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A long-acting selective neuropeptide Y2 receptor PEGylated peptide agonist reduces food intake in mice

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Abstract—Activation of the NPY2 receptor to reduce appetite while avoiding activation of the NPY1 and NPY5 receptors that stimulate feeding provides a pharmaceutical approach to modulate food intake. The naturally occurring peptide and development candidate PYY(3-36) is a non-selective NPY1, NPY2, and NPY5 agonist of limited in vivo duration of action. N-terminal modification with 20 kDa PEG of a selective NPY2 receptor agonist peptide results in a long-acting agent that outperforms PYY(3-36) in reducing food intake in mice. The results suggest that PEGylated, selective NPY2 peptide agonists offer a significantly improved therapeutic benefit over PYY(3-36) for obesity management.

Pharmaceutical modulation of the activity of NPY receptors offers an approach to the regulation of food intake and provides a potential strategy for the control of obesity and associated disorders.[1] The gut hormone PYY(3-36) is a potent in vitro NPY2 receptor agonist. PYY(3-36) reduces acute food intake in mice,[2–8] rats, rabbits, monkeys, and humans, and continuous dosing reduces body weight in rodents and rabbits.[9–16] Studies in NPY2 knockout mice and with an NPY2 antagonist suggest that the anorexigenic effect of PYY(3-36) is mediated though stimulation of the NPY2 receptor.[2,7] PYY(3-36) also activates the NPY1 and NPY5 receptors that stimulate feeding.[17,18]

The in vivo efficacy of PYY(3-36) is controversial, with some groups reporting that PYY(3-36) does not reduce food intake in a variety of preclinical rodent models.[19] The inconsistent efficacy observations for PYY(3-36) may reflect handling of the animals,[4] a poor pharmacokinetic profile in the rat, or concomitant activation of the NPY1 and NPY5 receptors. Receptor selectivity can be addressed with selective NPY2 receptor agonists,[20] and the pharmacokinetic properties of peptides can be improved with PEGylation.[21]

The contribution of PEGylation to efficacy of a selective NPY2 peptide agonist is assessed here using the selective NPY2 agonist PYY(24-36)-L31.[22] The peptide is PEGylated via a non-native Cys residue at either the N- or C-terminus with linear 5 or 20 kDa PEG, or with branched 40 kDa PEG (Table 1). The introduction of the Cys residue allows for site-specific conjugation with PEG derivatized with maleimide.[23]

PYY(24-36)-L31 binds and activates fully the NPY2 receptor (Figs. 1 and 2), with a K_i of 5 ± 1 nM and an EC_{50} of 6 ± 1 nM (Table 1).[24] K_i values at the NPY1 and NPY5 receptors are 210 ± 39 nM and >1000 nM, respectively (Table 1). These results are in accord with a previous study using a similar assay that reports an IC_{50} value of 3.9 nM for PYY(24-36)-L31 at the NPY2 receptor, with no binding observed at the NPY1 receptor.[22]

Modification of the C-terminus of PYY(24-36)-L31 with Cys or PEG is detrimental to in vitro receptor binding (Table 1). The introduction of Cys 37 decreases the NPY2 receptor EC_{50} and K_i values 27- and 40-fold, respectively, compared to PYY(24-36)-L31 (Table 1). C-terminal PEGylation exacerbates the decrease of in vitro affinity at the NPY2 receptor (Table 1), with the PEGylated derivatives exhibiting at least a 70-fold decrease in receptor binding and activation (Table 1).

Keywords: NPY2; Obesity; PEG; Peptide; PYY; NPY.
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Table 1. In vitro receptor activation and binding profile of reference compounds and PEGylated PYY(24-36)-L31 derivatives[a]

Peptide	EC_{50} (nM) NPY2	E/E_{max}[b] (%) NPY2	K_i (nM)		
			NPY2	NPY1	NPY5
Reference compound					
PYY(3-36)	0.3 ± 0.1	100	0.4 ± 0.1	21 ± 2	20 ± 2
PYY(24-36)-L31	6 ± 1	100	5 ± 1	210 ± 39	>1000
C-terminal modifications					
PYY(24-36)-L31-C37	160 ± 27	81	200 ± 24	>1000	>1000
PYY(24-36)-L31-PEG5	>1000		>1000	>1000	>1000
PYY(24-36)-L31-PEG20	>1000		>1000	>1000	>1000
PYY(24-36)-L31-PEG40	420 ± 50	66	680 ± 60	>1000	>1000
N-terminal modifications					
C23-PYY(24-36)-L31	33 ± 6	94	8.2 ± 1.4	>1000	>1000
PEG5-PYY(24-36)-L31	31 ± 13	96	15 ± 1	>1000	>1000
PEG20-PYY(24-36)-L31	50 ± 8	88	53 ± 10	620 ± 280	>1000
PEG40-PYY(24-36)-L31	31 ± 4	86	31 ± 4	>1000	>1000

