

PROTEIN TYROSINE-TYROSINE ANALOGS AND
METHODS OF USING THE SAME

[0001] The disclosure relates generally to biology and medicine, and more particularly it relates to Peptide Tyrosine-Tyrosine (PYY) analogs that can bind a neuropeptide Y (NPY) receptor such as the NPY2 receptor, as well as compositions including the same and their therapeutic use in treating obesity and obesity-related diseases and disorders such as type II diabetes (T2DM).

[0002] PYY is a member of the pancreatic polypeptide (PP) family and is involved in modulating food intake and energy expenditure following a meal (*see*, Tatemoto (1982) *Proc. Natl. Acad. Sci.* 79:2514-2518). PYY is secreted by L cells of the gastrointestinal track and has two main endogenous forms – PYY₁₋₃₆ (SEQ ID NO:1) and PYY₃₋₃₆ (SEQ ID NO:2). PYY₁₋₃₆ predominates over PYY₃₋₃₆ during fasting, whereas PYY₃₋₃₆ predominates over PYY₁₋₃₆ following feeding. Dipeptidyl peptidase-IV (DPP-IV) hydrolyzes PYY₁₋₃₆ at a Pro²-Ile³ bond to produce PYY₃₋₃₆, which is more selective for the NPY2 receptor than PYY₁₋₃₆.

[0003] Plasma PYY₃₋₃₆ concentration typically increases within 15 minutes of food intake, peaks within 60-90 minutes, and remains elevated for up to 6 hours before returning to baseline (*see*, Adrian *et al.* (1985) *Gastroenterology* 89:1070-1077; and De Silva & Bloom (2012) *Gut Liver* 6:10-20). In this manner, PYY₃₋₃₆ is believed to impact appetite via its direct central effect and also via its effect on gut motility (*i.e.*, its anorectic effect). Additionally, PYY₃₋₃₆ is believed to mediate insulin sensitivity to thereby help lower blood glucose (*i.e.*, its sensitization effect).

[0004] PYY₃₋₃₆ has been investigated as a potential therapeutic agent for body-weight regulation in view of its anorectic effects, especially for treating obesity and its associated diseases and disorders, including T2DM and cardiovascular diseases (*see, e.g.*, Intl. Patent Application Publication No. 2002/47712; and Schwartz & Morton (2002) *Nature* 418:595-597).

[0005] Unfortunately, exogenously administered PYY₃₋₃₆ has a short half-life (*e.g.*, about 10-15 minutes) due to proteases and other clearance mechanisms (*see*, Lluís *et al.* (1989) *Rev. Esp. Fisiol.* 45:377-384; and Torang *et al.* (2016) *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 310:R866-R874), which presents challenges when using it as a therapeutic agent.

With such a short half-life, PYY₃₋₃₆ should be administered at least once-daily to exert a therapeutic effect, which is inconvenient for an individual in need thereof. Efforts therefore have been made to increase PYY₃₋₃₆'s half-life and/or to increase its NPY2 receptor selectivity. For example, Rubinstein *et al.* describe PYY analogs having a 9-fluorenylmethoxy-carbonyl (Fmoc) or a 2-sulfo-9-fluorenyl-methoxycarbonyl (FMS) radical to increase half-life (*see*, Intl. Patent Application Publication No. WO 2004/089279). Moreover, DeCarr *et al.* describe PYY analogs having amino-terminally linked PEG moieties to increase half-life (*see*, DeCarr *et al.* (2007) *Bioorg. Med. Chem. Lett.* 17:1916-1919; *see also*, Ortiz *et al.* (2007) *J. Pharmacol. Exp. Ther.* 323:692-700). Furthermore, Kofoed *et al.* describe PYY analogs having albumin-binding side chains, at least one modified residue close to PYY's cleavage site (*e.g.* an N-methyl amino acid analog of an amino acid residue of interest), a N-glycine, and/or an arginine mimetic to increase half-life (*see*, Intl. Patent Application Publication No. WO 2011/033068).

[0006] Despite significant increases in understanding PYY₃₋₃₆'s role in metabolism, there remains a need for additional PYY analogs, especially PYY analogs having improved potency and selectivity at the NPY2 receptor.

[0007] As noted above, additional PYY analogs for therapeutic uses are needed. To address this need, the disclosure first describes PYY analogs that include a base amino acid sequence of (with respect to the numbering of native, human PYY₁₋₃₆ (SEQ ID NO:1)):

³PKPEX₇PX₉X₁₀DASPEEX₁₇X₁₈RYYX₂₂X₂₃LRHYLN₃₀LTRQRY³⁶ (Formula I), where X₇ is any amino acid with a functional group available for conjugation and the functional group is conjugated to a C₁₆-C₂₂ fatty acid, X₉ is E or G, X₁₀ is E or K, X₁₇ is L or W, X₁₈ is N or Q, X₂₂ is A or I, X₂₃ is E, D or S, and X₃₀ is E or W (SEQ ID NO:3), and where a carboxy-terminal (C-terminal) amino acid optionally is amidated.

[0008] In certain instances, the amino acid with the functional group available for conjugation at position X₇ can be C, D, E, K or Q. In particular instances, the amino acid with the functional group available for conjugation at position X₇ is K, and the amino acid sequence can be one of the following:

³PKPEKPGEDASPEEWQRYYAELRHYLNWLTRQRY³⁶ (SEQ ID NO:4),

³PKPEKPGEDASPEEWQRYYAELRHYLNELTRQRY³⁶ (SEQ ID NO:5),

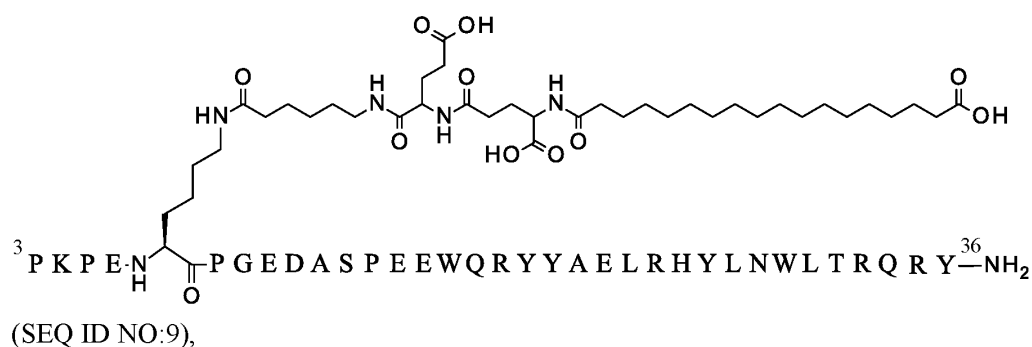
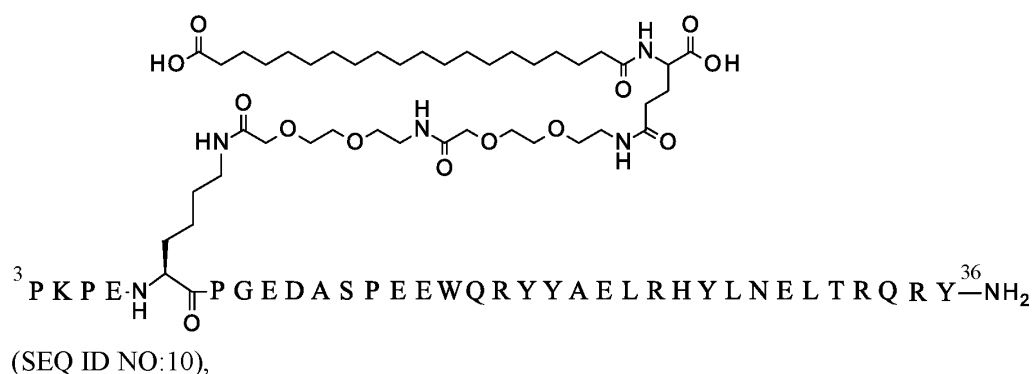
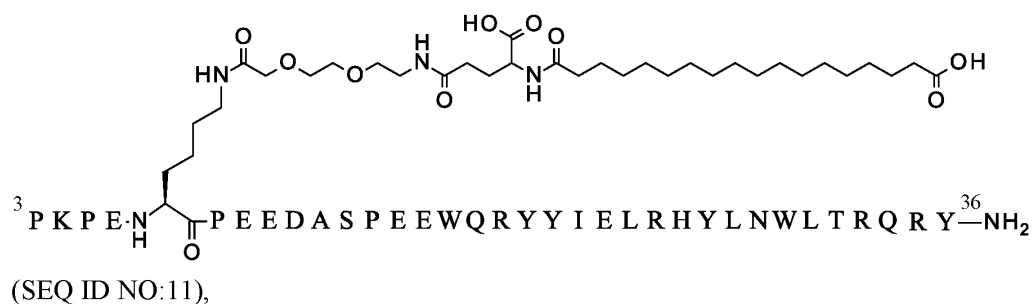
³PKPEKPEEDASPEEWQRYYYIELRHYLNWLTRQRY³⁶ (SEQ ID NO:6),

³PKPEKPGKDASPEEWNRYYADLRHYLNWLTRQRY³⁶ (SEQ ID NO:7), or

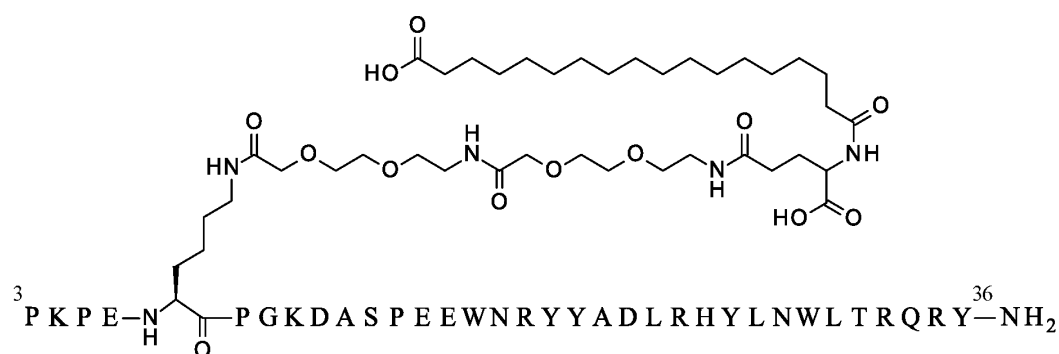
³PKPEKPGEDASPEELQRYYASLRHYLNWLTRQRY³⁶ (SEQ ID NO:8).

[0009] In some instances, the C₁₆-C₂₂ fatty acid is conjugated to the amino acid with the functional group available for conjugation via a linker. In certain instances, the C₁₆-C₂₂ fatty acid has a structure of -CO-(CH₂)_a-CO₂H, where _a is an integer between 16 to 22. In particular instances, the fatty acid is a C₁₈ diacid or a C₂₀ diacid such as palmitic acid, stearic acid, arachidic acid or eicosanoic acid, especially a saturated C₁₈ diacid or C₂₀ diacid. Likewise, and in some instances, the linker can be one or more units of [2-(2-amino-ethoxy)-ethoxy]-acetic acid (AEEA), aminohexanoic acid (Ahx), glutamic acid (E), γ -glutamic acid (γ E) or combinations thereof.

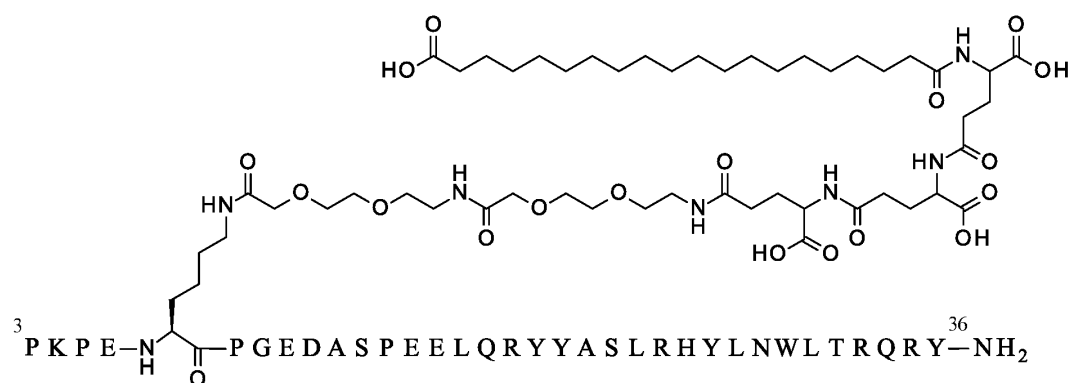
[0010] In particular instances, the PYY analog can be one of the following:



4



(SEQ ID NO:12), or



(SEQ ID NO:13).

[0011] In some instances, the base structure of the PYY analogs herein further can include the two amino-terminal (N-terminal) amino acids of native, human PYY₁₋₃₆ (SEQ ID NO:1), which subsequently can be processed *in vivo* to a PYY₃₋₃₆ analog (*i.e.*, the N-terminal “YP” residues of SEQ ID NO:1 can be cleaved *in vivo* from any one of the PYY analogs).

[0012] In some instances, the PYY analogs have a charge of greater than -2, especially -3 or -4.

[0013] In some instances, the PYY analogs have a binding affinity at the human NPY2 receptor that is greater than that of human PYY₃₋₃₆ (SEQ ID NO:2), such as from about 2-fold greater to about 10-fold greater, especially about 2-fold greater to about 3-fold greater.

[0014] In some instances, the PYY analogs have a half-life that is longer than that of human PYY₃₋₃₆ (SEQ ID NO:2), such as from about 5 hours to about 24 hours longer, especially about 12 hours.

[0015] Second, pharmaceutical compositions are described that include at least one PYY analog herein or a pharmaceutically acceptable salt thereof (*e.g.*, trifluoroacetate salts, acetate salts or hydrochloride salts) and a pharmaceutically acceptable carrier. In some instances, the pharmaceutical compositions further can include carriers, diluents and/or excipients.

[0016] Moreover, the pharmaceutical compositions can include an additional therapeutic agent such as, for example, other antidiabetic or weight loss agents, especially an incretin. In some instances, the incretin can be glucagon (GCG) or a GCG analog. In other instances, the incretin can be glucagon-like peptide-1 (GLP-1), GLP-1 (7-36)_{amide} or a GLP-1 analog. In other instances, the incretin can be gastric inhibitory peptide (GIP) or a GIP analog. In other instances, the incretin can be a dual receptor agonist such as oxyntomodulin (OXM) or an OXM analog, GLP-1/GCG or GIP/GLP-1. In other instances, the incretin can be an incretin analog having triple receptor activity (*i.e.*, incretin analogs with activity at each of the GIP, GLP-1 and GCG receptors). In other instances, the additional therapeutic agent can be a DPP-IV inhibitor.

[0017] Third, methods are described for using the PYY analogs herein, especially for using the PYY analogs to treat obesity and obesity-related diseases and disorders such as T2DM. The methods include at least a step of administering to an individual in need thereof an effective amount of a PYY analog as described herein or a pharmaceutically acceptable salt thereof.

[0018] In some instances, the PYY analog can be subcutaneously (SQ) administered to the individual. Likewise, and in some instances, the PYY analog can be administered daily, every other day, three times a week, two times a week, one time a week (*i.e.*, weekly), biweekly (*i.e.*, every other week), or monthly. In certain instances, the PYY analog can be administered SQ every other day, SQ three times a week, SQ two times a week, SQ one time a week, SQ every other week, or SQ once a month. In particular instances, the PYY analog is administered SQ one time a week (QW).

[0019] Alternatively, the PYY analog can be orally administered to the individual. As above, the PYY analog can be administered daily, every other day, three times a week, two times a week, one time a week (*i.e.*, weekly), biweekly (*i.e.*, every other week), or monthly. In certain instances, the PYY analog can be administered orally every other day, orally three times a week, orally two times a week, orally one time a week, orally every other

week, or orally once a month. In particular instances, the PYY analog is administered orally one time a week.

