Novel Selective Neuropeptide Y2 Receptor PEGylated Peptide Agonists Reduce Food Intake and Body Weight in Mice

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Selective activation of the NPY2 receptor to suppress appetite provides an approach to obesity management. Selective NPY2 PEGylated peptide agonists are described that consist of a peptide core corresponding to residues 25−36 of PYY and a nonpeptidic moiety at the peptide N-terminus that contributes to in vitro potency and in vivo efficacy and provides a PEGylation site. The lead peptide elicits a dose-dependent reduction of food intake in lean mice and of food intake, body weight, and fat mass in DIO mice.

Introduction

The naturally occurring gut hormone PYY(3−36)[a] is a development candidate for the management of obesity.[1] 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] PYY(3−36) does not reduce food intake in NPY2 knockout mice, and the effect of PYY(3−36) on feeding is blocked in the rat by the selective NPY2 antagonist BIIE0246, suggesting that the anorexigenic activity of PYY(3−36) is mediated specifically through the NPY2 receptor.[2,11] PYY(3−36) also activates the NPY1 and NPY5 receptors. This nonselective nature of PYY(3−36) is undesirable, since activation of the NPY1 and NPY5 receptors induces the undesired effect of increasing appetite.[17,18]

We have previously described an approach to attain selective NPY2 peptide agonists by abrogating NPY1 and NPY5 receptor affinity though N-terminal deletions of residues from PYY at the expense of NPY2 affinity and subsequently restoring NPY2 affinity with N-terminal nonpeptidic modifications.[19] The resulting peptides exhibit greater selectivity for the NPY2 receptor than previously described peptide agonists.[19]

Here, we describe new N-terminal modifying groups that allow for site-specific PEGylation to improve in vivo duration of action. In vivo PEG and N-terminal modifying group SAR is assessed using a lean mouse feeding model. The studies result in a novel, long-acting NPY2 receptor agonist that, when dosed once-daily in DIO mice, results in a significant reduction in food intake, body weight, and fat mass.

Results and Discussion

We have previously described a series of selective NPY2 receptor peptide agonists exemplified by **1** (Table 1).[19] The structural features of **1** include (i) the N-terminal modifying group, which improves in vitro NPY2 receptor affinity, and (ii) the peptide core corresponding to residues 25−36 of PYY, which provides the scaffold for NPY2 receptor affinity and selectivity. While peptide **1** of the initial series exhibits a favorable in vitro profile (Table 1), in vivo efficacy is limited (data not shown). This may reflect the poor pharmacokinetic properties expected of a small peptide such as **1**. Therefore, the amine of the N-terminal modifying group was changed to a thiol moiety suitable for site-specific derivatization with PEG, a modification that improves the exposure and duration of action of biologics.[20]

In Vitro Effects of PEGylation. Replacement of the amine group of **1** with a methylmercapto group to generate **2** results in a modest change of in vitro potency (Table 1). Replacement of the methylmercapto moiety with a thiol followed by PEGylation via a maleimide group with linear 5 or 20 kDa PEG or with branched 40 kDa PEG results in analogues that exhibit in vitro activity at the NPY2 receptor comparable to that of the non-PEGylated analogue **2** (Table 1). In general, PEG size or branching does not have a significant effect on in vitro NPY2 receptor binding or activation, with the EC_{50} and K_i at the NPY2 receptor within 2-fold of the values of the non-PEGylated peptides **1** and **2** (Table 1). The PEGylated peptides **3**−**5** maintain selectivity against the NPY1 and NPY5 receptors, with K_i for the NPY1 and NPY5 receptors above 1 μM (Table 1). The PEGylated peptides **3**−**5** display similar stimulation of the NPY2 receptor as assessed by cAMP accumulation (Figure 1A) and have similar affinities for the NPY2 receptor as assessed by the competitive displacement of PYY (Figure 1B).

