下步骤组成的用于改善胃泌酸调节素的曲线下面积 (AUC) 的方法 ：通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)，将聚乙二醇聚合物 (PEG 聚合物) 结合于所述胃泌酸调节素的氨基末端。

[0046] 在一种实施方式中，可以通过在针对 GLP-1 或胰高血糖素活性的所选测定法中确定肽的 EC_{50} 值来定量任何给定胰高血糖素类似物肽的 GLP-1 或胰高血糖素激动剂活性。如本领域技术人员将会非常了解的，EC50 值是浓度的度量，在特定的测定中，在该浓度下，达到化合物的最大活性的一半。在本说明书中，在用于 GLP-1 或胰高血糖素激动剂活性的测定中的 EC_{50} 值将分别称为 EC_{50}[GLP-1] 和 EC_{50}[Glu]。在比较不同化合物的 EC_{50} 值的情况下，应当理解的是，它们描述在相同的测定中在另外相同的条件下相关化合物的活性。

[0047] 胰高血糖素类似物肽的比率 EC_{50}[Glu]/EC_{50}[GLP-1] 可以大于胰高血糖素的比率 EC_{50}[Glu]/EC_{50}[GLP-1]。这可以解释为意味着，胰高血糖素类似物肽对于 GLP-1 受体比胰高血糖素具有更大的选择性。

[0048] 在另一种实施方式中，通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 通过将聚乙二醇聚合物 (PEG 聚合物) 结合于自由 OXM 的氨基末端 OXM 的 AUC、C_{max}、$t_{1/2}$、生物活性、或它们的任何组合的改善能够降低 OXM 的给药频率。在另一种实施方式中，本文提供了包括或由以下步骤组成的用于降低 OXM 的给药频率的方法 ：通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)，将聚乙二醇聚合物 (PEG 聚合物) 结合于 OXM 的氨基末端或赖氨酸残基。在另一种实施方式中，OXM 的反向聚乙二醇化有利于允许降低使用剂量。

[0049] 在另一种实施方式中，OXM 包含 SEQ ID NO:1 的氨基酸序列。在另一种实施方式中，OXM 由 SEQ ID NO:1 的氨基酸序列组成。在另一种实施方式中，SEQ ID NO:1 包括或由以下氨基酸 (AA) 序列组成 ：HSQGTFTSD YSKYLD SRJ AQDF VQ WLMNTKPvNRNNIA(SEQ ID NO:1)。在另

一种实施方式中，OXM 包含或由在 CAS No.62340-29-8 中描述的氨基酸序列组成。

[0050] 在另一种实施方式中，OXM 是人 OXM 或任何哺乳动物 OXM。在另一种实施方式中，OXM 还称为胰高血糖素 -37 或生物活性肠胰高血糖素。在另一种实施方式中，OXM 是双重 GLP-1/ 胰高血糖素受体激动剂。在另一种实施方式中，OXM 是 OXM 的生物活性片段。在另一种实施方式中，生物活性 OXM 从 SEQ ID NO:1 的氨基酸 30 延伸到氨基酸 37。在另一种实施方式中，生物活性 OXM 从 SEQ ID NO:1 的氨基酸 19 延伸到氨基酸 37。在另一种实施方式中，本发明的 OXM 对应于八肽，从其中删除两个 C 端氨基酸。在另一种实施方式中，本发明的 OXM 对应于 SEQ ID NO:1 的任何片段，其保留如在本文中所描述的 OXM 活性。

[0051] 在一种实施方式中，OXM 是指 SEQ ID NO:1 的肽的肽同源物。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 40% 同源性，如使用默认参数利用 National Center of Biotechnology Information(NCBI) 的 BlastP 软件所确定的。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 50% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 60% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 70% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 80% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。

[0052] 在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 90% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。在一种实施方式中，本发明的 OXM 氨基酸序列与在 SEQ ID NO:1 中提出的 OXM 序列具有至少 95% 同源性，如使用默认参数利用 NCBI 的 BlastP 软件所确定的。

[0053] 相比于野生型 OXM，本发明的 OXM 衍生物或变体包含数种氨基酸取代，和 / 或可以是聚乙二醇化或以其他修饰的（例如，重组地或化学地）。

[0054] 本文提供的 OXM 还包括上述 OXM 序列的任何类似物。通过本领域中众所周知的保守替换，可以独立地替换上述序列中的任何一个或多个氨基酸残基，即用类似化学类型的氨基酸来替换氨基酸，如用一种疏水性氨基酸替换另一种疏水性氨基酸。可替换地，可以进行非保守性氨基酸突变，这导致 OXM 的增强效应或生物活性。尤其是，OXM 被修饰以耐受二肽基肽酶 IV(DPP-IV) 的切割和失活。

[0055] OXM 的衍生物和变体及其产生方法公开于美国专利申请公开号 2011/0152182、美国专利申请公开号 2011/0034374、美国专利申请公开号 2010/0144617，所有这些均以引用方式结合于本文。

[0056] 在一种实施方式中，可以化学修饰本文提供的双重 GLP-1/ 胰高血糖素受体激动剂。在另一种实施方式中，可以化学修饰本文提供的 OXM。尤其是，可以修饰 OXM 的氨基酸侧链、氨基末端和 / 或羧酸末端。例如，OXM 可以经受以下一种或多种 :烷基化、二硫化物形成、金属络合、酰化、酯化、酰胺化、硝化、用酸处理、用碱处理、氧化或还原。用于执行这些过程的方法是本领域中众所周知的。尤其是，OXM 被提供为低级烷基酯、低级烷基酰胺、低级二烷基酰胺、其酸加成盐、羧酸盐或碱加成盐。尤其是，可以通过例如，酯化、酰胺化、酰化、氧化或还原，来衍生 OXM 的氨基末端或羧基末端。尤其是，可以衍生 OXM 的羧基末端以形成

酰胺部分。

[0057] 在一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂保持未修饰激动剂的生物活性。在另一种实施方式中，本发明的 OXM 保持未修饰 OXM 的生物活性。在一种实施方式中，本发明的长效 OXM 保持未修饰 OXM 的生物活性。在另一种实施方式中，本发明的长效 OXM 包含 OXM 生物活性。在另一种实施方式中，本发明的长效 OXM 的生物活性包括减少消化分泌。在另一种实施方式中，本发明的长效 OXM 的生物活性包括减少和延缓胃排空。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制在小肠中的进食蠕动模式。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制由五肽胃泌素刺激的酸分泌。在另一种实施方式中，本发明的长效 OXM 的生物活性包括增加胃生长抑素释放。在另一种实施方式中，本发明的长效 OXM 的生物活性包括增强肽 YY 的效应。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制生长素（ghrelin）释放。在另一种实施方式中，本发明的长效 OXM 的生物活性包括上调脂连素。在另一种实施方式中，本发明的长效 OXM 的生物活性包括减少游离脂肪酸。在另一种实施方式中，本发明的长效 OXM 的生物活性包括减少甘油三酯。在另一种实施方式中，本发明的长效 OXM 的生物活性包括减少胆固醇。在另一种实施方式中，本发明的长效 OXM 的生物活性包括刺激氨基比林积累和 cAMP 生产。在另一种实施方式中，本发明的长效 OXM 的生物活性包括结合 GLP-1 受体或胰高血糖素受体。在另一种实施方式中，本发明的长效 OXM 的生物活性包括通过活化腺苷酸环化酶来刺激 H+ 生产。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制组胺刺激的胃酸分泌。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制食物摄入量。在另一种实施方式中，本发明的长效 OXM 的生物活性包括刺激胰岛素释放。在另一种实施方式中，本发明的长效 OXM 的生物活性包括抑制外分泌胰腺分泌。在另一种实施方式中，本发明的长效 OXM 的生物活性包括增加胰岛素敏感度。在另一种实施方式中，本发明的长效 OXM 的生物活性包括降低葡糖糖水平。

[0058] 在一种实施方式中，术语 ″降低水平”是指相对于原始的、野生型、正常或对照水平降低约 1-10%。在另一种实施方式中，降低是约 11-20%。在另一种实施方式中，降低是约 21-30%。在另一种实施方式中，降低是约 31-40%。在另一种实施方式中，降低是约 41-50%。在另一种实施方式中，降低是约 51-60%。在另一种实施方式中，降低是约 61-70%。在另一种实施方式中，降低是约 71-80%。在另一种实施方式中，降低是约 81-90%。在另一种实施方式中，降低是约 91-95%。在另一种实施方式中，降低是约 96-100%。

[0059] 在一种实施方式中，术语 ″增加水平”或 ″延长 ″是指相对于原始的、野生型、正常或对照水平，增加约 1-10%。在另一种实施方式中，增加是约 11-20%。在另一种实施方式中，增加是约 21-30%。在另一种实施方式中，增加是约 31-40%。在另一种实施方式中，增加是约 41-50%。在另一种实施方式中，增加是约 51-60%。在另一种实施方式中，增加是约 61-70%。在另一种实施方式中，增加是约 71-80%。在另一种实施方式中，增加是约 81-90%。在另一种实施方式中，增加是约 91-95%。在另一种实施方式中，增加是约 96-100%。

[0060] 在另一种实施方式中，本发明进一步提供了用于在需要其的受试者中诱导葡萄糖耐受、改善血糖控制、或两者的方法，包括以下步骤：给予受试者有效量的由以下组成的组合物：通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 连接于聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂，以及药用载体。

[0061] 在另一种实施方式中，本发明进一步提供了用于在需要其的受试者中诱导葡萄糖耐受、改善血糖控制、或两者的方法，包括以下步骤：给予受试者有效量的由以下组成的组合物：通过 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 连接于聚乙二醇聚合物 (PEG 聚合物) 的胃泌酸调节素，以及药用载体。

[0062] 在一种实施方式中，本发明进一步提供了用于延长双重 GLP-1/ 胰高血糖素受体激动剂的生物半衰期的方法，由以下步骤组成：通过包括 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS) 的柔性连接基团，将激动剂结合于聚乙二醇聚合物 (PEG 聚合物)。

[0063] 在一种实施方式中，本发明进一步提供了用于延长胃泌酸调节素的生物半衰期的方法，包括以下步骤：以约 1:1:0.5 至约 1:1:3.5 的摩尔比率结合胃泌酸调节素、聚乙二醇聚合物 (PEG 聚合物) 和 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，摩尔比率是 1:1:10 的 OXM 相对于 PEG 相对于连接基团。在另一种实施方式中，范围是 1:1:5-1:1:9。在另一种实施方式中，范围是 1:1:3.7-1:1-4.9。

[0064] 在另一种实施方式中，本发明进一步提供了用于在受试者中减少食物摄入量、减少体重、或两者的方法，包括以下步骤：给予受试者通过柔性连接基团结合于聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂，其中所述柔性连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，受试者患有糖尿病。在另一种实施方式中，受试者是超重的。在另一种实施方式中，受试者患有肥胖症。

[0065] 在另一种实施方式中，本发明进一步提供了用于在受试者中减少食物摄入量、减少体重、或两者的方法，包括以下步骤：给予受试者通过柔性连接基团结合于聚乙二醇聚合物 (PEG 聚合物) 的胃泌酸调节素，其中所述柔性连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，受试者患有糖尿病。在另一种实施方式中，受试者是超重的。在另一种实施方式中，受试者患有肥胖症。

[0066] 在一种实施方式中，本文提供的 PEG-OXM 化合物诱导葡萄糖水平的显著减小而没有增加胰岛素水平。在另一种实施方式中，在给予单剂量的 PEG-OXM 化合物以后，本文提供的 PEG-OXM 化合物出人意料地降低葡萄糖水平以及降低空腹胰岛素水平 (参见本文中的实施例 7)。因此，在另一种实施方式中，本发明提供了用于在受试者中增加胰岛素敏感度的方法，包括以下步骤：给予受试者有效量的包含结合于聚乙二醇聚合物 (PEG 聚合物) 的双重 GLP-1/ 胰高血糖素受体激动剂的组合物。在另一种实施方式中，本发明出人意料地显示在受试者中在用本文提供的双重 GLP-1/ 胰高血糖素受体激动剂组合物进行急性治疗以后胰岛素敏感度的明显增加 (参见实施例 7)。在另一种实施方式中，通过连接基团，将激动剂结合于所述聚乙二醇聚合物 (PEG 聚合物)。在另一种实施方式中，激动剂是 OXM。在另一种实施方式中，连接基团是柔性连接基团。在另一种实施方式中，连接基团是 9- 芴基甲氧基羰基 (Fmoc) 或 2- 磺基 -9- 芴基甲氧基羰基 (FMS)。在另一种实施方式中，连接基团是不可断链的连接基团。在另一种实施方式中，连接基团是 N-(ε - 马来酰亚胺己酸) 琥珀酰亚胺酯 (EMCS)。

[0067] 在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括通过迷走神经间接机制来抑制胰腺分泌。在另一种实施方式中，本发明的长效双

重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括减少 hydromineral 转运通过小肠。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括刺激葡萄糖摄取。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括控制 / 刺激生长抑素分泌。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括减少食物摄入量和体重增加。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括减少肥胖。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括抑制胃口。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括诱导厌食。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括在超重和肥胖受试者中减少体重。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括诱导脂肪激素瘦素和脂连素的水平变化。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括在超重和肥胖受试者中除减少能量摄入以外还增加能量消耗。在另一种实施方式中，本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括降低血浆甘油三酯和增加的酮体。

[0068] 在一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括降低血浆甘油三酯和增加的酮体。在另一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括增加糖原异生基因 Pckl、Pgcl α 、和 Pdhal 的表达。在另一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括减少乙酰基 -CoA（丙酮酸脱羧的主要产物）、和丙二酰 -CoA 的肝储藏。在另一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括上调在肝脏中诱导脂肪酸氧化 (FAO) 的基因，包括 Fgf21 和 Cptla。在另一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括下调脂肪生成基因如 ChREBP。在另一种实施方式中，在急性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括上调 Ldlr 基因。

[0069] 在一种实施方式中，在慢性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括降低瘦素水平。在另一种实施方式中，在慢性治疗以后本发明的长效双重 GLP-1/ 胰高血糖素受体激动剂的生物活性包括增加 b- 羟基丁酸酯水平。

[0070] 在另一种实施方式中，通过 Fmoc 或 FMS，PEG 聚合物连接于胃泌酸调节素的氨基末端或赖氨酸残基。在另一种实施方式中，可互换地使用术语 ″ 附连 ″ 和 ″ 连接 ″。在另一种实施方式中，PEG 聚合物连接于 OXM 的 α - 氨基侧链。在另一种实施方式中，PEG 聚合物连接于 OXM 的 ε - 氨基侧链。在另一种实施方式中，PEG 聚合物连接于 OXM 的一个或多个 ε - 氨基侧链。在另一种实施方式中，PEG 聚合物包含巯基部分。

[0071] 在另一种实施方式中，PEG 是直链的。在另一种实施方式中，PEG 是支链的。在另一种实施方式中，PEG 具有 200 至 200,000Da 的分子量。在另一种实施方式中，PEG 具有 5,000 至 80,000Da 的分子量。在另一种实施方式中，PEG 具有 5,000 至 40,000Da 的分子量。在另一种实施方式中，PEG 具有 20,000Da 至 40,000Da 的分子量。

[0072] 在另一种实施方式中，长效 OXM 的制备是利用聚乙二醇化剂，是指能够与以下官能团反应的任何 PEG 衍生物 :例如但不限于在 Fmoc 或 FMS 部分的芴环处存在的 NH_2、OH、SH、

COOH、CHO、-N=C=O、-N=C=S、$-SO_2Cl$、$-SO_2CH=CH_2$、$--PO_2Cl$、$--(CH_2)xHal$。在另一种实施方式中，通常以其单甲氧基化形式来使用聚乙二醇化剂，其中在 PEG 分子的一个末端处仅一个羟基可用于结合。在另一种实施方式中，如果，例如，期望获得具有共价附连于单个 PEG 部分的两个肽或蛋白质残基的结合物，可以使用 PEG 的双官能形式，其中两个末端均可用于结合。

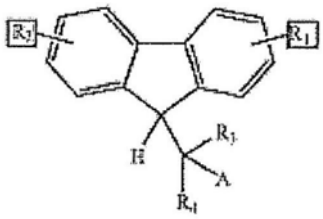
[0073] 在另一种实施方式中，支链 PEG 表示为 $R(PEG-OH)_m$，其中 R 表示中心核心部分如季戊四醇或甘油，以及 m 表示支链臂的数目。支链臂的数目 (m) 可以为 3 至 100 或更多。在另一种实施方式中，羟基受到化学修饰。在另一种实施方式中，支链 PEG 分子描述于美国专利号 6,113,906、5,919,455、5,643,575、和 5,681,567，其全部内容以引用方式结合于本文。

[0074] 在一种实施方式中，GLP-1/ 胰高血糖素受体激动剂是胃泌酸调节素。在另一种实施方式中，GLP-1/ 胰高血糖素受体激动剂是胃泌酸调节素变体。

[0075] 在另一种实施方式中，本发明提供了具有 PEG 部分的 OXM，该 PEG 部分并不直接附连于 OXM，如在标准聚乙二醇化步骤中，而是通过连接基团如 Fmoc 或 FMS 来附连 PEG 部分。在另一种实施方式中，连接基团对碱高度敏感，并且在温和的碱性条件下可除去。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM 与自由 OXM 具有等效活性。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM 比自由 OXM 具有更大的活性。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM 具有不同于自由 OXM 的活性。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM，不同于自由 OXM，具有中枢神经系统活性。在另一种实施方式中，可逆聚乙二醇化 OXM 穿过血 - 脑屏障并作用于下丘脑，以施加本文提供的生物活性。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM，不同于自由 OXM，不能通过血脑屏障进入脑。在另一种实施方式中，相比于自由 OXM，通过 Fmoc 或 FMS 连接于 PEG 的 OXM 具有延长的循环半衰期。在另一种实施方式中，通过 Fmoc 或 FMS 连接于 PEG 的 OXM 失去它的 PEG 部分以及 Fmoc 或 FMS 部分，从而回收自由 OXM。

[0076] 在另一种实施方式中，本发明提供了式 $(X)_n-Y$ 的化合物，其中 Y 是具有游离氨基、羧基、或羟基的 OXM 部分，以及 X 是式 (i) 的基团：

[0077]



[0078] 在另一种实施方式中，R_1 是包含蛋白质或聚合物载体部分的基团；聚乙二醇 (PEG) 部分；R_2 选自由以下组成的组：氢、烷基、烷氧基、烷氧基烷基、芳基、烷芳基、芳烷基、卤素、硝基、$-SO_3H$、$-SO_2NHR$、氨基、铵、羧基、PO_3H_2、和 OPO_3H_2；R 选自由以下组成的组：氢、烷基和芳基；R_3 和 R_4，相同或不同，各自选自由以下组成的组：氢、烷基和芳基；当上述基团连接于 OXM-Y 的氨基或羟基时 A 是共价键；n 是至少 1 的整数，以及其药用盐。

[0079] 在另一种实施方式中，术语 ″烷基 ″、″烷氧基 ″、″烷氧基烷基 ″、″芳基 ″、″烷芳基 ″和 ″芳烷基 ″用来指具有 1-8、优选 1-4 个碳原子的烷基，例如甲基、乙基、丙基、异丙基和丁基，以及具有 6-10 个碳原子的芳基，例如苯基和萘基。术语 ″卤素 ″包括溴基、氟基、

氯基和碘基。

[0080] 在另一种实施方式中，R_2、R_3 和 R_4 各自是氢以及 A 是 --OCO--，即 9- 芴基甲氧基羰基（在下文中 "Fmoc"）。在另一种实施方式中，R 是在芴环的位置 2 处的 $-SO_3H$，R_3 和 R_4 各自是氢，以及 A 是 --OCO-，即 2- 磺基 -9- 芴基甲氧基羰基（在下文中 "FMS"）。

[0081] 在另一种实施方式中，OXM 的聚乙二醇化以及 (PEG-Fmoc)n-OXM 或 (PEG-FMS)n-OXM 结合物的制备包括将 MAL-FMS-NHS 或 MAL-Fmoc-NHS 附连于 OXM 的胺成分，因而获得 MAL-FMS-OXM 或 MAL-Fmoc-OXM 结合物，然后用 PEG-SH 取代马来酰亚胺部分，从而分别产生 (PEG-FMS)n-OXM 或 (PEG-Fmoc)n-OXM 结合物。

[0082] 在另一种实施方式中，OXM 的聚乙二醇化包括使 MAL-FMS-NHS 或 MAL-Fmoc-NHS 和 PEG-SH 反应，因而形成 PEG-FMS-NHS 或 PEG-Fmoc-NHS 结合物，然后使它和 OXM 的胺组分反应，从而分别产生所期望的 (PEG-FMS)n-OXM 或 (PEG-Fmoc)n-OXM 结合物。在另一种实施方式中，肽 / 蛋白质如 OXM 的聚乙二醇化描述于美国专利号 7585837，其全部内容以引用方式结合于本文。在另一种实施方式中，肽 / 蛋白质如 OXM 的反向聚乙二醇化（使用 Fmoc 或 FMS）描述于美国专利号 7585837。

[0083] 在另一种实施方式中，可互换使用短语 " 长效 OXM" 和 " 反向聚乙二醇化 OXM"。在另一种实施方式中，反向聚乙二醇化 OXM 包括 PEG-FMS-OXM 和 PEG-Fmoc-OXM，在本文中由下式确定 :(PEG-FMS)n-OXM 或 (PEG-Fmoc)n-OXM，其中 n 是至少 1 的整数，以及通过至少 1 个氨基，将 OXM 连接于 FMS 或 Fmoc 基团。

[0084] 在另一种实施方式中，出人意料地，本文描述的长效 OXM，在其聚乙二醇化形式下和在其外周形式下均是活性的。在另一种实施方式中，出人意料地，(PEG-FMS)n-OXM 或 (PEG-Fmoc)n-OXM 的结构并使得这种结合物无活性。在另一种实施方式中，出人意料地，(PEG-FMS)n-OXM 或 (PEG-Fmoc)n-OXM 的构造并不使得 OXM 无活性。

[0085] 治疗应用

[0086] 在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来预防高血糖症，改善血糖控制，治疗选自由以下组成的组中的糖尿病 :非胰岛素依赖性糖尿病（在一种实施方式中，2 型糖尿病）、胰岛素依赖性糖尿病（在一种实施方式中，1 型糖尿病）、和妊娠糖尿病、或它们的任何组合。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来治疗 2 型糖尿病。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来增加对胰岛素的敏感性。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来减少胰岛素抗性。

[0087] 在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来抑制食欲。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来诱导饱感。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低体重。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来减少身体脂肪。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低体重指数。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来减少食品消耗量。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来治疗肥胖症。

在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来治疗与肥胖症有关的糖尿病。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来增加心率。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来增加基础代谢率 (BMR)。在另一种实施方式中，PEG-Fmoc- 胃泌酸调节素和 PEG-FMS- 胃泌酸调节素以及包含它们的药物组合物用来增加能量消耗。在另一种实施方式中，PEG-Fmoc- 胃泌酸调节素和 PEG-FMS- 胃泌酸调节素以及包含它们的药物组合物用来诱导葡萄糖耐受。在另一种实施方式中，PEG-Fmoc- 胃泌酸调节素和 PEG-FMS- 胃泌酸调节素以及包含它们的药物组合物用来诱导血糖控制。在一种实施方式中，血糖控制是指并非高的和 / 或并非波动的血糖水平和 / 或并非高的和 / 或并非波动的糖基化血红蛋白水平。

[0088] 在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来抑制体重增加。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低血糖水平 (图 4A 和 9)。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来减少热量摄取量。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低食欲。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来控制体重。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来诱导或促进体重减轻。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来保持以下的任何一种或多种 :希望的体重、希望的体重指数、希望的外形和良好的健康状况。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来控制脂类曲线。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低甘油三酯水平。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低甘油水平 (图 9D)。

[0089] 在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来降低胆固醇水平。在一种实施方式中，胆固醇水平的降低大于在给予天然 OXM 以后所观测到的降低 (图 9C)。在一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物将胆固醇水平降低 60-70%。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物将胆固醇水平降低 50-100%。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物将胆固醇水平降低 25-90%。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物将胆固醇水平降低 50-80%。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物将胆固醇水平降低 40-90%。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物用来增加 HDL 胆固醇水平。

[0090] 在一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物可以用于本文描述的目的而没有在给予过程中显著降低效用 (图 5A、6A、和 7)。在一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 1 天。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 2-6 天。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 1 周。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它

们的药物组合物保持有效持续 2 周。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 3 周。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 4 周。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 6 周。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 2 个月。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 4 个月。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 6 个月。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物保持有效持续 1 年或更长时间。