[a] All peptides are acetylated at the N-terminus and amidated at the C-terminus. PYY(24-36)-L31 has the sequence LRHYLNLLTRQRY-NH_2, corresponding to residues 24–36 of human PYY with the amino acid change of Val 31 to Leu.[20] C23 or C37 denotes PYY(24-36)-L31 with Cys at the N- or C-terminus, respectively. PEG5, PEG20, and PEG40 denote that the peptides are derivatized at either Cys 23 or Cys 37 with PEG of 5, 20, and 40 kDa, respectively. Values are means of experiments performed in triplicate ± standard error of the mean. Experiments were performed at least twice with equivalent results.

[b] Measured at 1 μM peptide concentration.

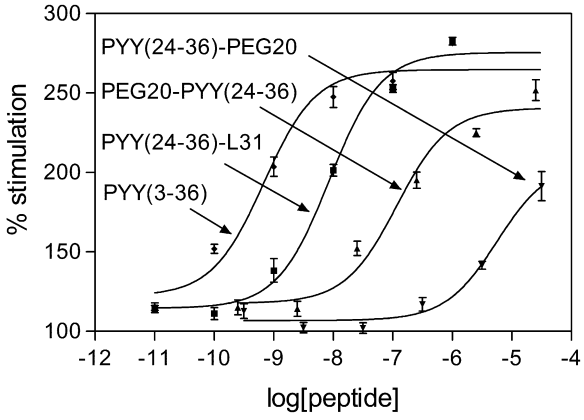

Figure 1. Stimulation of the human NPY2 receptor by human PYY(3-36), PYY(24-36)-L31, and PYY(24-36)-L31 analogs measured by GTPγ[S] accumulation.[24] C-terminal PEGylation is detrimental to receptor binding whereas N-terminal PEGylation is tolerated relatively well. Values are means of three values ± standard error of the mean. Experiments were performed at least twice with equivalent results.

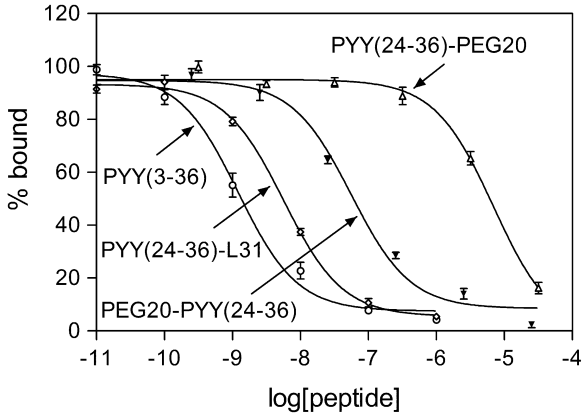

Figure 2. Binding at the human NPY2 receptor by human PYY(3-36), PYY(24-36)-L31, and PYY(24-36)-L31 analogs measured by the competitive displacement of ^{125}I-PYY.[24] C-terminal PEGylation is detrimental to receptor binding whereas N-terminal PEGylation is tolerated relatively well. Values are means of three values ± standard error of the mean. Experiments were performed at least twice with equivalent results.

Given the low in vitro potency, the C-terminally modified peptides were not studied in animals.

In contrast to the detrimental effects of the C-terminal modifications, N-terminal modifications of PYY(24-36)-L31 are tolerated (Figs. 1 and 2). The introduction of Cys 23 reduces the NPY2 receptor EC_{50} by approximately 6-fold to 33 nM, and the NPY2 receptor K_i by approximately 2-fold to 8 nM (Table 1). Derivatization of the Cys 23 variant with PEG causes relatively small further effects on in vitro potency (Table 1).