Effects of PEG Size on Acute Food Intake in Lean Mice. The in vivo activity of the PEGylated analogues on feeding was monitored in lean fasted−refed C57BL/6 mice and compared with PYY(3−36). The change in cumulative food intake was measured relative to control groups dosed subcutaneously with USP saline for PYY(3−36) or with PEG-Cys (in which the maleimide cross-linking group of mPEG-MAL is reacted with Cys) in USP saline of appropriate mass for the PEGylated peptides. A statistically significant difference in food intake is not induced by 5, 20, or 40 kDa PEG-Cys compared to USP saline (data not shown).

PYY(3−36) administered at 0.74 μmol/kg elicits a substantial reduction in food intake of 37 ± 3% at 4 h (Table 1 and Figure 2). The reduction in food intake is short in duration and does not persist to 24 h (Table 1 and Figure 2). These results are in accord with previous reports that PYY(3−36) reduces short-term food intake in mice.[2−8]

The 5 kDa PEG derivative **3** administered at 2.9 μmol/kg causes a reduction in food intake that is less than PYY(3−36),

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[a] Abbreviations: DIO, diet-induced obesity; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; NPY, neuropeptide Y; PEG, polyethylene glycol; PYY, peptide YY; SEM, standard error of the mean; TFA, trifluoroacetic acid; USP, United States Pharmacopeia.

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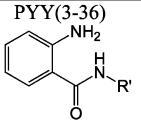
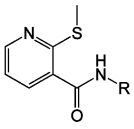
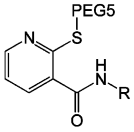
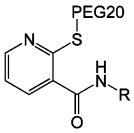
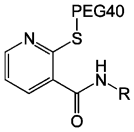
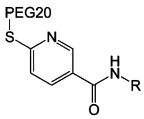
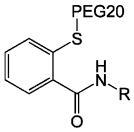
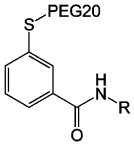
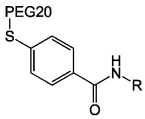
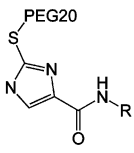
Table 1. In Vitro Receptor Activation and Binding Profile and In Vivo Effects on Food Intake[a]

Peptide	EC_{50} (nM)	K_i (nM)			Cumulative reduction in food intake[b] (%)			
	NPY2	NPY2	NPY1	NPY5	4 h	24 h	48 h	72 h
PYY(3-36)	0.3±0.1	0.4±0.1	21±2	20±2	-37±3*	-5±2	+3±2	+5±2
1 	3±1	4±2	>1000	>1000	n.d.	n.d.	n.d.	n.d.
2 	4±1	11±1	>1000	>1000	n.d.	n.d.	n.d.	n.d.
3 	6±1	7±1	>1000	>1000	-24±3*	-5±2	1±2	2±2
4 	25±6	41±8	4000	1600	-50±3*	-45±2*	-21±1*	-11±1*
5 	16±6	20±6	>1000	>1000	-4±5	-1±2	-1±2	0±1
6 	45±13	65±9	>1000	>1000	-29±2*	-24±2*	-12±1*	-5±1
7 	128±42	102±12	>1000	>1000	-16±3*	-3±1	-0±1	-1±1
8 	80±36	105±42	>1000	>1000	-26±2*	-23±2*	-12±2*	-6±1
9 	49±17	75±9	>1000	>1000	-31±3*	-18±2*	-8±2	-2±1
10 	46±11	52±16	>1000	>1000	-20±3*	-9±2	-4±2	-2±2