[0020] The methods also can include administering the at least one PYY analog in combination with an effective amount of an additional therapeutic agent such as a DPP-IV inhibitor or an incretin (*e.g.*, GCG or a GCG analog, GLP-1, GLP-1 (7-36)_{amide} or a GLP-1 analog, GIP or a GIP analog, OXM or an OXM analog, GIP/GLP-1, GLP-1/GCG, or an incretin having triple receptor activity). The DPP-IV inhibitor or incretin can be administered simultaneously, separately or sequentially with the PYY analog.

[0021] In some instances, the DPP-IV inhibitor or incretin can be administered with a frequency same as the PYY analog (*i.e.*, every other day, twice a week, or even weekly). In other instances, the DPP-IV inhibitor or incretin is administered with a frequency distinct from the PYY analog. In other instances, the DPP-IV inhibitor or incretin is administered QW. In still other instances, the PYY analog is administered SQ, and the DPP-IV inhibitor or the incretin can be administered orally.

[0022] In some instances, the individual is obese or overweight. In other instances, the individual is a person with diabetes (PwD), especially T2DM. In certain instances, the individual is obese with T2DM or overweight with T2DM.

[0023] The methods also may include steps such as measuring or obtaining the individual's weight and/or blood glucose and/or hemoglobin A1c (HbA1c) and comparing such obtained values to one or more baseline values or previously obtained values to assess the effectiveness of treatment.

[0024] The methods also may be combined with diet and exercise and/or may be combined with additional therapeutic agents other than those discussed above.

[0025] Fourth, uses are described for the PYY analogs herein in treating obesity and obesity-related diseases and disorders such as T2DM, which optionally can be administered simultaneously, separately or sequentially (*i.e.*, in combination) with a DPP-IV inhibitor and/or an incretin such as GCG or a GCG analog, GLP-1, GLP-1 (7-36)_{amide} or a GLP-1 analog, GIP or a GIP analog, OXM or an OXM analog, GIP/GLP-1, GLP-1/GCG, or even an incretin having triple receptor activity.

[0026] Fifth, uses are described for the PYY analogs herein in manufacturing a medicament for treating obesity and obesity-related diseases and disorders such as T2DM, where the medicament optionally may further include a DPP-IV inhibitor and/or an incretin

such as GCG or a GCG analog, GLP-1, GLP-1 (7-36)_{amide} or a GLP-1 analog, GIP or a GIP analog, OXM or an OXM analog, GIP/GLP-1, GLP-1/GCG, or even an incretin having triple receptor activity.

[0027] One advantage of the PYY analogs herein is that they not only can facilitate weight loss but also can lower glucose. In this manner, individuals, especially those susceptible to or having T2DM, can delay progressing to exogenous insulin and can maintain target HbA1c goals. Moreover, the PYY analogs herein can enhance glycemic control by improving insulin sensitization. Combined, GIP/GLP-1 and PYY analog can be used for both glucose control (incretin + potential insulin sensitizer) and weight loss (synergistic). In particular, the PYY analogs herein can cause an up to about 12% weight loss alone when administered to an individual in need thereof and can cause an up to about 25% weight loss in connection with an additional therapeutic agent such as an incretin when administered to an individual in need thereof.

[0028] Another advantage of the PYY analogs herein is that they can have a half-life of up to about 24 hours, thereby allowing for once weekly administration.

[0029] Another advantage of the PYY analogs herein is that they have increased physico-chemical stability and compatibility when compared to native, human PYY₃₋₃₆ (SEQ ID NO:2) and increased compatibility in a formulation with incretins when compared to native, human PYY₃₋₃₆ (SEQ ID NO:2).

[0030] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which the disclosure pertains. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the PYY analogs, pharmaceutical compositions and methods, the preferred methods and materials are described herein.

[0031] Moreover, reference to an element by the indefinite article “a” or “an” does not exclude the possibility that more than one element is present, unless the context clearly requires that there be one and only one element. The indefinite article “a” or “an” thus usually means “at least one.”

[0032] *Definitions*

[0033] As used herein, “about” means within a statistically meaningful range of a value or values such as, for example, a stated concentration, length, molecular weight, pH,

sequence identity, time frame, temperature, volume, *etc.* Such a value or range can be within an order of magnitude typically within 20%, more typically within 10%, and even more typically within 5% of a given value or range. The allowable variation encompassed by “about” will depend upon the particular system under study, and can be readily appreciated by one of skill in the art.

[0034] As used herein, “amino acid” means a molecule that, from a chemical standpoint, is characterized by the presence of one or more amine groups and one or more carboxylic acid groups and may contain other functional groups. As is known in the art, there is a set of twenty amino acids that are designated as standard amino acids and that are used as building blocks for most of the peptides/proteins produced by any living being.

[0035] As used herein, “amino acid with a functional group available for conjugation” means any natural or unnatural amino acid with a functional group that may be conjugated to a fatty acid by way of, for example, a linker. Examples of such functional groups include, but are not limited to, alkynyl, alkenyl, amino, azido, bromo, carboxyl, chloro, iodo and thiol groups. Additionally, examples of natural amino acids including such functional groups include C (thiol), D (carboxyl), E (carboxyl), K (amino) and Q (amide).

[0036] As used herein, “analog” means a compound, such as a synthetic peptide or polypeptide, that activates a target receptor and that elicits at least one *in vivo* or *in vitro* effect elicited by a native agonist for that receptor.

[0037] As used herein, “anorectic effect” means an ability of the PYY analogs herein to reduce appetite, resulting in lower food consumption and ultimately leading to weight loss. Anorectic effect also may refer to an ability of the PYY analogs herein to increase gut motility.

[0038] As used herein, “C₁₆-C₂₂ fatty acid” means a carboxylic acid having between 16 and 22 carbon atoms. The C₁₆-C₂₂ fatty acid suitable for use herein can be a saturated monoacid or a saturated diacid (“diacids” have a carboxyl group on each end).

[0039] As used herein, “AUC” means area under the curve.

[0040] As used herein, “effective amount” means an amount, concentration or dose of one or more PYY analogs herein, or a pharmaceutically acceptable salt thereof which, upon single or multiple dose administration to an individual in need thereof, provides a desired effect in such an individual under diagnosis or treatment (*i.e.*, may produce a clinically measurable difference in a condition of the individual such as, for example, a reduction in

blood glucose, a reduction in HbA1c, and/or a reduction in weight or body fat). An effective amount can be readily determined by one of skill in the art by using known techniques and by observing results obtained under analogous circumstances. In determining the effective amount for an individual, a number of factors are considered, including, but not limited to, the species of mammal, its size, age and general health, the specific disease or disorder involved, the degree of or involvement or the severity of the disease or disorder, the response of the individual, the particular PYY analog administered, the mode of administration, the bioavailability characteristics of the preparation administered, the dose regimen selected, the use of concomitant medication, and other relevant circumstances.

[0041] As used herein, “half-maximal effective concentration” or “EC₅₀” means a concentration of compound that results in 50% activation/stimulation of an assay endpoint, such as a dose-response curve (*e.g.*, cAMP).

[0042] As used herein, “in combination with” means administering at least one of the PYY analogs herein either simultaneously, sequentially or in a single combined formulation with one or more additional therapeutic agents.

[0043] As used herein, “incretin analog” means a peptide or polypeptide having structural similarities with, but multiple differences from, each of GIP, GLP-1, GCG and OXM, especially native, human GIP, GLP-1, GCG and OXM. Some incretin analogs also have affinity for and activity at two or even each of the GIP, GLP-1 and GCG receptors (*i.e.*, agonist activity at two receptors such as in OXM, GIP/GLP-1 or GLP-1/GCG, or even agonist activity at all three receptors).

[0044] As used herein, “individual in need thereof” means a mammal, such as a human, with a condition, disease, disorder or symptom requiring treatment or therapy, including for example, those listed herein. In particular, the preferred individual to be treated is a human.

[0045] As used herein, “long-acting” means that binding affinity and activity of a PYY analog herein continues for a period of time greater than native, human PYY₁₋₃₆ (SEQ ID NO:1) and/or native, human PYY₃₋₃₆ (SEQ ID NO:2), allowing for dosing at least as infrequently as once daily or even thrice-weekly, twice-weekly, once-weekly, or monthly. The time action profile of the PYY analogs herein may be measured using known pharmacokinetic test methods such as those described in the Examples below.

[0046] As used herein, “non-standard amino acid” means an amino acid that may occur naturally in cells but does not participate in peptide synthesis. Non-standard amino acids can be constituents of a peptide and often times are generated by modification of standard amino acids in the peptide (*i.e.*, via post-translational modification). Non-standard amino acids can include D-amino acids, which have an opposite absolute chirality of the standard amino acids above.

[0047] As used herein, “obese” or “obesity” means a condition in which an individual has a body mass index (BMI) that is $>30.0 \text{ kg/m}^2$. *See generally*, “Overweight & Obesity” by the Center for Disease Control and Prevention available at cdc.gov/obesity/adult/defining.html; and “Definitions & Facts for Adult Overweight & Obesity” by the National Institutes of Health at iddk.nih.gov/health-information/weight-management/adult-overweight-obesity/definition-facts.

[0048] As used herein, “obesity-related disease or disorder” means any diseases or disorders that are induced/exacerbated by obesity including, but not limited to, angina pectoris, cardiovascular disease, cholecystitis, cholelithiasis, congestive heart failure, dyslipidemia, fatty liver disease, fertility complications, glucose intolerance, gout, hypertension, hypothyroidism, hyperinsulinemia, insulin resistance, osteoarthritis, polycystic ovary syndrome (PCOS), pregnancy complications, psychological disorders, sleep apnea and other respiratory problems, stress urinary incontinence stroke, T2DM, uric acid nephrolithiasis (kidney stones), and cancer of the breast, colon, endometrium, esophagus, gall bladder, kidney, prostate and rectum.

[0049] As used herein, “overweight” means a condition in which an individual has a BMI that is about 25.0 kg/m^2 to $<30 \text{ kg/m}^2$. *See, id.*

[0050] As used herein, “PYY” means Peptide YY obtained or derived from any species, such as a mammalian species, especially a human. PYY includes both the native PYY (*i.e.*, full-length) and variations thereof (*i.e.*, additions, deletions and/or substitutions of native PYY). Specific PYYs include, but are not limited to, native, human PYY₁₋₃₆ (SEQ ID NO:1) and native, human PYY₃₋₃₆ (SEQ ID NO:2).

[0051] As used herein, “PYY analog” or “PYY analogs” means a PYY-like peptide or polypeptide that elicits one or more effects of native PYY at one or more NPY receptors such as the NPY2 receptor. In some instances, the PYY analogs herein can bind to a NPY receptor, especially the human NPY2 receptor, with higher or lower affinity but

demonstrate a longer half-life *in vivo* or *in vitro* when compared to native PYY, especially human PYY such as native, human PYY₁₋₃₆ (SEQ ID NO:1) and native, human PYY₃₋₃₆ (SEQ ID NO:2). In this manner, the PYY analogs herein are synthetic compounds that act as NPY2 receptor agonists.

[0052] As used herein, “saturated” means the fatty acid contains no carbon-carbon double or triple bonds.

[0053] As used herein, “sensitizing effect” means an ability of the PYY analogs herein to increase the effect of insulin and thereby help lower blood glucose.

[0054] As used herein, “treating” or “to treat” means attenuating, restraining, reversing, slowing or stopping progression or severity of an existing condition, disease, disorder or symptom.

[0055] Certain abbreviations are defined as follows: “ACR” refers to urine albumin/urine creatinine ratio; “amu” refers to atomic mass unit; “tBoc” refers to tert-butoxycarbonyl; “cAMP” refers to cyclic adenosine monophosphate; “DMF” refers to dimethylformamide; “DMSO” refers to dimethyl sulfoxide; “EIA/RIA” refers to enzyme immunoassay/radioimmunoassay; “hr” refers to hour; “HTRF” refers to homogenous time-resolved fluorescent; “IV” refers to intravenous; “kDa” refers to kilodaltons; “LC-MS” refers to liquid chromatography-mass spectrometry; “MS” refers to mass spectrometry; “OtBu” refers to O-tert-butyl; “Pbf” refers to NG-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; “RP-HPLC” refers to reversed-phase high performance liquid chromatography; “SQ” refers to subcutaneous; “SEM” refers to standard error of the mean; “TFA” refers to trifluoroacetic acid; and “Trt” refers to Trityl.

[0056] *PYY Analogs*

[0057] The PYY analogs herein have structural similarities to, but many structural differences, from native PYY peptides. For example, when compared to native, human PYY₁₋₃₆ (SEQ ID NO:1) and/or native, human PYY₃₋₃₆ (SEQ ID NO:2), the PYY analogs described herein include modifications at one or more of positions 3, 7, 9, 10, 17, 18, 22, 23, 30 and 31 with respect to the numbering of native, human PYY₁₋₃₆ (SEQ ID NO:1). In certain instances, exemplary amino acid sequences of the PYY analogs herein include (specific changes relative to corresponding residue of native, human PYY (SEQ ID NO:1) are in bold):

³PKPEX₇PX₉X₁₀DASPEEX₁₇X₁₈RYYX₂₂X₂₃LRHYLN_{X30}LTRQRY³⁶ (SEQ ID NO:3),
³PKPEKPGEDASPEEWQRYYAELRHYLNWLTRQRY³⁶ (SEQ ID NO:4),
³PKPEKPGEDASPEEWQRYYAELRHYLNELTRQRY³⁶ (SEQ ID NO:5),
³PKPEKPEEDASPEEWQRYYIELRHYLNWLTRQRY³⁶ (SEQ ID NO:6),
³PKPEKPGKDASPEEWNRYYADLRHYLNWLTRQRY³⁶ (SEQ ID NO:7), and
³PKPEKPGEDASPEELQRYYASLRHYLNWLTRQRY³⁶ (SEQ ID NO:8).

[0058] The PYY analogs herein result in sufficient activity at the human NPY2 receptor but insufficient activity at the NPY1, NPY4 and NPY5 receptors. Likewise, the PYY analogs herein have beneficial attributes relevant to their developability as therapeutic treatments, including improved solubility in aqueous solutions, improved chemical and physical formulation stability, extended pharmacokinetic profile, and minimized potential for immunogenicity.

[0059] In some instances, the PYY analogs herein are amidated at a C-terminal amino acid to affect stability. In addition to the changes described herein, the analogs may include one or more additional amino acid modifications, provided, however, that the analogs remain capable of binding to and activating the human NPY2 receptor.

[0060] The PYY analogs herein further include a fatty acid conjugated, for example, by way of a linker to a natural or unnatural amino acid with a functional group available for conjugation (*i.e.*, “acylation”). In some instances, the amino acid with a functional group available for conjugation can be C, D, E, K and Q. In particular instances, the amino acid with the functional group available for conjugation is K, where conjugation is to an ϵ -amino group of a K side chain.

[0061] Here, acylation of the PYY analogs is at position 7 when compared to native, human PYY₁₋₃₆ (SEQ ID NO:1). In this manner, the fatty acid can act as an albumin binder to provide for longer-acting analogs.

[0062] With respect to the fatty acid, it can be chemically conjugated to the functional group of the amino acid available for conjugation either by a direct bond or by a linker. The length and composition of the fatty acid impacts the half-life of the PYY analogs, the *in vivo* potency of the PYY analogs, and the solubility and stability of the PYY analogs. Conjugation to a C₁₆-C₂₂ saturated fatty monoacid or diacid thereby results in PYY analogs that exhibit desirable half-life, desirable *in vivo* potency, and desirable solubility and stability characteristics.