[0091] 在一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物可以用于本文描述的目的并且在给予首次剂量以后可以立刻见效（图 8A）。在另一种实施方式中，PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物在给予两个或更多剂量以后生效。

[0092] 在另一种实施方式中，利用 PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物（如上文中所描述的）的方法用于人类受试者，其患有可以通过 OXM 加以缓解、抑制、和 / 或治疗的疾病或病症。在另一种实施方式中，利用 PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物（如上文中所描述的）的方法是兽医方法。在另一种实施方式中，利用 PEG-Fmoc-OXM 和 PEG-FMS-OXM 以及包含它们的药物组合物（如上文中所描述的）的方法用于动物如农场动物、宠物、和实验动物。因此，在一种实施方式中，本发明的受试者是猫、犬、牛、猪、鼠、马等。

[0093] 在另一种实施方式中，本发明提供了用于在受试者中治疗或减轻通过 OXM 或包含其的药物制剂可治疗或可减轻的疾病的方法，该方法包括以下步骤：给予受试者治疗有效量的 PEG-Fmoc-OXM 和 / 或 PEG-FMS-OXM（如在本文中所描述的），从而在受试者中治疗或减轻通过 OXM 可治疗或可减轻的疾病。

[0094] 在另一种实施方式中，如在本文中所使用的，OXM、"肽"或"蛋白质"包括天然肽（降解产物、以合成方式合成的蛋白或重组蛋白）和肽模拟物（通常为以合成方式合成的蛋白）、以及类肽和半类肽（其是蛋白类似物），在一些实施方式中，其具有修饰，从而使得蛋白质在体内甚至更加稳定，或更加能够渗入细胞。

[0095] 在另一种实施方式中，修饰包括但不限于 N 端修饰、C 端修饰、肽键修饰，其包括但不限于 CH2-NH、CH2-S、CH2-S=O、O=C-NH、CH2-O、CH2-CH2、S=C-NH、CH=CH 或 CF=CH、主链修饰、和残基修饰。用于制备肽模拟化合物的方法是本领域中众所周知的并且例如详述于 Quantitative Drug Design, C.A. Ramsden Gd., Chapter17.2, F. Choplin Pergamon Press(1992)，其以引用方式结合于本文就像在本文中完整地提出一样。下文提供了在这方面的进一步详情。

[0096] 在另一种实施方式中，肽内的肽键 (-CO-NH-) 被取代。在一些实施方式中，肽键被 N- 甲基化键 (-N(CH3)-CO-) 取代。在另一种实施方式中，肽键被酯键 (-C(R)H-C-O-O-C(R)-N-) 取代。在另一种实施方式中，肽键被酮基亚甲基键 (-CO-CH2-) 取代。在另一种实施方式中，肽键被以下取代：α - 氮杂键 (-NH-N(R)-CO-)，其中 R 是任何烷基，例如，甲基，卡巴键 (-CH2-NH-)。在另一种实施方式中，肽键被羟基亚乙基键 (-CH(OH)-CH2-)

取代。在另一种实施方式中，肽键被硫代酰胺键（-CS-NH-）取代。在一些实施方式中，肽键被烯双键（-CH=CH-）取代。在另一种实施方式中，肽键被反式酰胺键（retro amide bond）（-NH-CO-）取代。在另一种实施方式中，肽键被肽衍生物（-N(R)-CH2-CO-）取代，其中 R 是天然存在于碳原子上的″正常″侧链。在一些实施方式中，这些修饰发生在沿着肽链的任何键处并且甚至同时发生在数个键（2-3 个键）处。

[0097] 在一种实施方式中，蛋白质的天然芳族氨基酸如 Tip、Tyr 和 Phe，被取代成合成的非天然酸如苯甘氨酸、TIC、萘基丙氨酸（Nol）、Phe 的环－甲基化衍生物、Phe 的卤化衍生物或邻甲基 -Tyr。在另一种实施方式中，本发明的肽包括一种或多种修饰氨基酸或一种或多种非氨基酸单体（例如脂肪酸、复杂的碳水化合物等）。

[0098] 在一种实施方式中，“一种氨基酸”或“多种氨基酸”应理解为包括 20 种天然发生的氨基酸；通常在体内翻译后修饰的那些氨基酸，包括例如，羟脯氨酸、磷酸丝氨酸和磷酸苏氨酸；和其他不常用氨基酸，包括但不限于 2- 氨基己二酸、羟赖氨酸、异锁链素、正缬氨酸、正亮氨酸和鸟氨酸。在一种实施方式中，″氨基酸″包括 D- 和 L- 氨基酸。

[0099] 在一种实施方式中，本发明的 OXM 用于治疗，其需要 OXM 为可溶形式。在另一种实施方式中，本发明的 OXM 包括一种或多种非天然或天然极性氨基酸，包括但不限于丝氨酸和苏氨酸，其能够增加蛋白质溶解度，这是由于它们的包含羟基的侧链。

[0100] 在一种实施方式中，以生化方式合成本发明的 OXM，如通过利用标准的固相技术。在另一种实施方式中，这些生化方法包括独有的固相合成、部分固相合成、片段缩合、或经典的溶液合成。

[0101] 在一种实施方式中，固相 OXM 合成步骤是本领域技术人员众所周知的并进一步描述于 John Morrow Stewart and Janis Dillaha Young, Solid Phase Protein Syntheses (2nd Ed., Pierce Chemical Company, 1984)。在另一种实施方式中，通过制备型高性能液相层析来纯化合成的蛋白质 [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] 并且其组成可以通过本领域技术人员已知的方法通过氨基酸测序来证实。

[0102] 在另一种实施方式中，重组蛋白技术用来产生本发明的 OXM。在一些实施方式中，重组蛋白技术用于产生大量的本发明的 OXM。在另一种实施方式中，重组技术描述于 Bitter et al., (1987) Methods in Enzymol. 153:516-544、Studier et al. (1990) Methods in Enzymol. 185:60-89、Brisson et al. (1984) Nature310:511-514、Takamatsu et al. (1987) EMBO J. 6:307-311、Coruzzi et al. (1984) EMBO J. 3:1671-1680、Brogli et al., (1984) Science224:838-843、Gurley et al. (1986) Mol. Cell. Biol. 6:559-565、 和 Weissbach& Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp421-463。

[0103] 在另一种实施方式中，利用编码本发明的 OXM 的多核苷酸来合成本发明的 OXM。在一些实施方式中，将编码本发明的 OXM 的多核苷酸连接到表达载体，包括顺式调节序列（例如，启动子序列）的转录调控。在一些实施方式中，顺式调节序列适用于引导本发明的 OXM 的组成型表达。

[0104] 在一种实施方式中，短语″多核苷酸″是指单链或双链核酸序列，其被分离并以以下形式提供：RNA 序列、互补性多核苷酸序列（cDNA）、基因组多核苷酸序列和 / 或复合多核

苷酸序列（例如，上述序列的组合）。

[0105] 在一种实施方式中，"互补性多核苷酸序列"是指这样的序列，其来自利用逆转录酶或任何其他 RNA 依赖性 DNA 聚合酶的信使 RNA 的逆转录。在一种实施方式中，可以随后利用 DNA 聚合酶在体内或体外扩增上述序列。

[0106] 在一种实施方式中，"基因组多核苷酸序列"是指这样的序列，其来源（分离）自染色体，因而它表示染色体的连续部分。

[0107] 在一种实施方式中，"复合多核苷酸序列"是指这样的序列，其是至少部分互补的和至少部分基因组的。在一种实施方式中，复合序列可以包括编码本发明的肽所需要的一些外显子序列，以及插入其间的一些内含子序列。在一种实施方式中，内含子序列可以是任何来源的，包括其他基因，以及通常将包括保守的剪接信号序列。在一种实施方式中，内含子序列包括顺式作用表达调节元件。

[0108] 在一种实施方式中，利用 PCR 技术、或本领域技术人员已知的任何其他方法或步骤来制备本发明的多核苷酸。在一些实施方式中，所述步骤涉及连接两个不同 DNA 序列（参见，例如，"Current Protocols in Molecular Biology"，eds. Ausubel et al.，John Wiley&Sons，1992）。

[0109] 在一种实施方式中，各种各样的原核或真核细胞可以用作宿主表达系统来表达本发明的 OXM。在另一种实施方式中，这些包括但不限于微生物，如用重组噬菌体 DNA、质粒 DNA 或粘粒 DNA 表达载体（其包含蛋白编码序列）转化的细菌；用重组酵母菌表达载体（其包含蛋白编码序列）转化的酵母菌；用重组病毒表达载体（例如，花椰菜花叶病毒，CaMV；烟草花叶病毒，TMV）感染的或用重组质粒表达载体如 Ti 质粒（其包含蛋白编码序列）转化的植物细胞系统。

[0110] 在一种实施方式中，使用非细菌表达系统（例如哺乳动物表达系统如 CHO 细胞）来表达本发明的 OXM。在一种实施方式中，用来在哺乳动物细胞中表达本发明的多核苷酸的表达载体是 pCI-DHFR 载体，其包含 CMV 启动子和新霉素抗性基因。

[0111] 在另一种实施方式中，在本发明的细菌系统中，可以有利地选择许多表达载体，其取决于所表达的蛋白质所期望的应用。在一种实施方式中，大量的 OXM 是所期望的。在一种实施方式中，期望这样的载体，其引导表达高水平的蛋白产物，可能作为与疏水性信号序列的融合物，其引导表达的产物进入细菌的周质或培养基中，其中蛋白产物容易被纯化。在一种实施方式中，某些融合蛋白设计有特定的切割位点以有助于蛋白质的回收。在一种实施方式中，适于这类操作的载体包括但不限于 pET 系列的大肠杆菌表达载体 [Studier et al，Methods in Enzymol. 185:60-89(1990)]。

[0112] 在一种实施方式中，使用酵母菌表达系统。在一种实施方式中，许多包含组成型或诱导型启动子的载体可以用于酵母菌，如在美国专利申请号 5,932,447 中公开的。在另一种实施方式中，使用了这样的载体，其促进外源 DNA 序列整合进入酵母菌染色体。

[0113] 在一种实施方式中，本发明的表达载体可以进一步包括另外的多核苷酸序列，其允许，例如，从单个 mRNA 翻译数种蛋白质，如内部核糖体进入位点 (IRES) 和用于启动子 - 嵌合蛋白的基因组整合的序列。

[0114] 在一种实施方式中，哺乳动物表达载体包括但不限于 pcDNA3、pcDNA3.1(+/-)、pGL3，pZeoSV2(+/-)、pSecTag2、pDisplay、pEF/myc/cyto、pCMV/myc/cyto、pCR3.1、

pSinRep5、DH26S、DHBB、pNMT1、pNMT41、pNMT81（其可获自 Invitrogen）、pCI（其可获自 Promega）、pMbac、pPbac、pBK-RSV 和 pBK-CMV（其可获自 Strategene）、pTRES（其可获自 Clontech）、以及它们的衍生物。

[0115] 在另一种实施方式中，本发明使用包含来自真核病毒如逆转录病毒的调节元件的表达载体。SV40 载体包括 pSVT7 和 pMT2。在另一种实施方式中，源自牛乳头瘤病毒的载体包括 pBV-1MTHA，以及源自 EB 病毒的载体包括 pHEBO、和 p205。其他示例性载体包括 pMSG、pAV009/A^+、pMT010/A^+、pMAMneo-5、杆状病毒 pDSVE、和任何其他载体，其允许在以下各项的指导下表达蛋白质：SV-40 早期启动子、SV-40 晚期启动子、金属硫蛋白启动子、小鼠乳腺肿瘤病毒启动子、劳氏肉瘤病毒启动子、多角体蛋白启动子、或在真核细胞中显示有效表达的其他启动子。

[0116] 在一种实施方式中，使用植物表达载体。在一种实施方式中，通过多种启动子来驱动双重 GLP-1/ 胰高血糖素受体激动剂编码序列（如 OXM）的表达。在另一种实施方式中，使用病毒启动子如 CaMV 的 35S RNA 和 19S RNA 启动子 [Brisson et al,Nature310:511-514(1984)]、或针对 TMV 的外壳蛋白启动子 [Takamatsu et al,EMBO J.6:307-311(1987)]。在另一种实施方式中，使用植物启动子，如，例如，RUBISCO 的小亚基 [Coruzzi et al,EMBOJ.3:1671-1680(1984)；和 Brogli et al,Science224:838-843(1984)] 或热激启动子，例如，大豆 hspl7.5-E 或 hspl7.3-B[Gurley et al,Mol.Cell.Biol.6:559-565(1986)]。在一种实施方式中，利用 Ti 质粒、Ri 质粒、植物病毒载体、直接 DNA 转化、微注射、电穿孔和技术人员众所周知的其他技术将构建物引入植物细胞中。参见，例如，Weissbach&Weissbach[Methods for Plant Molecular Biology,Academic Press,NY,Section VIII,pp421-463(1988)]。本发明还可以使用其他表达系统如昆虫和哺乳动物宿主细胞系统，这是本领域中众所周知的。

[0117] 可以理解的是，除了包含用于转录和翻译插入的编码序列的必要元件（编码蛋白质）之外，本发明的表达构建物还可以包括这样的序列，其设计成用来优化表达蛋白的稳定性、生产、纯化、产率或活性。

[0118] 在一些实施方式中，各种方法可以用来将本发明的表达载体引入宿主细胞系统。在一些实施方式中，这类方法一般地描述于 Sambrook et al.,Molecular Cloning:A Laboratory Manual,Cold Springs Harbor Laboratory,New York(1989,1992)；Ausubel et al.,Current Protocols in Molecular Biology,John Wiley and Sons,Baltimore,Md.(1989),Chang et al,Somatic Gene Therapy,CRC Press,Ann Arbor,Mich.(1995)；Vega et al.,Gene Targeting,CRC Press,Ann Arbor Mich.(1995)；Vectors:A Survey of Molecular Cloning Vectors and Their Uses,Butterworths,Boston Mass.(1988)；以及 Gilboa et at.[Biotechniques4(6):504-512,1986]，并且包括，例如，稳定或瞬时转染、脂质转染、电穿孔和用重组病毒载体感染。另外，关于阳性 - 阴性选择方法，参见美国专利号 5,464,764 和 5,487,992。

[0119] 在一种实施方式中，在有效条件下培养转化细胞，其允许表达大量的重组 OXM。在另一种实施方式中，有效的培养条件包括但不限于有效的培养基、生物反应器、温度、pH 和氧条件（其允许蛋白质生产）。在一种实施方式中，有效的培养基是指任何培养基，其中培养细胞以产生本发明的重组 OXM。在另一种实施方式中，培养基通常包括水溶液，其具有可同

化的碳、氮和磷源、以及适当的盐、矿物质、金属和其他营养成分，如维生素。在一种实施方式中，可以在常规发酵生物反应器、摇瓶、试管、微量滴定板和培养皿中培养本发明的细胞。在另一种实施方式中，在适合重组细胞的温度、pH 和氧含量下进行培养。在另一种实施方式中，培养条件在本领域普通技术人员的专业知识范围内。

[0120] 在一种实施方式中，取决于用于生产的载体和宿主系统，本发明产生的 OXM 保持在重组细胞内、分泌进入发酵培养基、分泌进入在两个细胞膜之间的间隙，如在大肠杆菌中的周质空间；或保留在细胞或病毒膜的外表面上。

[0121] 在一种实施方式中，在培养预定时间以后，进行重组 OXM 的回收。

[0122] 在一种实施方式中，本文中使用的短语″回收重组 OXM″是指收集包含 OXM 的整个发酵培养基而不需要另外的分离或纯化步骤。

[0123] 在一种实施方式中，利用各种各样的标准蛋白质纯化技术来纯化本发明的 OXM，例如但不限于亲和层析、离子交换层析、过滤、电泳、疏水性相互作用层析、凝胶过滤层析、反相层析、伴刀豆球蛋白 A 层析、层析聚焦和差别增溶。

[0124] 在一种实施方式中，为了促进回收，可以设计表达的编码序列以编码本发明的蛋白质以及融合可断链部分。在一种实施方式中，可以设计融合蛋白从而可以通过亲和层析来容易地分离蛋白质；例如，通过固定在对可断链部分具有亲和性的柱上。在一种实施方式中，将切割位点设计在蛋白质和可断链部分之间，并通过用适当的酶或试剂(其在该位点特异性地切割融合蛋白)进行处理，使蛋白质可以从层析柱释放[例如，参见 Booth et al.,Immunol.Lett.19:65-70(1988)；和 Gardella et ah,J.Biol.Chem.265:15854-15859(1990)]。在另一种实施方式中，以″基本上纯″的形式来获取本发明的 OXM。在另一种实施方式中，短语″基本上纯″是指这样的纯度，其允许在本文描述的应用中有效使用 OXM。

[0125] 在一种实施方式中，还可以利用体外表达系统来合成本发明的双重 GLP-1/ 胰高血糖素受体激动剂。在一种实施方式中，体外合成方法是本领域中众所周知的并且系统的成分是市售的。

[0126] 在另一种实施方式中，通过测量天然、重组和 / 或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)以及包含它们的药物组合物治疗或减轻疾病或病症(例如但不限于糖尿病、肥胖症、饮食紊乱、代谢障碍等)的能力来确定体外结合活性。在另一种实施方式中，通过所治疗的疾病的已知量度来推断体内活性。

[0127] 在另一种实施方式中，本发明的反向聚乙二醇化 OXM 的剂量包含 0.005 至 0.1 毫克 /kg OXM 肽。在另一种实施方式中，本发明的反向聚乙二醇化 OXM 的剂量包括 0.005 至 0.5 毫克 /kg OXM 肽。在另一种实施方式中，本发明的反向聚乙二醇化 OXM 的剂量包括 0.05 至 0.1 微克 OXM 肽。在另一种实施方式中，本发明的反向聚乙二醇化 OXM 的剂量包括 0.005 至 0.1 毫克 /kg OXM 肽(为注射溶液形式)。

[0128] 在另一种实施方式中，每天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 36 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 48 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 60 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 72 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 84 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施

方式中，每 96 小时一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 5 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 6 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 7 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 8-10 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 10-12 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 12-15 天一次给予反向聚乙二醇化 OXM 的剂量。在另一种实施方式中，每 15-25 天一次给予反向聚乙二醇化 OXM 的剂量。

[0129] 在另一种实施方式中，通过肌内 (IM) 注射、皮下 (SC) 注射、或静脉内 (IV) 注射，每周一次给予本发明的反向聚乙二醇化 OXM。

[0130] 在另一种实施方式中，可以将本发明的反向聚乙二醇化 OXM 提供给个体本身。在一种实施方式中，可以将本发明的反向聚乙二醇化 OXM 作为药物组合物的一部分（其中它与药用载体混合）提供给个体。

[0131] 在另一种实施方式中，″药物组合物″是指如在本文中所描述的长效 OXM 和其他化学成分如生理上合适的载体和赋形剂的制剂。药物组合物的目的是有助于将化合物给予生物体。在另一种实施方式中，反向聚乙二醇化 OXM 负责生物效应。

[0132] 在另一种实施方式中，本发明的任何组合物可以至少包含反向聚乙二醇化 OXM。在一种实施方式中，本发明提供了组合制剂。在一种实施方式中，″组合制剂″尤其在以下意义上定义″多部分的集合″，即可以独立地或通过使用与不同量的组合配合物的不同固定组合（即，同时地、并行地、分别地或顺序地），给予如上文所定义的组合配合物。在一些实施方式中，然后可以例如同时地或在时间上错开地给予多个部分集合中的部分，即对于多个部分集合的任何部分，在不同时间点并具有相等或不同的时间间隔。在一些实施方式中，可以将组合配合物总量的比率给予组合制剂中。在一种实施方式中，可以改变组合制剂，例如，用于应付待治疗的患者亚群的需要或单个患者的需要，其中不同的需要可以起因于特定的疾病、疾病的严重性、年龄、性别、或体重，如可以由本领域的技术人员容易确定的。

[0133] 在另一种实施方式中，短语″生理学上可接受的载体″和″药用载体″（其是可互换使用的）是指载体或稀释剂，其并不引起对生物体的显著刺激以及并不消除所给予化合物的生物活性和性能。这些短语包括佐剂。在一种实施方式中，在药用载体中包括的组分中的一种可以是例如聚乙二醇 (PEG)、生物相容性聚合物，其在有机介质和水介质中均具有较宽范围的溶解度 (Mutter et al.(1979)。

[0134] 在另一种实施方式中，″赋形剂″是指加入药物组合物的惰性物质以进一步促进长效 OXN 的给予。在一种实施方式中，赋形剂包括碳酸钙、磷酸钙、各种糖和多种类型的淀粉、纤维素衍生物、明胶、植物油和聚乙二醇。

[0135] 用于配制和给予药物的技术参见″Remington's Pharmaceutical Sciences,″Mack Publishing Co.,Easton,PA，最新版本，其以引用方式结合于本文。

[0136] 在另一种实施方式中，本发明的肽的适宜的给予途径，例如，包括口服、直肠、经粘膜、经鼻、肠或胃肠道外递送，其包括肌内、皮下和髓内注射以及鞘内、直接室内、静脉内、腹膜内、鼻内、或眼内注射。

[0137] 本发明还包括反向聚乙二醇化 OXM 用于制造用于通过针对上文描述的任何治疗方法的外周途径给予脑的药物的用途。外周途径的实例包括口服、直肠、胃肠道外给药，例

如静脉内、肌内、或腹膜内给药，粘膜给药，例如，颊部、舌下、鼻部、皮下或经皮给药，包括通过吸入来给药。以下给出用于药物的 OXM 的优选剂量。

[0138] 本发明提供了包含反向聚乙二醇化 OXM 和药用适宜载体的药物组合物，，其形式适用于口服、直肠、胃肠道外给药，例如静脉内、肌内、或腹膜内给药，粘膜给药例如颊部、舌下、鼻部、皮下或经皮给药，包括通过吸入来给药。如果处于单位剂型，则每单位剂量可以，例如，如下文所描述，或如基于以下给出的每 kg 剂量计算。

[0139] 在另一种实施方式中，以局部而不是全身方式来给予制剂，例如，通过将制剂直接注射进入患者身体的特定区域。在另一种实施方式中，将反向聚乙二醇化 OXM 配制成鼻内剂型。在另一种实施方式中，将反向聚乙二醇化 OXM 配制成注射剂型。

[0140] 本发明考虑了剂量范围的各种实施方式：以 0.01-0.5 毫克 /kg 体重 /3 天的范围给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分（仅提供在反向聚乙二醇化 OXM 组合物内 OXM 的重量，因为 PEG 的大小可以显著不同）。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重 /7 天的范围，给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重 /10 天的范围，给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重 /14 天的范围，给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。在另一种实施方式中，出人意料地，在反向聚乙二醇化 OXM 组合物中 OXM 的有效量是自由 OXM 的有效量的 1/4-1/10。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 50%。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 70%。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 75%。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 80%。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 85%。在另一种实施方式中，相比于自由 OXM，出人意料地，OXM 的反向聚乙二醇化能够将开具给患者的 OXM 的量限制至少 90%。

[0141] 在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重的范围，每 3 天一次给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分（仅提供在反向聚乙二醇化 OXM 组合物内的 OXM 重量，因为 PEG 的大小可以显著不同）。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重的范围，每 7 天一次给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重的范围，每 10 天一次给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。在另一种实施方式中，以 0.01-0.5 毫克 /kg 体重的范围，每 14 天一次给予在反向聚乙二醇化 OXM 组合物内的 OXM 肽成分。

[0142] 在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 将有效给药频率降低至少 2 倍并且将有效每周剂量减小至少 2 倍，从而限制不良事件的风险和增加对使用 OXM 疗法的依从。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 将有效给药频率降低至少 3 倍并将有效每周剂量减小至少 3 倍，从而限制不良事件的风险和增加对使用 OXM 疗法的依从。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 将有效给药频率降低至少 4 倍并且将有效每周剂量减小至少 4 倍，从而限制不良事件的风险和增加对使用 OXM 疗法的依从。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 将有效给药

频率降低至少 5 倍并且将有效每周剂量减小至少 5 倍，从而限制不良事件的风险和增加对使用 OXM 疗法的依从。在另一种实施方式中，相比于自由 OXM，反向聚乙二醇化 OXM 将有效给药频率降低至少 6 倍并且将有效每周剂量减小至少 6 倍，从而限制不良事件的风险和增加对使用 OXM 疗法的依从。在另一种实施方式中，有效给药频率和有效每周剂量是基于：(1) 在反向聚乙二醇化 OXM 组合物内给予的 OXM 成分的重量；以及 (2) 在自由 OXM（未修饰 OXM) 组合物内给予的 OXM 成分的重量。

