Neuropeptide Y (NPY) Y_4 Receptor Selective Agonists Based on NPY(32−36): Development of an Anorectic Y_4 Receptor Selective Agonist with Picomolar Affinity

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Received September 13, 2005

We have previously shown [Cys-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$, **1**, to be a moderately selective neuropeptide Y (NPY) Y_4 receptor agonist. Toward improving the selectivity and potency for Y_4 receptors, we studied the effects of dimerizing H-Trp-Arg-Nva-Arg-Tyr-NH_2 using various diamino-dicarboxylic acids containing either di-, tri-, or tetramethylene spacers. These parallel dimers, **2A**, **2B**, **3**, **4A**, and **4B**, and the corresponding linear tandem dimer and trimer analogues, **5** and **6**, had enhanced selectivity and affinity for Y_4 receptors compared to **1** (Table 1). Substitution of Trp and Nva with Tyr and Leu, respectively, as in 2,7-D/L-diaminosuberic acid derivatized dimer, **7**, resulted in a superior Y_4 selective agonist with picomolar affinity. Intraperitoneal (ip) injection of **7** potently inhibited food intake in fasted mice. Moreover, **7** (ip) inhibited the food intake in wild-type mice and not in $Y_4^{-/-}$ knock-out mice, confirming that the actions of **7** on food intake are not due to global effects, but specifically mediated Y_4 receptors.

Introduction

Neuropeptide Y (NPY), a 36-residue peptide amide isolated originally from the porcine brain, along with peptide YY (PYY) isolated from the intestine, and pancreatic polypeptide (PP) isolated from the pancreas, constitutes a family of homologous hormones.[1,2] Investigations to date have implicated these hormones in a wide variety of central and peripheral activities. These actions are mediated by at least six G-protein coupled receptors denoted as Y_1, Y_2, Y_3, Y_4, Y_5, and y_6, which have been shown to be coupled positively and negatively to intracellular Ca^{2+} and cAMP, respectively.

Investigations using specific receptor knock-out models have substantiated the involvement of these hormones in a wide spectrum of activities, and provide strong evidence for the involvement of Y_1, Y_2, and Y_5 receptors on feeding,[3−5] Y_1 receptors on vasoconstriction,[3] Y_2 receptors on angiogenesis,[6,7] Y_2 and Y_4 receptors on bone formation,[8,9] Y_2 and Y_5 receptors on seizure activity,[10] and Y_4 receptors on fertility.[11] In addition, Y_4 receptors have been shown to play a role in cardiac function,[12] glucose metabolism in chronic pancreatitis patients,[13] and mediation of intestinal absorption of electrolytes and water.[13]

The findings, as early as in 1979, that PP (iv) reduced food intake in subjects with Prader−Willi syndrome, a condition characterized by childhood hyperphagia and morbid obesity and associated with a deficiency in basal and postprandial PP concentrations, provided the first evidence that PP, the endogenous ligand for Y_4 receptors, may also play a role in the pathophysiology of obesity.[14] Consistent with these observations, it has been found that (1) plasma PP levels are reduced in other forms of human obesity[15,16] and are elevated in patients diagnosed with anorexia nervosa;[17] (2) mice overexpressing PP ate less and gained less body weight, and that this condition was reversed by PP antiserum;[18] and (3) systemic PP inhibited both the appetite and caloric intake in normal humans.[19] Moreover, a recent comprehensive investigation by Asakawa et al.[20] revealed that PP (iv) induces a state of negative energy balance by decreasing food intake and increasing energy expenditures in mice via decreasing the expression of orexigenic hormones, NPY, orexin, and ghrelin, while upregulating the anorexigenic urocortin and decreasing gastric emptying. It was also determined that daily administration of PP (ip) over 6−14 days ameliorated obesity and hyperlipidemia and improved glycemic control in ob/ob obese mice and FLS-obese mice. The latter mouse is known for fatty liver and liver enzyme abnormalities, and these conditions were also ameliorated by PP. Asakawa et al.[20] also found that the anorexigenic effects of peripheral PP to be more potent than PYY, leptin, CART, or α-MSH. These observations unequivocally demonstrate that postprandially released PP constitutes a powerful satiety agent and suggest that PP-based compounds may ultimately be developed to treat obesity. Since PP, unlike those of the centrally mediated effect of Y_1 and Y_5 ligands on food intake, exhibits anorectic effects on peripheral administration, even Y_4-peptidic agonists may prove invaluable as antiobesity drugs. However, native PP may not be an ideal candidate because it only has a