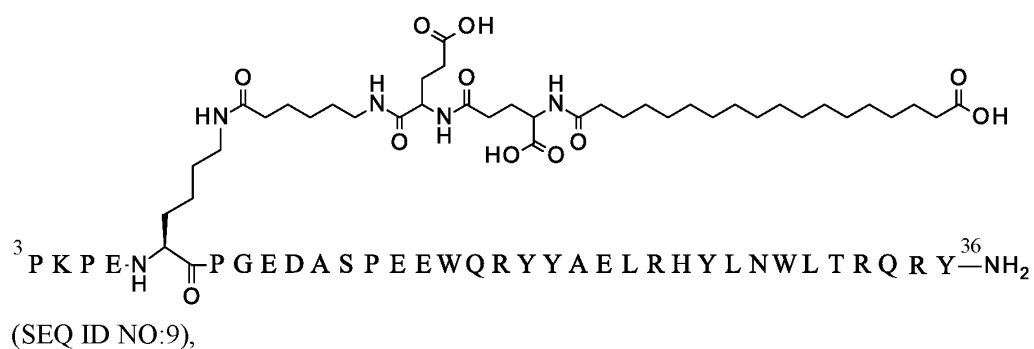
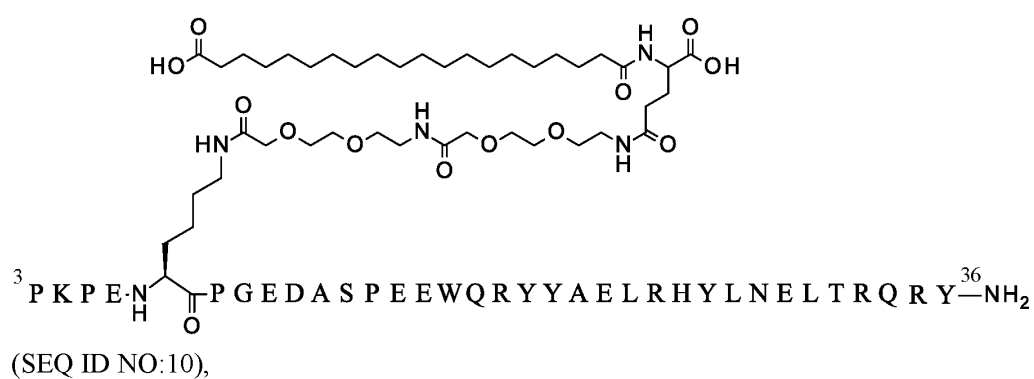
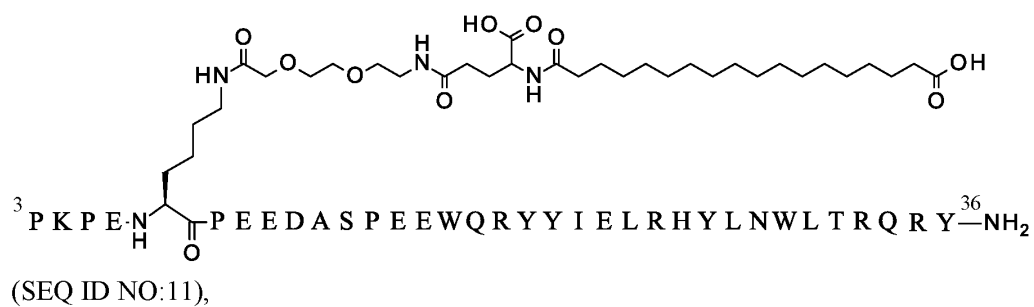
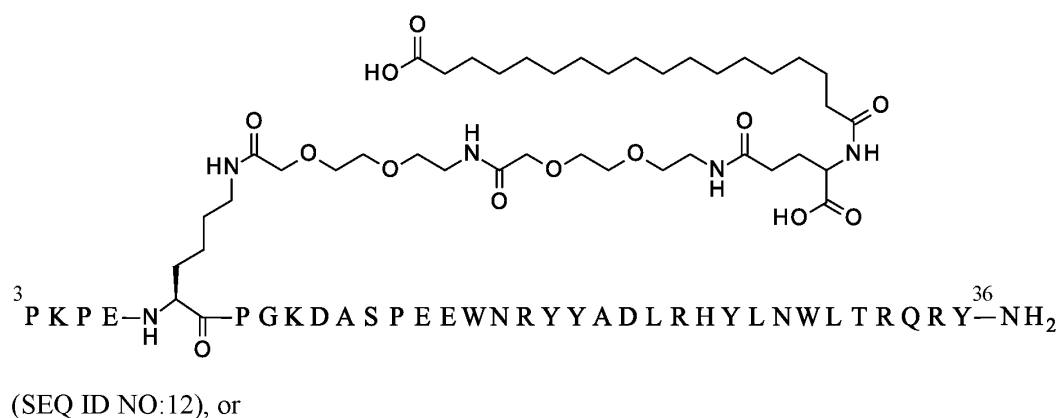
[0063] Exemplary saturated C₁₆-C₂₂ fatty acids for use herein include, but are not limited to, hexadecanoic acid (*i.e.*, palmitic acid, C₁₆ monoacid), hexadecanedioic acid (C₁₆ diacid), heptadecanoic acid (*i.e.*, margaric acid, C₁₇ monoacid), heptadecanedioic acid (C₁₇ diacid), stearic acid (C₁₈ monoacid), octadecanedioic acid (C₁₈ diacid), nonadecylic acid (*i.e.*, nonadecanoic acid, C₁₉ monoacid), nonadecanedioic acid (C₁₉ diacid), eicosanoic acid (*i.e.*, arachidic acid, C₂₀ monoacid), eicosanedioic acid (C₂₀ diacid), heneicosanoic acid (*i.e.*, heneicosylic acid, C₂₁ monoacid), heneicosanedioic acid (C₂₁ diacid), docosanoic acid (*i.e.*, behenic acid, C₂₂ monoacid), docosanedioic acid (C₂₂ diacid), and branched and substituted derivatives thereof. In certain instances, the C₁₆-C₂₂ fatty acid can be a saturated C₁₈ monoacid, a saturated C₁₈ diacid, a saturated C₁₉ monoacid, a saturated C₁₉ diacid, a saturated C₂₀ monoacid, a saturated C₂₀ diacid, and branched and substituted derivatives thereof. In particular instances, the C₁₆-C₂₂ fatty acid can be palmitic acid or hexadeconic acid, stearic acid or octadeconic acid, or arachidic acid or eicosanoic acid.

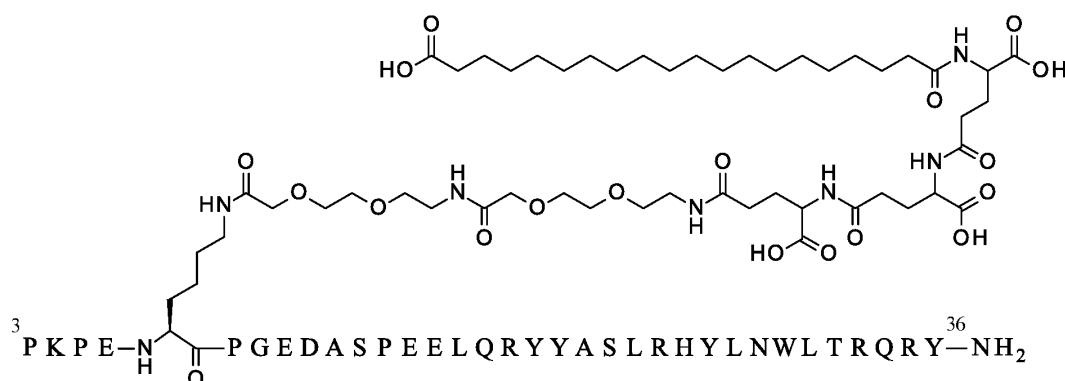
[0064] To assist in conjugating a fatty acid to a natural or unnatural amino acid with the functional group available for conjugation, the PYY analogs herein can include a linker. In some instances, the linker can be at least one of AEEA, Ahx, E or γ E, as well as combinations thereof.

[0065] When the linker includes the amino acids, it can have one to four E or γ E amino acid residues. In some instances, the linker can include one or two E and/or γ E amino acid residues. For example, the linker can include either one or two E and/or γ E amino acid residues. In other instances, the linker can include one to four amino acid residues (such as, for example, E or γ E amino acids) used in combination with AEEA or Ahx. Specifically, the linker can be combinations of E and γ E amino acid residues with AEEA or Ahx. In still other instances, the linker can be combinations of one or two γ E amino acid residues and one or two AEEA or Ahx. In particular instances, the linker can be an (AEEA)₂• γ E moiety, an Ahx•E• γ E moiety, or an AEEA• γ E moiety.

[0066] Exemplary linker-fatty acid moieties can include (AEEA)₂• γ E•C₂₀ diacid, Ahx•E• γ E•C₁₈ diacid, or AEEA• γ E•C₁₈ diacid. The structural features of these linker-fatty acid moieties result in analogs having improved half-life when compared to native, human PYY₁₋₃₆ (SEQ ID NO:1) or native, human PYY₃₋₃₆ (SEQ ID NO:2).

[0067] Taken together, exemplary PYY analogs are:





(SEQ ID NO:13).

[0068] Although the PYY analogs are described as having thirty-four amino acids like that of native, human PYY₃₋₃₆ (SEQ ID NO:2), it is contemplated that the PYY analogs herein can have an amino acid sequence based upon native, human PYY₁₋₃₆ (SEQ ID NO:1). That is, the PYY analogs can include the two N-terminal amino acids (*i.e.*, the “YP” residues of positions 1 and 2 of SEQ ID NO:1) of native, human PYY₁₋₃₆ (SEQ ID NO:1), which subsequently can be cleaved *in vivo* when administered to an individual as would occur when native, human PYY₁₋₃₆ (SEQ ID NO:1) is endogenously released.

[0069] Half-life of the PYY analogs herein may be measured using techniques known in the art including, for example, those described in the Examples below. Likewise, affinity of the PYY analogs herein for each of the various human NPY receptors (*e.g.*, NPY2R, NPY5R) may be measured using techniques known in the art for measuring receptor binding levels including, for example, those described in the Examples below, and is commonly expressed as an inhibitory constant (K_i) value. Moreover, activity of the PYY analogs herein at each of the receptors also may be measured using techniques known in the art, including, for example, the *in vitro* activity assays described below, and is commonly expressed as an EC_{50} value.

[0070] As a result of the modifications described above, the PYY analogs herein have a half-life that is longer than that of native, human PYY₃₋₃₆ (SEQ ID NO:2). For example, the PYY analogs can have a half-life from about 5 hours to about 24 hours, from about 6 hours to about 23 hours, from about 7 hours to about 22 hours, from about 8 hours to about 21 hours, from about 9 hours to about 20 hours, from about 10 hours to about 19 hours, from about 11 hours to about 18 hours, from about 12 hours to about 17 hours, from about 13 hours to about 16 hours, or even from about 14 hours to about 15 hours. Alternatively,

the PYY analogs herein can have a half-life that is about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, or even about 24 hours, especially about 12 hours.

[0071] Likewise, the PYY analogs herein have a binding affinity at the NPY2 receptor that is greater than that of native, human PYY₃₋₃₆ (SEQ ID NO:2), such as from about 2-fold to about 10-fold. Alternatively, the PYY analogs herein can have a binding affinity up to about 2-fold greater, about 3-fold greater, about 4-fold greater, about 5-fold greater, about 6-fold greater, about 7 fold-greater, about 8-fold greater, about 9-fold greater, or even about 10-fold greater, especially 2-fold greater to 3-fold greater, than that of native, human PYY₃₋₃₆ (SEQ ID NO:2).

[0072] *Pharmaceutical Compositions*

[0073] The PYY analogs herein can be formulated as pharmaceutical compositions, which can be administered by parenteral routes (*e.g.*, intravenous, intraperitoneal, intramuscular, subcutaneous or transdermal). Such pharmaceutical compositions and techniques for preparing the same are well known in the art. *See, e.g.*, Remington, "The Science and Practice of Pharmacy" (D.B. Troy ed., 21st Edition, Lippincott, Williams & Wilkins, 2006). In particular instances, the PYY analogs are administered SQ. Alternatively, however, the PYY analogs can be formulated in forms for other pharmaceutically acceptable routes such as, for example, tablets or other solids for oral administration; time release capsules; and any other form currently used, including creams, lotions, inhalants and the like.

[0074] To improve their *in vivo* compatibility and effectiveness, the PYY analogs herein may be reacted with any of a number of inorganic and organic acids/bases to form pharmaceutically acceptable acid/base addition salts. Pharmaceutically acceptable salts and common methodologies for preparing them are well known in the art (*see, e.g.*, Stahl *et al.*, "Handbook of Pharmaceutical Salts: Properties, Selection and Use," 2nd Revised Edition (Wiley-VCH, 2011)). Pharmaceutically acceptable salts for use herein include sodium, trifluoroacetate, hydrochloride and acetate salts.

[0075] The PYY analogs herein may be administered by a physician or self-administered using an injection. It is understood the gauge size and amount of injection volume can be readily determined by one of skill in the art. However, the amount of injection volume can be $\leq$ about 2 mL or even $\leq$ about 1 mL, and the needle gauge can be $\geq$ about 27 G or even $\geq$ about 29 G.

[0076] The disclosure also provides and therefore encompasses novel intermediates and methods useful for synthesizing the PYY analogs herein, or a pharmaceutically acceptable salt thereof. The intermediates and PYY analogs herein can be prepared by a variety of methodologies that are well known in the art. For example, a method using chemical synthesis is illustrated in the Examples below. The specific synthetic steps for each of the routes described may be combined in different ways to prepare the PYY analogs described herein. The reagents and starting materials are readily available to one of skill in the art.

[0077] The PYY analogs herein are generally effective over a wide dosage range. Exemplary doses of the PYY analogs herein or of pharmaceutical compositions including the same can be milligram (mg), microgram (μ g), nanogram (ng) or picogram (pg) amounts per kilogram (kg) of an individual. In this manner, a daily dose can be from about 1 μ g to about 100 mg.

[0078] Here, the effective amount of the PYY analog in a pharmaceutical composition can be a dose of about 0.25 mg to about 5.0 mg. One of skill in the art, however, understands that in some instances the effective amount (*i.e.*, dose/dosage) may be below the lower limit of the aforesaid range and be more than adequate, while in other cases the effective amount may be a larger doses and may be employed with acceptable side effects.

[0079] In addition to the PYY analog, the pharmaceutical composition also can include an additional therapeutic agent, especially other antidiabetic or weight loss agents. In some instances, the additional therapeutic agent can be at least one of an incretin or a DPP-IV inhibitor. Exemplary incretins include, but are not limited to, GCG, GLP-1, GLP-1 (7-36)_{amide}, GIP, OXM, a GCG analog, a GLP-1 analog, a GIP analog, an OXM analog, a GIP/GLP-1, a GLP-1/GCG, or even an incretin analog having triple receptor activity.

[0080] In this manner, the pharmaceutical composition can include an effective amount of a PYY analog of SEQ ID NO:9 and an incretin or a DPP-IV inhibitor, an effective amount of a PYY analog of SEQ ID NO:10 and an incretin or a DPP-IV inhibitor, an effective amount of a PYY analog of SEQ ID NO:11 and an incretin or a DPP-IV inhibitor,

an effective amount of a PYY analog of SEQ ID NO:12 and an incretin or a DPP-IV inhibitor, or an effective amount of a PYY analog of SEQ ID NO:13 and an incretin or a DPP-IV inhibitor.

[0081] In those instances in which the incretin is GLP-1 or a GLP-1 analog, it can be GLP-1 or a GLP-1 analog such as albiglutide, dulaglutide, liraglutide, semaglutide, or combinations thereof, especially dulaglutide.