[0143] 在另一种实施方式中，本发明的方法包括增加患有需要 OXM 疗法的慢性疾病的患者的依从性。在另一种实施方式中，通过反向聚乙二醇化 OXM（如上文中所描述的)，本发明的方法能够降低 OXM 的给药频率。在另一种实施方式中，术语依从性包括顺从性 (adherence)。在另一种实施方式中，本发明的方法包括通过减少 OXM 的给药频率来增加需要 OXM 疗法的患者的依从性。在另一种实施方式中，由于使得 OXM 更加稳定和更加有效的反向聚乙二醇化，降低 OXM 的给药频率。在另一种实施方式中，由于增加 OXM 的 T1/2，降低 OXM 的给药频率。在另一种实施方式中，由于减小 OXM 的血液清除率，降低 OXM 的给药频率。在另一种实施方式中，由于增加 OXM 的 T1/2，降低 OXM 的给药频率。在另一种实施方式中，由于增加 OXM 的 AUC 量度，降低 OXM 的给药频率。

[0144] 在另一种实施方式中，每天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每两天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每三天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每四天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每五天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每六天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每周一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每 7-14 天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每 10-20 天一次给予受试者反向聚乙二醇化 OXM，在另一种实施方式中，每 5-15 天一次给予受试者反向聚乙二醇化 OXM。在另一种实施方式中，每 15-30 天一次给予受试者反向聚乙二醇化 OXM。

[0145] 在另一种实施方式中，每天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每两天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每三天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每四天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每五天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每六天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每周一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每 7-14 天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每 10-20 天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每 5-15 天一次给予受试者聚乙二醇化 OXM。在另一种实施方式中，每 15-30 天一次给予受试者聚乙二醇化 OXM。

[0146] 在一种实施方式中，在给予单剂量的 PEG-OXM 变体以后，本文提供的聚乙二醇化 OXM 变体出人意料地降低葡萄糖以及降低空腹胰岛素水平。在另一种实施方式中，本文提供的聚乙二醇化 OXM 变体导致增加受试者对胰岛素的敏感性（参见实施例 6)。

[0147] 在一种实施方式中，口服给予包括单位剂型，其包括片剂、胶囊剂、锭剂、咀嚼片剂、混悬剂、乳剂等。这样的单位剂型包含安全和有效量的本发明的 OXM，在一种实施方式中，其各自为约 0.7 或 3.5mg 至约 280mg/70kg，或在另一种实施方式中，为约 0.5 或 10mg 至约 210mg/70kg。适用于制备用于口服给予的单位剂型的药用载体是本领域中众所周知的。

在一些实施方式中，片剂通常包含常规药学上相容的佐剂作为惰性稀释剂，如碳酸钙、碳酸钠、甘露醇、乳糖和纤维素；粘合剂如淀粉、明胶和蔗糖；崩解剂如淀粉、海藻酸和交联羧甲纤维素；润滑剂如硬脂酸镁、硬脂酸和滑石粉。在一种实施方式中，助流剂如二氧化硅可以用来改善粉末混合物的流动特性。在一种实施方式中，可以针对外观添加着色剂，如 FD&C 染料。甜味剂和增香剂，如阿司帕坦、糖精、薄荷醇、薄荷油、和水果香料，是用于咀嚼片剂的有用的佐剂。胶囊剂通常包含一种或多种上文公开的固体稀释剂。在一些实施方式中，载体成分的选择取决于次要考虑事项如味道、成本、和储存稳定性，其对于本发明的目的而言并不是关键的，并且可以由本领域技术人员容易地确定。

[0148] 在一种实施方式中，口服剂型包含预定的释放曲线。在一种实施方式中，本发明的口服剂型包括延长释放片剂、胶囊剂、锭剂或咀嚼片剂。在一种实施方式中，本发明的口服剂型包括缓慢释放片剂、胶囊剂、锭剂或咀嚼片剂。在一种实施方式中，本发明的口服剂型包括立即释放片剂、胶囊剂、锭剂或咀嚼片剂。在一种实施方式中，按照本领域技术人员已知的长效 OXM 的希望的释放曲线来配制口服剂型。

[0149] 在另一种实施方式中，用于本发明方法的组合物包括溶液或乳剂，在另一种实施方式中其是包含安全和有效量的本发明化合物以及可选地其他化合物的水溶液或乳剂，用于局部鼻内给药。在一些实施方式中，组合物包含约 0.001% 至约 10.0%w/v 的主题化合物，更优选地约 00.1% 至约 2.0，其用于通过鼻内途径来全身递送化合物。

[0150] 在另一种实施方式中，通过静脉内、动脉内、皮下或肌内注射液体制剂来给予药物组合物。在另一种实施方式中，液体制剂包括溶液、混悬剂、分散体、乳剂、油等。在一种实施方式中，静脉内给予药物组合物，因而将该组合物配制成适用于静脉内给予的形式。在另一种实施方式中，动脉内给予药物组合物，因而将该组合物配制成适用于动脉内给予的形式。在另一种实施方式中，肌内给予药物组合物，因而将该组合物配制成适用于肌内给予的形式。

[0151] 另外，在另一种实施方式中，将药物组合物局部给予身体表面，因而将该组合物配制成适用于局部给予的形式。适宜的局部制剂包括凝胶剂、软膏剂、乳膏剂、洗剂、滴剂等。对于局部给予，本发明的化合物结合于另外适当的一种或多种治疗剂，其制备和用作处于生理上可接受的稀释剂(有或没有药物载体)中的溶液、混悬剂、或乳剂。

[0152] 在一种实施方式中，通过本领域中众所周知的过程来制备本发明的药物组合物，例如，通过常规的混合、溶解、粒化、糖衣丸制作、磨细、乳化、胶囊化、包埋或冷冻干燥过程。

[0153] 在一种实施方式中，使用一种或多种生理学上可接受的载体(包括赋形剂和助剂，其促进 OXM 处理成可以药用的制剂)以常规方式配制根据本发明使用的药物组合物。在一种实施方式中，制剂取决于所选的给予途径。

[0154] 在一种实施方式中，在水溶液中配制本发明的注射剂。在一种实施方式中，在生理相容性缓冲液中配制本发明的注射剂，如 Hank 氏溶液、林格氏液、或生理盐缓冲液。在一些实施方式中，对于经粘膜给予，在制剂中使用适合渗透屏障的渗透剂。这样的渗透剂在本领域中通常是已知的。

[0155] 在一种实施方式中，本文描述的制剂配制成用于胃肠道外给予，例如，通过推注或连续输注。在另一种实施方式中，以单位剂型来提供用于注射的制剂，例如，在安瓿或多剂量容器中，其可选地具有添加的防腐剂。在另一种实施方式中，组合物是处于油性或水性载

体中的混悬剂、溶液或乳剂，并且包含配制剂如悬浮剂、稳定剂和 / 或分散剂。

[0156] 在另一种实施方式中，组合物还包含防腐剂，如苯扎氯铵和硫柳汞等；螯合剂，如依地酸钠等；缓冲剂如磷酸盐、柠檬酸盐和乙酸盐；等渗剂如氯化钠、氯化钾、甘油、甘露醇等；抗氧化剂如抗坏血酸、乙酰半胱氨酸、焦亚硫酸钠等；芳香剂；粘度调节剂，如聚合物，包括纤维素及其衍生物；从及聚乙烯醇和酸以及碱，以根据需要调节这些含水组合物的 pH。在一些实施方式中，组合物还包含局部麻醉剂或其他活性物质。上述组合物可以用作喷雾剂、雾剂、滴剂等。

[0157] 在一种实施方式中，用于胃肠道外给予的药物组合物包括水溶性形式的活性制剂的水溶液。另外，在一些实施方式中，长效 OXM 的混悬剂被制备成适当的油性或水基的注射混悬剂。在一些实施方式中，适宜的亲脂性溶剂或载体包括脂肪油如芝麻油、或合成脂肪酸酯如油酸乙酯、甘油三酯或脂质体。在一些实施方式中，水性注射混悬剂包含增加悬浮液粘度的物质，如羧甲基纤维素钠、山梨醇或葡聚糖。在另一种实施方式中，悬浮液还包含适宜的稳定剂或增加长效 OXM 的溶解度的试剂，以便于制备高浓度溶液。

[0158] 在另一种实施方式中，可以在小泡，尤其是脂质体中递送活性化合物（参见 Langer, Science249:1527-1533(1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler(eds.), Liss, New York, pp. 353-365(1989); Lopez-Berestein, ibid., pp. 317-327；一般地参见同上）。

[0159] 在另一种实施方式中，配制以控释系统递送的药物组合物用于静脉内输注、可植入渗透泵、透皮贴剂、脂质体、或其他给予方式。在一种实施方式中，使用泵（参见 Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201(1987); Buchwald et al., Surgery88:507(1980); Saudek et al, N. Engl. J. Med321:574(1989)）。在另一种实施方式中，可以使用聚合物材料。在又一种实施方式中，可以将控释系统放置在治疗靶的邻近，即，脑，因而仅需要全身剂量的一部分（参见，例如，Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138(1984)）。在 Langer(Science249:1527-1533(1990)) 的综述中讨论了其他控释系统。

[0160] 在一种实施方式中，长效 OXM 为粉末形式，用于在使用前用适宜的载体，例如，无菌、无热原水基溶液，加以配制。在一些实施方式中，配制组合物用于雾化和吸入给予。在另一种实施方式中，将组合物包含在具有附连的雾化装置的容器中。

[0161] 在一种实施方式中，利用，例如，常规的栓剂基质如可可脂或其他甘油酯，将本发明的制剂配制成直肠组合物如栓剂或滞留型灌肠剂。

[0162] 在一种实施方式中，适用于本发明上下文的药物组合物包括这样的组合物，其中包含有效量的长效 OXM 以达到预期的目的。在另一种实施方式中，治疗有效量是指长效 OXM 的量可有效地预防、缓解或减轻疾病的症状或延长所治疗的受试者的存活时间。

[0163] 在一种实施方式中，治疗有效量的确定在本领域技术人员的能力范围内。

[0164] 组合物还包含防腐剂，如苯扎氯铵和硫柳汞等；螯合剂，如依地酸钠等；缓冲剂如磷酸盐、柠檬酸盐和乙酸盐；等渗剂如氯化钠、氯化钾、甘油、甘露醇等；抗氧化剂如抗坏血酸、乙酰半胱氨酸、焦亚硫酸钠等；芳香剂；粘度调节剂，如聚合物，包括纤维素及其衍生物；以及聚乙烯醇和酸以及碱，来根据需要调节这些含水组合物的 pH。组合物还包含局部麻醉剂或其他活性物质。组合物可以用作喷雾剂、雾剂、滴剂等。

[0165] 可以用作药用载体或其成分的物质的一些实例是糖，如乳糖、葡萄糖和蔗糖；淀粉，如玉米淀粉和马铃薯淀粉；纤维素及其衍生物，如羧甲基纤维素钠、乙基纤维素、和甲基纤维素；粉状黄芪胶；麦芽；明胶；滑石粉；固体润滑剂，如硬脂酸和硬脂酸镁；硫酸钙；植物油，如花生油、棉籽油、芝麻油、橄榄油、玉米油和可可油；多元醇如丙二醇、甘油、山梨醇、甘露醇、和聚乙二醇；海藻酸；乳化剂，如吐温™商标乳化剂、润湿剂，如月桂基硫酸钠；着色剂；增香剂；压片剂；稳定剂；抗氧化剂；防腐剂；无热原水；等渗盐水；和磷酸盐缓冲溶液。连同化合物一起使用的药用载体的选择基本上由有待给予的化合物来确定。在一种实施方式中，如果将要注射主题化合物，药用载体是无菌生理盐水，具有血液相容的悬浮剂，其 pH 已被调节至约 7.4。

[0166] 另外，组合物进一步包含粘合剂（例如，阿拉伯胶、玉米淀粉、明胶、卡波姆、乙基纤维素、瓜尔胶、羟丙基纤维素、羟丙基甲基纤维素、聚乙烯吡咯烷酮）、崩解剂（例如玉米淀粉、马铃薯淀粉、海藻酸、二氧化硅、交联羧甲纤维素钠、交聚维酮、瓜尔胶、淀粉羟乙酸钠）、各种 pH 和离子强度的缓冲剂（例如，Tris-HCl、乙酸盐、磷酸盐）、添加剂如白蛋白或明胶以防止吸收到表面、洗涤剂（例如，吐温 20、吐温 80、泊洛沙姆 F68、胆酸盐）、蛋白酶抑制剂、表面活性剂（例如月桂基硫酸钠）、渗透促进剂、增溶剂（例如，甘油、聚乙烯甘油）、抗氧化剂（例如，抗坏血酸、焦亚硫酸钠、丁羟茴醚）、稳定剂（例如羟丙基纤维素、羟丙基甲基纤维素）、粘度增加剂（例如卡波姆、胶态二氧化硅、乙基纤维素、瓜尔胶）、甜味剂（例如阿司帕坦、柠檬酸）、防腐剂（例如，硫柳汞、苄醇、对羟基苯甲酸酯类）、润滑剂（例如硬脂酸、硬脂酸镁、聚乙二醇、月桂基硫酸钠）、流动助剂（例如胶态二氧化硅）、增塑剂（例如酞酸二乙酯、柠檬酸三乙酯）、乳化剂（例如卡波姆、羟丙基纤维素、月桂基硫酸钠）、聚合物涂层（例如，泊洛沙姆或泊洛沙胺(poloxamine)）、涂层和成膜剂（例如乙基纤维素、丙烯酸酯、聚甲基丙烯酸酯）和 / 或佐剂。

[0167] 用于糖浆剂、酏剂、乳剂和混悬剂的载体的典型成分包括乙醇、甘油、丙二醇、聚乙二醇、液体蔗糖、山梨醇和水。对于混悬剂，典型的悬浮剂包括甲基纤维素、羧甲基纤维素钠、纤维素（例如 Avicel™、RC-591）、黄芪胶和海藻酸钠；典型的润湿剂包括卵磷脂和聚氧化乙烯山梨聚糖（例如聚山梨酯 80）。典型的防腐剂包括羟苯甲酸甲酯和苯甲酸钠。在另一种实施方式中，口服液体组合物还包含一种或多种成分如甜味剂、增香剂和着色剂(如上文公开的)。

[0168] 组合物还包括活性物质结合到聚合物化合物的颗粒制剂，如聚乳酸、聚乙醇酸、水凝胶等中或其上，或结合到脂质体、微乳液、胶束、单层或多层囊泡、红细胞空壳、或原生质球体上)。这样的组合物将影响物理状态、溶解性、稳定性、体内释放速率、和体内清除速率。

[0169] 本发明还包括涂有聚合物（例如泊洛沙姆或泊洛沙胺）的颗粒组合物，和结合至针对组织特异性受体、配体或抗原的抗体，或结合至组织特异性受体的配体的化合物。

[0170] 在一种实施方式中，通过共价连接水溶性聚合物如聚乙二醇、聚乙二醇和聚丙二醇的共聚物、羧甲基纤维素、葡聚糖、聚乙烯醇、聚乙烯吡咯烷酮或聚脯氨酸，来修饰化合物。在另一种实施方式中，相比于相应的未修饰化合物，在静脉内注射以后，修饰的化合物在血液中显示显著更长的半衰期。在一种实施方式中，修饰还增加化合物在水溶液中的溶解度，消除聚集，增强化合物的物理和化学稳定性，以及大大降低化合物的免疫原性和反应性。在另一种实施方式中，相比于未修饰的化合物，通过较低频率地或以较低剂量给予这类

聚合物 - 化合物结合物来实现所期望的体内生物活性。

[0171] 在另一种实施方式中，最初可以由体外测定来估计有效量或剂量的制剂。在一种实施方式中，可以在动物模型中配制剂量并且这样的信息可以用来更精确地确定在人类中的用量。

[0172] 在一种实施方式中，可以通过标准制药步骤来确定长效激动剂（如 OXM）（如在本文中所描述的）在体外、在细胞培养物或实验动物中的毒性和治疗效果。在一种实施方式中，获自这些体外和细胞培养物测定和动物研究的数据可以用于配制用于人类的剂量范围。在一种实施方式中，剂量根据采用的剂型和使用的给予途径而变化。在一种实施方式中，可以由个人医师根据患者的病情来选择精确的配方、给予途径和剂量。[参见例如，Fingl, et al., (1975)"The Pharmacological Basis of Therapeutics", Ch. 1p. 1]。

[0173] 在一种实施方式中，取决于待治疗病症的严重性和响应性，可以单次或多次给予剂量，其中疗程持续数天至数周或直到治愈或减轻疾病状态。

[0174] 在一种实施方式中，待给予的组合物的量当然将取决于待治疗的受试者、疾病的严重性、给予方式、处方医师的判断等。

[0175] 在一种实施方式中，还制备配制在相容的药物载体中的包括本发明的制剂的组合物，放置在适当的容器中，并标示用于治疗指定的病症。

[0176] 在另一种实施方式中，通过全身给予来给予聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）。在另一种实施方式中，通过静脉内、肌内或皮下注射来给予聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）。在另一种实施方式中，聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）是与复杂的有机赋形剂和稳定剂如非离子型表面活性试剂（即表面活性剂）、各种糖、有机多元醇和 / 或人血清白蛋白结合的冷冻干燥制剂。在另一种实施方式中，药物组合物包括处于注射用无菌水中的冷冻干燥聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）。在另一种实施方式中，药物组合物包括处于注射用无菌 PBS 中的冷冻干燥的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）。在另一种实施方式中，药物组合物包括处于注射用无菌 0.9%NaCl 中的冷冻干燥的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）。

[0177] 在另一种实施方式中，药物组合物包括聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）和复合载体如人血清白蛋白、多元醇、糖、和阴离子型表面活性稳定剂。参见，例如，WO89/10756（Hara et al. - 包含多元醇和对羟基苯甲酸酯）。在另一种实施方式中，药物组合物包含反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）和乳糖酸以及乙酸盐 / 甘氨酸缓冲液。在另一种实施方式中，药物组合物包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）和氨基酸，如精氨酸或谷氨酸，其增加干扰素成分在水中的溶解度。在另一种实施方式中，药物组合物包含冷冻干燥的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）以及甘氨酸或人血清白蛋白 (HSA)、缓冲剂（例如乙酸盐）和等渗剂（例如 NaCl）。在另一种实施方式中，药物组合物包含冷冻干燥的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂（如在本文中所描述的）

以及磷酸盐缓冲剂、甘氨酸和 HSA。

[0178] 在另一种实施方式中，当放置在 pH 为约 4 至 7.2 的缓冲溶液中时，稳定了包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物。在另一种实施方式中，使用氨基酸作为稳定剂以及在一些情况下使用盐（如果氨基酸并不包含带电荷侧链）来稳定包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物。

[0179] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物是液体组合物，其包含按重量计约 0.3% 至 5% 的为氨基酸的稳定剂。

[0180] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物提供剂量准确性和产品安全性。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物提供生物活性的稳定的液体制剂，用于注射应用。在另一种实施方式中，药物组合物包含非冷冻干燥的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)。

[0181] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物提供液体制剂，其允许以液态长时间存储，从而有利于在给予以前的储存和运输。

[0182] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含固体脂质作为基质材料。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的注射药物组合物包含固体脂质作为基质材料。在另一种实施方式中，Speiser(Speiser and al.,Phann.Res.8(1991)47-54) 描述了通过喷射冻凝来生产脂质微颗粒，接着脂质纳米颗粒用于口服给予 (Speiser EP0167825(1990))。在另一种实施方式中，身体良好地耐受使用的脂质（例如由存在于用于胃肠外营养的乳液中的脂肪酸构成的甘油酯）。

[0183] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物为脂质体形式 (J.E.Diederichs and al.,Pharm./nd.56(1994)267-275)。

[0184] 在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含聚合物微颗粒。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的注射药物组合物包含聚合物微颗粒。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含纳米颗粒。在另一种实施方式中，包含反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含脂质体。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化 OXM (如在本文中所描述的)的药物组合物包含脂质乳液。在另一种实施方式中，包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含微球体。在另一种实施方式中，包含聚乙二醇化或反向

聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包含脂质纳米颗粒。在另一种实施方式中,包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包括包含两亲脂质的脂质纳米颗粒。在另一种实施方式中,包含聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂(如在本文中所描述的)的药物组合物包括包含药物、脂质基质和表面活性剂的脂质纳米颗粒。在另一种实施方式中,脂质基质具有至少 50%w/w 的甘油单酯含量。

[0185] 在一种实施方式中,以包装或分配装置来提供本发明的组合物,如 FDA 批准的试剂盒,其包含含有长效双重 GLP-1/ 胰高血糖素受体激动剂的一种或多种单位剂型。例如,在一种实施方式中,包装包括金属或塑料箔,如泡眼包装。在一种实施方式中,包装或分配装置伴随有用于给药的说明书。在一种实施方式中,包装或分配器装有与容器有关的通告,其形式为由管理药品生产、使用或销售的政府机构规定的形式,该通告反映了该机构批准该组合物的形式或人或兽医给药。在一种实施方式中,该通告是由美国食品和药物管理局批准的处方药物的标识或批准产品插入物的标识。

[0186] 在一种实施方式中,可以理解的是,可以连同另外的活性剂一起将本发明的聚乙二醇化或反向聚乙二醇化双重 GLP-1/ 胰高血糖素受体激动剂提供给个体,以与使用每种试剂本身进行治疗相比实现改进的治疗效果。在另一种实施方式中,针对与联合疗法有关的不利的副作用,采取措施(例如,互补剂的剂量和选择)。

[0187] 本领域技术人员,在考察以下非限制性实施例之后,可以清楚本发明的另外的目的、优点、和新特点。另外,如上文描述的以及如在随附权利要求部分中要求的,本发明的多个实施方式和方面的每一种在以下实施例中找到实验支持。

[0188] 实施例

[0189] 总体上,在本文中使用的术语和在本发明中使用的实验室步骤包括分子技术、生物化学技术、微生物学技术和重组 DNA 技术。这类技术在文献中充分地说明。参见,例 如,"Molecular Cloning:A laboratory Manual"Sambrook et al.,(1989);"Current Protocols in Molecular Biology"VolumesI-III Ausubel,R.M.,ed.(1994);Ausubel et al.,"Current Protocols in Molecular Biology",John Wiley and Sons,Baltimore,Maryland(1989);Perbal,"A Practical Guide to Molecular Cloning",John Wiley&Sons,New York(1988);Watson et al.,"Recombinant DNA",Scientific American Books,New York;Birren et al.(eds)"Genome Analysis:A Laboratory Manual Series",Vols.1-4,Cold Spring Harbor Laboratory Press,New York(1998);如在美国专利号 4,666,828、4,683,202、4,801,531、5,192,659 和 5,272,057 中阐述的方法;"Cell Biology:A Laboratory Handbook",Volumes I-III Cellis,J.E.,ed.(1994);"Culture of Animal Cells-A Manual of Basic Technique"by Freshney,Wiley-Liss,N.Y.(1994),Third Edition;"Current Protocols in Immunology"Volumes I-III Coligan J.E.,ed.(1994);Stites et al.(eds),"Basic and Clinical Immunology"(8th Edition),Appleton&Lange,Norwalk,CT(1994);Mishell and Shiigi(eds),"Selected Methods in Cellular Immunology",W.H.Freeman and Co.,New York(1980);可用的免疫测定广泛地描述于专利和科学文献,参见,例如,美国专利号 3,791,932、3,839,153、3,850,752、3,850,578、3,853,987、3,867,517、3,879,262、3,901,654、3,935,074、3,984,533、3,996,345、

4,034,074、4,098,876、4,879,219、5,011,771 和 5,281,521；"Oligonucleotide Synthesis"Gait,M.J.,ed.(1984)；"Nucleic Acid Hybridization"Hames,B.D.,and Higgins S.J.,eds.(1985)；"Transcription and Translation"Hames,B.D.,and Higgins S.J.,eds.(1984)；"Animal Cell Culture"Freshney,R.I.,ed.(1986)；"Immobilized Cells and Enzymes"URL Press,(1986)；"A Practical Guide to Molecular Cloning"Perbal,B.,(1984)and"Methods in Enzymology"Vol.1-317,Academic Press；"PCR Protocols:A Guide To Methods And Applications",Academic Press,San Diego,CA(1990)；Marshak et al.,"Strategies for Protein Purification and Characterization-A Laboratory Course Manual"CSHL Press(1996)；所有这些均以引用方式结合于本文。贯穿本文件中提供了其他一般性参考文献。