[a] All peptides are amidated at the C terminus. R′ of **1** has the sequence RHYLNLVTRQRY−NH_2, corresponding to residues 25−36 of human PYY. R of **2**−**10** has the sequence RHYLNLLTRQRY−NH_2, corresponding to residues 25−36 of human PYY with the amino acid change of Val31 to Leu. PEG5, PEG20, and PEG40 denote that the peptides are derivatized with PEG of 5, 20, and 40 kDa, respectively. Values are the mean of experiments performed in triplicate ± SEM. Experiments were performed at least twice with equivalent results. [b] For fasted−refed mice dosed with 0.74 μmol/kg PYY(3−36) or 2.9 μmol/kg of **2**−**10**. The reduction in food intake is reported relative to USP saline for PYY(3−36) or PEG-Cys of appropriate mass for peptides **2**−**10**. Values are the mean of experiments performed on 20 mice ± SEM: n.d., not determined. The asterisk (∗) denotes $p < 0.05$.

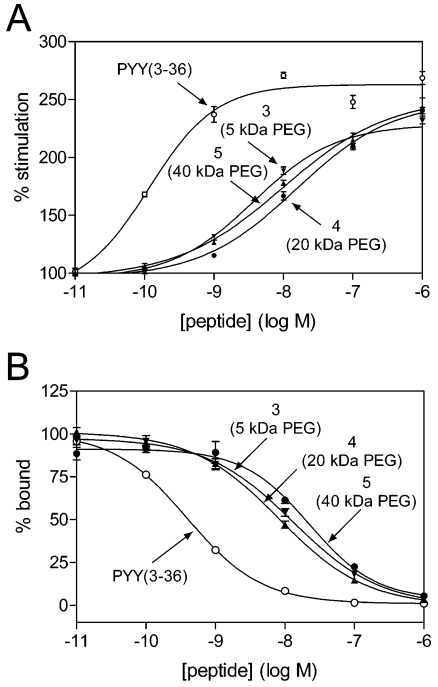


Figure 1. NPY2 receptor activation and binding by human PYY(3−36) (○) and the PEGylated analogues **3** (5 kDa PEG, ▼), **4** (20 kDa PEG, ●), and **5** (40 kDa PEG, ▲): (A) NPY2 receptor stimulation by PYY(3−36) and the PEGylated analogues **3**−**5**, measured by cAMP accumulation; (B) NPY2 receptor binding by PYY(3−36) and the PEGylated analogues **3**−**5**, measured by competitive displacement of ^{125}I-labeled PYY. Values are the mean of experiments performed in triplicate ± SEM. Three independent experiments were performed with equivalent results.

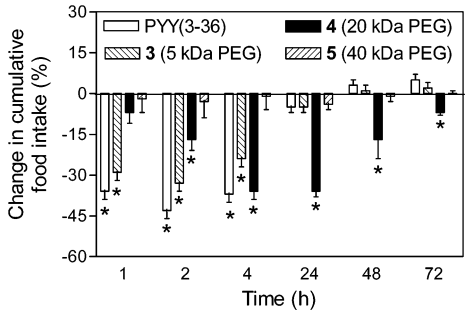


Figure 2. Effect of varying PEG size on food intake in lean C57BL/6 mice. Mice were administered a single subcutaneous dose of PYY(3−36) (0.74 μmol/kg), **3**, **4**, or **5** (2.9 μmol/kg) or PEG (2.9 μmol/kg) in USP saline. Food intake was measured relative to USP saline for PYY-(3−36) or PEG-Cys for **3**−**5**. The asterisk (∗) denotes $p < 0.05$.

with a 24 ± 3% reduction in food intake at 4 h that does not persist to 24 h (Figure 2). The 40 kDa PEG derivative **5** is inactive (Figure 2). Thus, the 5 kDa and 40 kDa PEG derivatives **3** and **5** do not offer improved in vivo efficacy over unmodified PYY(3−36).