[0082] *Methods of Making and Using the PYY Analogs*

[0083] The PYY analogs herein can be synthesized via any number of peptide synthesis methods known in the art using standard manual or automated solid-phase synthesis procedures. Automated peptide synthesizers are commercially available from, for example, Applied Biosystems (Foster City, CA) and Protein Technologies Inc. (Tucson, AZ). Reagents for solid-phase synthesis are readily available from commercial sources. Solid-phase synthesizers can be used according to the manufacturer's instructions for blocking interfering groups, protecting amino acids during reaction, coupling, deprotecting and capping of unreacted amino acids.

[0084] Typically, an N- α -carbamoyl-protected amino acid and the N-terminal amino acid on the growing peptide chain attached to a resin are coupled at room temperature in an inert solvent such as DMF, N-methylpyrrolidone or methylene chloride in the presence of coupling agents such as diisopropyl-carbodiimide and 1-hydroxybenzotriazole. The N- α -carbamoyl protecting group is removed from the resulting peptide resin using a reagent such TFA or piperidine, and the coupling reaction is repeated with the next desired N- α -protected amino acid to be added to the peptide chain. Suitable amine protecting groups are well known in the art and are described, for example, in Green & Wuts, "Protecting Groups in Organic Synthesis," (John Wiley and Sons, 1991). The most commonly used examples include tBoc and Fmoc. After completion of synthesis, peptides are cleaved from the solid-phase support with simultaneous side chain deprotection using standard treatment methods under acidic conditions.

[0085] One of skill in the art will appreciate that the peptide chains described herein are synthesized with a C-terminal carboxamide. For the synthesis of C-terminal amide peptides, resins incorporating Rink amide MBHA or Rink amide AM linkers typically are used with Fmoc synthesis, while MBHA resin is generally used with tBoc synthesis.

[0086] Crude peptides typically are purified using RP-HPLC on C8 or C18 columns using water-acetonitrile gradients in 0.05 % to 0.1 % TFA. Purity can be verified by analytical RP-HPLC. Identity of peptides can be verified by MS. Peptides can be solubilized in aqueous buffers over a wide pH range.

[0087] One use of the PYY analogs herein is for reducing blood glucose and/or body weight in individuals, especially individuals who are overweight or obese and have T2DM. Administering a PYY analog as described herein can result in glycemic control by improving insulin sensitization and weight loss. As such, the PYY analogs herein show glucose lowering efficacy with the added benefit of weight reduction, such that individuals can delay progression to insulin and maintain target HbA1c goals.

[0088] The methods can include the steps described herein, and these maybe be, but not necessarily, carried out in the sequence as described. Other sequences, however, also are conceivable. Moreover, individual or multiple steps may be carried out either in parallel and/or overlapping in time and/or individually or in multiply repeated steps. Furthermore, the methods may include additional, unspecified steps.

[0089] Such methods therefore can include selecting an individual who is overweight and has T2DM or is predisposed to the same. Alternatively, the methods can include selecting an individual who is obese and has T2DM or is predisposed to the same.

[0090] The methods also can include administering to the individual an effective amount of at least one PYY analog as described herein, which may be in the form of a pharmaceutical composition as also described herein. In some instances, the at least one PYY analog/pharmaceutical composition can include an additional therapeutic agents such as an incretin or a DPP-IV inhibitor.

[0091] The concentration/dose/dosage of the at least one PYY analog and optional incretin or DPP-IV inhibitor are discussed elsewhere herein.

[0092] With regard to a route of administration, the at least one PYY analog or pharmaceutical composition including the same can be administered in accord with known methods such as, for example, orally; by injection (*i.e.*, intra-arterially, intravenously, intraperitoneally, intracerebrally, intracerebroventricularly, intramuscularly, intraocularly, intraportally or intralesionally); by sustained release systems, or by implantation devices. In certain instances, the at least one PYY analog or pharmaceutical composition including the same can be administered SQ by bolus injection or continuously.

[0093] With regard to a dosing frequency, the at least one PYY analog or pharmaceutical composition including the same can be administered daily, every other day, three times a week, two times a week, one time a week (*i.e.*, weekly), biweekly (*i.e.*, every other week), or monthly. In certain instances, the at least one PYY analog or pharmaceutical composition including the same is administered SQ every other day, SQ three times a week, SQ two times a week, SQ one time a week, SQ every other week or SQ monthly. In particular instances, the at least one PYY analog or pharmaceutical composition including the same is administered SQ one time a week (QW).

[0094] Alternatively, the at least one PYY analog or pharmaceutical composition including the same can be orally administered. As above, and with regard to dosing frequency, the at least one PYY analog or pharmaceutical composition including the same can be administered daily, every other day, three times a week, two times a week, one time a week (*i.e.*, weekly), biweekly (*i.e.*, every other week), or monthly. In certain instances, the at least one PYY analog or pharmaceutical composition including the same is administered orally every other day, orally three times a week, orally two times a week, orally one time a week, or orally every other week. In particular instances, the PYY analog is administered orally one time a week.

[0095] With regard to those instances in which the at least one PYY analog or pharmaceutical composition including the same is administered in combination with an effective amount of an incretin, the incretin can be GCG or a GCG analog, GLP-1, GLP-1 (7-36)_{amide} or a GLP-1 analog, GIP or a GIP analog, OXM or an OXM analog, GIP/GLP-1, GLP-1/GCG, or even an incretin having triple receptor activity. The GCG, GCG analog, GLP-1, GLP-1 (7-36)_{amide}, GLP-1 analog, GIP, GIP analog, OXM, OXM analog, GIP/GLP-1, GLP-1/GCG, or incretin having triple receptor activity can be administered simultaneously, separately or sequentially with the at least one PYY analog or pharmaceutical composition including the same.

[0096] Moreover, the GCG, GCG analog, GLP-1, GLP-1 (7-36)_{amide}, GLP-1 analog, GIP, GIP analog, OXM, OXM analog, GIP/GLP-1, GLP-1/GCG, or incretin having triple receptor activity can be administered with a frequency same as the at least one PYY analog or pharmaceutical composition including the same (*i.e.*, every other day, twice a week, or even weekly). Alternatively, the GCG, GCG analog, GLP-1, GLP-1 (7-36)_{amide}, GLP-1 analog, GIP, GIP analog, OXM, OXM analog, GIP/GLP-1, GLP-1/GCG, or incretin having

triple receptor activity can be administered with a frequency distinct from the at least one PYY analog or pharmaceutical composition including the same. In other instances, the GCG, GCG analog, GLP-1, GLP-1 (7-36)_{amide}, GLP-1 analog, GIP, GIP analog, OXM, OXM analog, GIP/GLP-1, GLP-1/GCG, or incretin having triple receptor activity is administered QW. In still other instances, the PYY analog is administered SQ, and the GCG, GCG analog, GLP-1, GLP-1 (7-36)_{amide}, GLP-1 analog, GIP, GIP analog, OXM, OXM analog, GIP/GLP-1, GLP-1/GCG, or incretin having triple receptor activity can be administered orally.

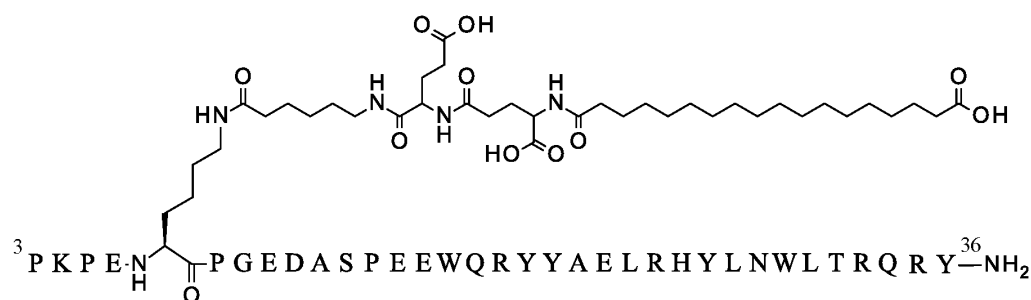
[0097] It is further contemplated that the methods may be combined with diet and exercise and/or may be combined with additional therapeutic agents other than those discussed above.

EXAMPLES

[0098] The following non-limiting examples are offered for purposes of illustration, not limitation.

[0099] Example 1: PYY Analog 1.

[0100] One PYY analog incorporating the inventive concept can have a structure of:



(SEQ ID NO:9).

[0101] Here, the N-terminus is free, and the C-terminal amino acid is amidated as a C-terminal primary amide. The K at position 7 is chemically modified through conjugation to the ϵ -amino group of the K side chain at position 7 with (Ahx-E-(γ E)-CO-(CH₂)₁₆-COOH.

[0102] The PYY analog according to SEQ ID NO:9 is generated by solid-phase peptide synthesis using Fmoc/t-Bu strategy on a SymphonyX Automated Peptide Synthesizer (PTI

Protein Technologies Inc.) starting from RAPP AM-Rink Amide resin (H40023 Polystyrene AM RAM, Rapp polymere GmbH). Amino acid couplings are performed using 10 equivalents of amino acid, 0.9 M diisopropylcarbodiimide (DIC) and 0.9 M Oxyma (1:1:1 molar ratio) in DMF for 3h at 25°C. Deprotections are carried out using 25% piperidine solutions in DMF.

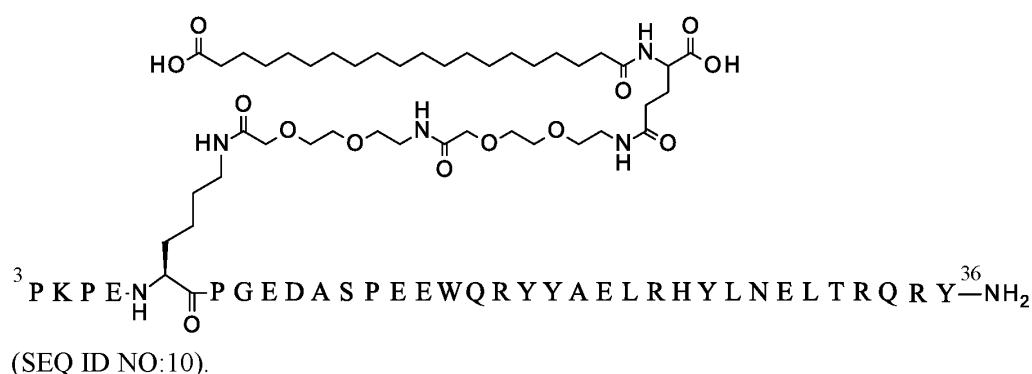
[0103] After elongating the peptide-resin as described above, the MTT protecting group present in K at position 7 is removed using 30% Hexafluoroisopropanol (HFIP) in dichloromethane (DCM). Additional coupling/deprotection cycles using a Fmoc/t-Bu strategy to extend the K at position 7 side chain involve Fmoc-6-aminohexanoic acid (Chem-Impex International Catalog#02490), Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OH)-OtBu (ChemPep Catalog #100703) and HOOC-(CH₂)₁₆-COOtBu. In all couplings, 3 equivalents of the building block are used with PyBOP (3 equiv) and DIEA (6 equiv) in DMF for 3 h at 25°C.

[0104] Concomitant cleavage from the resin and side chain protecting group removal are carried out in a solution containing TFA : triisopropylsilane : 1,2-ethanedithiol : methanol : thioanisole 80:5:5:5:5 (v/v) for 2 h at 25°C followed by precipitation with cold ether. Crude peptide is purified to >99% purity (15-20% purified yield) by RP-HPLC on a Phenyl Hexyl Column (Phenomenex, Luna; 5 µm, 100Å), where suitable fractions are pooled and lyophilized.

[0105] The purity of the PYY analog is examined by analytical RP-HPLC, and identity is confirmed using LC/MS (observed: $M+3H^+/3 = 1659.2$ (+/-0.2); calculated: $M+3H^+/3 = 1659.2$; observed: $M+4H^+/4 = 1244.6$ (+/-0.2); calculated: $M+4H^+/4 = 1244.6$; observed: $M+5H^+/5 = 995.9$ (+/-0.2); calculated: $M+5H^+/5 = 995.9$).

[0106] Example 2: PYY Analog 2.

[0107] One PPY analog incorporating the inventive concept can have a structure of:



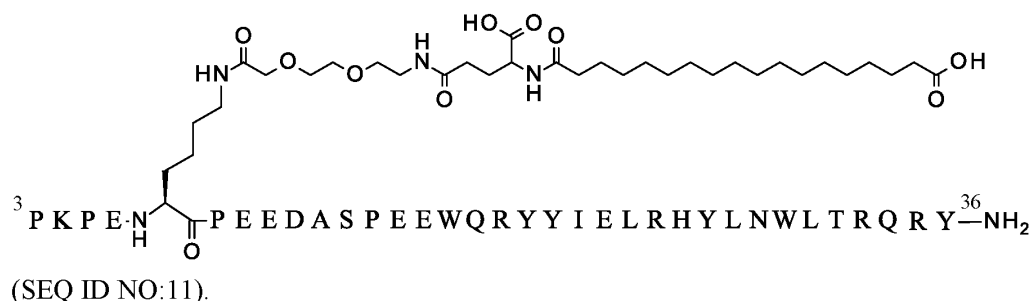
[0108] As in Example 1, the N-terminus is free, and the C-terminal amino acid is amidated as a C-terminal primary amide. In contrast, however, the K at position 7 is chemically modified through conjugation to the ϵ -amino group of the K side chain with ([2-(2-amino-ethoxy)-ethoxy]-acetyl)₂-(γ E)-CO-(CH₂)₁₈-COOH.

[0109] The PYY analog according to SEQ ID NO:10 is generated by solid-phase peptide, similar to that described above in Example 1. Thus, Fmoc-NHPEG₂-CH₂COOH and HOOC-(CH₂)₁₈-COOtBu is attached to the side chain after MTT cleavage using 3 equivalents of the building block with PyBOP (3 equiv) and DIEA (6 equiv) in DMF for 3 h at 25°C.

[0110] The purity of the PYY analog is examined by analytical RP-HPLC, and identity is confirmed using LC/MS (observed: $M+3H^+/3 = 1665.4$ (+/-0.2); calculated: $M+3H^+/3 = 1665.5$; observed: $M+4H^+/4 = 1249.3$ (+/-0.2); calculated $M+4H^+/4 = 1249.4$; observed: $M+5H^+/5 = 999.7$ (+/-0.2); calculated: $M+5H^+/5 = 999.7$).

[0111] Example 3: PYY Analog 3.

[0112] One PYY analog incorporating the inventive concept can have a structure of:



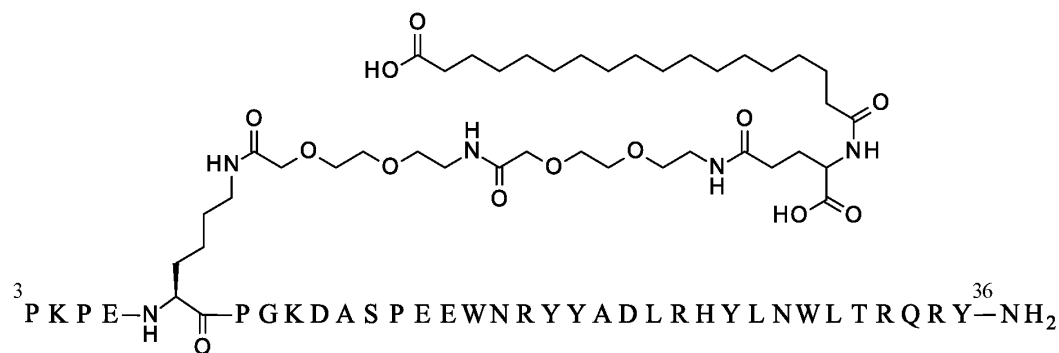
[0113] As in Example 1, the N-terminus is free, and the C-terminal amino acid is amidated as a C-terminal primary amide. In contrast, however, the K at position 7 is chemically modified through conjugation to the ϵ -amino group of the K side chain with ([2-(2-amino-ethoxy)-ethoxy]-acetyl)-(γ E)-CO-(CH₂)₁₆-COOH.