Table 1. C-Terminal Pentapeptide Parallel and Tandem Dimer Analogues of NPY

no.	structures[a]
1	[Cys-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
2A	Pim[-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
2B	Pim[-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
3[b]	Adp[-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
4A	Sub[-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
4B	Sub[-Trp-Arg-Nva-Arg-Tyr-NH_2]$_2$
5	H-[Trp-Arg-Nva-Arg-Tyr]$_2$-NH_2
6	H-[Trp-Arg-Nva-Arg-Tyr]$_3$-NH_2
7[b]	Sub[-Tyr-Arg-Leu-Arg-Tyr-NH_2]$_2$

[a] Adp, 2, 5-D/L-diaminoadipic acid dimer; Pim, 2, 6-D/L-diaminopimelic acid dimer; Sub, 2, 7-D/L-diaminosuberic acid dimer. [b] Isomers could not be separated.

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10.1021/jm050907d CCC: $33.50
Published on Web 03/30/2006

Table 2. Affinities of C-Terminal Pentapeptide Parallel and Tandem Dimer Analogues of NPY for Cloned "Y"-Receptors (see Experimental Section for details

peptides	K_i, nM Y_4[a]	Y_1	Y_2	Y_5	selectivity for Y_4: $Y_4/Y_1/Y_2/Y_5$
NPY(PP)	(0.08 ± 0.01)[b]	0.50 ± 0.02	0.20 ± 0.02	2.20 ± 0.03	
1[c]	30 ± 10	340 ± 60	>3000	770 ± 80	1/10/110/30
2A	4.3 ± 0.2	120 ± 10	310 ± 20	2160 ± 240	1/30/70/500
2B	4.1 ± 0.6	140 ± 10	260 ± 40	2280 ± 140	1/40/60/550
3	4.2 ± 0.3	140 ± 10	360 ± 30	330 ± 30	1/34/85/78
4A	4.1 ± 0.4	90 ± 10	390 ± 10	120 ± 10	1/20/100/30
4B	2.0 ± 0.2	80 ± 10	$530 \pm 8 = 90$	280 ± 30	1/40/270/140
5[c]	5.3 ± 0.8	220 ± 80	1050 ± 260	250 ± 90	1/50/200/50
6	3.9 ± 0.2	30 ± 3	50 ± 10	160 ± 30	1/10/14/40
7	0.05 ± 0.01	7.50 ± 0.90	890 ± 90	>1000	1/150/17800/20000

[1] Y_4 receptor affinities of analogues were determined using ^{125}I-hPP. Y_1, Y_2, and Y_5 receptor affinities were determined using ^{125}I-NPY. [2] hPP was used instead of NPY. [3] From ref 27.

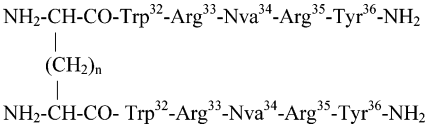

Figure 1. Structure of diamino-dicarboxylic acid derived dimer peptides. $n = 2$: 2,5-D/L-diaminoadipic acid (Adp) dimer; $n = 3$: 2,6-D/L-diaminopimelic acid (Pim) dimer; $n = 4$: 2,7-D/L-diaminosuberic acid (Sub) dimer.

half-life of ~6 min[21] and exhibits high affinity for Y_5 receptors.[22] In this regard, although numerous Y_1 and Y_5 receptor selective ligands and a few Y_2 receptor selective compounds have been developed,[1,2,23,24] no peptidic or nonpeptidic Y_4 receptor selective ligands have been developed to date.

We have previously demonstrated that Y_1 receptor selective antagonists, GR231118 developed by Daniel and co-workers[25] and compounds **1** and **5** developed by us (Table 1), also exhibit potent Y_4 agonist activity.[26,27] Our structure−activity studies with **1** have resulted in the development of a series of Y_4 receptor selective agonists, and one of these agonists, **7**, binds selectively to Y_4 receptors with picomolar affinity (Table 2) and inhibits food intake in fasted mice upon intraperitoneal (ip) administration. These investigations are described in this manuscript.