The in vivo activity of the N-terminal PEGylated variants on feeding was monitored in lean C57BL/6 mice using the fasted-refed model and compared with PYY(3-36).[25] Food intake was measured relative to control groups dosed subcutaneously with USP saline for PYY(3-36) or with PEG-Cys in USP saline of appropriate mass for the PEGylated peptides. No statistically significant difference in food intake was observed for mice treated with the different PEG control substances (Fig. 3).[26]

PYY(3-36) induces a marked reduction in food intake in mice at times close to dosing. PYY(3-36) administered at 2.5 μmol/kg (10 mg/kg) elicits a substantial reduction in food intake of 42% at 4 h, with a small albeit statistically significant reduction in food intake of 9% at 24 h

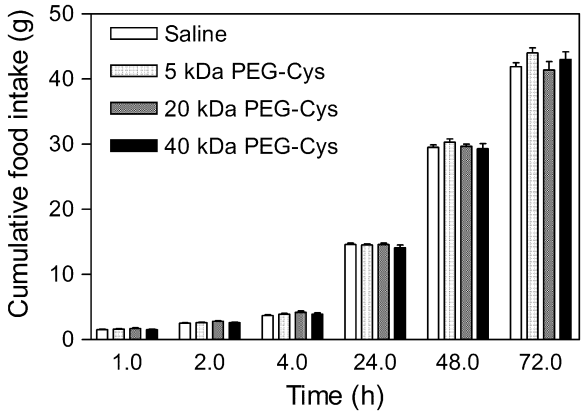

Figure 3. Negligible effect of PEG on cumulative food intake. Mice were administered a single subcutaneous dose of PEG-Cys formulated in USP saline at 5.6 μmol/kg, and cumulative food intake was measured over 72 h. A statistically significant difference in food intake is not induced by 5, 20 or 40 kDa PEG-Cys compared to USP saline. Values are means of experiments performed on 20 mice ± standard error of the mean. Experiments were performed twice with equivalent results.

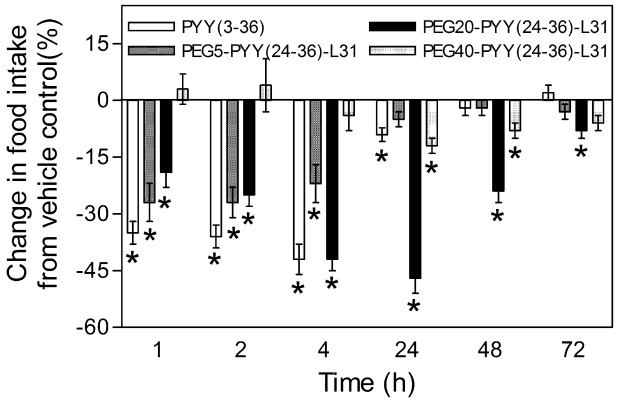

Figure 4. Reduction in cumulative food intake caused by subcutaneous administration of PYY(3-36) or PEGylated PYY(24-36)-L31 derivatives. Mice were administered a single subcutaneous dose of PYY(3-36) at 2.5 μmol/kg (10 mg/kg) or a PEGylated PYY(24-36)-L31 derivative at 5.6 μmol/kg. The 20 kDa PEG PYY(24-36)-L31 derivative exhibits a significantly prolonged duration of action relative to PYY(3-36). The asterisk denotes $p \leqslant 0.05$. Values are means of experiments performed on 20 mice ± standard error of the mean. Experiments were performed twice with equivalent results.

(Fig. 4). These results are in accord with previous reports that PYY(3-36) reduces food intake in mice.[2–8]

PYY(24-36)-L31 administered at 5.6 μmol/kg (10 mg/kg) causes only a small reduction in food intake that is less than PYY(3-36), with a 6–9% reduction in food intake over 1–24 h (data not shown).

PEG5-PYY(24-36)-L31 administered at 5.6 μmol/kg causes a reduction in food intake that is less than PYY(3-36), with a 22% reduction in food intake at 4 h, and little effect at 24 h (Fig. 4). PEG40-PYY(24-36)-L31 is inactive in vivo up to 4 h, although a small but statistically significant decrease in food intake of 12% and 8% is seen at 24 and 48 h, respectively (Fig. 4). Thus, neither the unmodified peptide nor the 5 and 40 kDa PEG derivatives offer improved in vivo efficacy over unmodified PYY(3-36).