[0114] The PYY analog according to SEQ ID NO:11 is generated by solid-phase peptide, similar to that as described above in Example 1. Thus, Fmoc-NHPEG₂-CH₂COOH and HOOC-(CH₂)₁₆-COOtBu are attached to the side chain after MTT cleavage using 3 equivalents of the building block with PyBOP (3 equiv) and DIEA (6 equiv) in DMF for 3 h at 25°C.

[0115] The purity of the PYY analog is examined by analytical RP-HPLC, and identity is confirmed using LC/MS (observed: $M+3H^+/3 = 1664.7$ (+/-0.2); calculated: $M+3H^+/3 = 1664.9$; observed: $M+4H^+/4 = 1248.9$ (+/-0.2); calculated: $M+4H^+/4 = 1248.9$; observed: $M+5H^+/5 = 999.3$ (+/-0.2); calculated: $M+5H^+/5 = 999.3$).

[0116] Example 4: PYY Analog 4.

[0117] One PYY analog incorporating the inventive concept can have a structure of:



(SEQ ID NO:12).

[0118] As in Example 1, the N-terminus is free, and the C-terminal amino acid is amidated as a C-terminal primary amide. In contrast, however, the K at position 7 is chemically modified through conjugation to the ϵ -amino group of the K side chain with ([2-(2-amino-ethoxy)-ethoxy]-acetyl)-(γ E)-CO-(CH₂)₁₆-COOH.

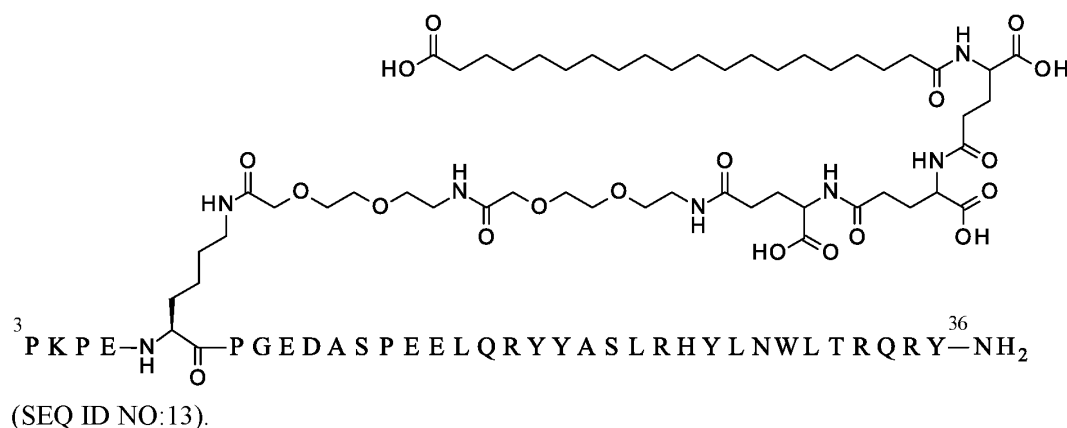
[0119] The PYY analog according to SEQ ID NO:12 is generated by solid-phase peptide, similar to that as described above in Example 1. Thus, Fmoc-NHPEG₂-CH₂COOH and HOOC-(CH₂)₁₆-COOtBu are attached to the side chain after MTT cleavage using 3

equivalents of the building block with PyBOP (3 equiv) and DIEA (6 equiv) in DMF for 3 h at 25°C.

[0120] The purity of the PYY analog is examined by analytical RP-HPLC, and identity is confirmed using LC/MS (observed: $M+3H^+/3 = 1664.8$ (± 0.2); calculated: $M+3H^+/3 = 1665.5$; observed: $M+4H^+/4 = 1248.9$ (± 0.2); calculated: $M+4H^+/4 = 1249.4$; observed: $M+5H^+/5 = 998.9$ (± 0.2); calculated: $M+5H^+/5 = 995.7$).

[0121] Example 5: PYY Analog 5.

[0122] One PYY analog incorporating the inventive concept can have a structure of:



[0123] As in Example 1, the N-terminus is free, and the C-terminal amino acid is amidated as a C-terminal primary amide. In contrast, however, the K at position 7 is chemically modified through conjugation to the ϵ -amino group of the K side chain with $[(2-(2\text{-amino-ethoxy})\text{-ethoxy})\text{-acetyl}]_2\text{-(}\gamma\text{E)}_3\text{-CO-(CH}_2\text{)}_{18}\text{-COOH}$.

[0124] The PYY analog according to SEQ ID NO:13 is generated by solid-phase peptide, similar to that as described above in Example 1. Thus, Fmoc-NHPEG₂-CH₂COOH and HOOC-(CH₂)₁₈-COOtBu are attached to the side chain after MTT cleavage using 3 equivalents of the building block with PyBOP (3 equiv) and DIEA (6 equiv) in DMF for 3 h at 25°C.

[0125] The purity of the PYY analog is examined by analytical RP-HPLC, and identity is confirmed using LC/MS (observed: $M+3H^+/3 = 1732.2$ (± 0.2); calculated: $M+3H^+/3 = 1732.3$; observed: $M+4H^+/4 = 1299.4$ (± 0.2); calculated: $M+4H^+/4 = 1299.5$; observed: $M+5H^+/5 = 1039.7$ (± 0.2); calculated: $M+5H^+/5 = 1039.8$).

[0126] Example 6: *In Vitro* Activity of PYY Analogs.

[0127] (1) *In vitro* binding to hNPY1, hNPY2, hNPY4, and hNPY5 receptors

[0128] Purpose:

[0129] To assess the *in vitro* binding affinity (K_i) of the PYY analogs of Examples 1 to 5 in the absence of bovine serum albumin (BSA) to the following human (h) receptors: hNPY1R, hNPY2R, hNPY4R and hNPY5R. Competitive radioligand binding assays with membranes prepared from cell lines overexpressing each of the recombinant receptors and the relevant [^{125} I]-labeled peptides are used in a scintillation proximity assay (SPA) method. The binding affinity for the associated native peptides, PYY₁₋₃₆ (SEQ ID NO:1), PYY₃₋₃₆ (SEQ ID NO:2) and Pancreatic Polypeptide₁₋₃₆ (PP₁₋₃₆; SEQ ID NO:14), is determined in each assay as a control.

[0130] Methods:

[0131] PYY analogs, native, human PYY₁₋₃₆ and control PYY₃₋₃₆ are synthesized at Lilly Research Laboratories (Indianapolis, IN, USA) and are characterized by LC/MS, NMR, and LC/UV analysis (99.5% purity). Peptide contents are estimated at 80% powder mass. The peptides are prepared as 10 mM stock solution in 100% DMSO and kept frozen at -20°C until just prior to testing in the assays.

[0132] For hNPY1R, transient overexpression is performed using CHO cells. Stably transfected cell lines are prepared for hNPY2R and hNPY4R by subcloning receptor cDNA into pcDNA3.1 expression plasmid and transfecting into human embryonic kidney (HEK) 293 cells followed by selection with Geneticin. hNPY5R cloning is performed at Multispan, Inc. (Hayward, CA).

[0133] For the preparation of hNPY1R, hNPY2R, mNPY2R and hNPY4R crude cell membranes, two different methods (described below) are utilized. hNPY5R membranes are purchased from Multispan, Inc. (#MCG1275).

[0134] Method 1 – For hNPY2R and hNPY4R membranes, frozen cell pellets are lysed on ice in 10 mL hypotonic homogenization buffer containing 50 mM Tris HCl, pH 7.5, and Roche Complete™ Protease Inhibitors with EDTA (#1169749001) per gram of wet cell paste. The cell suspension is disrupted using a glass Potter-Elvehjem homogenizer fitted with a Teflon® pestle for 25 strokes. The homogenate is centrifuged at 4°C at 1100 x g for 10 minutes. The supernatant is collected and stored on ice while the pellets are resuspended

in homogenization buffer and rehomogenized as described above. The homogenate is centrifuged at 1100 x g for 10 minutes. The second supernatant is combined with the first supernatant and centrifuged at 35000 x g for 1 hour at 4°C. The resulting membrane pellet is resuspended in homogenization buffer containing protease inhibitors at approximately 1 to 3 mg/mL, quick frozen in liquid nitrogen, and stored as aliquots in a -80°C freezer until use. Protein concentration is determined using a BCA protein assay kit (Pierce, #23225) with BSA as a standard.

[0135] Method 2 – For hNPY1R membranes, frozen cell pellets are lysed on ice in 5 mL hypotonic homogenization Buffer containing 25 mM Tris HCl, pH 7.5, 1 mM MgCl₂, 25 units/mL DNase I (Invitrogen, #18047-019) and Roche Complete™ Protease Inhibitors without EDTA (#11836170001) per gram of wet cell paste. The cell suspension is disrupted using a glass Potter-Elvehjem homogenizer fitted with a Teflon® pestle for 25 strokes. The homogenate is centrifuged at 4°C at 1800 x g for 15 minutes in a 50 mL conical tube. The supernatant is collected and stored on ice while the pellets are resuspended in homogenization buffer and rehomogenized as described above, except DNase I is not used in the homogenization buffer. The homogenate is centrifuged at 1800 x g for 15 minutes. The second supernatant is combined with the first supernatant and centrifuged at 25000 x g for 30 minutes at 4°C. The resulting membrane pellet is resuspended in homogenization buffer containing protease inhibitors at approximately 2 mg/mL, aliquoted, and stored in a -80°C freezer until use. Protein concentration is determined using a BCA protein assay kit (Pierce, #23225) with BSA as a standard.

[0136] General binding assay methods – The equilibrium dissociation constants (K_d) for the various receptor/radioligand interactions are determined from saturation binding analysis using the same reagents and buffers as described below for compound testing. The K_d values determined for the receptor preparations used in this study are as follows: hNPY2R, 0.0047 nM; hNPY1R, 0.07 nM; hNPY4R, 0.084 nM; and hNPY5R, 0.896 nM.

[0137] hNPY1R receptor binding protocol – The receptor binding affinity (K_i) of PYY analog peptides and PYY₁₋₃₆ for hNPY1R is determined from a competitive radioligand binding assay with human recombinant [¹²⁵I]-PYY₁₋₃₆ (#NEX341, 2200 Ci/mmol) obtained from Perkin Elmer (Waltham, MA). The assay is performed with a SPA method using polyvinyltoluene (PVT) wheat germ agglutinin-coupled SPA beads (#RPNQ0001, Perkin Elmer). Assay buffer (25 mM HEPES, pH 7.5, 1 mM MgCl₂, 2.5 mM CaCl₂, and 0.2%

w/v Bacitracin (RPI, #32000)) is used for preparation of reagents. PYY analogs and PYY₁₋₃₆ are thawed and 3-fold serially diluted in 100% DMSO (10 point concentration response curves) using a Tecan Evo liquid handler. A 20-fold step-down dilution of peptide into assay buffer is made to reduce the level of DMSO and peptide concentration prior to addition into the assay plate. Next, 5 μ L serially diluted peptide or DMSO is transferred into a Corning® 3632 clear bottom assay plate containing 45 μ L assay buffer or unlabeled PYY₁₋₃₆ control (nonspecific binding or NSB, at 10 nM final concentration). Then, 50 μ L [¹²⁵I]-PYY₁₋₃₆ (0.05 nM final concentration) and 50 μ L hNPY1R membranes (1.0 μ g/well) are added. The final addition is 50 μ L of WGA SPA beads (50 μ g/well). Final DMSO concentration are 0.125%. Plates are sealed and mixed on a plate shaker (setting 6) for 1 minute and read with a PerkinElmer Trilux MicroBeta® scintillation counter after 10 hours of incubation/bead settling time at room temperature. Final assay concentration ranges for peptides tested in response curves are: PYY analogs (2.5 μ M to 0.13 nM) and PYY₁₋₃₆ (10 nM to 0.5 pM).

[0138] hNPY2 receptor binding protocol – The receptor binding affinity (K_i) of PYY analog peptides and PP₁₋₃₆ for hNPY2R is determined from a competitive radioligand binding assay as described above for hNPY1R. Final assay concentration ranges for peptides tested in response curves are: PYY analogs (0.1 μ M to 5 pM) and PYY₃₋₃₆ (10 nM to 0.5 pM).

[0139] hNPY4R receptor binding protocol – The receptor binding affinity (K_i) of PYY analog peptides and PP₁₋₃₆ for hNPY4R is determined from a competitive radioligand binding assay as described above for hNPY1R. Final assay concentration ranges for peptides tested in response curves are: PYY analogs (2.5 μ M to 0.13 nM) and PYY₁₋₃₆ (10 nM to 0.5 pM).

[0140] hNPY5R receptor binding protocol – The receptor binding affinity (K_i) of PYY analog peptides and PYY₁₋₃₆ for hNPY5R is determined from a competitive radioligand binding assay as described above for hNPY1R. Final assay concentration ranges for peptides tested in response curves are: PYY analogs (1 μ M to 10 pM) and PYY₁₋₃₆ (1 μ M to 10 pM).

[0141] Data analysis for NPY receptor binding assays – Raw count per minute (CPM) data for concentration curves of PYY analogs, PYY₁₋₃₆, PYY₃₋₃₆, or PP₁₋₃₆ are converted to percent specific inhibition by subtracting nonspecific binding (NSB, binding in the

presence of excess unlabeled PYY₁₋₃₆, PYY₃₋₃₆, or PP₁₋₃₆, respectively) from the individual CPM values and dividing by the total binding signal, also corrected by subtracting nonspecific binding, as shown in the equation below:

$$\% \text{ Specific Inhibition} = 100 - \left[\frac{\text{CPM for Analog or Control} - \text{CPM for NSB}}{\text{CPM for Total Binding} - \text{CPM for NSB}} \times 100 \right].$$

[0142] Data are analyzed using four-parameter (curve maximum, curve minimum, IC₅₀, Hill slope) nonlinear regression routines (Genedata Screener, version 13.0.5, Genedata AG, Basal, Switzerland). The affinity (K_i) is calculated from the relative IC₅₀ value based upon the equation $K_i = IC_{50} / (1 + D/K_d)$ where D = the concentration of radioligand in the experiment, IC₅₀ is the concentration causing 50% inhibition of binding, and K_d is the equilibrium binding affinity constant of the radioligand determined from saturation binding analysis (listed above). A qualifier (>) indicates that the data did not reach 50% inhibition, compared to maximum binding in the absence of competitor, whereby the K_i is calculated using the highest concentration of the compound tested in the assay.

[0143] Reported values for K_i are calculated as the geometric mean as shown below:

$$\text{Geometric Mean} = 10^{(\text{Arithmetic Mean of Log}_{10} K_i \text{ Values})}.$$

[0144] Standard error of the mean (SEM) is calculated using the delta method as shown below:

$$\text{SEM} = \text{Geometric Mean} \times \frac{\text{SD of log transformed data}}{\text{Square root of } n} \times \ln(10),$$

where SD is the standard deviation, n is the number of independent runs, and ln(10) is the natural logarithm of 10.

[0145] Selectivity of peptides for hNPY2R (Y2) versus hNPY5R (Y5), hNPY4R (Y4), and/or hNPY1R (Y1) are calculated by dividing by the hNPY2R results in nM.

[0146] Results:

[0147] Table 1: *In Vitro* Binding (K_i) to hNPY1R, hNPY2R, hNPY4R and hNPY5R.