Results and Discussion:

All peptides used in this study were >94% homogeneous as determined by reversed phase analytical chromatography using two different solvent systems and had the expected mass (see Supporting Information). Diamino-dicarboxylic acid peptides (Figure 1) were synthesized using the corresponding N-α-di-Boc-D/L-diamino-dicarboxylic acids, and the two isomers were separated by reversed phase chromatography, but were not identified as to which peak corresponded to the D- and L-isomers, respectively. In the case of compounds **3** and **7**, the isomers could not be separated and they were therefore used as D/L mixtures.

We have previously reported **1** to be a selective Y_1 receptor antagonist relative to the Y_2 receptors.[26] However, reinvestigation of its properties after cloning of Y_4 and Y_5 receptors revealed that **1** is a moderately selective Y_4 agonist (Table 3).[27] This was in contrast to the corresponding monomer, which exhibited weak affinity for "Y" receptors (not shown). It appears therefore that dimerization may be stabilizing the bioactive conformation under physiological conditions. We therefore reasoned that the Y_4 receptor affinity and selectivity could further be enhanced via optimizing the spacing and orientation of the two peptide chains. Toward this end, we synthesized dimers using 2, 6-D/L-diamino pimelic acid (Pim, **2A**, **2B**), 2, 5-D/L-diaminoadipic acid (Adp, **3**), and 2,7-D/L-diamino-suberic acid (Sub, **4A**, **4B**) (Figure 1, Table 1).[28] It should be noted that trimethylene (anti) and di-/tetramethylene (syn) spacers would place the two peptide chains in opposite directions.[29]

Table 3. Agonist (EC_{50}, nM) or Antagonist (K_b in bold, nM) Potencies of the C-Terminal Pentapeptide Parallel and Tandem Dimer Analogues of NPY in Cells Expressing Cloned "Y"-Receptors[a]

peptides	EC_{50} or K_b (bold), nM Y_4	Y_1	Y_2	Y_5
NPY (PP)	(0.10 ± 0.01)[a]	0.60 ± 0.03	1.70 ± 0.20	9.90 ± 0.10
1[b]	570 ± 90[c]	**1730 ± 320**	ND[e]	ND
2A	200 ± 20[c]	no effect[d]	no effect	no effect
2B	220 ± 30[c]	no effect	no effect	no effect
3	460 ± 20[c]	no effect	no effect	no effect
4A	430 ± 110[c]	no effect	no effect	no effect
4B	370 ± 40[c]	1410 ± 830	no effect	no effect
5[b]	290 ± 50[c]	**320 ± 20**	ND	ND
6[b]	230 ± 20[c]	no effect*	1180 ± 560	no effect*
7	14.8 ± 0.5[c]	240 ± 30	no effect*	no effect*

[a] PP was used instead of NPY as a control in Y_4 cells. [b] From ref 27. [c] These analogues and hPP inhibited forskolin stimulated cAMP production in Y_4 cells to the same maximal extent. Therefore, these analogues are full Y_4 agonists. [d] No effect, no agonist/antagonist activity at = 20 000 nM, and those indicated with "*"also did not exhibit any agonist activity, but their antagonist effects were not determined. [e]ND, not determined.

