Antiobesity Effect of a Short-Length Peptide YY Analogue after Continuous Administration in Mice

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S Supporting Information

ABSTRACT: Gastrointestinal peptides such as peptide YY (PYY) can regulate appetite, which is relevant to the study of obesity. The intraperitoneal bolus administration of PYY_{3–36} and a 12-amino acid PYY analogue, benzoyl-[Cha^{27,28,36},Aib³¹]PYY_{25–36} (**1**), showed similar anorectic activity by activating the Y2 receptor (Y2R). However, food intake inhibition and body weight loss were not observed upon continuous subcutaneous administration of **1** with osmotic pumps in diet-induced obese (DIO) mice. N-Terminal elongation of **1**, together with amino acid substitution at position 24, led to a hydrophilic 14-amino acid peptide, Ac-[D-Hyp²⁴,Cha^{27,28,36},Aib³¹]PYY_{23–36} (**18**), that showed higher affinity and more potent agonist activity for Y2R and a robust anorectic activity with potency similar to that of PYY_{3–36}. In addition, the continuous subcutaneous administration of **18** at 0.3 mg/(kg·day) induced significant body weight loss in DIO mice. These results suggest that a short-length PYY analogue can be a lead compound for antiobesity therapy in a sustained-release formulation.

KEYWORDS: Peptide YY (PYY), Y2 receptor (Y2R), Antiobesity, Anorectic effect, Short-length PYY analogue

Several gastrointestinal peptides are known to regulate appetite, such as ghrelin, glucagon-like peptide-1, and peptide YY (PYY). Peptide YY is synthesized and released from endocrine L cells in the distal gut into the circulation in response to food intake. Peptide YY is cleaved into PYY_{3–36} by the enzyme dipeptidyl peptidase IV. Peripheral administration of PYY_{3–36} has been shown to reduce food intake and body weight gain in rodents.¹ When administered intravenously to humans, PYY_{3–36} has been shown to reduce calorie intake. It was reported that the plasma PYY levels of fasting obese subjects are lower than those in fasting lean subjects,² and PYY levels are affected by the duration of a fasting period.³ The postprandial increases in plasma PYY levels are more blunted in obese subjects compared to those in lean subjects.^{2,4,5} Peptide YY knockout mice display an obese phenotype, which is reversed by administration of exogenous PYY.⁶ Thus, PYY administration has been explored as an attractive concept for antiobesity therapy.

N-Terminally truncated PYY analogues, which are composed of 12 amino acids with an N-terminal benzoyl group, were shown to possess potent agonist activity for the neuropeptide Y Y2 receptor (Y2R) and strict selectivity for Y2R over receptors Y1 and Y5.⁷ It is well-known that short-length peptides are readily degraded by proteases and are rapidly excreted by renal clearance. To circumvent these issues, a simple modification with polyethylene glycol (PEG) (i.e., PEGylation) is often performed, as found in PEGylated PYY_{13–36}, which causes decreased food intake, body weight loss, and improved glucose metabolism in rodents.^{8–10} However, few studies except for our previous study¹¹ have been reported on the small-sized PYY analogues, which show anorectic activity without any

modification (e.g., PEG, albumin, or long-chain alkyl). Small peptides with a simple structure are suitable for a sustained-release formulation such as poly(lactic-co-glycolic acid), which is the most commonly considered formulation with advantages of reduced dose frequency and better medication compliance of patients. The aim of this study was to establish a proof-of-concept for a sustained-release formulation of PYY analogues.

Recently, we found that a short-length PYY, benzoyl-[Cha^{27,28,36},Aib³¹]PYY_{25–36} (**1**), designed from a 12-mer PYY analogue (benzoyl-[Ala²⁶,Ile^{28,31}]PYY_{25–36}) with low metabolic stability in mouse serum, showed more potent anorectic activity than PYY_{3–36} after intraperitoneal bolus administration at 250 and 500 nmol/kg (ca. 0.43 and 0.86 mg/kg for **1**) in lean mice.¹¹ The substitution of the unnatural amino acid residue Cha for Tyr at position 27, 36, or at both positions improved serum stability; notably, a double-substituted analogue was completely resistant to serum proteases in 20% mouse serum/saline for 30 min. Subsequent investigation of the N-terminal modifications and amino acid substitutions at positions 27, 28, 30, and 31 in terms of anorectic activity led to the discovery of **1** containing Cha²⁸ and Aib³¹ residues. However, continuous subcutaneous administration of **1** at 1 mg/(kg·day) did not reduce the body weight ($\Delta BW = 0.4\%$) in diet-induced obese (DIO) mice in contrast to that of PYY_{3–36} ($\Delta BW = -11.2\%$) (Table 1). For pharmacokinetic studies, **1** was injected intravenously and subcutaneously in C57BL/6J mice at 1 mg/kg. Plasma levels of **1** were determined by liquid