The 20 kDa PEG derivative **4** administered at 2.9 μmol/kg induces a higher reduction in food intake to PYY(3−36) of 50 ± 3% at 4 h, which is sustained throughout the 4−24 h period and remains significant (21 ± 1%) at 48 h (Figure 2). The effects in lean mice of **4** on feeding are dose-dependent (Figure 3). Peptide **4** administered at 0.58 μmol/kg induces a comparable reduction in food intake at 4 h to PYY(3−36) at 0.74 μmol/kg that remains significant at 24 h, in contrast to PYY(3−36) (Figure 3). The 20 kDa PEG derivative therefore imparts superior in vivo efficacy with a longer duration of action than PYY(3−36).

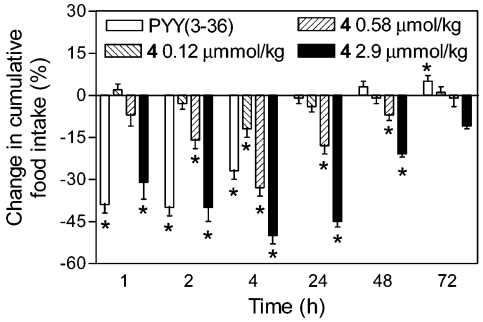


Figure 3. Dose-dependent reduction of food intake in lean C57BL/6 mice by peptide **4**. Mice were administered a single subcutaneous dose of PYY(3−36) (0.74 μmol/kg), 20 kDa PEG-Cys (2.9 μmol/kg), or **4** at 0.12, 0.58, or 2.9 μmol/kg in USP saline. Food intake was measured relative to USP saline for PYY(3−36) or PEG-Cys for **4**. The asterisk (∗) denotes $p < 0.05$.

In Vitro and In Vivo SAR of the N-Terminal Modifying Group. The PEG comparison studies described above used the N-terminal modifying group 2-mercaptonicotinic acid. The use of 4-mercaptonicotinic acid (**5**), mercaptobenzoic acid isomers (**7**−**9**), or mercaptoimadazole (**10**) followed by derivatization with 20 kDa PEG provides selective NPY2 receptor agonists with a 6-fold range of in vitro potencies (Table 1).

The derivatives were screened for in vivo activity in lean mice at 2.9 μmol/kg using the lean mouse fasted−refed model (Table 1). The peptide that is most potent in vitro, **4**, is also the most potent in terms of reducing food intake and duration of action in lean mice (Table 1). The least potent peptide in vitro (**7**) exhibits the least in vivo efficacy, and intermediate efficacy and moderate duration of action are observed for peptides **8**−**10** that also exhibit intermediate in vitro potency at the NPY2 receptor (Table 1). In vitro activity at the NPY2 receptor therefore correlates with in vivo efficacy in lean mice.

Effect in DIO Mice of Daily Dosing on Body Weight and Composition. Peptide **4** was selected for weight loss and body composition studies in DIO mice. Once-daily subcutaneous administration of **4** results in a dose-dependent reduction in body weight in DIO mice, with a loss of approximately 7% body weight following dosing at 2.9 μmol/kg over 7 days (Figure 4A). Food intake is reduced for the treated animals during the first 3 days of treatment and then increases on day 4 to levels seen for the untreated control groups (Figure 4B). The initial reduction and subsequent restoration of food intake are also observed with PYY(3−36) upon continuous dosing in mice[5,7,8] and are seen with other anorexigenic agents such as the small-molecule type 1 cannabinoid receptor antagonist rimanobant (SR141716).[21]

The body-weight loss is due to a reduction in fat mass, as assessed using NMR analysis of body composition (Figure 4C). There is also an apparent slight loss in lean tissue mass (Figure 4D). The change in lean tissue mass is not, however, statistically significant ($p = 0.2$).

Conclusion

The lead optimization of the selective NPY2 receptor peptide agonist **1** revolved around different nonpeptidic N-terminal modifying groups and changes in PEG size. These studies identify **4**, which is a potent and selective NPY2 receptor agonist that exhibits significant in vivo efficacy through the reduction of food intake in lean mice and the reduction of food intake, body weight, and fat mass in DIO mice.