As anticipated, dimerization with diamino-dicarboxylic acids as in analogues **2A**, **2B**, **3**, **4A**, and **4B** enhanced the affinity for Y_4 receptors 7−14 times relative to the Cys-Cys dimer **1** (Table 2, and Figure 1S in Supporting Information). Moreover, these analogues exhibited higher selectivity for Y_4 receptors than **1**, especially relative to Y_1 and Y_5 receptors, and retained modest selectivity relative to Y_2 receptors. To determine whether the observed Y_4 selectivity based on receptor affinities also extended to functional activities, we investigated the effects of **2A**, **2B**, **3**, **4A**, and **4B** on forskolin-stimulated cAMP synthesis in cells expressing Y_1, Y_2, Y_4, and Y_5 receptors. These studies revealed that these analogues are full agonists at the Y_4 receptors since they inhibited cAMP synthesis to the same maximal extent as the intact hPP and did not exhibit any Y_4 receptor antagonist effects at <20 000 nM (Table 3, and Figure 2S in Supporting Information). Moreover, the selectivity of these analogues for Y_4 receptors was further exemplified in the functional studies. All analogues, except **4B**, exhibited neither agonist nor antagonist activities in any of the other "Y" receptors at = 20 000 nM. Even **4B** exhibited only very weak agonist activity at Y_1 receptors. It is, however, surprising that all the diamino-dicarboxylic acid bridging groups used in **2A**, **2B**, **3**, **4A**, and **4B** enhanced the Y_4 receptor affinity to the same degree despite differences in the disposition of monomers with regard to the spacing and the orientation. This may be because the diamino-dicarboxylic acids induced similar conformational changes and thus imparted the same degree of Y_4 receptor selectivity and affinity. Alternatively, the presence of multiple methylene

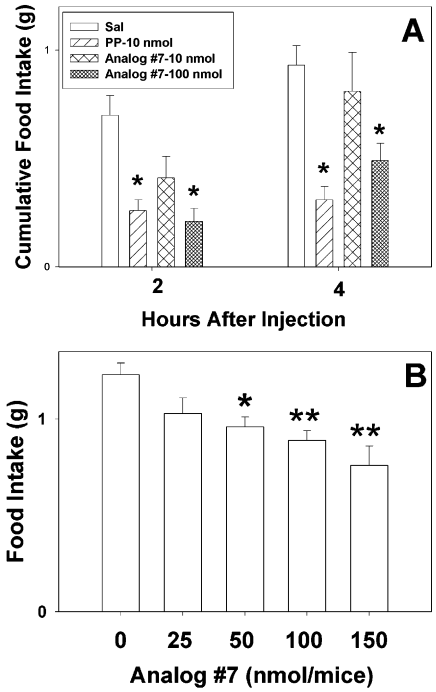

Figure 2. (A) Peripheral treatment (bolus, ip) with hPP (10 nmol/mouse) or analogue **7** (100 nmol/mouse) significantly inhibited the food intake by fasted mice ($n = 8$ per group). * = $p > 0.05$ vs.saline. Although analogue **7** (10 nmol/mouse) exhibited a tendency to inhibit the food intake, it did not reach significant levels. (B) Analogue **7** dose-dependently inhibited the food intake by fasted mice. Increasing doses of analogue **7** were injected (bolus, ip) to fasted mice ($n = 8$ per group), and the 4 h food intake was monitored. * = $p < 0.05$ vs saline; ** = $p < 0.01$ vs.saline.

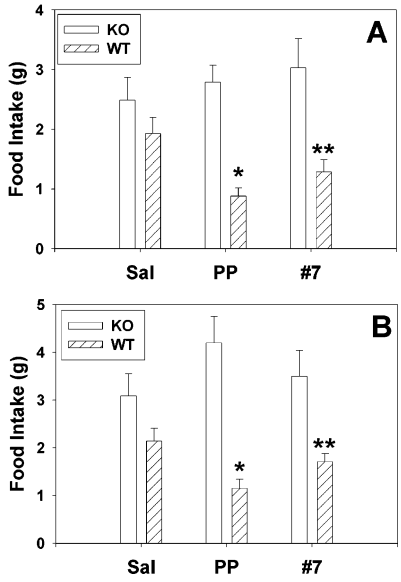

Figure 3. hPP (10 nmol/mouse, bolus, ip) and analogue **7** (100 nmol/mouse) significantly inhibited 2 h (A) and 4 h (B) food intake by fasted wild-type mice (WT) and not by fasted Y_4 receptor knock-out (KO) mice ($n = 7$ per group). * = $p < 0.01$ vs saline; ** = $p < 0.05$ vs saline.