Peptide	hNPY2R (nM)	hNPY5R (nM)	hNPY4R (nM)	hNPY1R (nM)	Fold (Y5/Y2)	Fold (Y4/Y2)	Fold (Y1/Y2)
PP ₁₋₃₆	--	--	0.07	--	--	--	--
PYY ₁₋₃₆	0.007	0.37	5	0.06	52	714	8.6
PYY ₃₋₃₆	0.008	3.6	27	7	450	3375	875
Example 1	0.011	59	626	256	5363	56909	23272
Example 2	0.070	>1000	>1840	>1460	>14285	>26286	>20857
Example 3	0.009	36.4	112	>1550	4044	12444	>193750
Example 4	0.005	ND	280	>148	NA	56000	>29600

Example 5	0.030	ND	560	>1530	NA	18666	>51000
Ref. 1*	0.016	189	--	--	11812	--	--
Ref. 2*	0.021	112	--	--	5333	--	--
Ref. 3*	0.013	469	--	--	36076	--	--

* Known PYY analog for comparison; *see*, Intl. Patent Application Publication No. WO 2016/198682, where Ref. 1 corresponds to Compound 4 therein, Ref. 2 corresponds to Compound 21 therein, and Ref. 3 corresponds to Compound 32 therein.

ND – not detected

NA – not applicable

[0148] As shown above, the PYY analogs of Examples 1-5 are highly selective for the hNPY2 receptor, even demonstrating reduced binding affinity to hNPY5, hNPY4 and hNPY1 receptors versus native, human PYY₃₋₃₆ (SEQ ID NO:2).

[0149] (2) *In vitro* cAMP activity on the human NPY2 receptor

[0150] Purpose:

[0151] To determine the *in vitro* functional activity of the PYY analogs of Examples 1 to 5 compared to native, human PYY₃₋₃₆ by measuring inhibition of forskolin-induced intracellular cAMP production in HEK 293 cells overexpressing the recombinant human NPY2 receptor.

[0152] Methods:

[0153] PYY analogs and human PYY₃₋₃₆ (SEQ ID NO:2) are synthesized, characterized and stored as described above in the receptor binding assays.

[0154] Receptor cloning – A stably transfected cell line is prepared hNPY2 receptor by subcloning receptor cDNA into pcDNA3.1 expression plasmid and transfecting it into HEK 293 cells followed by selection with Geneticin. Aliquots of cells (1×10^7 cells/mL) at passage 9 are made and kept frozen in the vapor phase of a liquid nitrogen tank. These frozen aliquots are used at the time of the assay. Cells maintain greater than 95% viability over several months.

[0155] hNPY2R cAMP assay – Inhibition of forskolin-induced cAMP production by PYY analogs or PYY₃₋₃₆ is measured using HEK 293 cells overexpressing recombinant hNPY2R. Frozen aliquots of cells are thawed in a 37°C water bath. Cells are transferred to a 50 mL tube with 10 mL of culture medium (MEM cell culture medium from Life Tech 11090-081 with 10% FBS from Life Tech 10082-147, 1 mM L-Glutamine from Life Tech 25030-081, 1xNEAA from Life Tech 11140-050, 1 mM sodium pyruvate from Life Tech 11360-070, 1x antibiotics-antimycotics from Life Tech 15240-062) and are centrifuged 5 minutes at 1500 rpm in a Beckman tabletop centrifuge. The supernatant is removed and the cell pellet is resuspended in 10 mL of cell culture medium followed by passage through

a 40 μm strainer. An accurate count of cell number and cell viability is determined using a Vi-Cell Analyzer from Beckman-Coulter (Vi-Cell XR 2.03). 8000 cells per well are plated into a white 384 well assay plate (Corning, Poly-D-Lysine coated, white/opaque, cat# 356661) using a Combi-Tip Dispenser (Thermo Scientific). Plates are centrifuged 1 second at 1000 rpm and incubated 18 to 20 hours at 37°C in a 5% CO₂-controlled incubator. Culture medium is removed from the assay plates by flicking gently on paper towels. 10 μL of assay buffer [1X HBSS (Hyclone, #SH3026801), 20 mM HEPES, pH 7.5 (Hyclone #SH30237.01), 0.1% w/v Casein (CTL Scientific Supply Corp., #440203H), 500 μM IBMX (Sigma-Aldrich #I5876)] is added to the wells using the Combi-Tip Dispenser followed by centrifugation for 10 seconds at 1500 rpm. A concentration response curve (20 point) at 2-fold dilutions is prepared in 100% DMSO using acoustic dispensing technology (Labcyte Echo 550). The cells are treated with PYY analogs or human PYY₃₋₃₆ for 45 minutes at 37°C (final DMSO concentration = 1%), then stimulated with 1 μM forskolin (Sigma-Aldrich, #F6886) for 45 minutes at 37°C. The intracellular cAMP is quantified using a CisBio cAMP-G_i Dynamic Kit (#62AM9PEB). Briefly, cAMP levels within the cell are detected using the HTRF kit reagents by adding cAMP-d2 conjugate in cell lysis buffer (10 μL) followed by adding the antibody anti-cAMP-Eu³⁺-Cryptate, also in cell lysis buffer (10 μL). The resulting competitive assay is incubated for at least 60 minutes at room temperature, then read on a PerkinElmer Envision™ instrument with excitation at 320 nm and emission at 665 nm and 620 nm. Final assay concentration ranges for peptides tested in response curves are: PYY analogs (0.1 μM to 0.2 pM) and human PYY₃₋₃₆ (10 nM to 0.02 pM). A standard curve of known cAMP concentrations (0.5 μM to 1 pM) is prepared in assay buffer. Wells in the absence of added competitor or with an excess of added human PYY₃₋₃₆ are included on each plate as Maximum Response and Inhibitor Controls, respectively.

[0156] Data analysis for hNPY2 receptor cAMP assay – Time-resolved fluorescence emission is used to calculate a fluorescence ratio (665 nm/620 nm), which is inversely proportional to the amount of cAMP present. Signals for PYY analogs and human PYY₃₋₃₆ are converted to nM cAMP per well using a cAMP standard curve plotted as relative response units (emission at 665 nm/620 nm*10,000, y-axis) versus concentration of cAMP (x-axis).

[0157] The amount of cAMP generated (nM) in each well is converted to a percent of the maximal response observed with forskolin only as shown in the equation below:

$$\% \text{ Specific Inhibition} = \frac{\text{Peptide-Inhibitor Control}}{\text{Maximum Response-Inhibitor Control}}$$

where Inhibitor Control is the cAMP produced in the presence of added excess human PYY₃₋₃₆, Maximum Response is the cAMP produced in the presence of forskolin only, and Peptide is the cAMP produced in the presence of test peptide.

[0158] Percent specific inhibition (y-axis) is plotted against the concentration of competitor (x-axis) and analyzed using a four-parameter (curve top, curve bottom, IC₅₀, Hill slope) nonlinear regression routine (Genedata Screener, version 13.0.5, Genedata AG, Basal, Switzerland) as defined below:

$$y = \text{bottom} + \frac{\text{top-bottom}}{1 + \left(\frac{x}{\text{IC}_{50}}\right)^{\text{Hill slope}}}$$

[0159] The relative IC₅₀ value represents the concentration causing 50% inhibition of forskolin-induced cAMP production.

[0160] Reported values for IC₅₀ are calculated as the geometric mean as shown below:

$$\text{Geometric Mean} = 10^{(\text{Arithmetic Mean of Log}_{10} \text{IC}_{50} \text{ Values})}$$

[0161] Standard error of the mean (SEM) is calculated using the delta method as shown below:

$$\text{SEM} = \text{Geometric Mean} \times \frac{\text{SD of log transformed data}}{\text{Square root of } n} \times \ln(10),$$

where SD is the standard deviation, n is the number of independent runs, and ln(10) is the natural logarithm of 10.

[0162] Results:

[0163] Table 2: *In Vitro* cAMP Activity on the hNPY2 Receptor.

Peptide	hNPY2R EC ₅₀ (nM)
PYY ₁₋₃₆	0.12
PYY ₃₋₃₆	0.15
Example 1	0.07
Example 2	1.23
Example 3	0.15
Example 4	0.06
Example 5	0.55
Ref. 1*	0.30
Ref. 2*	0.23

Ref. 3*	0.32
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* Known PYY analog for comparison; see, Intl. Patent Application Publication No. WO 2016/198682, where Ref. 1 corresponds to Compound 4 therein, Ref. 2 corresponds to Compound 21 therein, and Ref. 3 corresponds to Compound 32 therein.

[0164] As shown above, results from the cAMP assay demonstrate functional activity of the PYY analogs of Examples 1-5 on the hNPY2 receptor, with Example 2 showing the weakest potency at 12-fold lower potency versus human PYY₃₋₃₆.

[0165] (3) *In vitro* GTP γ S activity on the hNPR2 receptor

[0166] Purpose:

[0167] To assess receptor-mediated activation of G-proteins by the PYY analogs of Examples 1 to 5. Receptor-mediated activation can be measured using the non-hydrolyzable GTP analog, GTP γ [³⁵S]. Agonist-mediated stimulation of G-protein-coupled receptors results in the activation of membrane-associated G $\alpha\beta\gamma$ -protein heterotrimeric complexes. This represents a first step in transducing extracellular signals to modify intracellular pathways. Herein, a GTP γ [³⁵S] functional assay is used to assess the potency of various PYY analogs at the hNPY2 receptor.

[0168] Methods:

[0169] PYY analogs, human PYY₁₋₃₆ (SEQ ID NO:1) and control peptide PYY₃₋₃₆ (SEQ ID NO:2) are synthesized, characterized and stored as described above in the receptor binding assay.

[0170] For each test peptide, concentration response curves (CRC) with 1/3 log dilutions (logarithmic concentrations from -6.52 to -12.52) are completed with a Hamilton NIMBUS liquid handler in assay buffer (20mM HEPES, pH 7.4, 100mM NaCl, 6mM MgCl₂, 1 mM EDTA) supplemented with 0.2% Bacitracin (U.S. Biologicals #11805) and 0.5% DMSO. Final assay concentrations of Bacitracin and DMSO are 0.05% and 0.125%, respectively. hNPY2R membranes (Multispan #HTS066M) are prepared to a concentration of 7.5ug/mL in assay buffer supplemented with 20 uM GDP (Sigma #G-7127) and 6 ug/mL Saponin (Sigma #S-4521) and hNPY2R membranes are incubated at room temperature for 20 minutes prior to addition to the assay. GTP γ S[³⁵S] (PerkinElmer #NEG030H) is prepared in assay buffer to a concentration of 0.6nM. WGA SPA beads (PerkinElmer #RPNQ0001) are prepared at concentration of 12mg/mL in assay buffer. The assay is performed in a 96-well plate (Costar #3604) by first adding 100 uL to the hNPY2R membranes, then 50 uL of CRC solution, then 50 uL of GTP γ S[³⁵S] solution for a final volume of 200 uL. The plate is covered and placed on an orbital shaker (175 rpm for 45 minutes) at room

temperature. Then, 25 uL of SPA beads are added and plate is re-sealed and vortexed to mix, then placed back on orbital shaker for 3 hours at room temperature. The plate is then centrifuged for 5 minutes at 500 rpm and counted on a PerkinElmer 2450 Microplate counter for 1 minute per well. Basal binding (CPM) is determined in the absence of PYY analog or human PYY₃₋₃₆ and is used to calculate a percent above basal value for each concentration of peptide with the following equation: $(\text{PYY analog, or human PYY}_{3-36}, \text{CPM} - \text{Basal CPM}) / (\text{Basal CPM}) * 100$. EC₅₀ (nM) values is determined by subjecting the logarithm of concentrations and percent of basal values to non-linear regression analysis (log(agonist) vs. response--Variable slope (four parameters)) in GraphPad Prism 7.0 using the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$. Geometric mean and standard error of the mean are calculated from EC₅₀ (nM) values using the column statistics function in GraphPad Prism 7.0.

[0171] Results:

[0172] Table 3: *In Vitro* GTPγS Activity on the hNPY2 Receptor.

Peptide	hNPY2R EC ₅₀ (nM)
PYY ₁₋₃₆	0.42
PYY ₃₋₃₆	0.43
Example 1	0.08
Example 2	1.83
Example 3	0.06
Example 4	0.04
Example 5	0.16
Ref. 1*	0.22
Ref. 2*	0.13
Ref. 3*	0.11

* Known PYY analog for comparison; see, Intl. Patent Application Publication No. WO 2016/198682, where Ref. 1 corresponds to Compound 4 therein, Ref. 2 corresponds to Compound 21 therein, and Ref. 3 corresponds to Compound 32 therein.

[0173] As shown above, results from the GTPγ[³⁵S] functional assay demonstrate activity of the PYY analogs on the hNPY2 receptor, with Example 2 showing the weakest potency at 4-fold lower potency versus human PYY₃₋₃₆.

[0174] (4) Pharmacokinetics

[0175] Purpose:

[0176] To investigate the pharmacokinetic properties of the PYY analogs.

[0177] Methods:

[0178] LC/MS – Plasma concentrations of various PYY analogs are determined by LC/MS methods. The methods measure the whole compound; peptide plus linked time extension. For the assay, PYY analogs and an internal standard are extracted from mouse, rat or monkey plasma (50 µL) using methanol with 0.1% formic acid. The samples are centrifuged and supernatant is transferred to a Thermo Protein Precipitation Plate. The samples are loaded on a Sep-Pak tC18 SPE µElution Plate that is conditioned with methanol and 0.1% formic acid in water. The SPE columns are washed twice with 0.1% formic acid in water. The compounds are then eluted using Formic Acid/Water/acetonitrile (0.1:15:85), which are then dried and reconstituted prior to injecting an aliquot (10 µL) onto a Thermo Acclaim PepMap100 C18, 300 µm x 5 mm trap column and Thermo Easy Spray PepMap C18, 75 µm x 15 cm column for LC/MS analysis. The column effluent is directed into a Thermo Q-Exactive Plus Mass Spectrometer for detection and quantitation.

[0179] Pharmacokinetics of PYY analogs in CD-1 mice – The plasma pharmacokinetics of the PYY analogs are evaluated in male CD-1 mice following a single subcutaneous dose of 200 nmol/kg. Blood samples are collected from 2 animals per time point over 168 hours. Since non-serial sampling is used to evaluate the kinetics of the PYY analogs in mice, the mean concentration versus time data are used to tabulate the pharmacokinetic parameters for the PYY analogs following a single subcutaneous dose of 200 nmol/kg. Plasma concentrations of PYY analogs are detected through 120 hours following a single subcutaneous administration of 200 nmol/kg.

[0180] Pharmacokinetics of PYY analogs in SD rats – The plasma pharmacokinetics of the PYY analogs are evaluated in male Sprague Dawley rats following a single subcutaneous dose of 50 nmol/kg. Blood samples are collected from 2 animals per time point over 168 hours. Since serial sampling was used to evaluate the kinetics of the PYY analogs in rats, individual animal concentration versus time data were used to tabulate the pharmacokinetic parameters for the PYY analogs following a single subcutaneous dose of 50 nmol/kg. Plasma concentrations of PYY analogs are detected through 120 hours following a single subcutaneous administration of 50 nmol/kg.

[0181] Pharmacokinetics of PYY analogs in cynomolgus monkeys – The plasma pharmacokinetics of the PYY analogs are evaluated in male and female cynomolgus monkeys following a single subcutaneous dose of 50 nmol/kg. Blood samples are collected over 504 hours. Since serial sampling is used to evaluate the kinetics of the PYY analogs

in monkeys, individual animal concentration versus time data are used to tabulate the pharmacokinetic parameters for the PYY analogs following a single subcutaneous dose of 50 nmol/kg. Plasma concentrations of PYY analogs are detected through 504 hours following a single subcutaneous administration of 50 nmol/kg.