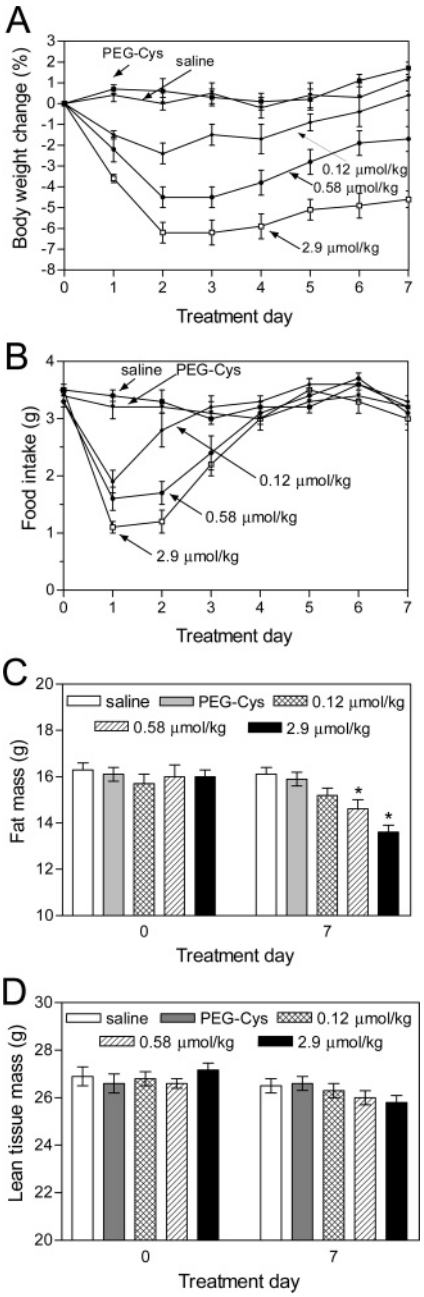


Figure 4. Effects on DIO mice of once-daily, subcutaneous dosing with **4**: (A) dose-dependent reduction in body weight; (B) dose-dependent reduction in food intake; (C) dose-dependent reduction in body fat measured with NMR; (D) apparent slight dose-dependent reduction in muscle mass measured with NMR ($p = 0.2$). Mice were administered USP saline, 20 kDa PEG-Cys (2.9 μmol/kg), or **4** at 0.12, 0.58 or 2.9 μmol/kg. The asterisk (*) denotes $p < 0.05$.

Materials and Methods

PYY(3−36). Human PYY(3−36) was purchased from American Peptide Co. Inc., Sunnyvale, CA (catalog number 48-0-33).

Peptide Synthesis. Peptides were synthesized with solid-phase Fmoc chemistry on Rink amide resin with an ABI 433A synthesizer. The following side chain protecting groups were used: O^{t}Bu for Asp and Glu; ^{t}Bu for Ser, Th, and Tyr; Boc for Lys; Trt for Asn, His, and Gln; Pmc for Arg. The amine and thiol moieties of the N-terminal modifying groups of **1** and **2**−**10** were protected during coupling with Fmoc and Trt, respectively. N-Terminal modifying groups were obtained from Aldrich (**1**−**9**) and Interchim (**10**). Amino acids were activated with HBTU/DIEA. The Fmoc group was deprotected with 20% piperdine in NMP. For N-terminally modified peptides **1**−**10**, a 10-fold molar excess of the modifying group over resin was activated with HBTU/DIEA and coupled as per a normal amino acid except that the coupling reaction proceeded overnight. Prior to cleavage, the resin was washed with NMP and dichloromethane and dried under a stream of argon. The peptide was cleaved with 84.6% TFA, 4.4% H_2O, 4.4% phenol, 4.4% thioanisole, and 2.2% ethandithiol for 2 h and filtered though glass wool directly into 40 mL of cold (4 °C) methyl *tert*-butyl ether. The precipitate was collected by centrifugation, washed by resuspension in 40 mL of cold methyl *tert*-butyl ether, collected with centrifugation, and dried under a stream of Ar. The crude peptide was suspended in 8 M guanidine hydrochloride, and an equal volume of 0.1% (v/v) TFA (aq) was added. Purification was by reversed-phase C_{18} HPLC (Varian Dynamax R000832221C) and a linear water/acetonitrile gradient (0.5%/min) containing 0.1% TFA. Purity (>98%) was confirmed with analytical C_{18} and cation-exchange HPLC. Identity was confirmed with electrospray mass spectrometry, and in each case the expected and observed masses agreed to within 1 Da.