groups in diamino-dicarboxylic acid dimers may strengthen the association with lipid membranes or facilitate interaction of one of the message sequences (monomer) with the receptors. The latter possibility seems more plausible because the previously developed dimer, **5**, of this peptide unit exhibited similar affinity and selectivity for Y_4 receptors as the parallel dimers, **2A**, **2B**, **3**, **4A**, and **4B**, investigated here (Table 2 and 3).[27] To substantiate this observation, we also synthesized the corresponding tandem trimer **6**, which had similar affinity and selectivity for Y_4 receptors (Table 2 and 3). At present, however, we do not know which one of these possibilities is correct. If lipid interactions are important, then elongation of the monomer with an idealized amphipathic helix as has been done in glucagon[29] or even acylation of monomer with fatty acids should enhance Y_4 receptor affinities. These possibilities remain to be investigated. However, we already know that (1) NPY contains a central amphipathic α-helical region that interacts with lipids;[30,31] (2) this region only plays a role in orienting the active site for interaction with the receptors;[32,33] and (3) this segment could be replaced by 8-aminooctanoic acid or 6-aminohexanoic without much loss in receptor affinities.[32,33]

We have previously determined that the purported potent Y_1 receptor selective antagonist, Bis(29/31′, 29′/31) {[Glu[29], Pro[30], Dpr,[31] Tyr[32], Leu[34]]NPY(28−36)-NH_2]}, GR231118, exhibits subnanomolar affinity to Y_4 receptors.[27] Therefore, we hypothesized that substitution of Trp[32] and Nva[34] in the diamino-dicarboxylic derivatized pentapeptide dimers (Table 1) with Tyr and Leu, respectively, as in GR231118, may further enhance the Y_4 affinity.[27] As expected these substitutions resulted in analogue **7** with picomolar affinity to Y_4 receptors, an increase of 40−80 times over the Trp and Nva substituted pentapeptide dimers (Table 1). Moreover, its Y_4 selectivity relative to all the other "Y" receptors was substantially enhanced. Analogue **7** also potently inhibited forskolin-stimulated cAMP synthesis in Y_4 cells, and did not exhibit any Y_2 or Y_5 agonist activity at < 20 000 nM and behaved as a weak agonist in Y_1 cells. It should be noted that **7** is 150 times more selective for Y_4 receptors relative to Y_1 receptors. This increased affinity of **7** to Y_4 receptors may be due to its ability to further stabilize the bioactive conformation via additional hydrogen bonding possibility through the hydroxyl group of Tyr[32]. In this regard, our recent 2D NMR and molecular modeling studies with the monomer analogues of G231118, [Pro[30], Tyr[32], Leu[34]]NPY(28−36)-NH_2 (BW1911U90) and [Pro[30], Tyr[32], Leu[34]]NPY(28−36)-OMe, have revealed that the Tyr[32] hydroxyl group is in fact involved in hydrogen bonding in these analogues.[34] Moreover, Daniel and co-workers[35] have previously shown that Tyr[32] plays a crucial role in the stabilization of the conformation of yet an another C-terminal analogue of NPY, [Tyr[32], Leu[34]]NPY(27−36)-NH_2, through additional hydrogen bonding.

Implications of PP in the pathophysiology of human obesity,[14−17] and the findings that PP could attenuate food intake in mice[18,20] and humans,[16,19] led us to investigate the effects of the most potent and highly selective Y_4 receptor agonist, analogue **7**, on food intake. Analogue **7** (ip) (100 nmol/mice) significantly inhibited the 2 and 4 h food intake in fasted mice (Figure 2A). This effect of analogue **7** was comparable to that of intact PP (10 nmol/mouse). Analogue **7** also exhibited a tendency to inhibit the 2 h food intake at a lower dose (10 nmol/), but this effect did not reach significant levels. Moreover, **7** inhibited the food intake in a dose-dependent manner suggesting that these effects of **7** are receptor mediated (Figure 2B). To unambiguously prove the involvement of Y_4 receptors in the inhibitory effects of **7** on food intake, we also repeated the studies in $Y_4^{-/-}$ mice.[11] As shown in Figure 3, both PP (10 nmol/, ip) and **7** (100 nmol/) significantly inhibited the 2 and 4 h food intake in wild-type mice compared to saline treatment but had no effects at all on the food intake in $Y_4^{-/-}$ mice. These observations reaffirm the specificity of **7** for Y_4 receptors and show that the inhibitory effect of **7** on food intake is a receptor mediated event. It should also be noted that $Y_4^{-/-}$ mice,

regardless of the treatment, exhibited a tendency to consume more food, but this did not reach significant levels compared to the food intake by saline-wild type group. However, this is not surprising because postprandially released PP will not be able to relay the satiety signal in $Y_4^{-/-}$ mice.