[0190] 材料和方法

[0191] PEG_{40}-Fmoc-OXM 和 PEG_{40}-FMS-OXM 合成

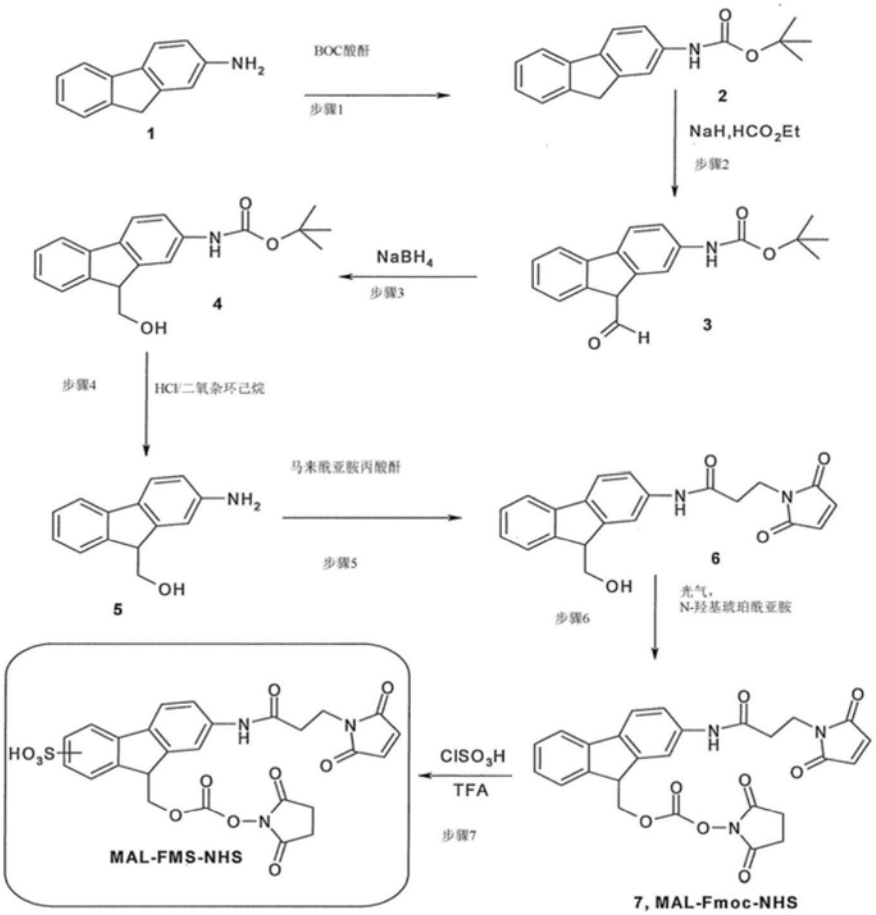
[0192] OXM 合成：在整个肽链组装中采用 FMOC 策略 (Almac Sciences，苏格兰) 通过固相法来合成序列为 HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA(SEQ ID NO:1) 的胃泌酸调节素。使用以下步骤来组装肽序列：(1) 加帽：利用处于 DMF(Rathburn) 中的 0.5M 乙酸酐 (Fluka) 溶液来将树脂加帽；(2) 脱保护：利用处于 DMF(Rathburn) 中的 20%v/v 哌啶 (Rathburn) 溶液从增长的肽链中除去 Fmoc 保护基团；以及 (3) 氨基酸耦合：利用处于 DMF(Rathburn) 中的 1M HOBt(Carbosynth) 溶液和处于 DMF(Rathburn) 中的 1M DIC(Carbosynth) 溶液来活化处于 DMF(Rathburn) 中的 0.5M 氨基酸 (Novabiochem) 溶液。每次耦合使用 4 当量的每种氨基酸。

[0193] 从树脂上切割粗制肽，并通过在三异丙基硅烷 (Fluka)、水、二甲基硫化物 (Aldrich)、碘化铵 (Aldrich) 和 TFA(Applied Biosystems) 的混合物中搅拌 4 小时来除去保护基团。然后通过沉淀从冷乙醚中收集粗制肽。

[0194] 肽纯化：将粗制肽溶解于乙腈 (Rathburn)/ 水 (MilliQ)(5:95) 中并加载到制备型 HPLC 柱。层析参数如下：柱：Phenomenex Luna C18250mm x30mm,15μm,300A；流动相 A：水 +0.1%v/v TFA(Applied Biosystems)；流动相 B：乙腈 (Rathburn)+0.1%v/v TFA(Applied Biosystems)；紫外检测：214 或 220nm；梯度：25%B 至 31%B，超过 4 倍柱体积；以及流率为 43mL/ 分钟。

[0195] 阶段 2- 连接基团合成 -MAL-FMS-NHS 连接基团的合成

[0196]



[0197] 化合物2-5的合成是基于由Albericio等人在Synthetic Communication,2001,31(2),225-232中描述的步骤,其全部内容以引用方式结合于本文。

[0198] 2-(Boc-氨基)芴(2):在冰浴中,通过磁力搅拌,将2-氨基芴(18g,99mmol)悬浮于二氧杂环己烷:水(2:1)(200ml)和2N NaOH(60ml)的混合物中。然后添加Boc_2O(109mmol,1.1当量),并在室温下持续搅拌。通过TLC(Rf=0.5,己烷/乙酸乙酯,2:1)来监测反应,并通过添加2N NaOH来将pH维持在9-10之间。在反应完成以后,用1M KHSO4将悬浮液酸化至pH=3。过滤固相并用冷水(50ml)、二氧杂环己烷-水(2:1)洗涤,然后在将它用于下一步骤中之前和甲苯一起共沸两次。

[0199] 9-甲酰基-2-(Boc-氨基)芴(3):在3颈RBF中,将NaH(60%,在油中;330mmol,3.3当量)悬浮于无水THF(50ml)中,然后经20分钟,滴加处于无水THF(230ml)中的来自步骤2的2-(Boc-氨基)芴(28g;100mmol)溶液。观测到稠密的黄色淤浆,然后在室温下在氮气下搅拌混合物10分钟。滴加甲酸乙酯(20.1m,250mmol,2.5当量)(小心:气体逸出)。淤浆变成淡棕色溶液。搅拌溶液20分钟。通过TLC(Rf=0.5,己烷/乙酸乙酯,1:1)来监测反应,并且当仅观测到微量起始材料时,用冰水(300ml)将它淬灭。在减压下蒸发混合物,直到除去大部分THF。用乙酸处理产生的混合物至pH=5。将获得的白色沉淀物溶解于乙酸乙酯中并分离有机层。用乙酸乙酯提取水层并合并所有有机层,然后用饱和碳酸氢钠、盐水洗涤,并经$MgSO_4$干燥。在过滤和除去溶剂以后,获得黄色固体。该物质用于下一步骤。

[0200] 9-羟基甲基-2-(Boc-氨基)芴(4):将化合物3悬浮于MeOH(200ml)中,然后经15分钟分批添加硼氢化钠。搅拌混合物30分钟(小心:放热反应和气体逸出)。通过

TLC(Rf=0.5,己烷 /EtOAc,1:1) 来监测反应,并完成反应。添加水 (500ml) 并用乙酸将 pH 调节至5。后处理涉及用乙酸乙酯提取两次,用碳酸氢钠和盐水洗涤合并的有机层,经 $MgSO_4$ 干燥,过滤并浓缩至干燥。利用庚烷 /EtOAc(3:1) 通过快速层析法来纯化获得的粗制物,从而产生黄色泡沫 (36g,97.5% 纯度,在 ^{1}H-NMR 中观测到微量乙酸乙酯和乙醚)。

[0201] 9- 羟基甲基 -2- 氨基芴 (5) :将化合物 4 加入处于二氧杂环已烷中的 4NHC1 的冰冷溶液。允许反应混合物达到室温并搅拌过夜。获得淡黄色沉淀物。在 0℃下冷却悬浮液并进一步搅拌 5 小时。在此时间之后,过滤固体并用 DCM(5x30ml) 充分洗涤。在干燥以后,获得浅黄色固体 (20g,96.5% 纯度),其中经 3 个步骤总产率为 80%。

[0202] 9- 羟基甲基 -2-(氨基 -3- 马来酰亚胺丙酸酯) 氟 (6) :将 9- 羟甲基 -2- 氨基芴 (5,5.5g,26mmol) 和马来酰亚胺丙酸酐 (6.93g,26mmol) 放置在配备有搅拌器、回流冷凝器和氮气鼓泡器的 250ml RBF 中。在 85℃下回流反应混合物 25 小时。在此时间之后,TLC(Rf=0.25,己烷 /EtOAc,1:4) 显示反应完成。在真空下浓缩反应混合物以产生黄色固体。通过柱层析来纯化产物。

[0203] MAL-Fmoc-NHS(7) :在具有顶置搅拌的清洁干燥 500ml RBF 中加入处于无水 THF(55ml) 中的三光气 (1.58g,0.35 当量) 以在环境温度下形成溶液。用冰 / 水浴,将溶液冷却至约 0℃,然后在氮气下在 0℃下,经 10 分钟,滴加处于无水 THF(19ml) 中的 NHS(0.67g,0.38 当量) 溶液。搅拌所得溶液 30 分钟。在 0℃下经 10 分钟,滴加处于无水 THF(36ml) 中的另外部分的 NHS(1.34g,0.77 当量),然后搅拌 15 分钟。

[0204] 将化合物 6(5.5g,1 当量)、无水 THF(55ml) 和吡啶 (3.07ml,2.5 当量) 搅拌在一起以形成悬浮液。在 0-5℃下将上述悬浮液分批加入 NHS 溶液,然后通过除去冰浴来允许达到室温。在 20 小时以后,停止反应 (起始材料仍然存在,如果反应推进到完成,则观测到二聚体杂质(dimmer impurity))。过滤反应混合物,并向滤液添加 4% 盐水 (200ml) 和 EtOAc(200ml)。在分离以后,用 5% 柠檬酸 (220ml) 和水 (220ml) 洗涤有机层。然后浓缩有机层,以产生 7.67g 粗制 MAL-Fmoc-NHS。利用梯度环已烷 /EtOAc(70:30 至 40:60)通过柱层析来纯化该物质。在真空下浓缩包含产物的馏分以产生 3.47g(45%) 的 MAL-Fmoc-NHS。

[0205] MAL-FMS-NHS(测试反应): 向处于三氟乙酸 (10ml) 中的 MAL-Fmoc-NHS(100mg,0.2mmol) 溶液中添加氯磺酸 (0.5ml)。在 15 分钟以后,添加冰冷乙醚 (90ml) 并沉淀产物。通过离心来收集该物质,用乙醚洗涤并在真空下干燥。获得 41.3mg(35%) 米黄色固体。

[0206] 阶段 3- 结合

[0207] PEG-Fmoc-OXM 结合 :以 1:1:1 的摩尔比率进行 PEG、Fmoc 和 OXM 的结合,例如,将 PEG40-SH(44mg,在 4.4ml 水中,相当于 1.0μmol) 加入肽 (4.5mg,相当于 1.0μmol) 并添加 NaHCO3(1M,0.1ml)。在搅拌下,添加 Fmoc(Almac,10mg/ml,在 DMF 中,50μl)。在室温下搅拌反应 24 小时。

[0208] PEG-FMS-OXM 结合 :使用以下试剂以 PEG、连接基团和 OXM 的 1:1:1 摩尔比率进行所有结合 :PEG_{40}-SH 和 PEG_{30}-SH(NOF)、FMS(Almac)、EMCS(Termo Scientific)、OXM(Almac)。将 PEG_{40}-SH 溶解于 0.1M 磷酸钠缓冲液 (Sigma)(pH7.2) 至 10mg/mL 的浓度。将溶液加入 1 当量的纯化 OXM 肽 (Almac)。将 MAL-FMS-NHS(Almac) 连接基团溶解于 DMF 至 10mg/mL 的浓度,将 1 当量加入反应。搅拌混合物 30 分钟。利用冰乙酸 (Fisher) 中和溶液至 pH4。

过滤中和的混合物 (0.45μm) 并利用制备型层析加以分离。过滤反应混合物并通过制备 HPLC(Phenomenex Luna C18) 加以纯化,冷冻干燥并冷冻保存。

[0209] 层析参数如下 :柱 :Phenomenex Luna C18(2)250mm x30mm,15μm 制备型,100A ;流动相 A :水 (MilliQ)+0.1%v/v TFA(Applied Biosystems) ;流动相 B :水 / 乙腈 (Rathburn) (25:75)+0.1%v/v TFA(Applied Biosystems) ;紫外检测 :214nm ;梯度 :10%B 至 65%B,经 41 分钟 ;以及速率 :43mL/ 分钟。

[0210] 利用氨基酸分析 (AAA) 或碱性水解来确定 OXM 含量。以 20mg/ml 的浓度将限定量的冷冻干燥 OXM 结合物溶解于水中。然后确定在 280nm 处的吸光度,然后利用 ε 280=29,700 按照在 280nm 处的吸光度来计算浓度。通过酸水解等分部分,接着定量氨基酸分析,来精确定量肽的浓度 ;理想分数是这样的分数,其在 280nm 处计算的吸光度和肽含量之间具有紧密的一致性。

[0211] 基于 cAMP 细胞诱导的测定

[0212] 以 200,000 个细胞 /ml 的密度,将过表达 GLP-1 受体的 CHO-K1 细胞 (Millipore HTS163C2) 接种于 96 孔半区白板 (Greiner) 中并在 37℃下温育 24 小时。在使用或不使用 1% 大鼠血清 (Bio reclamation) 的条件下,用逐步升高浓度的 OXM(ALMAC)、PEG40-EMCS-OXM 和 PEG40-Fmoc-OXM 温育细胞。通过 HTRF 测定 (Cisbio62AM4PEB) 来定量细胞的 cAMP 浓度,并通过 PRISM 软件来分析 EC50 参数。

[0213] 药物代谢动力学研究

[0214] PEG_{40}-Fmoc-OXM 的药代代谢动力学曲线的评估如下 :静脉内 (IV) 或皮下 (SC) 给予雄性 Wistar 大鼠单剂量的天然 OXM(n=9,278μg/kg) 或 PEG_{40}-Fmoc-OXM(n=6,278μg/kg 肽当量)。在交替的时间点,3 只动物 / 组的同龄组进行取血。利用市售的 ELISA 试剂盒 (Cat#S-1393,Bachem) 来分析 OXM 血清浓度。

[0215] IP 葡萄糖耐受试验

[0216] 将 C57BL/6 雄性小鼠禁食过夜并称重,然后利用便携式血糖仪通过尾静脉采样来测量血糖水平。对小鼠 IP 注射 PBS(载体)、OXM(333nmol/kg)、PEG_{40}-EMCS-OXM(不可逆的聚乙二醇化 OXM,333nmol/kg 体重肽含量) 和 PEG40-Fmoc-OXM(202nmol/kg 体重肽含量) 以及 PEG_{40}-OSU(546nmol/kg) 作为对照。在给予试验物质 (载体、OXM 和 PEG_{40}-Osu) 以后 15 分钟或在给予 PEG_{40}-Fmoc-OXM 以后 120 分钟,IP 给予葡萄糖 (1.5gr/kg)。利用便携式血糖仪在给予葡萄糖以前以及在给予葡萄糖以后 10、20、30、60 和 120 分钟,通过尾静脉采样来测量血糖水平。

[0217] 饮食诱导的肥胖症小鼠模型

[0218] 研究 1 :在到达以后,将 C57BL/6J 小鼠 (4-6 周龄,Harlan UK Limited,Bicester,Oxon,UK) 分组安置在聚丙烯笼中。所有动物随时可自由获得高脂肪饮食 (D12451 ;45% 千卡源自脂肪 ;Research Diets,New Jersey,USA) 和自来水。以正相 12 小时明暗周期 (在 07:00 时照明) 维持动物。将动物暴露于适当的饮食,持续至少 6 个月 (直到平均体重大约是 50g)。随后,在聚丙烯笼中单独地安置动物另外两周并置于反相照明 (关灯 8 小时,09:30-17:30)。在单个安置第二周期间,动物开始每日一次的处置方案和 7 天基线期。随后,给予小鼠载体或试验药物,如以下表 1 所示 :

[0219] 表 1

[0220]

组	治疗(sc)	频率	n
A	载体(PBS)	每日两次	10
B	OXM5000nmol/kg 体重(PBS)	每日两次	10
C	西布曲明 20mg/kg (PBS)	每日两次	10
D	PEG40-FMS-OXM5000nmol/kg 体重(柠檬酸盐缓冲液)	第 1、3、5、7 天	10
E	556mg/kg(27.8mg/ml)PEG-SH (柠檬酸盐缓冲液)	第 1、3、5、7 天	10

[0221] 每日测量体重和食物摄入量,直到第 8 天。在第 12 天进行体重的最终测量。在 PBS 中配制 OXM 和西布曲明,同时在 147mM NaCl 10mM 柠檬酸盐缓冲液(pH6)中配制 PEG40-FMS-OXM 和 PEG-SH。通过碱性水解来确定在 PEG40-FMS-OXM 中的 OXM 含量。

[0222] 研究 2 :如针对研究 1 所描述的,进行研究 2。在基线期以后,按照在表 2 中描述的以下设计,给予动物 :

[0223]

表 2 组	治疗 (SC)	频率	n
A	载体 (PBS)	每日两次	8
B	OXM 5000 nmol/kg 体重 (PBS)	每日两次	8
C	PEG40-FMS-OXM 1000 nmol/kg 体重 (柠檬酸盐缓冲液)	第 1、4、7 天	9
D	PEG40-FMS-OXM 5000 nmol/kg 体重 (柠檬酸盐缓冲液)	第 1、4、7 天	9
E	PEG40-FMS-OXM 8000 nmol/kg 体重 (柠檬酸盐缓冲液)	第 1、7 天	9
F	PEG40-EMCS-OXM 1000 nmol/kg 体重(柠檬酸盐缓冲液)	第 1、4、7 天	9
G	PEG40-EMCS-OXM 5000 nmol/kg 体重(柠檬酸盐缓冲液)	第 1、4、7 天	9
H	PEG40-EMCS-OXM 8000 nmol/kg 体重(柠檬酸盐缓冲液)	第 1、7 天	9
I	PEG30-FMS-OXM 5000 nmol/kg 体重 (柠檬酸盐缓冲液)	第 1、4、7 天	9
J	PEG40-SH (柠檬酸盐缓冲液)	第 1、4、7 天	9
K	西布曲明	每日两次	8

[0224] 每日测量体重和食物摄入量,直到第 14 天。

[0225] 研究 3 :如针对研究 1 和 2 所描述的,进行研究 3,其中一个区别在于 :在实验开始时小鼠的体重为 45-46g。在基线期以后,按照在下面表 3 中描述的设计,给予动物 :

[0226] 表 3

[0227]

组	治疗(sc)	n
A	PEG5-FMS-OXM6000nmol/kg ;第 1、8、15 天	7
B	PEG30-FMS-6000nmol/kg ;第 1、8、15 天	7

C	PEG40-FMS-OXM6000nmol/kg ;第 1、8、15 天	7
D	PEG60-FMS-OXM6000nmol/kg ;第 1、8、15 天	7
E	载体(PBS sc)	7
F	利拉鲁肽(200μg/kg 每日两次)在 PBS 中	7

[0228] 数据和统计分析

[0229] 在 PBS 中配制 OXM 和西布曲明,同时在 147mM NaCl 10mM 柠檬酸盐缓冲液(pH6)中配制 PEG40-EMCS-OXM、PEG40-FMS-OXM 和 PEG-SH。通过 AAA 来确定在 PEG40-FMS-OXM 和 PEG40-EMCS-OXM 中的 OXM 含量。

[0230] 体重和食物摄入量表示为平均值 ±SEM。通过 ANCOVA 来分析体重、体重增加、每日和平均食物摄入量数据以及累积食物摄入量,其中基线作为协变量,接着进行适当的比较(双尾)以确定与对照组的显著差异。P<0.05 被认为是统计显著的。基线是在基线期期间体重或平均食品或水消耗的第 1 天值。

[0231] 实施例 1

[0232] PEG-Fmoc-OXM 的合成和表征

[0233] 在整个肽链组装中,采用 Fmoc 策略通过固相法来合成 OXM 肽。通过制备 HPLC,利用 Phenomenex Luna C18(250x30mm) 柱,通过利用在溶液 A(0.1%TFA+H_2O) 和 B(0.1%TFA+MeCN) 之间的梯度,来纯化肽。肽纯度高于 95%,分子量是 4449Da(通过 MALDI 测得)。在有 $NaHCO_3$ 存在的情况下,通过 Fmoc 连接基团,进行 OXM 肽与 PEG_{40}-SH 的结合。在室温下搅拌反应混合物 24 小时,接着过滤,并通过制备 HPLC(Jupiter C5) 加以纯化。通过 MALDI 来分析结合物分子量并通过 AAA 来分析 OXM 肽含量。平均 OXM 肽含量是 189μgOXM/1mg PEG_{10}-Fmoc-OXM 结合物,132μgOXM/1mg PEG_{20}-Fmoc-OXM 结合物,61μgOXM/1mg PEG_{40}-Fmoc-OXM 结合物,以及 40μg OXM/1mg PEG_{40}-FMS-OXM 结合物。

[0234] 实施例 2

[0235] 相比于天然 OXM,PEG10-Fmoc-OXM、PEG20-Fmoc-OXM 和 PEG40-Fmoc-OXM 的药代动力学曲线

[0236] 在雄性 Wistar 大鼠中,相比于 PEG_{10}-Fmoc-OXM 和 PEG_{20}-Fmoc-OXM,估计了 OXM 的药代动力学曲线。给予动物单个 SC 注射的天然 OXM(278μg/kg 肽)、PEG_{10}-Fmoc-OXM(278μg/kg 肽含量)或 PEG_{20}-Fmoc-OXM(278μg/kg 肽含量)。在指定的时间间隔,测量化合物的血清浓度(市售的 ELISA,示于图 1 的 PK 曲线以及常规的非区室化 PK 参数总结于表 3)。结合于 PEG_{10} 和 PEG_{20} 的 OXM 的可逆聚乙二醇化导致天然 OXM 的半衰期延长(对于天然 OXM 为 0.15 小时 ;对于 PEG_{10}-Fmoc-OXM 为 16.16 小时,以及对于 PEG_{20}-Fmoc-OXM 为 27.38 小时)。如通过 AUC 参数所反映的,暴露增加约 450 倍(对于 PEG_{10}-Fmoc-OXM) 和约 2210 倍(对于 PEG_{20}-Fmoc-OXM)。因此,相比于 PEG_{10},OXM 与 PEG_{20} 的可逆结合导致更加延长的效果。为了进一步表征通过 Fmoc 连接基团可逆地结合于 PEG_{40} 的 OXM 的 PK 曲线,对雄性 Wistar 大鼠 IV 或 SC 注射天然 OXM 或 PEG_{40}-Fmoc-OXM(278μg/kg 肽含量),并在指定的时间点分析血清浓度(利用市售的 ELISA,示于图 2 中的 PK 曲线和常规的非区室化 PK 参数总结于表 4)。

结果表明，可逆聚乙二醇化延长 OXM 肽的半衰期 100 倍，并显著增加暴露，如通过 AUC 参数反映的。此外，天然肽的生物利用度仅为 4.37%，而 PEG_{40}-Fmoc-OXM 的给予导致 84% 的生物利用度。

[0237] 表 3：在大鼠中，在 SC 给予以后，OXM、PEG_{10}-Fmoc-OXM 以及 PEG_{20}-Fmoc-OXM 的非区室化 PK 参数。

[0238]

	AUC hr*ng/ml	T1/2 term. hr	MRT hr
OXM	3.2	0.15	0.3
PEG_{10}-Fmoc-OXM	1456	16.16	20.6
PEG_{20}-Fmoc-OXM	7079	27.38	37.2

[0239] 表 4：在大鼠中，在 IV 或 SC 给予以后，OXM 和 PEG_{40}-Fmoc-OXM 的 PK 参数。

[0240]

	给药途径	AUC hr*ng/ml	T1/2 term. hr	MRT hr	F %
OXM	IV	72.44	0.44	0.414	100
	SC	3.34	0.69	0.913	.374
PEG_{40}-Fmoc-OXM	IV	435,73	23.3	24.3	100
	SC	656,65	30.4	57.7	4.68

[0241] 实施例 3

[0242] 通过 OXM 和可逆聚乙二醇化 OXM 诱导 cAMP

[0243] 为了相比于 PEG40-Fmoc-OXM、和 PEG40-EMCS-OXM（非可逆聚乙二醇化 OXM）来评估 OXM 的体外活性，用逐步升高浓度的不同化合物来温育过表达 GLP-1 受体的 CHO-K1 细胞，接着进行 cAMP 定量。相比于具有可比较的体外活性的 PEG_{40}-Fmoc-OXM 和 PEG_{40}-EMCS-OXM，天然 OXM 显示改善的活性（对于 OXM、PEG40-EMCS-OXM 和 PEG40-Fmoc-OXM，EC50 分别为 2.53x10-9、2.07x10-6 和 5.87x10-7，图 3）。重要的是，OXM 聚乙二醇化并不完全取消由 OXM 诱导的 GLP-1 受体活化。另外，虽然在血清中 OXM 的温育导致降低的活性，可能是由于肽的部分蛋白水解，但在存在和不存在大鼠血清的条件下对于 PEG_{40}-Fmoc-OXM 和 PEG_{40}-EMCS-OXM 获得可比较的活性，表明聚乙二醇化掩盖 OXM 上潜在的蛋白水解位点。