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Table 1. Biological Activities of Peptide YY (PYY) Analogues Substituted at the N-Terminus and at Positions 22, 23, 24, and 26

R-AA22-AA23-AA24-Arg-AA26-Cha-Cha-Asn-Leu-Aib-Thr-Arg-Gln-Arg-Cha-NH ₂												
compound	R	AA22	AA23	AA24	AA26	binding affinity		agonist activity		% food intake inhibition ^c		% body weight reduction ^d
						IC ₅₀ (nM) ^a	EC ₅₀ (nM) ^b	continuous [mg/(kg·day)]		continuous [mg/(kg·day)]		
								1	0.3			
PYY _{3–36}						0.57	0.28	100	48		−11.2	
1	Benzoyl				His	7.4 (4.3—13)	2.0 (1.2—3.3)	ND	ND		0.4	
2	Benzoyl				Abu	5.7 (3.5—9.3)	4.6 (3.2—6.5)	ND	ND		ND	
3	Isobutanoyl				Abu	7.3 (4.2—13)	4.2 (3.1—5.6)	ND	ND		ND	
4	Ac			Leu	Abu	17 (9.0—31)	6.4 (5.3—7.8)	ND	ND		ND	
5	Ac		Ser	Leu	Abu	8.8 (5.0—15)	3.0 (2.0—4.4)	ND	ND		ND	
6	Ac	Ala	Ser	Leu	Abu	6.8 (4.0—12)	4.6 (2.7—8.0)	ND	ND		ND	
7	H	Ala	Ser	Leu	Abu	2.7 (1.9—3.9)	2.5 (1.9—3.4)	ND	ND		ND	
8	H	Ala	Ser	Leu	His	1.7 (1.3—2.3)	0.74 (0.53—1.0)	ND	ND		ND	
9	Ac		Ser	D-Leu	Abu	11 (7.1—17)	1.3 (1.0—1.8)	ND	ND		ND	
10	Ac		D-Ser	D-Leu	Abu	23 (13—39)	3.8 (3.1—4.6)	ND	ND		ND	
11	Ac		D-Ser	Leu	Abu	12 (7.0—19)	5.5 (3.5—8.6)	ND	ND		ND	
12	Ac		Ser	MeAla	His	5.4 (3.7—7.9)	1.2 (0.91—1.7)	ND	ND		ND	
13	Ac		Ser	D-MeAla	His	2.8 (2.0—4.0)	0.59 (0.47—0.75)	28	ND		ND	
14	Ac		Ser	Sar	His	2.8 (2.4—3.3)	1.0 (0.66—1.6)	13	ND		ND	
15	Ac		Ser	Aib	His	2.0 (1.4—2.7)	0.55 (0.38—0.81)	51	ND		ND	
16	Ac		Ser	Gly	His	4.8 (3.6—6.2)	1.3 (0.88—1.9)	ND	ND		ND	
17	Ac		Ser	D-Pro	His	1.5 (1.2—1.9)	0.61 (0.38—0.98)	33	ND		ND	
18	Ac		Ser	D-Hyp	His	0.70 (0.61—0.80)	0.20 (0.13—0.29)	79	47		−10.2	

^aIC₅₀ values of peptide analogues correspond to concentrations required to displace the binding of radiolabeled ligand by 50%. ^bEC₅₀ values of agonist activities correspond to concentrations of peptide analogues that induced 50% of the maximum [³⁵S]GTPγS binding. ^cPercentage inhibition of food intake after 3-day continuous administration of peptide analogues at 0.3 or 1 mg/(kg·day) compared to that after injection with PYY_{3–36} at 1 mg/(kg·day) (defined as 100% food intake inhibition) in male C57BL/6J mice (*n* = 5 per group). ^dPercentage reduction of body weight after 6–7-day continuous administration of peptide analogues at 1 mg/(kg·day) compared to vehicle in male C57BL/6J DIO mice (*n* = 6 per group). ND: not determined.