PEGylation. Peptides were cross-linked to PEG conjugated to maleimide via the thiol group of the N-terminal modifying group. Peptides were incubated with a 2-fold molar excess of 5 kDa mPEG-MAL (Nektar Therapeutics, 2D2M0H01), 20 kDa mPEG-MAL (Nektar Therapeutics, 2D2M0P01), or 40 kDa mPEG2-MAL (Nektar Therapeutics, 2D3X0T01) in 100 mM Tris, pH 8, for 2 h. PEGylated peptides were purified with cation-exchange HPLC using a SP-5PW column (TosaHaas 07575) equilibrated in 10 mM HCl, 20% methanol, and a linear NaCl gradient. The product was dialyzed against water and lypholized. Purity (>97%), including the absence of free peptide, was confirmed with analytical C_{18} and cation-exchange HPLC.

Preparation of PEG-Cys. Cys was used to block the maleimide moiety of the PEG reagent for use as a control for in vivo studies. The PEG reagent (5 or 20 kDa mPEG-MAL or 40 kDa mPEG2-MAL) was incubated overnight with a 2-fold molar excess of Cys in Tris, pH 7.4, at room temperature, dialyzed against water, and lyophilized.

Peptide Concentration. Peptide amount was determined with amino acid analysis by the W. M. Keck Foundation Biotechnology Facility, Yale University, New Haven, CT.

Receptor Binding and Activation Assays. NPY2 receptor [^{35}S]-GTPγ[S] functional cAMP accumulation assays and NPY1, NPY2, and NPY5 receptor ^{125}I-labeled PYY displacement assays were performed using the human receptors as described previously.[19]

Fasted−Refed Feeding Studies in C57BL/6 Lean Mice. Lean C57BL/6 male mice (Taconic Farms, Inc.) 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 (standard chow pellet diet) continuously available. A fasted−refed study included 20 mice 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 peptide in USP saline. Control groups were dosed with USP saline or PEG-Cys in USP saline. Preweighed food was provided 30 min after dosing. The significance of differences in food consumption was evaluated by analysis of variance followed by Fisher's PLSD post hoc analysis (StatView, SAS Inc.).

Body-Weight Loss Studies in C57BL/6 DIO Mice. Male C57BL/6 mice (Taconic Farms, Inc.) were fed a high-fat diet containing 45% calories from fat (Research Diets, D12451) for 20−22 weeks and had an average body weight over 5 standard deviations greater than mice fed a standard laboratory diet (5% calories from fat). A treatment group comprised 10 mice per treatment group with body weights of approximately 44 g (standard deviation, 0.4 g). Mice were kept in standard animal rooms under controlled temperature and humidity and a 12/12 h light−dark cycle. Food and water were continuously available. Animals were adapted to the grid floors for 4 days and sham-dosed with study vehicle for another 4 days before recording of 2 days of baseline body weight and 24 h of food consumption. Mice were assigned to treatment groups based on their body weight so that the initial mean and

SEM of body weight were similar. Animals were administered peptide in USP saline subcutaneously once daily before the dark phase, and body weight and food consumption were measured. On the final day, body fat and lean tissue composition were measured with quantitative NMR with a Bruker Minispec. The animals were then euthanized by CO_2 inhalation, and body weight was measured.

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