[0244] 实施例 4

[0245] 可逆聚乙二醇化长效 OXM 诱导的葡萄糖耐受

[0246] 为了评估 OXM 或 PEG_{40}-Fmoc-OXM 的体内活性，应用了 IPGTT 模型。对禁食过夜的 C57BL/6 小鼠 IP 注射 OXM 肽或 PEG_{40}-Fmoc-OXM，接着 IP 注射葡萄糖，然后通过血糖仪测量来自尾静脉的血糖水平。在葡萄糖 IP 注射（1.5gr/kg）以前 IP 给予 OXM(333nmol/kg)、PEG_{40}-EMCS-OXM（非可逆聚乙二醇化 OXM，333nmol/kg 体重肽含量）和 PEG40-Fmoc-OXM(202nmol/kg 体重肽含量)，15 分钟（OXM 和 PEG_{40}-EMCS-OXM），或 2 小时（PEG_{40}-Fmoc-OXM）。葡萄糖耐受的诱导与载体组相比较。作为 PEG_{40} 效应的对照，给予对照组 PEG_{40}-Osu(546nmol/kg)。虽然，和载体组相比，OXM 肽对葡萄糖耐受具有较小的影响，但给予具有甚至更低 OXM 摩尔含量的 PEG_{40}-Fmoc-OXM 导致诱导的葡萄糖耐受（图 4）。出人意料地，不可逆聚乙二醇化的给予导致诱导葡萄糖耐受，这表明聚乙二醇化 OXM 具有体内药理学活性。

[0247] 实施例 5

[0248] 在 DIO 小鼠中，可逆聚乙二醇化长效 OXM 降低体重并抑制食物摄入量

[0249] 在 DIO 小鼠中，在 SC 注射天然 OXM、和可逆聚乙二醇化 OXM 以后，进一步评估了 OXM 的药理学活性。在研究 1 中，给予雄性 DIO 小鼠 (n=10/ 组)5000nmol/kg 体重的 OXM，每日两次，或包含 5000nmol/kg 体重 OXM 的 PEG_{40}-FMS-OXM，每隔一天，持续给药 7 天。每日测量体重和食物摄入量，持续 8 天，其中在第 12 天最终测量体重。每天两次注射 OXM 导致中等程度降低体重 (和载体对照组相比，在第 8 天，体重减轻 6%) 和统计上显著抑制食物摄入量。在另一方面，给予 PEG_{40}-FMS-OXM(其具有相同的 OXM 肽含量 / 剂量，但每隔一天加以注射) 导致显著的体重减轻 (相比于 PEG-SH 对照组，在第 8 天，体重减轻 24%) 并且显示食物摄入量的显著抑制 (图 4)。西布曲明，神经递质再摄取抑制剂，其用作阳性对照，将体重降低 15.6%。值得注意的是，在 PEG_{40}-FMS-OXM 组中，体重的减轻是一致的，直到在第 12 天 (其是在最后剂量之后的 5 天) 的最后测量，其表明可逆聚乙二醇化 OXM 的长效作用 (图 5)。

[0250] 因为在 IPGTT 模型中，PEG_{40}-EMCS-OXM 诱导葡萄糖耐受，所以重要的是，在体重和食物摄入量的方面，比较不可逆聚乙二醇化 OXM 和可逆聚乙二醇化 OXM 的功效。因此，设计后续的研究来解决此问题 (研究 2，在材料和方法中)。虽然每 3 天给予 5000nmol/kg 体重的 PEG_{40}-FMS-OXM(共注射 3 次) 导致体重的显著减轻，但以相同频率注射 5000nmol/kg 体重的 PEG_{40}-EMCS-OXM 则导致对体重微不足道的影响。值得注意的是，在第 1 天，单次注射 8000nmol/kg 体重的 PEG_{40}-FMS-OXM 导致明显的体重减轻，持续 6 天。出人意料地，给予 5000nmol/kg 体重的 PEG_{30}-FMS-OXM 导致体重减轻上升，其表明，和 PEG_{40}-FMS-OXM 相比，改善的功效 (图 6)。

[0251] OXM 是用于治疗代谢障碍如糖尿病和肥胖症的潜在肽，如在超重和肥胖的健康受试者中通过天然 OXM 所获得的体重减轻所说明的 (Wynne et al, 2005)。另外，由于在体内肽的较短半衰期和较低稳定性，需要重复地每日给予超生理剂量，以在人体中实现药理学效应。本专利提供了在生理条件下通过可逆地聚乙二醇化起作用的 OXM 用于稳定 OXM 的有效方法，从而导致长效。出人意料地，修饰的 OXM，聚乙二醇化形式，是活性的并且并不仅仅是前体药物。

[0252] 相比于天然 OXM，在大鼠中，可逆聚乙二醇化 OXM 显示优异的药代动力学曲线，具有显著增加的暴露和延长的半衰期。当比较可逆地结合于 OXM 的具有各种分子量的 PEG 对 OXM-PK 曲线的影响时，相比于 PEG_{10} 或 PEG_{20}，PEG_{40}- 结合物显示优异的延长效应。因此，在药理学研究中，进一步估计了 PEG_{40}(图 1 和 2)。重要的是，在 SC 给予 PEG40-Fmoc-OXM 以后，OXM 的生物利用度从 4.37% 显著地增加到 84.6%，这有助于增加可逆聚乙二醇化肽的暴露 (表 2)。相比于天然 OXM，PEG_{40}-Fmoc-OXM 改善了葡萄糖耐受，如在禁食过夜的 C57BL/6 小鼠 IPGTT 模型中所评估的。在此模型中，结合于 PEG_{40} 的不可逆聚乙二醇化 OXM(PEG_{40}-EMCS-OXM) 显示与 PEG40-Fmoc-OXM 可比较的葡萄糖耐受诱导活性。针对 PEG_{40}-EMCS-OXM 和 PEG_{40}-Fmoc-OXM 观测到的体外活性进一步支持这个结果，其中 OXM 的常规聚乙二醇化并不完全消除 OXM 与它的受体的结合 (由于位阻干扰针对聚乙二醇化肽所观测到的现象)，因此并不导致生物活性的完全丧失 (图 3 和 4)。

[0253] 接着，在 DIO 小鼠中，相比于天然 OXM，评估了 PEG_{40}-FMS-OXM 对体重和食物摄入量的影响。SC 注射 5000nmol/kg 天然 OXM (每日给予两次)，在给予 7 天以后，导致体重和

食物摄入量中等程度降低。相比之下，和对照相比，在第 8 天，每隔一天注射 5000nmol/kg PEG_{40}-FMS-OXM 导致体重和食物摄入量的显著降低（对于 OXM 和 PEG_{40}-FMS-OXM，体重分别降低 6% 和 24.9%，图 5）。总而言之，考虑到，相比于给予天然 OXM 的组，在针对 PEG_{40}-FMS-OXM 研究期间给予的 OXM 的累积剂量几乎低 4 倍，所以 PEG_{40}-FMS-OXM 呈现延长的抗肥胖症效应和改善的功效。

[0254] 在 IPGTT 测试中，不可逆聚乙二醇化 OXM，PEG_{40}-EMCS-OXM，显示改善的葡萄糖耐受。因此，有必要，在 DIO 模型中，相比于可逆地聚乙二醇化 OXM 和天然 OXM，来评估常规聚乙二醇化的食品调节活性。每 3 天给予 5000nmol/kg PEG_{40}-EMCS-OXM 导致可以忽略不计的体重降低，虽然给药后长达 3 天的食物摄入量的抑制是明显的（图 6）。食物摄入量的中度抑制可能来自 OXM 在胃肠道中的直接活性并与在 IPGTT 模型中观测到的外周活性相关。因为 OXM 食品调节活性涉及穿过血脑屏障和结合于在 ARC 中的神经元上的受体，所以，必要的是，OXM 渗透到 CNS 的能力将不会消除。与这种化合物缺少降低 DIO 小鼠体重的能力相对，PEG_{40}-EMCS-OXM 的所观测到的外周生物活性表明，与 PEG 部分的共价结合限制了 PEG_{40}-EMCS-OXM 穿过 BBB 进入 ARC（其是在下丘脑中 OXM 的潜在作用位点）的能力。相比之下，以相同频率注射 5000nmol/kg PEG_{40}-FMS-OXM 显著降低体重并且将食物摄入量抑制 20%，如在第 12 天测得的。值得注意的是，每周一次注射 8000mnol/kg PEG_{40}-FMS-OXM 导致类似的体重减轻 20%，这表明，在人体中，通过每周一次注射或甚至更低频率地给予可逆聚乙二醇化 OXM，可以实现显著的体重减轻。

[0255] 可逆聚乙二醇化策略的目的是克服在常规聚乙二醇化中经常观测到的活性丧失，同时保持药物的延长效应。在显著地失去或甚至消除聚乙二醇化前药生物活性的情况下，先前证明了应用可逆聚乙二醇化的优点（美国专利号 7585837）。然而，并不知道，相比于保留其生物活性的共价不可逆聚乙二醇化药物，可逆地聚乙二醇化前药的功效如何。这是特别相关的，因为，当评估结合物血液浓度时，预计共价聚乙二醇化肽的 PK 曲线优于可逆聚乙二醇化肽，这是由于肽从结合物中缓慢释放。在体外以及在体内 IPGTT 模型中，PEG_{40}-EMCS-OXM 显示出活性。因而，可能的是，在 SC 给予小鼠以后，该分子还将诱导饱感和降低体重。然而，这显示是不正确的，因为在 DIO 小鼠中 PEG_{40}-EMCS-OXM 未能降低体重而 PEG_{40}-FMS-OXM 则具有显著的效果。关于 OXM 的外周活性和 OXM 的 CNS 活性的贡献，以前的出版物提供了相互矛盾的数据。在一方面，通过先前 ARC 内给予 GLP-1R，$exendin_{9-39}$，ip OXM 的抑制食欲的作用被阻断，表明 OXM 的 CNS 相关活性的重要性 (Dakin et al2004)。然而，口服递送表达 OXM 的双歧杆菌到超重的小鼠导致体重减轻，同时在该小鼠的血浆中并没有检测到 OXM，这表明，胃肠细胞的直接激活对于 OXM 的体重减轻活性是重要的 (Long et al,2010)。如上所述，由于缺少关于 OXM 的作用方式和聚乙二醇化的影响的信息，所以不可能相比于天然 OXM 和共价结合的聚乙二醇化 OXM 来预测可逆地聚乙二醇化 OXM 的功效如何。

[0256] 相比于 PEG_{40}-FMS-OXM，PEG_{30}-FMS-OXM 的优异功效（如在研究 2 中所表明的）是令人惊讶的。虽然并没有评估 PEG_{30}-FMS-OXM 的 PK 曲线，PEG_{40}-FMS-OXM 的 PK 曲线明显优于 PEG_{10}-FMS-OXM 和 PEG_{20}-FMS-OXM。可能的是，PEG_{30} 的使用会提供有利的 PEG 大小，其在一方面显著降低结合 PEG_{30}-FMS-OXM 的肾清除率，同时有助于 OXM 从结合物的水解速率，这使得用于诱发它的药理活性所需要的 OXM 能够持续存在。

[0257] 研究 3

[0258] 在此研究中，评估了 OXM 与各种尺寸的 PEG 的结合。如在先前研究中所表明的，每周一次给予 PEG30-FMS-OXM 和 PEG40-FMS-OXM 对体重具有显著的影响。出人意料地，和载体组相比，PEG5-FMS-OXM 完全丧失了诱导体重减轻的能力，而 PEG60-FMS-OXM 则诱导比 PEG30-FMS-OXM 甚至更显著的降低。在此研究中，在 PEG30-FMS-OXM 和 PEG40-FMS-OXM 之间在体重减轻方面的差异是在实验可变范围内并且它并不显著。

[0259] 实施例 6

[0260] 在用可逆聚乙二醇化 OXM 治疗的肥胖小鼠中改善的血糖和血脂曲线

[0261] 材料和方法

[0262] 用于饮食诱导肥胖症 (DIO) 小鼠模型的实验步骤

[0263] 在 RenaSci Ltd Company(Nottingham,UK) 得到 DIO 模型。将 C57BL/6J 小鼠 (4-6 周龄 ,Harlan UK Limited,Bicester,Oxon,UK) 暴露于高脂肪饮食 (D12451 ;45% 千卡源自脂肪 ;Research Diets,New Jersey,USA) 持续至少 6 个月 (直到平均体重大约为 50g)。在给予药物之前两周，将动物单独圈养和置于反相照明 (关灯 8 小时，09:30-17:30)。在单独圈养的第一周期间 (处理期)，动物开始每日一次执行方案，以及在第二周期间 (基线期)，通过皮下途径，它们被给予适当的载体，每日两次或每周一次，如在治疗期期间它们被给予的)。7 组 (n=8)DIO 小鼠给药持续 29 天，具体如下 ：

[0264]

组	治疗 (SC)	频率
A	PEG40-SH (662mg/kg)	每周一次 (1、8、15、22、29)
B	PEG40-EMCS-OXM (6,000nmol/kg)	每周一次 (1、8、15、22、29)
C	PEG30-EMCS-OXM (6,000nmol/kg)	每周一次 (1、8、15、22、29)
D	PEG40-FMS-OXM (6,000nmol/kg)	每周一次 (1、8、15、22、29)
E	PEG30-FMS-OXM (6,000nmol/kg)	每周一次 (1、8、15、22、29)
F	载体 (PBS)	每日两次
G	OXM (6,000nmol/kg ;PBS)	每日两次

[0265] 在基线和治疗期期间，每日记录食物摄入量、水摄入量和体重。在两周基线以后的第 1 和第 22 天，所有小鼠被禁食过夜。在第 2 和第 23 天，小鼠经受口服葡萄糖耐受试验 (OGTT)。每只动物给予载体或测试化合物并在 60 分钟以后给予 D- 葡萄糖 (2g/kg po)。在给予载体或测试化合物 (B1) 之前立即地以及在葡萄糖负荷 (B2) 之前立即地取得基线血液样品。在葡萄糖给予以后 10、20、30、45、60 和 120 分钟获取另外的血液样品。从尾静脉取得所有血液样品 (大约 20μl)。制备血浆样品并利用 Thermoelectron Infinity 葡萄糖试剂 (TR15421) 和 Alpco 小鼠超敏感胰岛素 ELISA(80-INSMSU-E10) 分别测定葡萄糖 (n=2) 和胰岛素 (n=1)。在第 30 天，通过心脏穿刺来收集末端血浆样品 (在第 29 天的最后剂量

以后 24 小时）并利用小鼠超敏感胰岛素 ELISA(80-INSMSU-E10)、Thermoelectron Infinity 葡萄糖试剂 (TR15421)、Thermoelectron Infinity 胆固醇试剂 (TR13421) 以及 Sigma 甘油三酯试剂盒 (TR0100) 来测定胰岛素、葡萄糖、胆固醇和甘油三酯。在末端血液采样以后记录最终尸体重量并在 -20℃下冷冻尸体。

[0266] 用于身体成分研究的实验步骤：

[0267] 利用标准化学分析技术来确定尸体的身体脂肪、蛋白质、水和灰分水平。仅测量脂肪、蛋白质、水和灰分含量，这是因为其他成分（主要为碳水化合物）构成小于 2% 的总身体成分。通过冷冻干燥小鼠尸体至恒重来确定尸体水分。然后用实验室研磨机研磨干燥的尸体，准备用于随后的分析。利用改进的 Soxhlet 提取方案（石油醚，在 40-60℃下），使用 Tecator Soxtec2050 系统 (Foss UK Ltd, Wheldrake, UK)，按照制造商推荐的方案，来确定冷冻干燥样品的尸体脂肪。利用微量 Kjeldahl 步骤，利用 Tecator2012 消化块和 2200 蒸馏装置 (Foss UK Ltd)，来确定冷冻干燥样品的尸体蛋白质。利用马弗灰化炉，通过在高温下灼烧冷冻干燥样品，来确定残余尸体灰分。

[0268] 数据和统计分析：

[0269] 体重、食物摄入量和水摄入量表示为平均值 ±SEM。通过 ANCOVA 来分析体重、体重增加、每日和平均食品和水摄入量数据以及累积食物摄入量，其中基线作为协变量，接着进行适当的比较（双尾）以确定与对照组的显著差异。$P<0.05$ 被认为是统计显著的。基线是在基线期期间体重或平均食品或水消耗的第 1 天值。

[0270] 通过一般线性模型来分析末端血浆胰岛素、胆固醇和甘油三酯，其中治疗作为因子以及取血顺序和基线体重作为协变量，接着进行适当的比较（双尾）以确定与相关载体组的显著差异。如果合适的话，使用对数变换和 / 或稳健回归技术。

[0271] 每种身体成分参数（脂肪、蛋白质、水和灰分）的数据表示为 g/ 尸体和 % 总量。还分析了最终尸体重量，作为直接比较。通过稳健回归来进行分析，其中治疗作为因子以及在基线处的体重作为协变量，接着进行适当的多重比较检验（双尾）来比较每个治疗组与相关载体组的影响。

[0272] 结果

[0273] 在 30 天期间，相比于每天两次注射天然胃泌酸调节素的组的 17% 体重减轻，每周注射可逆 PEG30(PEG30-FMS-OXM(6,000nmol/kg；柠檬酸盐缓冲液)) 或可逆 PEG40(PEG40-FMS-OXM(6,000nmol/kg；柠檬酸盐缓冲液)) 分别提供 28% 和 23% 的体重减轻（图 7），同时连同可逆 PEG30 一起注射的净胃泌酸调节素的累积剂量仅为 8.6%（对于 30 天期）。非可逆地聚乙二醇化 OXM(PEG40-EMCS-OXM 和 PEG30-EMCS-OXM) 在减少体重方面甚至是较少有效的。

[0274] 在 DIO 小鼠中，在每周注射可逆聚乙二醇化 OXM(PEG30-FMS-OXM 或 PEG40-FMS-OXM) 以后的葡萄糖耐受可与在第 2 天（图 8A）和在第 23 天（图 8B）通过每天两次注射天然胃泌酸调节素所诱发的葡萄糖耐受相比较。

[0275] 另外，在 DIO 小鼠中，每周一次给予可逆聚乙二醇化 OXM 改善了血糖和脂类曲线，通过末端葡萄糖的减少（图 9A）、末端胰岛素的减少（图 9B）、末端胆固醇的减少（图 9C）、和末端甘油的减少（图 9D）来证明。

[0276] 最后，DIO 小鼠的身体成分分析证明，由用可逆聚乙二醇化 OXM 治疗的小鼠所显示

的体重减轻来自特定的脂肪减少（图 10）。

[0277] 一并考虑，在不同的毒理学啮齿动物模型中，反向聚乙二醇化表明是安全和可耐受的。反向聚乙二醇化还能够延长 OXM 半衰期，同时保持它渗透靶组织（例如穿透 BBB）的潜力。

[0278] 可逆地聚乙二醇化 OXM 证明了优异的长效性能，从而支持在人体中每周一次注射。通过特定的脂肪减少，可逆地聚乙二醇化 OXM 减轻体重（身体成分评估）。可逆地聚乙二醇化 OXM 改善了血糖和血脂曲线。预期可逆地聚乙二醇化 OXM 会为肥胖症和 II 型糖尿病患者提供长期疗法(通过它对血糖活性和脂肪损失的令人印象深刻的效果)。

[0279] 实施例 7

[0280] 可逆聚乙二醇化 OXM 对葡糖糖水平和胰岛素分泌的影响

[0281] 用于饮食诱导肥胖症 (DIO) 小鼠模型的实验步骤：

[0282] 在 RenaSci Ltd Company(Nottingham,UK) 得到 DIO 模型。将 57BL/6J 小鼠 (4-6 周龄，Harlan UK Limited,Bicester,Oxon,UK) 暴露于高脂肪饮食 (D12451 ;45% 千卡源自脂肪 ;Research Diets,New Jersey,USA) 持续至少 6 个月（直到平均体重大约是 50g)。在药物给予之前两周，单独圈养动物，其中它们开始驯化期。在第一周，处理期，动物开始每日一次执行方案，并在第二周期间，基线期，通过皮下途径，给予它们适当的载体（每日两次或每周一次，如在治疗期期间给予它们的）。在基线和治疗期期间，每日记录食物摄入量、水摄入量和体重。在第 1 天的上午，给予小鼠，接着禁食过夜。在第 2 天，在给予之后 24 小时 (A-E 组）或在上午给药之前 (F-H 组），针对空腹血糖和空腹胰岛素对小鼠采样。所有血液样品（大约 20μl) 取自尾静脉。制备血浆样品并利用 Thermoelectron Infinity 葡萄糖试剂 (TR15421) 和 Alpco 小鼠超敏感胰岛素 ELISA(80-INSMSU-E10) 分别测定葡萄糖 (n=2) 和胰岛素 (n=1)。

[0283] 结果

[0284] 在这组实验中，进行了两个独立的体内研究。第一项实验包括 8 组 (n=8)DIO 小鼠，其给药持续 2 天，具体如下：

[0285]

组	治疗(SC)	频率
A	PEG40-SH（662mg/kg）	在第 1 天单次注射
B	PEG40-EMCS-OXM（6,000nmol/kg）	在第 1 天单次注射
C	PEG-30-EMCS-OXM（6,000nmol/kg）	在第 1 天单次注射
D	PEG40-FMS-OXM（6,000nmol/kg）	在第 1 天单次注射
E	PEG30-FMS-OXM（6,000nmol/kg）	在第 1 天单次注射
F	载体(PBS)	每日两次
G	OXM（6,000nmol/kg ;PBS）	每日两次
H	利拉鲁肽(200μg/kg）	每日两次

[0286] 第二项实验包括 7 组 (n=7)DIO 小鼠,其给药 2 天,具体如下 :

[0287]

组	治疗(SC)	频率
A	PEG60-SH (947.4mg/kg)	在第 1 天单次注射
B	PEG5-FMS-OXM6000nmol/kg	在第 1 天单次注射
C	PEG30-FMS-6000nmol/kg	在第 1 天单次注射
D	PEG40-FMS-OXM6000nmol/kg	在第 1 天单次注射
E	PEG60-FMS-OXM6000nmol/kg	在第 1 天单次注射
F	载体(PBS)	每日两次
G	利拉鲁肽(200μg/kg 每日两次),在 PBS 中	每日两次

[0288] 在这两项研究中,当和载体相比时,给予单剂量的所有 PEG-OXM 变体 :PEG40-EMCS-OXM、PEG30-EMCS-OXM、PEG40-FMS-OXM、PEG30-FMS-OXM(实 验 #1) 或 PEG30-FMS-OXM、PEG40-FMS-OXM 和 PEG60-FMS-OXM(实验 #2) 会产生明显和显著减小的空腹血糖 (图 11 和 12)。在实验 # 1 中,载体组 (PEG40-SH) 显示 9.5mM 的葡糖糖水平而 PEG-OXM 治疗组则显示 5.18 至 5.8mM 的葡糖糖水平。在实验 # 2 中 (除了 PEG5-OXM 组),对于 PEG-OXM 治疗组还观测到相同减小的葡糖糖水平,显示葡萄糖从载体组的 11.9mM 减小到 PEG-OXM 治疗组的 5-5.7mM。此效应与实验 # 1 中空腹血浆胰岛素水平的减小相关 :从载体组的 2.8ng/ml 减小到 PEG-OXM 治疗组的 1.4-1.9ng/ml,如图 11 所示。在实验 # 2 中,空腹胰岛素水平是 0.99ng/ml,而 PEG-OXM(除了 PEG5-OXM) 的空腹胰岛素水平则是 0.78 至 0.91ng/ml。

[0289] 在这两项实验中,当和载体 (PBS) 相比时,利拉鲁肽显著降低空腹血糖 ;在实验 # 1 中,9.3mM 载体降至 6.06mM 以及在实验 # 2 中 11.5mM 载体降至 6.7mM。连同葡萄糖的这种减小一起,该治疗组显示血浆胰岛素的显著增加 :在实验 # 1 中,从 2.5ng/ml 载体增加至 4.4ng/ml 以及在实验 # 2 中从 1.98ng/ml 增加至 3ng/ml。在实验 # 1 中分析了 OXM 天然肽,相比于载体,其在葡萄糖和胰岛素水平方面并未显示任何显著差异,这可能是由于它的较短的血清半衰期和从身体非常快速的清除。