Another point to be noticed in our studies presented here is that although PP and **7** exhibited comparable picomolar affinities to Y_4 receptors, **7** was less potent than PP in the cAMP assay as well as in feeding experiments. At this point we do not know the reasons for these differences. However, the binding studies were done with membranes in low [Na^+], whereas cAMP studies were done in cells in physiological medium containing high [Na^+]. Moreover, we have previously shown that the binding of C-terminal analogues of NPY including GR231118 to Y_4 receptors are attenuated by high [Na^+], and their efficacies compared favorably with their affinities determined in high [Na^+] medium.[36] On the other hand, high [Na^+] had little or no effect on the binding of intact PP to Y_4 receptors.[36] It appears plausible that intact PP, unlike the C-terminal analogues of NPY such as GR231118 and **7**, has sufficient tertiary structure to overcome the disruption of ionic and polar interactions by high [Na^+] and is therefore able to retain high potency even in vitro and in vivo functional assays. In addition, agonist binding to Y_4 receptor also shows selective sensitivity to modulators of Na^+ transporters suggesting Y_4 receptor association with ion transporters and exchangers.[37] It remains to be determined whether alleosteric participation of Na^+ transporters also play a role in the observed differences in the efficacies of intact PP and **7**.

In summary, we have developed a series of highly selective and potent Y_4 receptor selective agonists, with one of the compounds, **7**, exhibiting picomolar affinity to Y_4 receptors. These compounds may prove useful in delineating Y_4 receptor-mediated activities and may provide a framework for the development of Y_4 receptor selective ligands, which may have clinical utility in treating various disorders including obesity, bone formation, intestinal absorption, glucose metabolism, and fertility.

Experimental Section

Peptide Synthesis. All peptides were synthesized by the standard stepwise t-Boc solid-phase method and purified by reversed phase chromatography according to our recently published procedures.[38] Briefly, the protected amino acids were assembled sequentially on *p*-methylbenzhydrylamine resin (0.45 mmol amino group) using an automated Applied Biosystem instrument employing a program supplied by the manufacturers for single coupling procedures. All amino acids were coupled using 4.4 equiv of preformed 1-hydroxybenzotriazole esters. To obtain parallel dimers, N-α-di-Boc-cystine or N-α-di-Boc-D/L-diamino-dicarboxylic acids (0.5 equiv) were coupled to the N-terminus of the protected pentapeptide peptide resin in the presence of equivalent quantities of DIC, HOBT, and DIEA. This coupling was performed manually and was generally complete within 10−18 h. At the end of the synthesis, the N-α-Boc group was removed as programmed. N^{in}-CHO, if present, was then removed with 20% piperidine−DMF, and the free peptides were obtained by treating the peptide resins (∼0.25 mmol) with HF (∼10 mL) containing 5% *p*-cresol for about an hour at −2 to −4 °C. In the case of peptides with Trp or Cys, the HF reaction mixture also contained ∼2.5% dimethyl sulfide. The residue after HF cleavage was washed repeatedly with diethyl ether, extracted with 30% acetic acid (2 × 15 mL), diluted to 10%, and lyophilized. Peptides were purified to homogeneity by semipreparative reversed phase chromatography on a Waters HPLC system with a Vydac C_{18} column (10 mm × 250 mm, 300 Å pore size, 10 μ particle size) using a gradient of 20−50%B over 60 min at a flow rate of 4.7 mL/min (A = 0.1% TFA−H_2O, B = 0.1% TFA in 90% CH_3-CN:10% H_2O) as described by us for NPY.[38] All peptides were characterized by analytical reversed phase chromatography using two different solvent systems and mass spectral analyses (Table 1S in Supporting Information).