[0182] Results:

[0183] Table 4: Mean Pharmacokinetic Parameters Following a Single Subcutaneous Dose to Male CD-1 Mice.

Peptide	Dose (nmol/kg)	T _{1/2} (hr)	T _{max} (hr)	C _{max} (nmol/L)	AUC _{inf} (hr*nmol/L)	CL/F (mL/hr/kg)
Example 1	200	15	6	1140	29795	6.71
Example 2	200	27	12	1014	56198	3.56
Example 3	200	26	12	1360	52751	3.79
Example 4	200	15	12	916	31257	6.40

Abbreviations: AUC_{inf} = area under the curve from 0 to infinity; CL/F = clearance divided by bioavailability (F); C_{max} = maximum concentration; T_{max} = time at maximal concentration; T_{1/2} = half-life.

[0184] Table 5: Mean Pharmacokinetic Parameters Following a Single Subcutaneous Dose to Male SD Rats.

Peptide	Dose (nmol/kg)	T _{1/2} (hr)	T _{max} (hr)	C _{max} (nmol/L)	AUC _{inf} (hr*nmol/L)	CL/F (mL/hr/kg)
Example 1	50	22	12	221	8621	5.83
Example 3	50	38	12	399	21094	2.39
Example 4	50	19	8	219	8289	6.41

Abbreviations: AUC_{inf} = area under the curve from 0 to infinity; CL/F = clearance divided by bioavailability (F); C_{max} = maximum concentration; T_{max} = time at maximal concentration; T_{1/2} = half-life.

[0185] Table 6: Mean Pharmacokinetic Parameters Following a Single Subcutaneous Dose to Cynomolgus Monkeys.

Peptide	Dose (nmol/kg)	T _{1/2} (hr)	T _{max} (hr)	C _{max} (nmol/L)	AUC _{inf} (hr*nmol/L)	CL/F (mL/hr/kg)
Example 1	50	101	9	500	64552	0.775
Example 2	50	131	18	583	123173	0.408
Example 3	50	148	9	599	105839	0.509

Abbreviations: AUC_{inf} = area under the curve from 0 to infinity; CL/F = clearance divided by bioavailability (F); C_{max} = maximum concentration; T_{max} = time at maximal concentration; T_{1/2} = half-life.

[0186] Results:

[0187] These data demonstrate that the above compounds have a pharmacokinetic profile suitable for once weekly administration.

[0188] (5) Solubility & Stability

[0189] Purpose:

[0190] To determine the soluble pH ranges and stability of the PYY analogs

[0191] Methods:

[0192] Visual solubility range assessment – Lyophilized PYY analog powders are reconstituted in water at 4 mg/mL concentrations, and the pH is adjusted with citric acid/phosphate buffer to pH 4. pH of the system is titrated up with 0.5 N NaCl to pH 8 and then titrated down with 0.5 N HCl to pH 4.

[0193] Thermal stability evaluation – PYY analog solutions in 10 mM or 20 mM sodium phosphate, pH 7.0 at 1 mg/mL or 2 mg/mL concentration are prepared and incubated at 4°C and 40°C for 4 weeks. The samples at 4-week time point are analyzed by size exclusion chromatography (SEC) and RP-HPLC.

[0194] SEC method – Performed using a TOSOH TSKgelG2000SWx1, 7.8 mm ID x 30 cm, 5 µm column, with mobile phase composition of 50 mM sodium phosphate, 300 mM NaCl, pH 7.0 with 20% acetonitrile over 30 minutes with a flow rate of 0.5 mL/min, λ – 214 nm.

[0195] RP method – Performed using a Cortecs C18, 2.7 µm, 4.6 x 50 mm column, 20% - 45% acetonitrile /water with 0.085% TFA over 10 minutes with a flow rate 1 mL/min, λ - 214 nm.

[0196] Results:

[0197] Table 7: Solubility and Thermal Stability of the PYY Analogs.

Peptide	Solubility (pH range)	Thermal Stability (10 or 20 mM Phosphate, pH 7.0)			
		RP % Main Peak		SEC % Main Peak	
		4°C	40°C	4°C	40°C
Example 1	> 5.7	95.8	92.9	98.9	95.7
Example 2	> 5.5	99.7	99.3	99.9	99.6
Example 3	> 6.1	99.5	97.1	95.2	92.4
Example 4	> 6.8	96.8	97.5	99.6	98.7
Example 5	> 5.8	96.8	95.2	99.7	97.5

[0198] As shown above, all the peptides are soluble at pH > 7.0. The stability, as assessed by RP and SEC, suggests that these peptides are relative stable under the aggressive thermal stress.

[0199] Example 7: *In Vivo* Effects of PYY.

[0200] (1) *In vivo* effects on food intake and body weights in normal mice

[0201] Purpose:

[0202] To compare the effect of the PYY analogs of Examples 1 to 5 to reduce body weight and suppress food intake in normal mice after a single injection.

[0203] Methods:

[0204] Male C57Bl/6 mice from Envigo RMS (Indianapolis, IN) are maintained on a chow diet (5008; LabDiet, St. Louis, MO) and single housed in a temperature-controlled facility (74.0°F; 23.3°C) with a normal 12:12-hour light cycle and free access to food and water. At 9-10 weeks of age, non-fasted body weights and initial food weights are recorded, and animals are administered a single subcutaneous injection of vehicle or peptide, followed by daily measurements of body weight and food intake for 3 days post dose. Area under the curve analysis (AUC) is calculated for both body weight and food intake versus vehicle. Example 4 at 30 nmol/kg is used as benchmark of 100% efficacy for body weight and food intake in each run of the assay.

[0205] Results:

[0206] Table 8: Changes in Body Weight and Food Intake for 3 Days in C57/Bl6 Mice Following a Single Dose of PYY Analog.

Peptide	Dose (nmol/kg, SC)	Δ Body Weight (%)**	Δ Food Intake (%)**
Example 1	30	89	103
	100	108	105
Example 2	100	71	71
	300	117	78
Example 3	30	98	93
	100	111	100
Example 4	3	22	12
	10	41	48
	30	100	100
Example 5	100	70	99
	300	129	118
Ref. 1*	100	87	79
	300	127	104
Ref. 2*	100	62	67
	300	110	85
Ref. 3*	100	50	78
	300	104	99

* Known PYY analog for comparison; see, Intl. Patent Application Publication No. WO 2016/198682, where Ref. 1 corresponds to Compound 4 therein, Ref. 2 corresponds to Compound 21 therein, and Ref. 3 corresponds to Compound 32 therein.

** Example 4 at 30 nmol/kg (AUCs) set at 100% efficacy

[0207] As shown above, reductions in both body weight and food intake demonstrate efficacy of the PYY analogs *in vivo*, where comparisons on doses required for full efficacy demonstrate improvements in efficacy.

[0208] (2) *In vivo* effects on food intake and body weights in diet-induced obese mice

[0209] Purpose:

[0210] To investigate the effect of daily dosing of the PYY analogs of Examples 1-5 to reduce body weight, either alone or in combination with a GLP-1 receptor agonist, over a two-week period in diet-induced obese (DIO) mice.

[0211] Methods:

[0212] DIO male C57Bl/6 mice (Taconic) at 20 weeks of age are maintained on a 60% fat diet upon arrival (D12492; Research Diets, New Brunswick, NJ). Animals are individually housed in a temperature-controlled facility (74.0°F; 23.3°C) with a 12-hour light/dark cycle (lights on 22:00) and free access to food and water. After a one-week acclimation period of daily vehicle dosing, non-fasted body weights are measured, and animals are randomized by body weight into experimental groups (n=6) and administered daily subcutaneous injections of vehicle, a GLP-1 receptor agonist (GLP-1 RA; SEQ ID NO:15), PYY analogs, or combinations of PYY analogs plus the GLP-1 RA. After 2 weeks of dosing, non-fasted body weights are recorded and changes in average body weight versus vehicle are calculated. To determine additive or synergistic effects of PYY analogs in combination with a GLP-1 RA, efficacy above that of the GLP-1 RA alone (net effect) is calculated.

[0213] Results:

[0214] Table 9: Changes in Body Weight in a 2-Week Study in Diet-Induced Obese Mice with PYY Analogs Alone or in Combination with a GLP-1 Receptor Agonist.

Peptide	Dose (nmol/kg, SC)	Δ Body Weight (%)	
		PYY Alone	PYY + GLP-1RA*
Example 1	1	-4	-18
	3	-6	-26
	10	-15	-31
Example 2	3	ND	-1
	10	ND	-6
	30	2	-11
Example 3	1	-3	-16
	3	-6	-28
	10	-23	-30

Example 4	0.3	0	-3
	1	-1	-12
	3	-4	-17
	10	-12	-21
Example 5	1	-2	-2
	3	0	-11
	10	-2	-18
	30	-3	ND

* Efficacy above that of a GLP-1 receptor agonist (GLP-1 RA)

[0215] As shown above, reductions in body weight with the PYY analogs, both alone and in combination with the GLP-1 RA, demonstrate efficacy of the PYY analogs *in vivo*, where comparisons are made on the magnitude of weight loss.

[0216] (3) *In vivo* effects on body weight and glucose in diabetic and obese (db/db) mice

[0217] Purpose:

[0218] To investigate the effect of daily dosing of the PYY analogs of Examples 1 to 5 to reduce body weight and blood glucose levels over a ten-day period in obese and diabetic mice (db/db).

[0219] Methods:

[0220] Lep^{db/db} (db/db) male mice from Envigo RMS (Indianapolis, IN) are maintained on a chow-style diet (5008; LabDiet, St. Louis, MO) and housed 5 animals per cage in a temperature-controlled facility (74.0°F; 23.3°C) with a normal 12:12-hour light cycle and free access to food and water. At 8-9 weeks of age, body weights and blood glucose levels using Accu-Check® Glucometers (Roche Diabetes Care, Inc., Indianapolis, IN) are measured, followed by daily subcutaneous injections of vehicle or peptide. After 10 days of dosing, body weights and blood glucose levels are measured and changes versus vehicle treatment are calculated.

[0221] Results:

[0222] Table 10: Effects on Body Weight and Blood Glucose in db/db Mice Treated for 10 Days.

Peptide	Dose (nmol/kg, SC)	Δ Body Weight (%)	Δ Glucose (%)
Example 1	1	-1	7
	3	-1	-10
	10	-10	-56
	30	-14	-59

Example 2	30	-5	-40
	100	-9	-60
	300	-17	-67
Example 3	1	0	0
	3	-5	-7
	10	-9	-50
	30	-15	-53
Example 4	3	-2	-23
	10	-12	-61
	30	-17	-62
Example 5	10	-4	-24
	30	-11	-59
	100	-17	-66

[0223] As shown above, reductions in body weight and blood glucose levels with the PYY analogs demonstrate efficacy of the PYY analogs *in vivo*, where comparisons are made on the magnitude of weight loss and glucose lowering.

[0224] In conclusion, the PYY analogs herein show selectivity toward NPY2R. They also show dose-dependent reductions in body weight, as reflected in normal mice, diet-induced obese mice and db/db mice, as well as dose-dependent improvement in blood glucose in db/db mice, with the PYY analogs of Examples 1, 3 and 4 being the most efficacious, in line with the *in vitro* profile.

SEQUENCES

SEQ ID NO:1 – Human PYY₁₋₃₆

YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY

SEQ ID NO:2 – Human PYY₃₋₃₆

IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY

SEQ ID NO:3 – PYY Analog

PKPEX₇PX₉X₁₀DASPEEX₁₇X₁₈RYX₂₂X₂₃LRHYLN₃₀LTRQRY

SEQ ID NO:4 – PYY Analog

PKPEKPGEDASPEEWQRYYAELRHYLNWLTRQRY

SEQ ID NO:5 – PYY Analog

PKPEKPGEDASPEEWQRYYAELRHYLNELTRQRY

SEQ ID NO:6 – PYY Analog

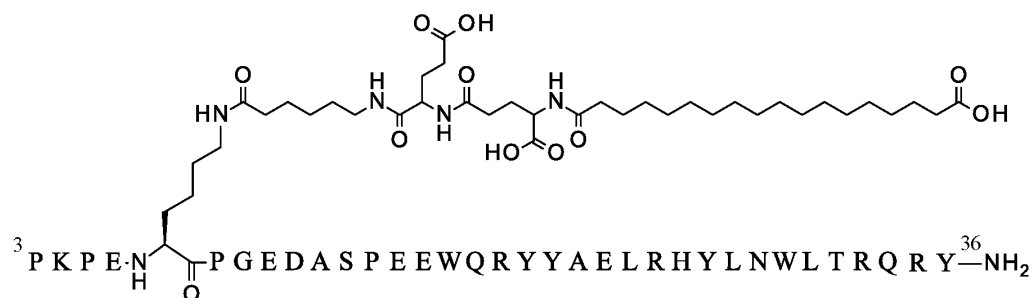
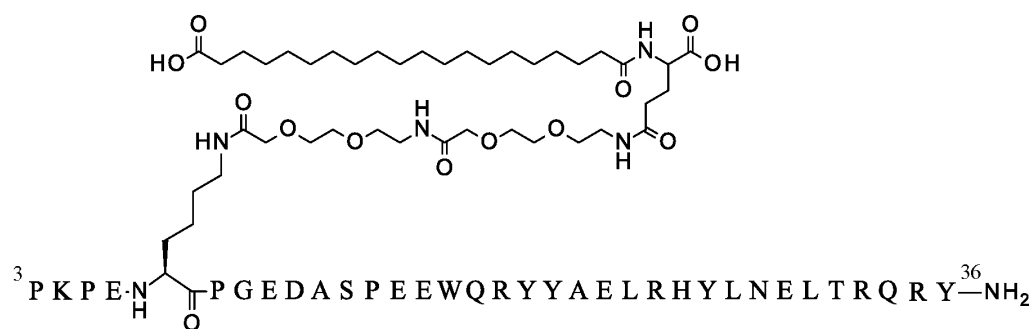
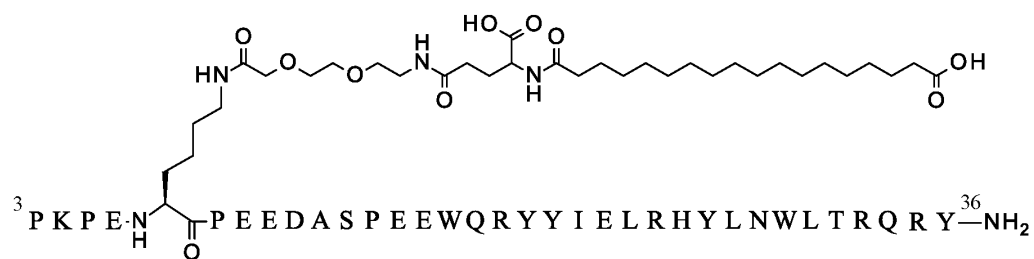
PKPEKPEEDASPEEWQRYYIELRHYLNWLTRQRY