[0290] 来自 DIO 小鼠的这两个独立实验的结果显示了,PEG-OXM 化合物诱导葡糖糖水平的显著减小但没有增加胰岛素水平,如在利拉鲁肽给予以后所观测到的,以及如从先前的关于 OXM 天然肽显示的数据所预期的。葡糖糖水平的这种意外降低连同空腹胰岛素水平的降低一起表明,单剂量的 PEG-OXM 导致增加动物对胰岛素的敏感性(在急性暴露以后,并且并非由于慢性治疗)。

[0291] 虽然本文已说明和描述了本发明的某些特点,但本领域技术人员现在将可以想到许多改进、代替、变化、和等效物。因此,应当明了,所附权利要求旨在覆盖落在本发明的真正精神内的所有这样的改进和变化。

[0001]

序列表

<110> 普罗勒生物科技有限公司

<120> 长效GLP-1/胰高血糖素受体激动剂

<130> P-74757-PC

<140> PCT/US12/999999
<141> 2012-06-04

<150> 61/492,448
<151> 2011-06-02

<150> 61/624,589
<151> 2012-04-16

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1               5                  10                  15


Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr Lys Arg Asn
            20                  25                  30


Arg Asn Asn Ile Ala
        35
```

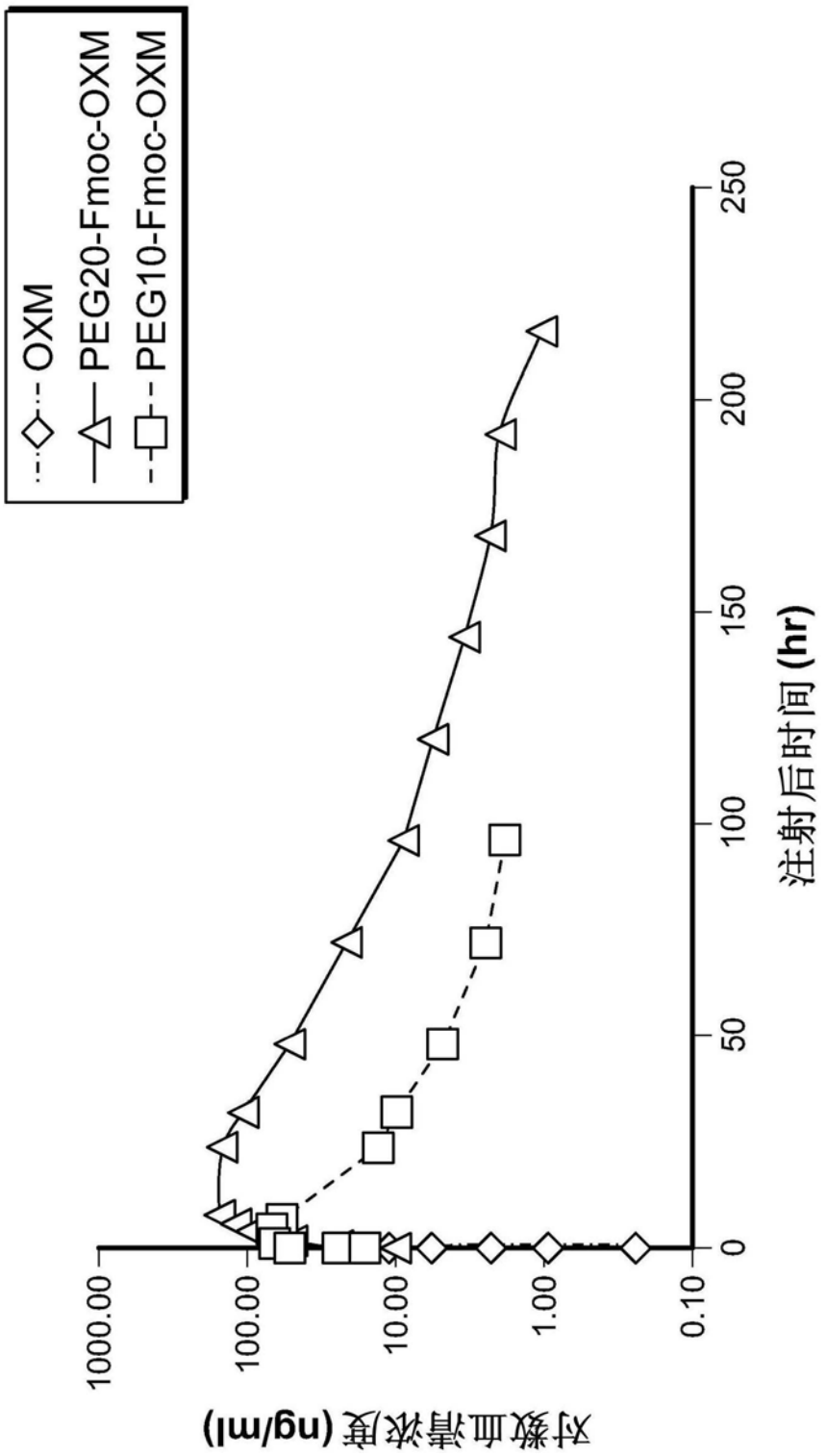

OXM
PEG20-Fmoc-OXM
PEG10-Fmoc-OXM
1000.00
100.00
10.00
1.00
0.10
对数血清浓度 (ng/ml)
0
50
100
150
200
250
注射后时间 (hr)

图 1

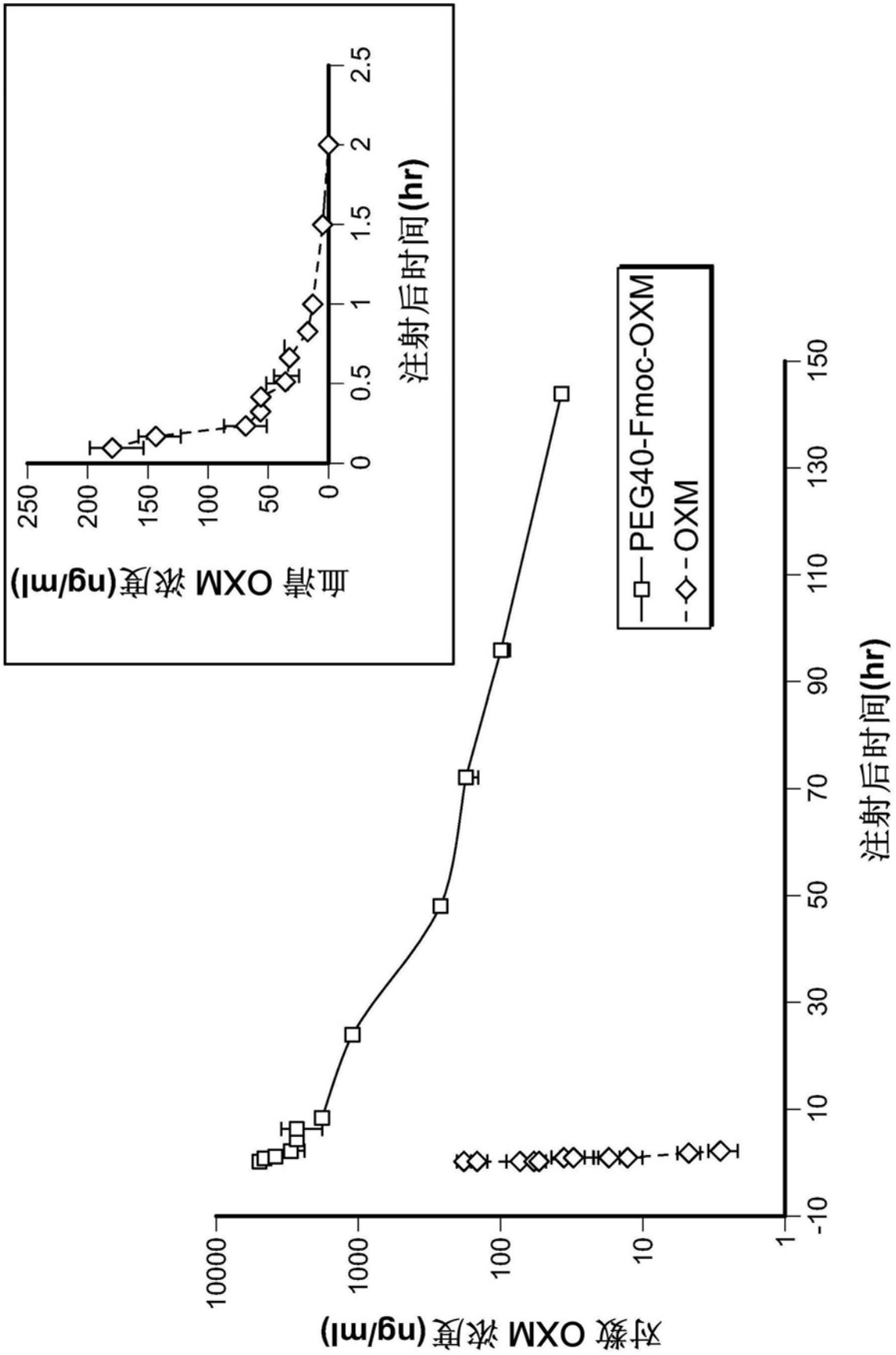

对数 OXM 浓度(ng/ml)
10000
1000
100
10
1
-10
10
30
50
70
90
110
130
150
注射后时间(hr)
PEG40-Fmoc-OXM
OXM
血清 OXM 浓度(ng/ml)
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
注射后时间(hr)

图 2A

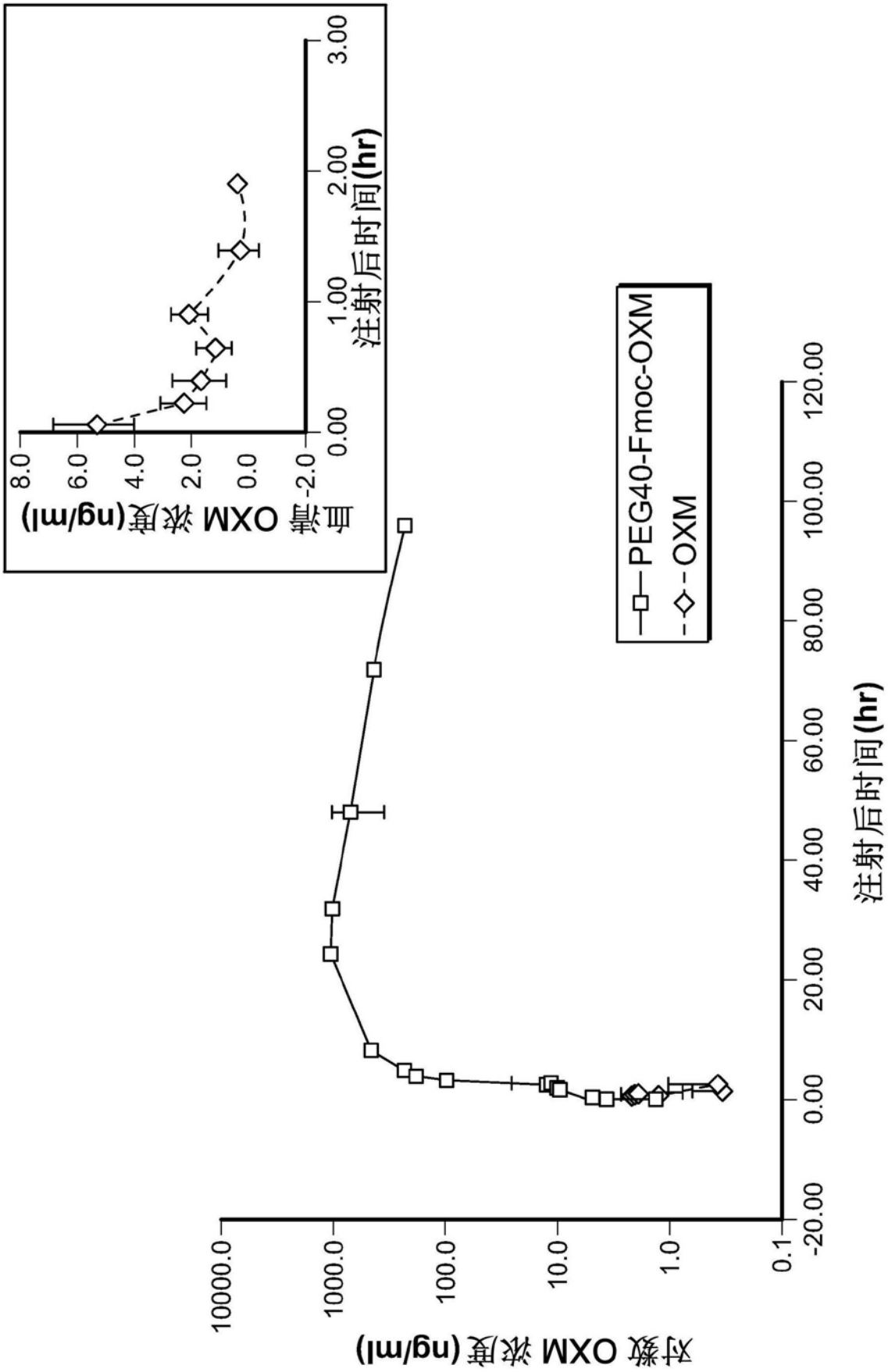

血清 OXM 浓度(ng/ml)
8.0
6.0
4.0
2.0
0.0
-2.0
0.00
1.00
2.00
3.00
注射后时间(hr)
PEG40-Fmoc-OXM
OXM
对数 OXM 浓度(ng/ml)
10000.0
1000.0
100.0
10.0
1.0
0.1
-20.00
0.00
20.00
40.00
60.00
80.00
100.00
120.00
注射后时间(hr)

图 2B

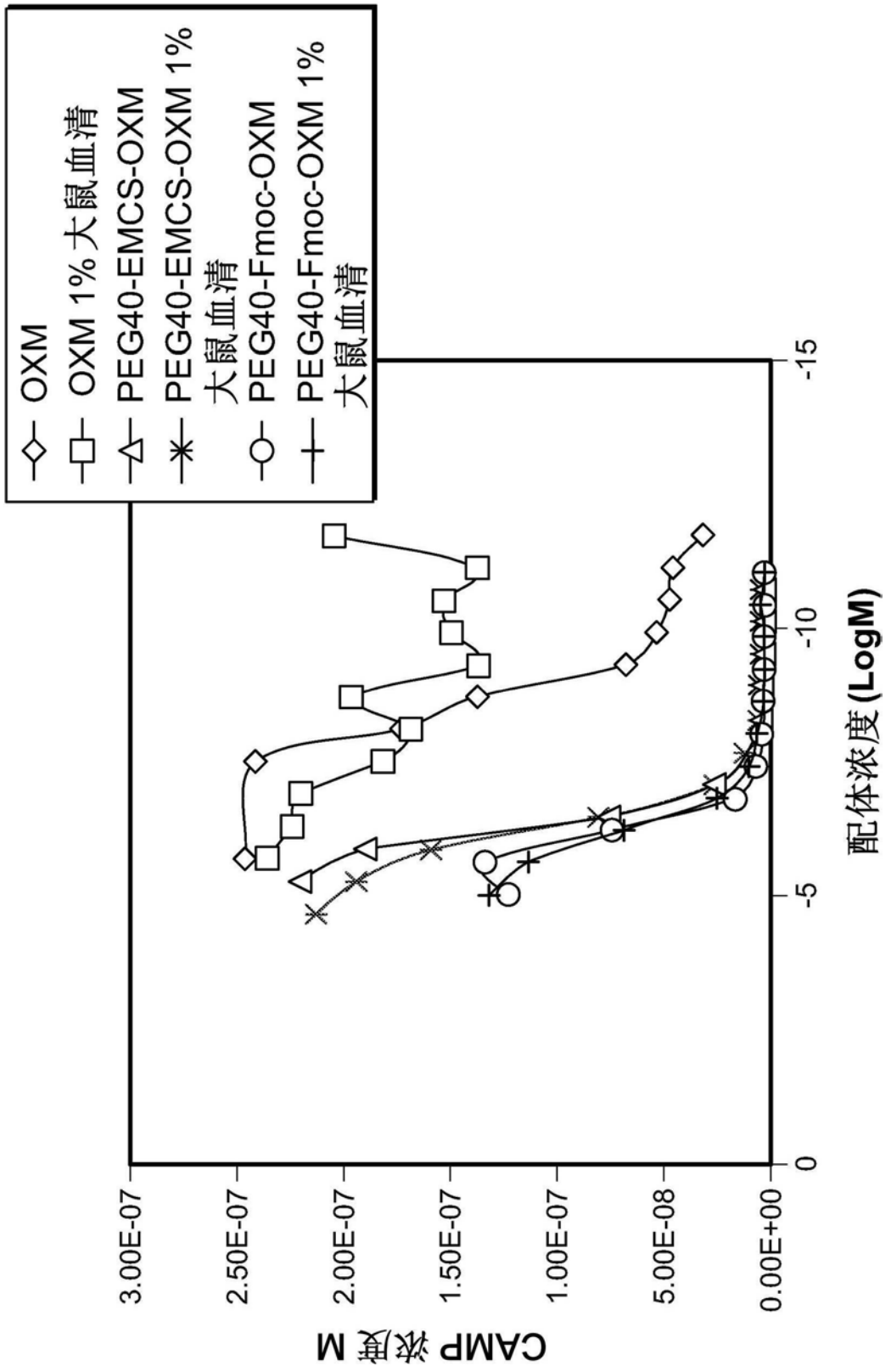

OXM
OXM 1% 大鼠血清
PEG40-EMCS-OXM
PEG40-EMCS-OXM 1% 大鼠血清
PEG40-Fmoc-OXM
PEG40-Fmoc-OXM 1% 大鼠血清
cAMP 浓度 M
3.00E-07
2.50E-07
2.00E-07
1.50E-07
1.00E-07
5.00E-08
0.00E+00
0
-5
-10
-15
配体浓度 (LogM)

图 3

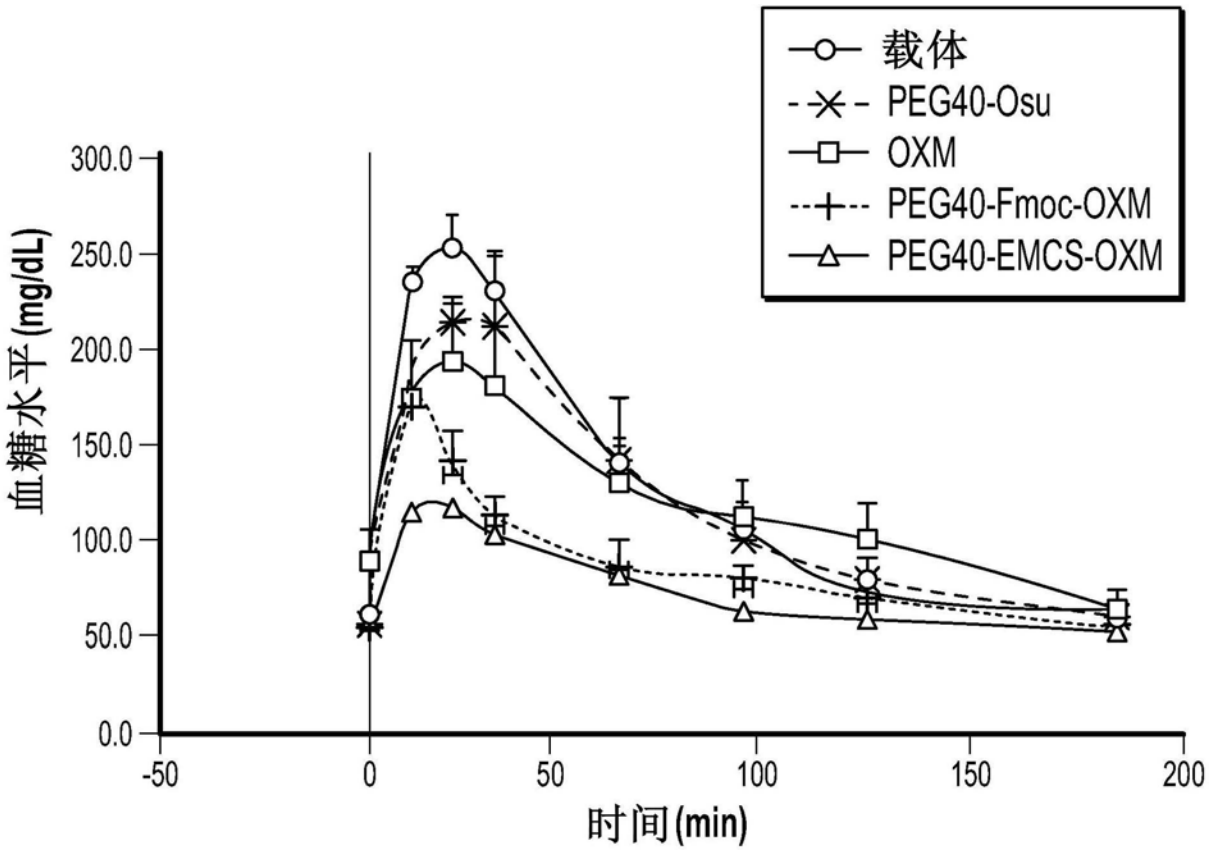

载体
PEG40-Osu
OXM
PEG40-Fmoc-OXM
PEG40-EMCS-OXM
血糖水平 (mg/dL)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50
0
50
100
150
200
时间 (min)

图 4A

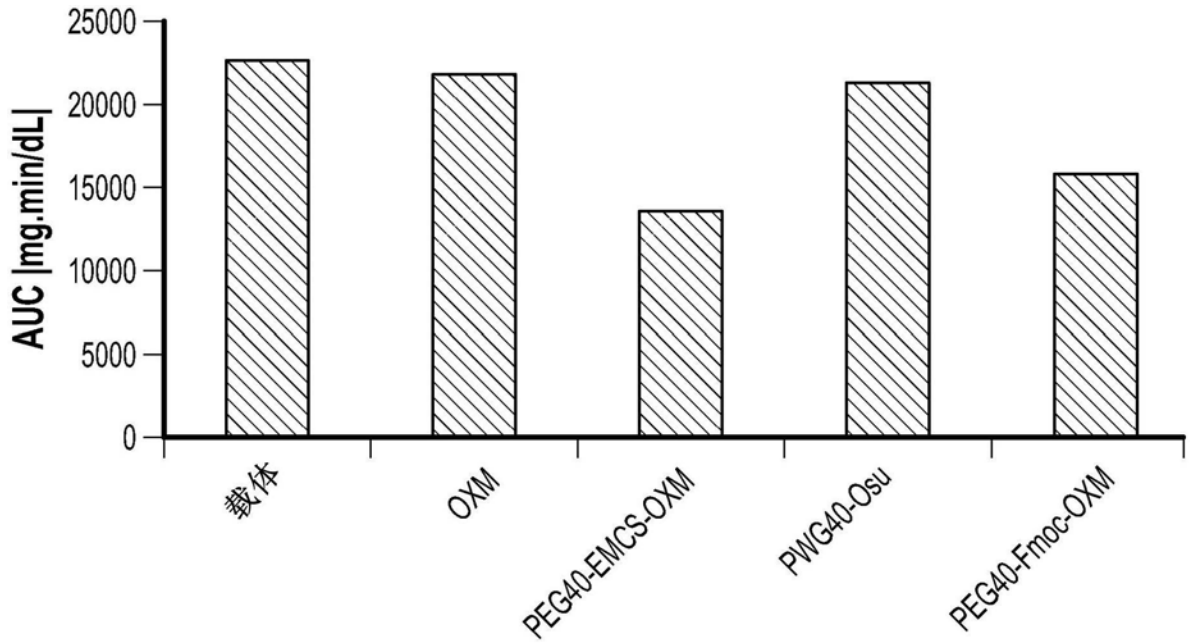

AUC |mg.min/dL|
25000
20000
15000
10000
5000
0
载体
OXM
PEG40-EMCS-OXM
PWG40-Osu
PEG40-Fmoc-OXM

图 4B

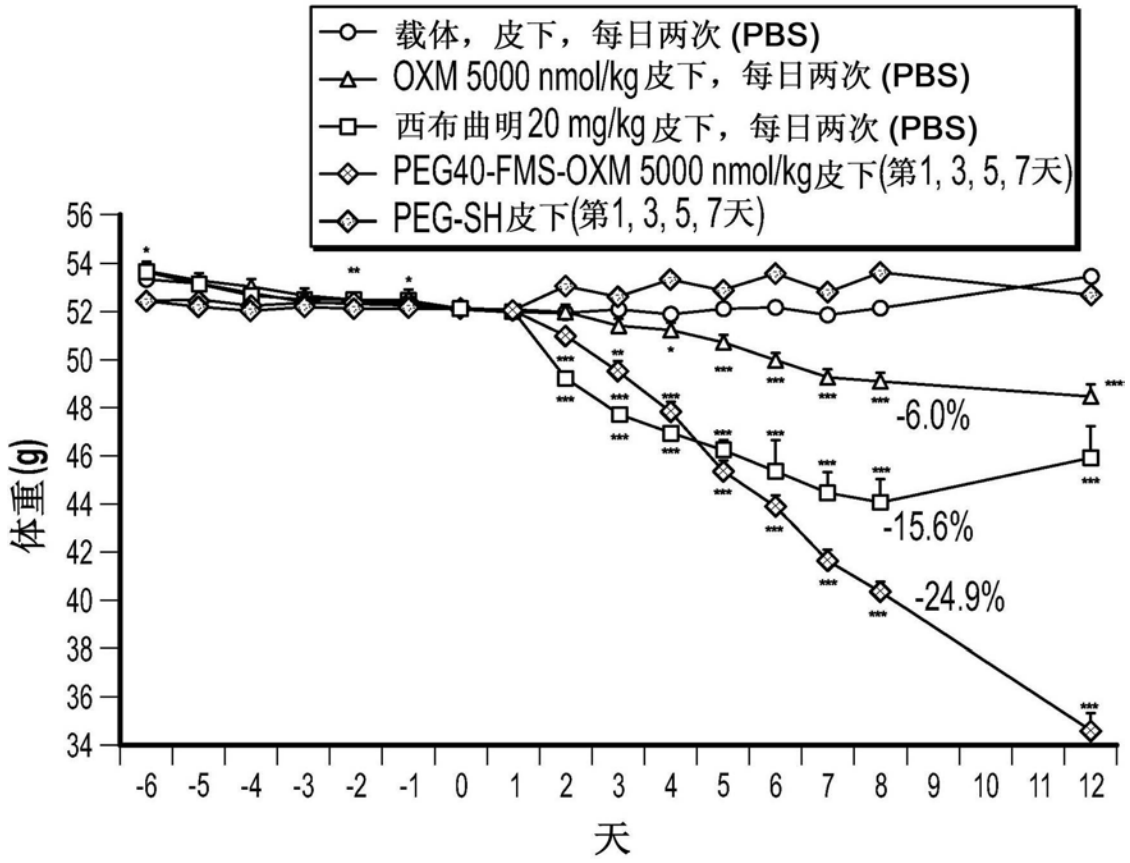