Radioligand Binding and cAMP Assays. Generation of CHO or HEK-293 cells permanently transfected with cloned human NPY Y_1, Y_2, Y_4 or a rat-human hybrid Y_5 (rhY_5) receptors have been described in detail previously.[27] Radioligand binding and cAMP assay procedures have also been reported.[27] Briefly, the competition binding assays were carried out by incubating 5−10 μg of CHO cell membrane protein with 0.2 nM ^{125}I-PYY (Dupont-NEN, Boston, MA, 2200 Ci/mmol) and various concentrations of competing peptides in 200 μL of binding buffer (50 mM HEPES, pH 7.3, 0.1% bovine serum albumin, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) for 90 min at room temperature. Bound and unbound radioligands were then separated by filtration through 0.3% polyethyleneimine pretreated glass fiber filters in a 96-well format. Each filter was then washed three times with phosphate-buffered saline and subsequently counted in a γ-counter. ^{125}I-PP was used as the radioligand in the investigations with Y_4 cell membranes. The binding in the presence of 1 μM NPY (or PP in the case of Y_4 receptors) was taken as the nonspecific binding.

For cAMP determinations, CHO (Y_2, Y_4, and Y_5 receptors) or 293 (Y_1 receptors) cells were seeded into 96-well plates at a density of 30 000 cells per well. After 48 h, the cells were rinsed with Hank's balanced salt solution (Life Technologies) and preincubated for 20 min at 37 °C with assay buffer (Hank's balanced salt solution supplemented with 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin, 4 mM $MgCl_2$, and 1 mM isobutyl-1-methylxanthine). In the case of antagonism experiments, the preincubation medium also contained the antagonist (100 nM). The medium was then replaced with the assay buffer containing 2.5 μM forskolin for CHO cells or 5.0 μM for HEK293 cells and various concentrations of peptides. After 10 min incubation at 37 °C, the assay buffer was removed and the cells lysed with ethanol. The ethanol was evaporated, and cAMP content in each well was determined using the ^{125}I-cAMP Flash Plate kit (Dupont-NEN) according to the protocols provided by the manufacturer.

Each point in the binding assay was done in duplicate, and each peptide was tested in three separate experiments. Each point in the cAMP assay was carried out in triplicate, and each assay was repeated at least three times. All the data were analyzed by nonlinear regression analysis (GraphPad Prism Software, San Diego, CA).

Feeding Studies. 7−8 week old C57BL/6 male mice (Harlan Laboratories, Indianapolis, IN) were housed in individual cages in a temperature-controlled room (25 °C) under 12-h light/dark cycle. Mice had free access to standard chow and water. Animals were also acclimated to daily ip saline injections. After one week of acclimatization, animals were fasted for 18 h the night before the experiment, and saline (0.1 mL) or peptides (10 or 100 nmol/mice) in saline (0.1 mL) were injected (ip). Food was provided, and the 2 and 4 h food intakes were monitored. To determine whether **7** exhibits a dose-dependent inhibition on food intake, we repeated the experiments in 18 h fasted mice, injected saline (0.1 mL) or increasing doses of **7** (25, 50, 100, or 150 nmol/mice, ip), and monitored the 4 h food intake.

To unambiguously determine the involvement of Y_4 receptors on the inhibitory effects of **7** on food intake, experiments were also conducted in $Y_4^{-/-}$ mice in the laboratories of Herzog and co-workers.[9,11] Generation and characterization of C57BL/6 $Y_4^{-/-}$ mice have already been described.[9,11] Nine week old male $Y_4^{-/-}$ mice and wild type mice (n = 7 per group) were individually housed and used in a double blind cross-over fashion for injecting the drugs and monitoring of food intake. The three injections were separated by one week each. For all experiments, mice were fasted for 18 h and then injected (ip) with either 0.10 mL of saline, PP (10 nmol) in 0.10 mL of saline, or **7** (100 nmol) in 0.10 mL of saline per mouse. After injection, 2 and 4 h food intake were monitored.

Statistical significance was determined by ANOVA, with individual means being compared post-hoc by Tukey's corrected *t*-test.

Animal procedures were approved by University of Cincinnati IACUC committee.

Acknowledgment. This research was supported in part by NIH grant GM47122.

Supporting Information Available: Analytical data and figures depicting Y_4 receptor affinity and agonist activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050907D