PEG20-PYY(24-36)-L31 administered at 5.6 μmol/kg induces a comparable reduction in food intake to PYY(3-36) of 42% at 4 h (Fig. 3). In contrast to PYY(3-36), the effect is sustained from 4 to 24 h and remains significant (24%) at 48 h. Derivatization of PYY(24-36)-L31 with 20 kDa PEG, therefore, imparts superior in vivo efficacy with a longer duration of action than unmodified PYY(3-36). This finding provides preclinical proof-of-concept that PEGylated, selective NPY2 peptide agonists reduce food intake in mice.

Larger PEG derivatives may induce a greater improvement of in vivo efficacy than smaller PEG derivatives due to lower clearance.[27] This is not the case, however, for the present peptide. The 40 kDa PEG derivative is expected to have the longest in vivo lifetime and plasma exposure but provides the least effect on feeding. This finding highlights the role that PEG architecture beyond size may play in determining exposure at the drug target site and subsequent in vivo efficacy, and perhaps especially for receptors protected by the blood–brain barrier such as the NPY2 receptor.

We conclude that a selective NPY2 agonist peptide that is site-specifically derivatized with 20 kDa PEG to improve in vivo lifetime outperforms PYY(3-36) in a mouse feeding model. PYY(3-36) is currently a clinical candidate for the management of obesity.[1] The present results suggest that a long-acting, selective NPY2-receptor PEGylated peptide agonist may offer a significantly improved therapeutic benefit over PYY(3-36).

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23. Ac-PYY(24-36)-L31 [also called *N*-acetyl [Leu^{28}, Leu^{31}] NPY(24-36)],[21] human PYY(3-36), and human PYY were purchased from American Peptide Co. Inc., Sunnyvale, CA, USA. Other peptides were synthesized with solid-phase Fmoc/HBTU chemistry with an ABI 433A synthesizer. Final purification was by C_{18} HPLC using a linear H_2O/CH_3CN gradient containing 0.1% (v/v) TFA. Purity (>98%) was confirmed with analytical C_{18} HPLC and identity was confirmed with MALDI mass spectrometry. PEGylated peptides were prepared by crosslinking mPEG via a Cys residue introduced at either the N- or C-terminus of the peptide in 100 mM Tris, pH 8, for 2 h and purified with reversed phase C_{18} HPLC. Linear 5 kDa mPEG-MAL (Nektar Therapeutics 2D2M0H01), linear 20 kDa mPEG-MAL (Nektar Therapeutics 2D2M0P01), and branched 40 kDa mPEG2-MAL (Nektar Therapeutics 2D3X0T01) conjugated to maleimide were used. The absence of free peptide was confirmed with analytical C_{18} HPLC and PEGylation confirmed with SDS–PAGE. Peptide amount was determined with amino acid analysis performed by the W.M. Keck Foundation Biotechnology Facility, Yale University, CT.
24. Assays were performed using human receptors and data fit to a single-site binding model as described previously.[20] NPY2 receptor [^{35}S]GTPγ[S] functional assays and ^{125}I-PYY displacement assays were performed in a SPA format with membrane prepared from KAN-TS cells as a source of endogenous NPY2 receptor.[20] NPY1 and NPY5 receptor ^{125}I-PYY displacement assays were performed in a filter-binding format using membranes prepared from SK-N-MC cells as a source of endogenous NPY1 receptor or HEK293 cells expressing recombinant NPY5 receptor.[20]
25. Lean C57BL/6 male mice were acclimated for a minimum of a week with controlled temperature and humidity on a 12 h light/dark cycle. Mice were housed in pairs in cages with a grid floor with water and food continuously available. A fasted-refed study included 20 mice (10 cages) per treatment group with an average body weight of approximately 22 g. The mice were fasted overnight with water available during the dark phase and dosed subcutaneously with PYY(3-36) or the PEGylated PYY(24-36)-L31 derivatives in USP saline. Control groups were dosed with USP saline or PEG-Cys[26] in USP saline. Pre-weighed food was returned to the cage 30 min after dosing. The significance of differences in food consumption was evaluated by analysis of variance (StatView, SAS Inc.).
26. Cys was used to block the maleimide moiety of the PEG reagent for use as a control for in vivo studies. A 2-fold molar excess of Cys and the PEG reagent (5 or 20 kDa mPEG-MAL, or 40 kDa mPEG2-MAL) were incubated overnight in Tris, pH 7.4, at room temperature, dialyzed against water, and lyophilized.
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