载体，皮下，每日两次 (PBS)
OXM 5000 nmol/kg皮下，每日两次 (PBS)
西布曲明20 mg/kg皮下，每日两次 (PBS)
PEG40-FMS-OXM 5000 nmol/kg皮下(第1, 3, 5, 7天)
PEG-SH皮下(第1, 3, 5, 7天)
体重(g)
天
-6.0%
-15.6%
-24.9%

图 5A

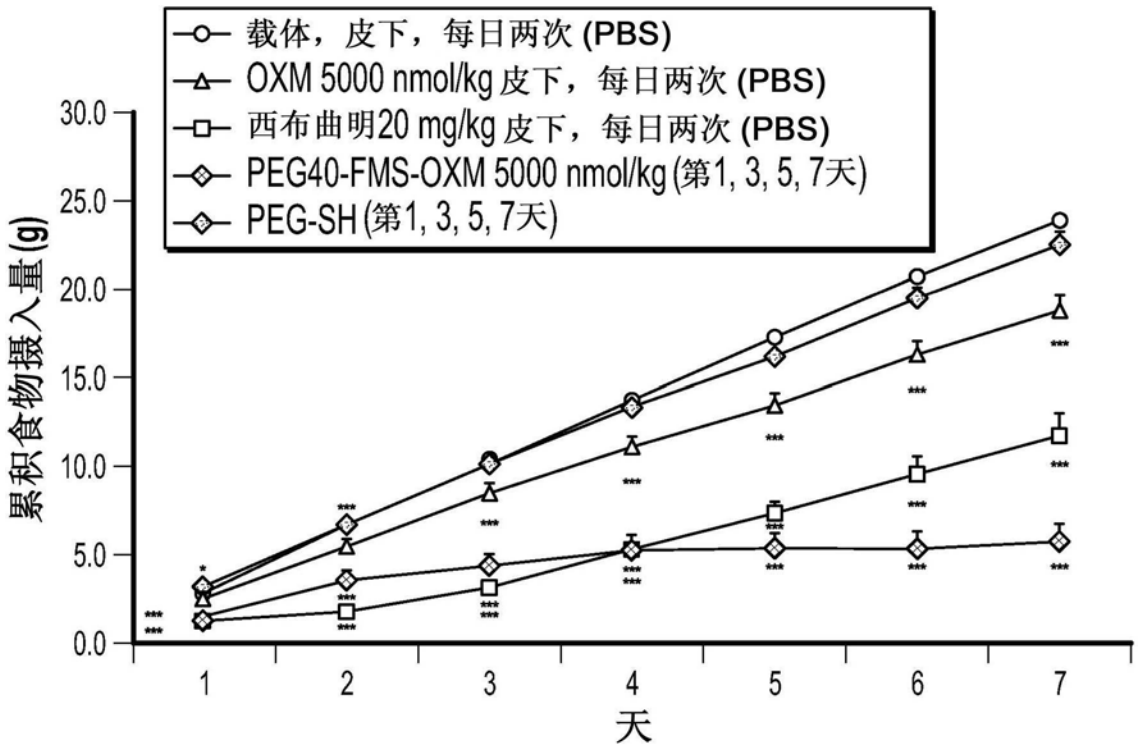

载体，皮下，每日两次 (PBS)
OXM 5000 nmol/kg 皮下，每日两次 (PBS)
西布曲明20 mg/kg 皮下，每日两次 (PBS)
PEG40-FMS-OXM 5000 nmol/kg (第1, 3, 5, 7天)
PEG-SH (第1, 3, 5, 7天)
累积食物摄入量(g)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1
2
3
4
5
6
7
天

图 5B

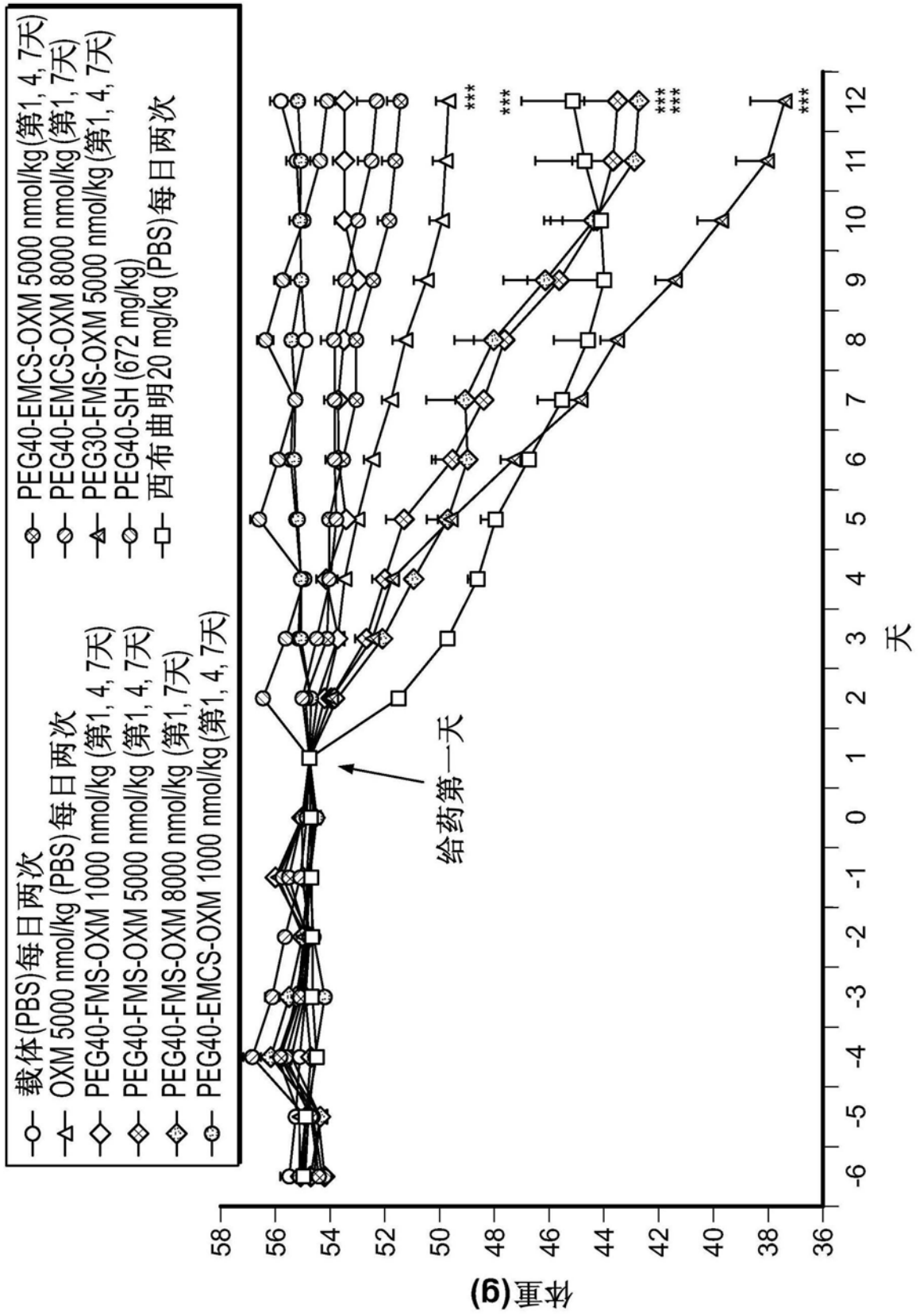

载体(PBS)每日两次
OXM 5000 nmol/kg (PBS)每日两次
PEG40-FMS-OXM 1000 nmol/kg (第1, 4, 7天)
PEG40-FMS-OXM 5000 nmol/kg (第1, 4, 7天)
PEG40-FMS-OXM 8000 nmol/kg (第1, 7天)
PEG40-EMCS-OXM 1000 nmol/kg (第1, 4, 7天)
PEG40-EMCS-OXM 5000 nmol/kg (第1, 4, 7天)
PEG40-EMCS-OXM 8000 nmol/kg (第1, 7天)
PEG30-FMS-OXM 5000 nmol/kg (第1, 4, 7天)
PEG40-SH (672 mg/kg)
西布曲明20 mg/kg (PBS)每日两次
给药第一天
体重(g)
天
***

图 6A

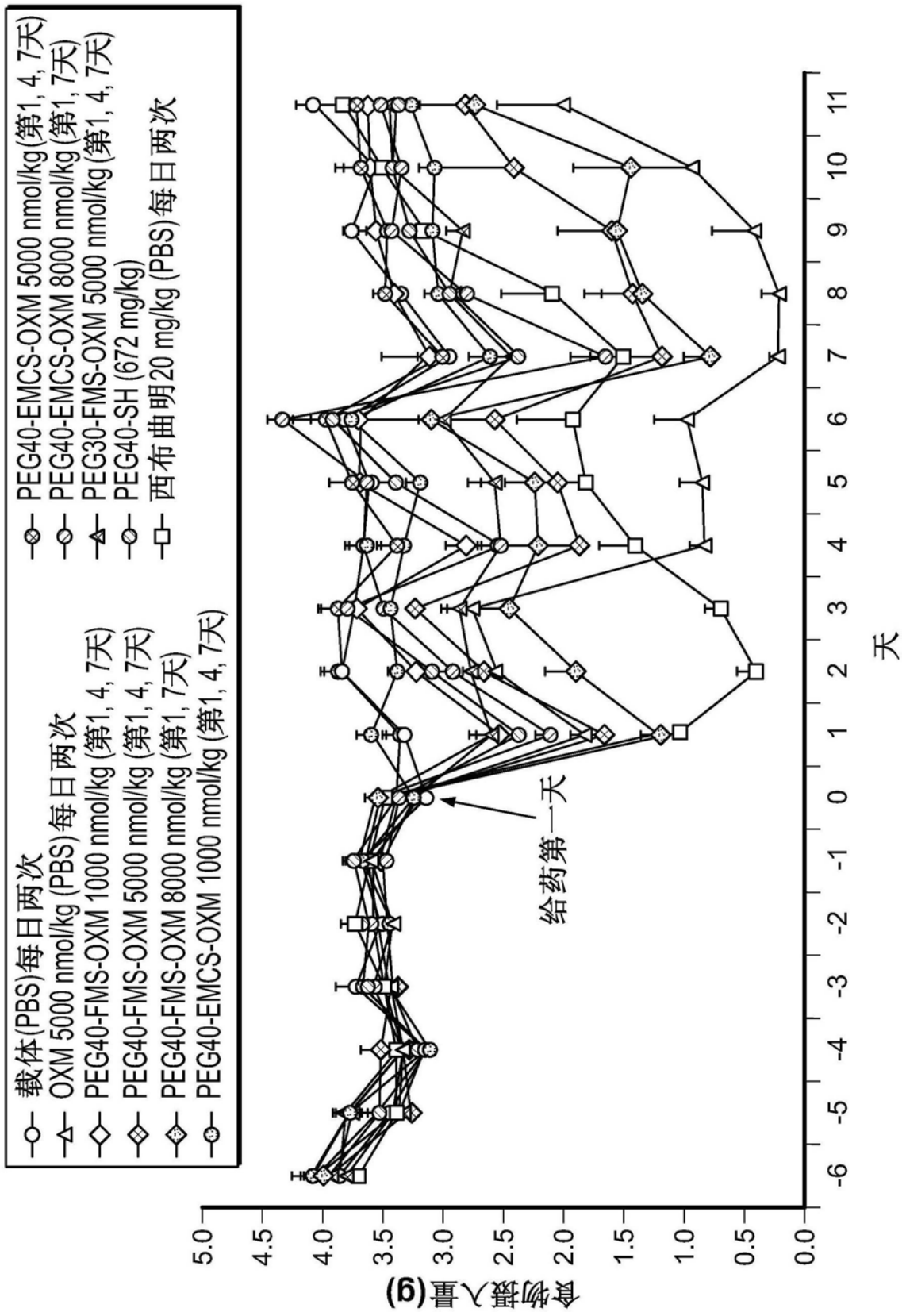

载体(PBS)每日两次
OXM 5000 nmol/kg (PBS)每日两次
PEG40-FMS-OXM 1000 nmol/kg (第1, 4, 7天)
PEG40-FMS-OXM 5000 nmol/kg (第1, 4, 7天)
PEG40-FMS-OXM 8000 nmol/kg (第1, 7天)
PEG40-EMCS-OXM 1000 nmol/kg (第1, 4, 7天)
PEG40-EMCS-OXM 5000 nmol/kg(第1, 4, 7天)
PEG40-EMCS-OXM 8000 nmol/kg (第1, 7天)
PEG30-FMS-OXM 5000 nmol/kg (第1, 4, 7天)
PEG40-SH (672 mg/kg)
西布曲明20 mg/kg (PBS)每日两次
给药第一天
药物摄入量(g)
天

图 6B

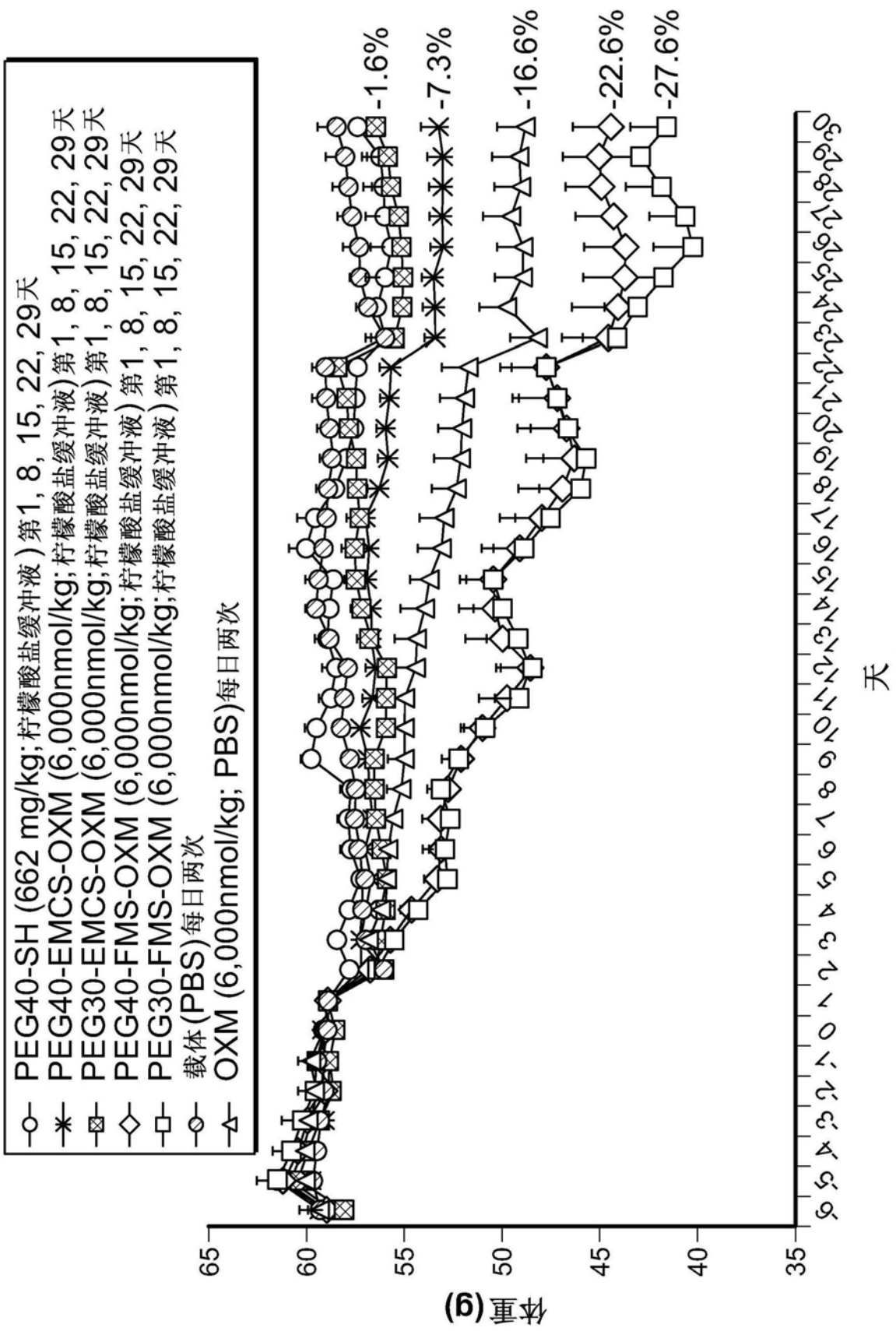

PEG40-SH (662 mg/kg;柠檬酸盐缓冲液)第1, 8, 15, 22, 29天
PEG40-EMCS-OXM (6,000nmol/kg;柠檬酸盐缓冲液)第1, 8, 15, 22, 29天
PEG30-EMCS-OXM (6,000nmol/kg;柠檬酸盐缓冲液)第1, 8, 15, 22, 29天
PEG40-FMS-OXM (6,000nmol/kg;柠檬酸盐缓冲液)第1, 8, 15, 22, 29天
PEG30-FMS-OXM (6,000nmol/kg;柠檬酸盐缓冲液)第1, 8, 15, 22, 29天
载体(PBS)每日两次
OXM (6,000nmol/kg; PBS)每日两次
-1.6%
-7.3%
-16.6%
-22.6%
-27.6%
体重(g)
天

图 7

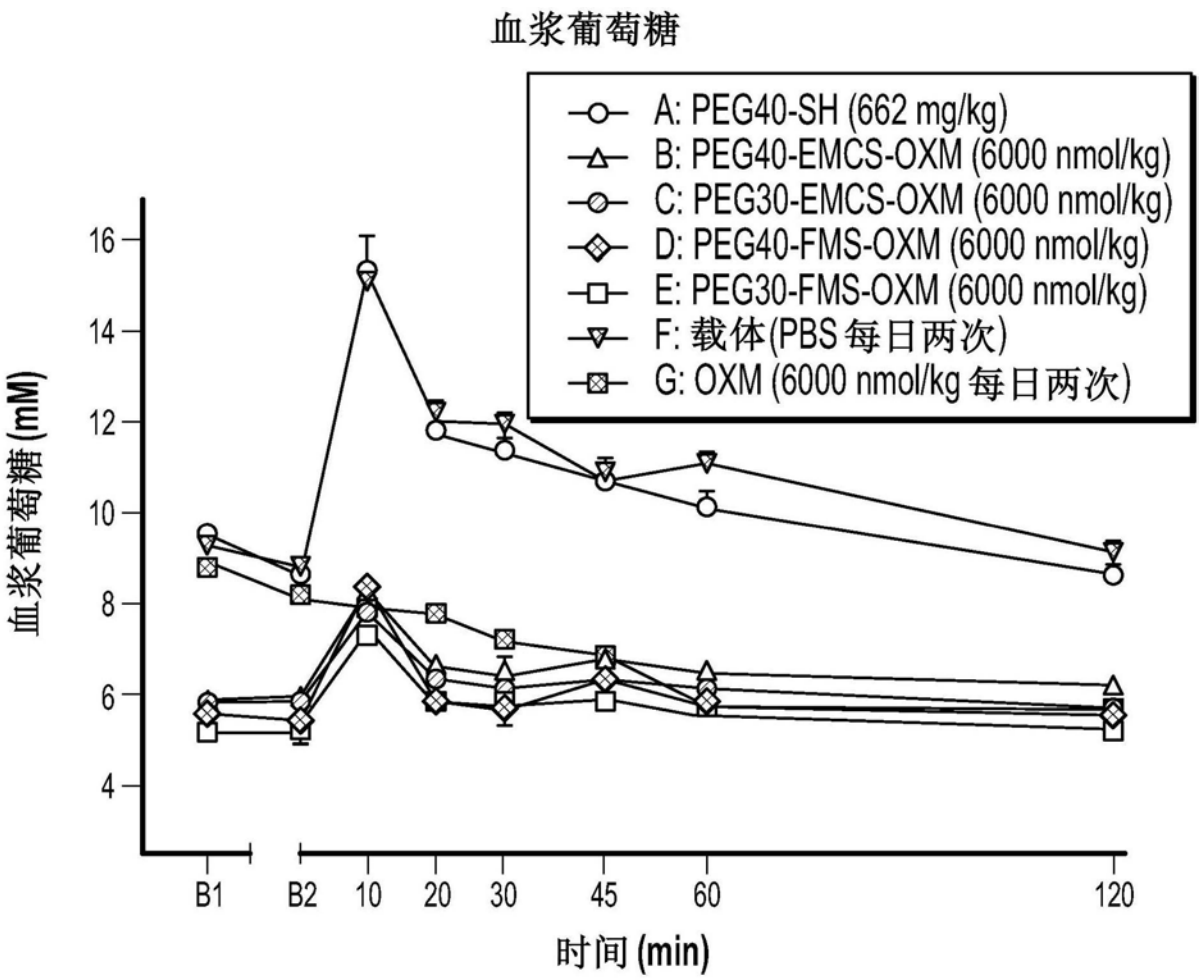

血浆葡萄糖
A: PEG40-SH (662 mg/kg)
B: PEG40-EMCS-OXM (6000 nmol/kg)
C: PEG30-EMCS-OXM (6000 nmol/kg)
D: PEG40-FMS-OXM (6000 nmol/kg)
E: PEG30-FMS-OXM (6000 nmol/kg)
F: 载体(PBS 每日两次)
G: OXM (6000 nmol/kg 每日两次)
血浆葡萄糖 (mM)
16
14
12
10
8
6
4
B1
B2
10
20
30
45
60
120
时间 (min)