SEQ ID NO:7 – PYY Analog

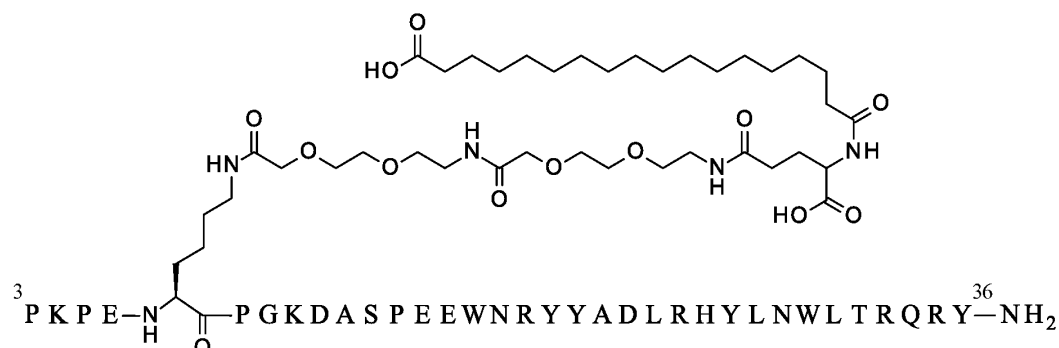
PKPEKPGKDASPEEWNRYYADLRHYLNWLTRQRY

SEQ ID NO:8 – PYY Analog

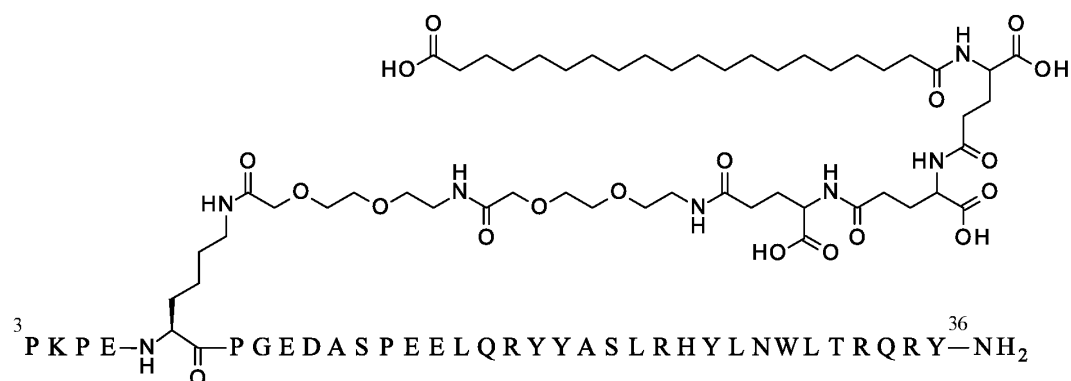
PKPEKPGEDASPEELQRYYASLRHYLNWLTRQRY

SEQ ID NO:9 – PYY AnalogSEQ ID NO:10 – PYY AnalogSEQ ID NO:11 – PYY Analog

SEQ ID NO:12 – PYY Analog



SEQ ID NO:13 – PYY Analog



SEQ ID NO:14 – PP₁₋₃₆

APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY

SEQ ID NO:15 – GLP-1 RA

HGEFTFTSDVSSYLEEQAAKEFIAWLVKGRGGGGGSGGGGSGGGGSESKYGPPC
PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDG
VEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKT
ISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
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LG

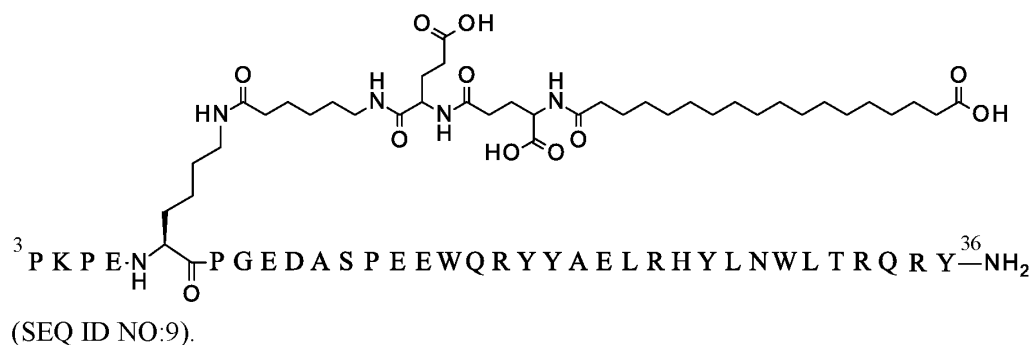
CLAIMS

The invention claimed is:

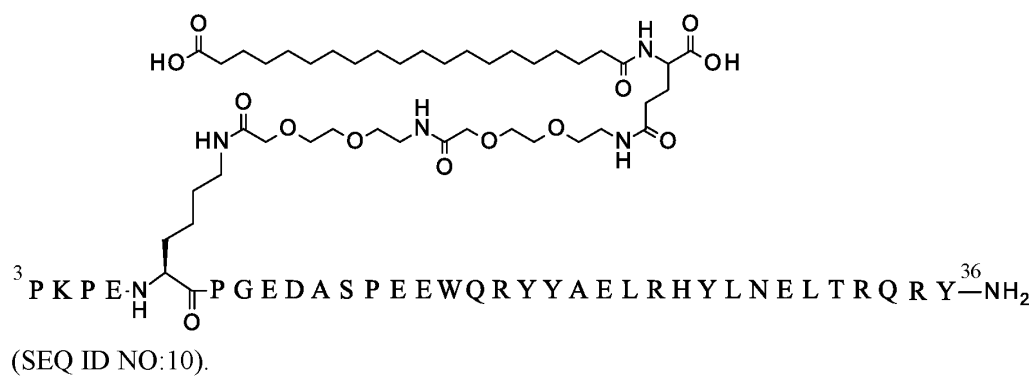
1. A Peptide Tyrosine-Tyrosine (PYY) analog comprising an amino acid sequence of:
PKPEX₇PX₉X₁₀DASPEEX₁₇X₁₈RYYX₂₂X₂₃LRHYLN₃₀LTRQRY (Formula I),
wherein X₇ is any amino acid with a functional group available for conjugation and
the functional group is conjugated to a C₁₆-C₂₂ fatty acid,
wherein X₉ is E or G,
wherein X₁₀ is E or K,
wherein X₁₇ is L or W,
wherein X₁₈ is N or Q,
wherein X₂₂ is A or I,
wherein X₂₃ is E, D or S,
wherein X₃₀ is E or W (SEQ ID NO:3), and
wherein a C-terminal amino acid is optionally amidated.
2. The PYY analog of Claim 1, wherein X₇ is selected from the group consisting of C,
D, E, K and Q.
3. The PYY analog of Claim 1, wherein X₇ is K and conjugation to the C₁₆-C₂₂ fatty
acid is through an epsilon-amino group of a K side chain.
4. The PYY analog of Claim 1, wherein the amino acid sequence is selected from the
group consisting of:
PKPEKPGEDASPEEWQRYYAELRHLYLNWLTRQRY (SEQ ID NO:4);
PKPEKPGEDASPEEWQRYYAELRHLYLNELTRQRY (SEQ ID NO:5);
PKPEKPEEDASPEEWQRYYIELRHLYLNWLTRQRY (SEQ ID NO:6);
PKPEKPGKDASPEEWNRYYADLRHYLNWLTRQRY (SEQ ID NO:7); and
PKPEKPGEDASPEELQRYYASLRHYLNWLTRQRY (SEQ ID NO:8).

5. The PYY analog of any one of Claims 1 to 4, wherein the C₁₆-C₂₂ fatty acid is selected from the group consisting of a hexadecanoic acid, a hexadecanedioic acid, a heptadecanoic acid, a heptadecanedioic acid, a stearic acid, an octadecanedioic acid, a nonadecylic acid, a nonadecanedioic acid, an eicosanoic acid, an eicosanedioic acid, a heneicosanoic acid, a heneicosanedioic acid, a docosanoic acid, a docosanedioic acid, and branched and substituted derivatives thereof.
6. The PYY analog of Claim 5, wherein the C₁₆-C₂₂ fatty acid is a C₁₈-C₂₀ fatty acid.
7. The PYY analog of Claim 6, wherein the C₁₈-C₂₀ fatty acid is a straight-chain fatty acid having a formula of CO-(CH₂)_x-CO₂H, and wherein x is 18 or 20.
8. The PYY analog of Claim 7, wherein the C₁₈-C₂₀ fatty acid is selected from the group consisting of palmitic acid, stearic acid, arachidic acid and eicosanoic acid.
9. The PYY analog of any one of Claims 1 to 8, wherein the C₁₆-C₂₂ fatty acid is conjugated to the amino acid with the functional group available for conjugation via a linker.
10. The PYY analog of Claim 9, wherein the linker can be one or more units selected from the group consisting of [2-(2-amino-ethoxy)-ethoxy]-acetic acid (AEEA), aminohexanoic acid (Ahx), glutamic acid (E), gamma glutamic acid (γE) and combinations thereof.

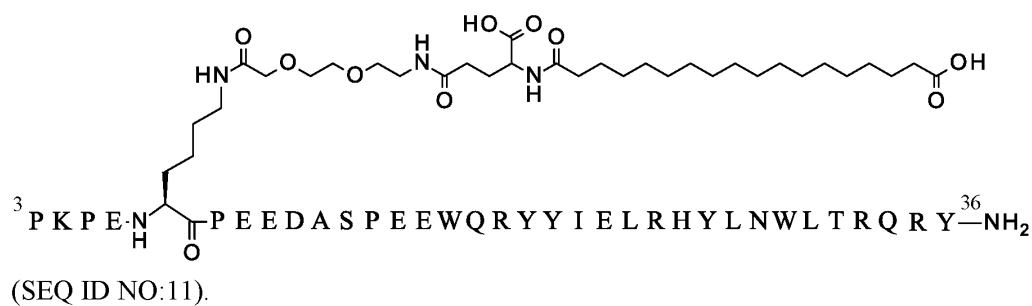
11. A Peptide Tyrosine-Tyrosine (PYY) analog comprising:



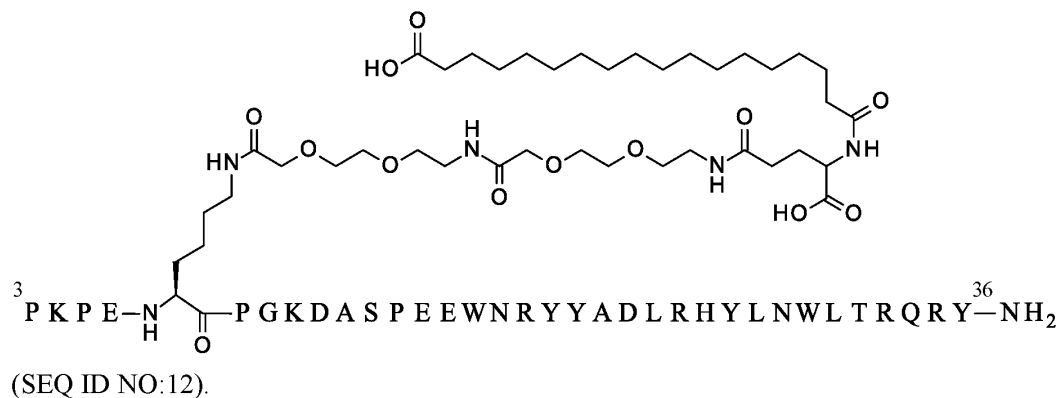
12. A Peptide Tyrosine-Tyrosine (PYY) analog comprising:



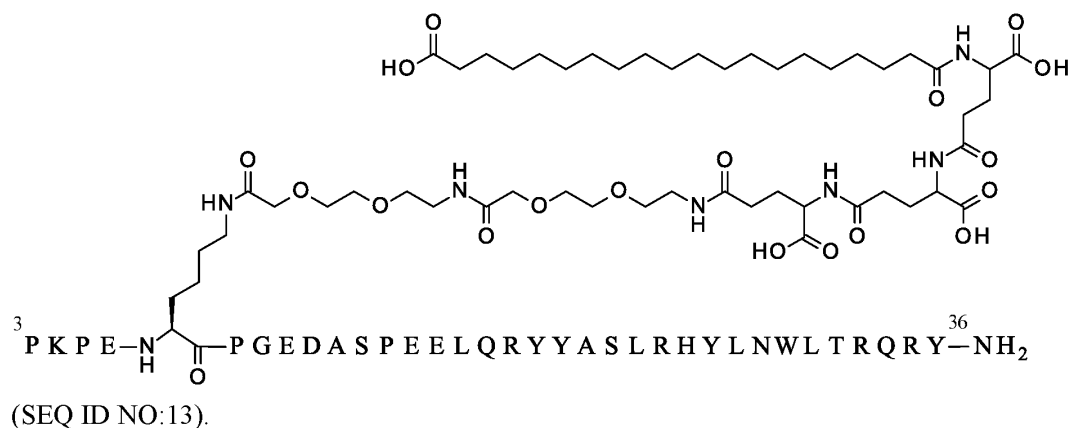
13. A Peptide Tyrosine-Tyrosine (PYY) analog comprising:



14. A Peptide Tyrosine-Tyrosine (PYY) analog comprising:



15. A Peptide Tyrosine-Tyrosine (PYY) analog comprising:



16. The PYY analog of any one of Claims 1 to 15, wherein the PYY analog has a charge of greater than -2.

17. The PYY analog of any one of Claims 1 to 16, wherein the PYY analog has a binding affinity at a NPY2 receptor that is greater than that of PYY₃₋₃₆ (SEQ ID NO:2).

18. The PYY analog of any one of Claims 1 to 17, wherein the PYY analog has a half-life that is greater than that of human PYY₃₋₃₆ (SEQ ID NO:2).

19. A pharmaceutical composition comprising:
at least one Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a salt thereof; and
one or more pharmaceutically acceptable carriers, diluents and excipients.
20. The pharmaceutical composition of Claim 19 further comprising an additional therapeutic agent.
21. The pharmaceutical composition of Claim 20, wherein the additional therapeutic agent is an incretin selected from the group consisting of glucagon (GCG), a GCG analog, glucagon-like peptide-1 (GLP-1), GLP-1₇₋₃₆-amide, a GLP-1 analog, gastric inhibitory peptide (GIP), a GIP analog, oxyntomodulin (OXM), an OXM analog, a GIP/GLP-1, a GLP-1/GCG, or an incretin analog having triple receptor activity.
22. The pharmaceutical composition of Claim 20, wherein the additional therapeutic agent is a dipeptidyl peptidase-IV (DPP-IV) inhibitor.
23. A method of treating obesity or an obesity related disease or disorder, the method comprising a step of:
administering to an individual in need thereof an effective amount of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof.
24. The method of Claim 23, wherein the PYY analog or pharmaceutically acceptable salt thereof is subcutaneously (SQ) administered to the individual.
25. The method of Claim 23, where the PYY analog or pharmaceutically acceptable salt thereof is orally administered to the individual.
26. The method of Claim 24 or 25, wherein the PYY analog or pharmaceutically acceptable salt thereof is administered daily, every other day, three times a week, two times a week, one time a week, or biweekly.

27. The method of Claim 23, wherein the PYY analog or pharmaceutically acceptable salt thereof is administered SQ one time a week (QW).
28. The method of Claim 23, wherein the PYY analog or pharmaceutically acceptable salt thereof is administered orally one time a week.
29. The method of any one of Claims 23 to 28 further comprising administering an additional therapeutic agent.
30. The method of Claim 29, wherein the additional therapeutic agent is an incretin selected from the group consisting of glucagon (GCG), a GCG analog, glucagon-like peptide-1 (GLP-1), GLP-1₇₋₃₆-amide, a GLP-1 analog, gastric inhibitory peptide (GIP), a GIP analog, oxyntomodulin (OXM), an OXM analog, a GIP/GLP-1, a GLP-1/GCG, or an incretin analog having triple receptor activity.
31. The method of Claim 29, wherein the additional therapeutic agent is a dipeptidyl peptidase-IV (DPP-IV) inhibitor.
32. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for treating obesity.
33. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for treating an obesity-related disease or disorder.
34. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for treating type II diabetes mellitus.
35. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for manufacturing a medicament for treating obesity.

36. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for manufacturing a medicament for treating an obesity-related disease or disorder.

37. Use of a Peptide Tyrosine-Tyrosine (PYY) analog of any one of Claims 1 to 18 or a pharmaceutically acceptable salt thereof for manufacturing a medicament for treating type II diabetes mellitus.