图 8

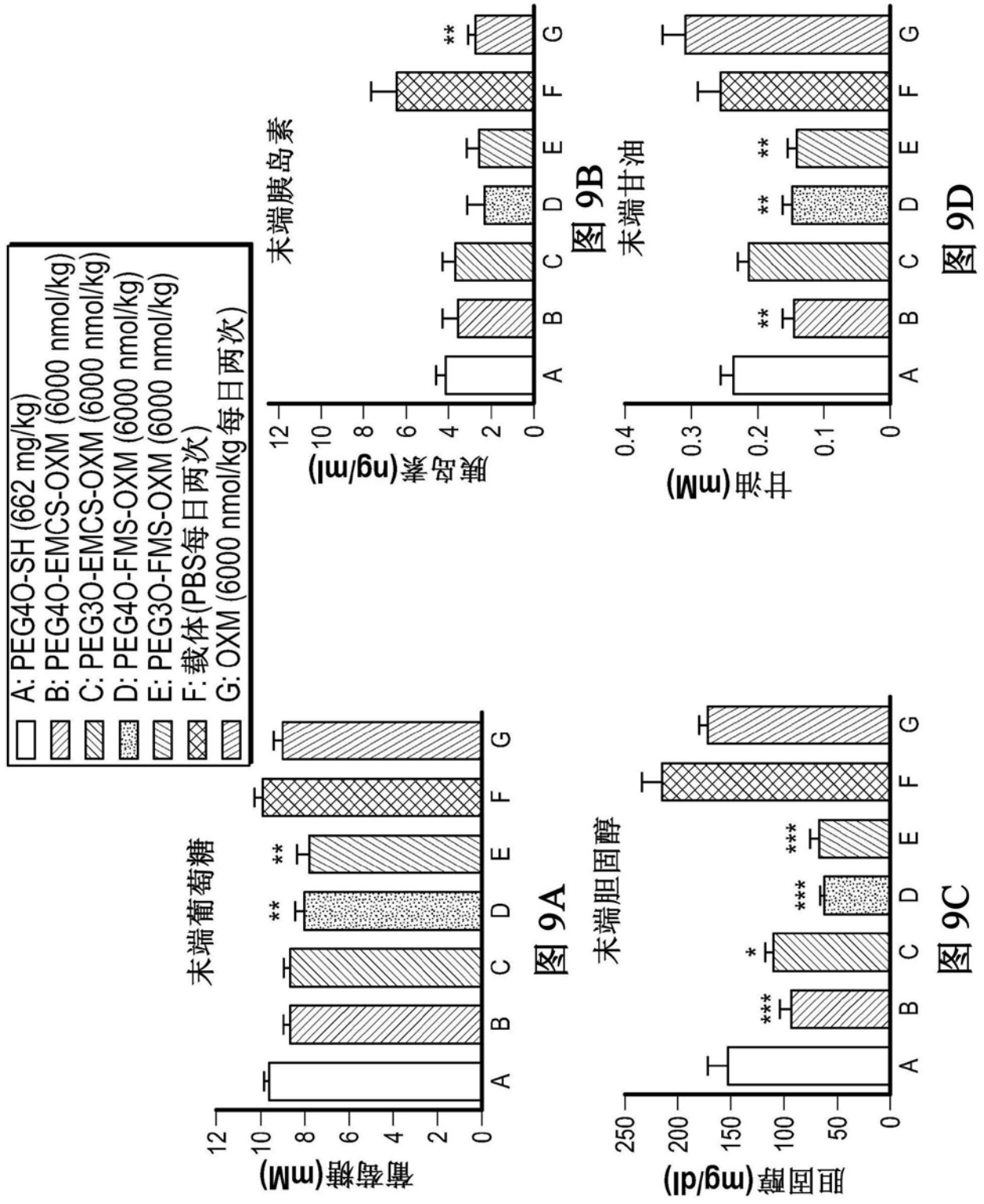


图 9A 图 9B 图 9C 图 9D

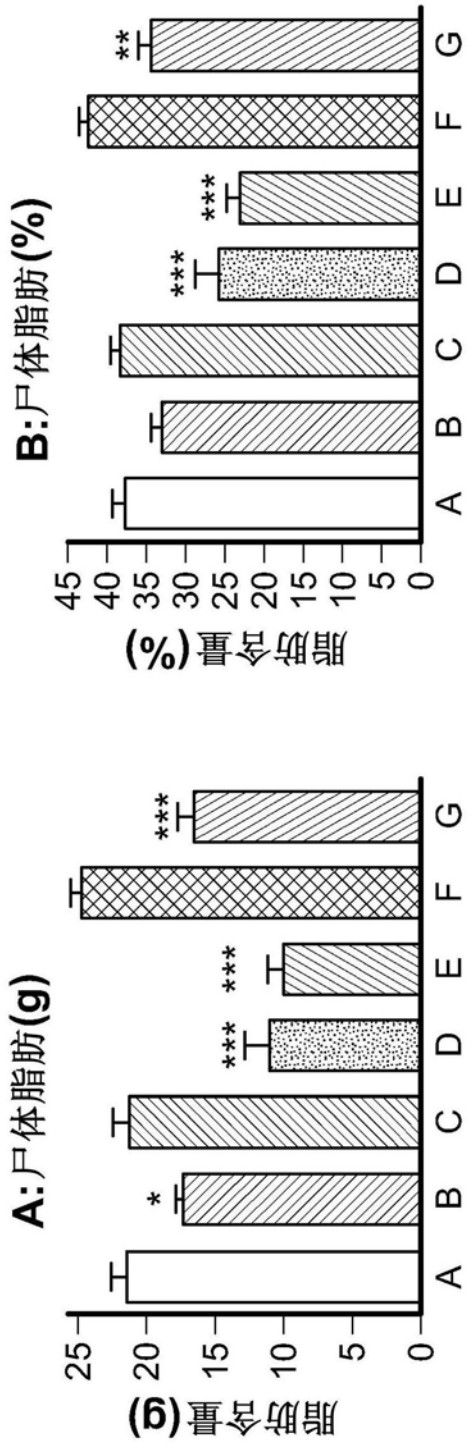

A:尸体脂肪(g)
脂肪含量(g)
0
5
10
15
20
25
A B C D E F G
*
***
***
***

图 10A

B:尸体脂肪(%)
脂肪含量(%)
0
5
10
15
20
25
30
35
40
45
A B C D E F G
***
***
**

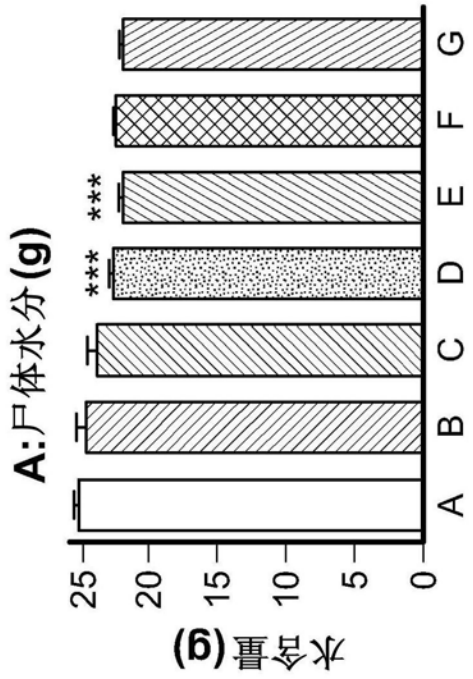
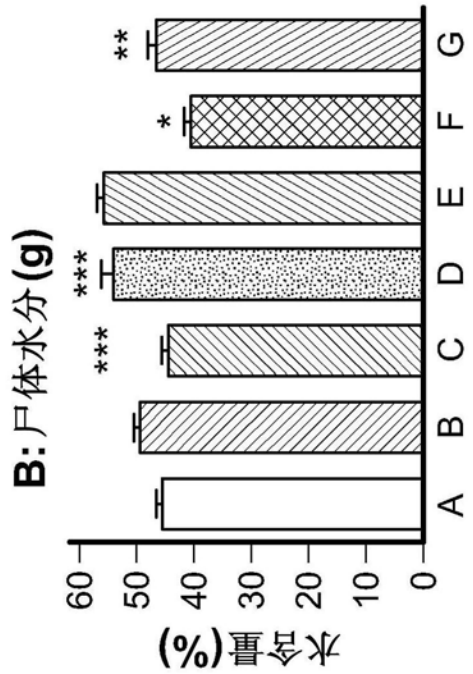
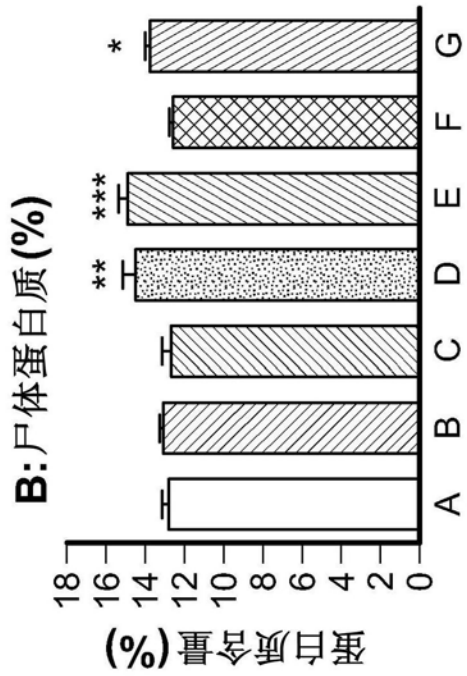
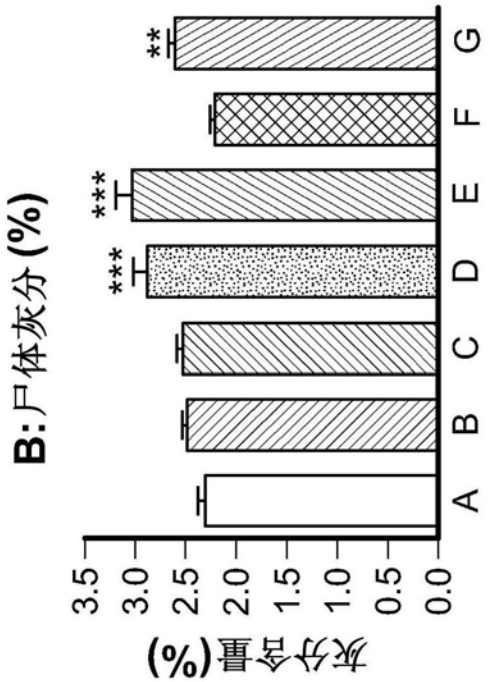

A:尸体水分(g)
水含量(g)
0
5
10
15
20
25
A B C D E F G
***
***
***

图 10B


B:尸体水分(g)
水含量(%)
0
10
20
30
40
50
60
A B C D E F G
***
***
*
**


B:尸体蛋白质(%)
蛋白质含量(%)
0
2
4
6
8
10
12
14
16
18
A
B
C
D
E
F
G
**
***
**
*


B:尸体灰分(%)
灰分含量(%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
A
B
C
D
E
F
G
***
***
**

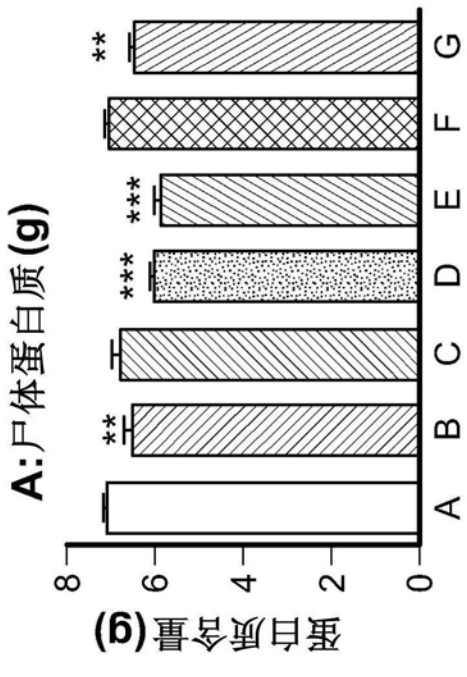

A:尸体蛋白质(g)
蛋白质含量(g)
0
2
4
6
8
A
B
C
D
E
F
G
**
***
***
**

图 10C

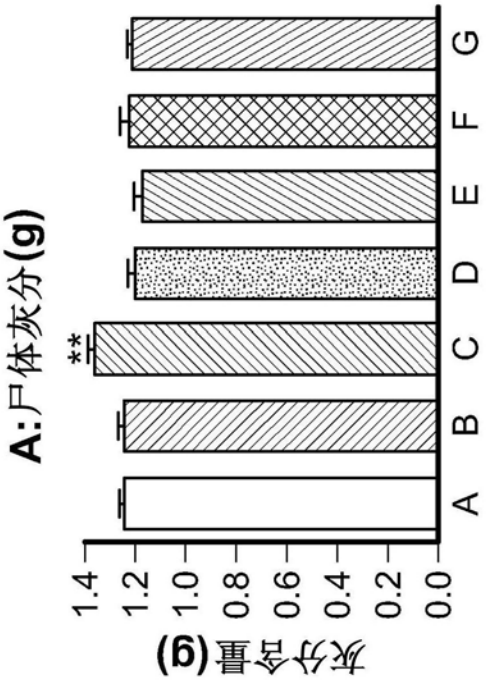

A:尸体灰分(g)
灰分含量(g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
A
B
C
D
E
F
G
**

图 10D

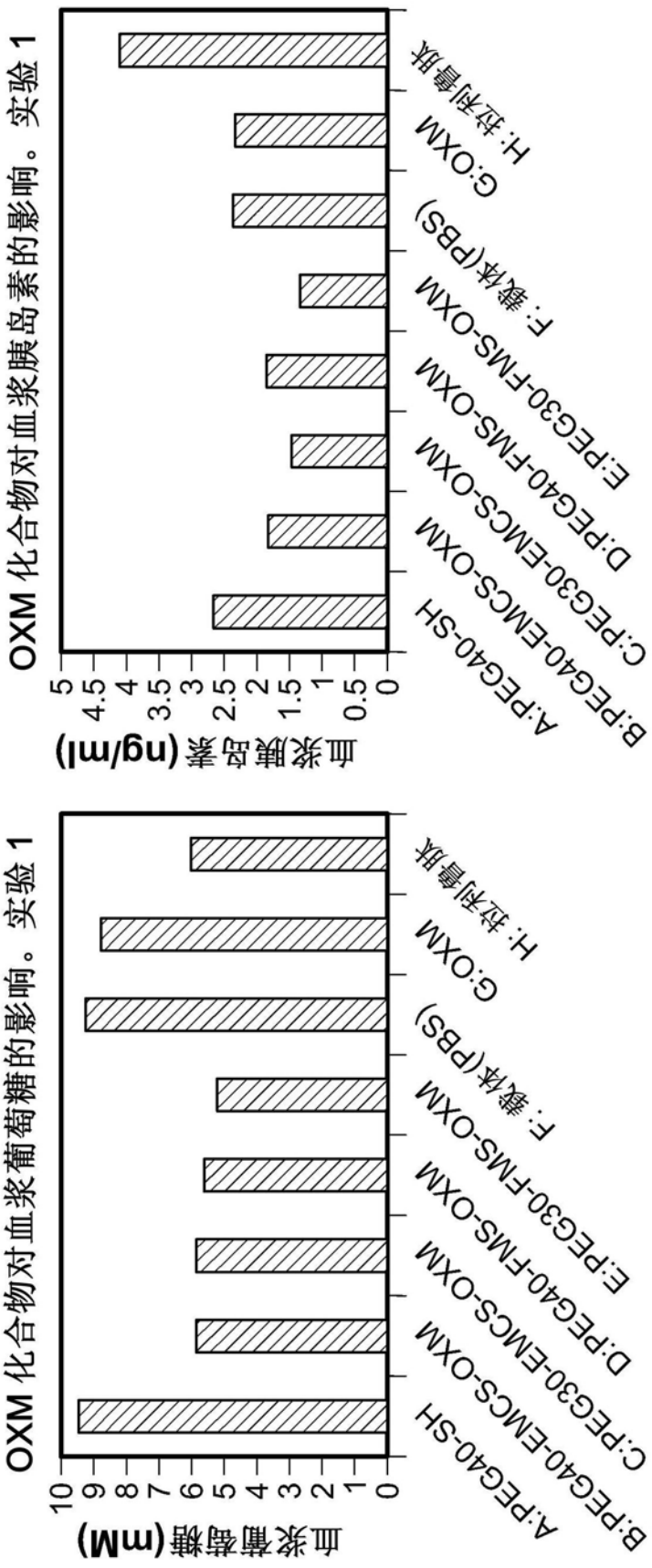

OXM 化合物对血浆胰岛素的影响。实验 1
血浆胰岛素 (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
A:PEG40-SH
B:PEG40-EMCS-OXM
C:PEG30-EMCS-OXM
D:PEG40-FMS-OXM
E:PEG30-FMS-OXM
F: 载体(PBS)
G:OXM
H: 拉利鲁肽
OXM 化合物对血浆葡萄糖的影响。实验 1
血浆葡萄糖 (mM)
0
1
2
3
4
5
6
7
8
9
10
A:PEG40-SH
B:PEG40-EMCS-OXM
C:PEG30-EMCS-OXM
D:PEG40-FMS-OXM
E:PEG30-FMS-OXM
F: 载体(PBS)
G:OXM
H: 拉利鲁肽

图 11

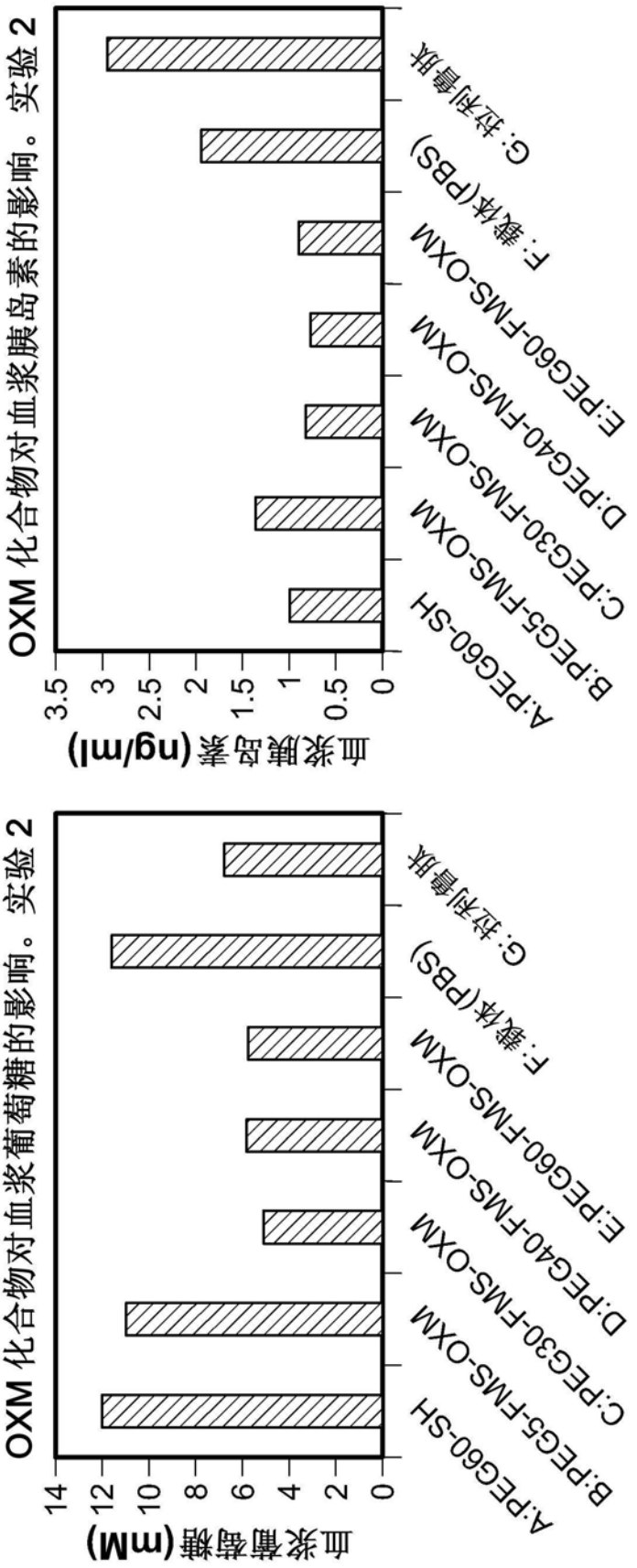

OXM 化合物对血浆胰岛素的影响。实验 2
血浆胰岛素 (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
A:PEG60-SH
B:PEG5-FMS-OXM
C:PEG30-FMS-OXM
D:PEG40-FMS-OXM
E:PEG60-FMS-OXM
F:载体(PBS)
G:拉利鲁肽
OXM 化合物对血浆葡萄糖的影响。实验 2
血浆葡萄糖 (mM)
0
2
4
6
8
10
12
14
A:PEG60-SH
B:PEG5-FMS-OXM
C:PEG30-FMS-OXM
D:PEG40-FMS-OXM
E:PEG60-FMS-OXM
F:载体(PBS)
G:拉利鲁肽

图 12

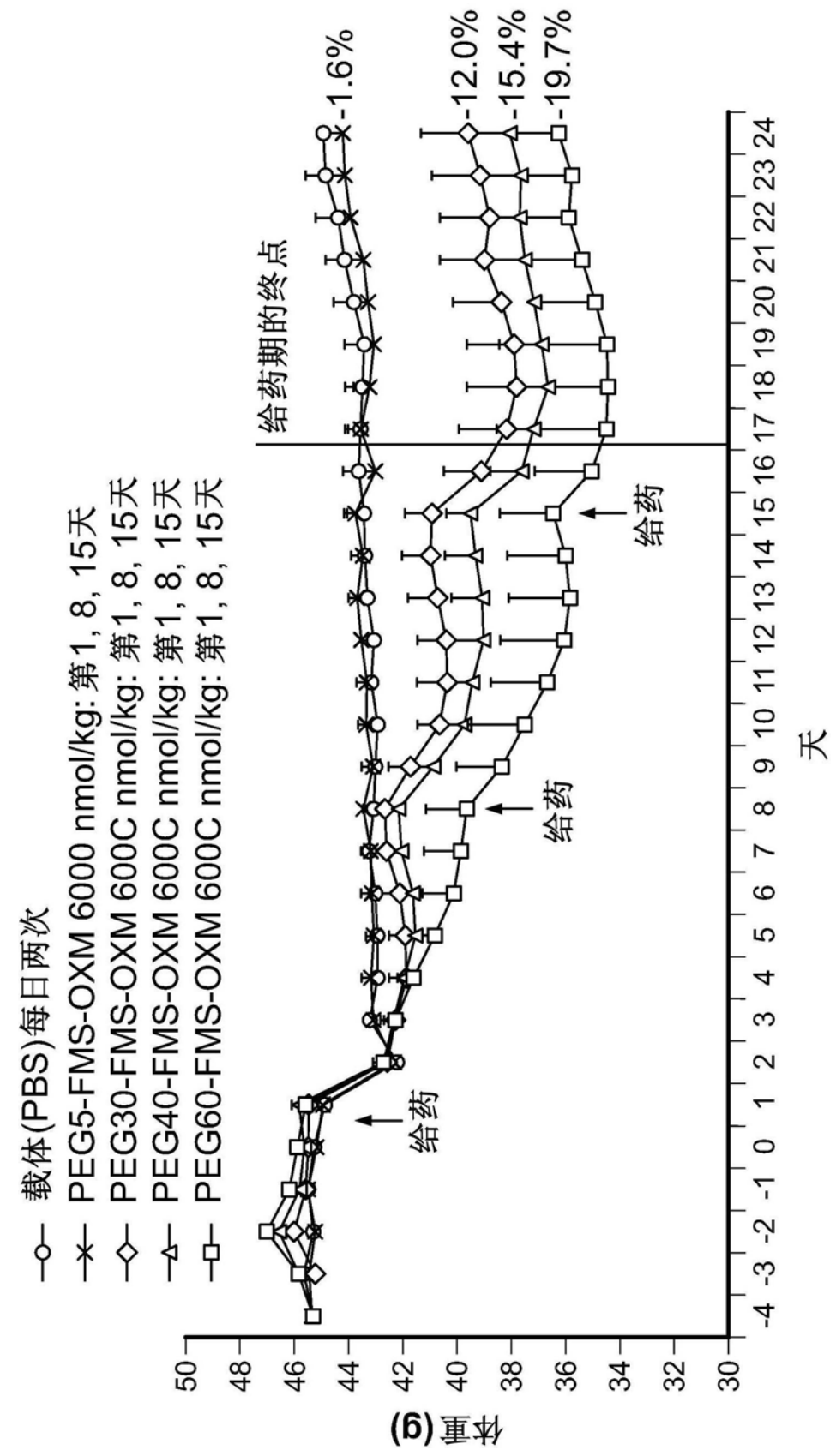

载体(PBS)每日两次
PEG5-FMS-OXM 6000 nmol/kg: 第1, 8, 15天
PEG30-FMS-OXM 600C nmol/kg: 第1, 8, 15天
PEG40-FMS-OXM 600C nmol/kg: 第1, 8, 15天
PEG60-FMS-OXM 600C nmol/kg: 第1, 8, 15天
给药期的终点
-1.6%
-12.0%
-15.4%
-19.7%
给药
给药
给药
体重(g)
天

图 13