chromatography–tandem mass spectrometry (LC/MS/MS). The mean absorption time (MAT) of **1** was relatively long, 2.72 h [mean residence time (MRT)_{sc}: 3.17 h, MRT_{iv}: 0.45 h], demonstrating the slow absorption rate of **1** from subcutaneous tissues. We hypothesized that the lack of an antiobesity effect of **1** was caused by its high hydrophobicity, leading to low bioavailability (BA, 5.9%) and long MAT. Moreover, the binding affinity and agonist activity of **1** for Y2R were ten times lower than those of PYY_{3–36} (Table 1). To obtain potent PYY analogues, we determined the effects of substitution and introduction of amino acids to improve hydrophilicity and presumably affect peptide conformation on the binding affinity and agonist activity for Y2R, and anorectic activity upon continuous administration in lean mice.

Preliminary structure–activity relationship studies on short-length PYY analogues revealed that replacement of His²⁶ with an unnatural amino acid, 2-aminobutyric acid (Abu), improved the anorectic activity and slightly decreased the in vitro activities (data not shown). As can be seen in Table 1, the Abu²⁶-substituted analogue (**2**) and the parent peptide **1** showed similar binding affinities. Replacement of the benzoyl group with an isobutanoyl group (**3**) at the N-terminus of **2**, which mimicked the Leu side chain at position 24 of PYY_{3–36}, did not greatly change the in vitro activities. The elongation of Ac-Leu²⁴ (**4**) slightly decreased the binding affinity for Y2R compared to **1**. However, the elongation of Ac-Ser²³-Leu²⁴ (**5**) restored the Y2R affinity reduced by the replacement with Ac-Leu²⁴. Elongation of Ac-Ala²²-Ser²³-Leu²⁴ (**6**) further improved the binding affinity, and that of H-Ala²²-Ser²³-Leu²⁴ (**7**)

improved it to an even greater extent. Reversion of Abu²⁶ to His (**8**) restored the agonist activity to the subnanomolar range, i.e., to a PYY_{3–36}-comparable range. A basic and/or hydrophilic moiety in the N-terminal region of the peptides is considered to contribute to a higher affinity for Y2R by direct or indirect interaction. Compound **5** derivatives with the corresponding D-amino acids at positions 23 and/or 24 (**9–11**), which were expected to avoid cleavage by proteases at the N-terminal region, showed in vitro activities comparable to those of **5**. Based on the results from this small number of peptides, the L-configuration at position 23 and the D-configuration at position 24 were considered ideal for potentiating the in vitro activity of the 14-amino acid PYY analogues. The effect of substitutions at position 24 with several amino acids, which were presumed to affect the peptide conformation, was reflected in the improved binding affinities and agonist activities of **12–17**. Although **1** was ineffective on food intake in DIO mice after continuous subcutaneous administration, D-MeAla²⁴ (**13**), Aib²⁴ (**15**), and D-Pro²⁴ (**17**) analogues showed anorectic activity in lean mice after continuous administration at 1 mg/(kg·day) for 3 days. The introduction of a hydroxyl group at the D-Pro side chain at position 24 of **17** to improve hydrophilicity, i.e., a D-Hyp²⁴ analogue (**18**), showed higher affinity and a potent agonist activity for Y2R, which were approximately equivalent to those of PYY_{3–36}. Compound **18** exerted a potent, i.e., PYY_{3–36}-comparable, anorectic activity on lean mice after continuous administration at 0.3 and 1 mg/(kg·day) (Table 1).

The pharmacokinetics of **1** and **18** was examined using mice as described above. As expected, the increased hydrophilicity of

18 resulted in short MAT and improved BA (Table 2). The MRT of **18** after subcutaneous administration was short (0.32

Table 2. Pharmacokinetic Parameters of **1 and **18** after Intravenous and Subcutaneous Administration in Mice^a**

compound	1	18
iv		
C _{5 min} (nmol/mL)	7548.3	934.3
AUC _{0–24h} (nmol·h/mL)	3562.8	180.5
MRT (h)	0.45	0.18
Vd _{ss} (mL/kg)	73	533
CL _{total} (mL/h/kg)	162	2968
sc		
C _{max} (nmol/mL)	75.6	98.1
t _{max} (h)	0.25	0.17
AUC _{0–24h} (nmol·h/mL)	209.1	33.6
MRT (h)	3.17	0.32
MAT (h)	2.72	0.14
BA (%)	5.9	18.6

^aPeptides were administered intravenously or subcutaneously to C57BL/6J mice at 1 mg/kg. Blood samples were collected at 5, 10, 15, and 30 min, and at 1, 3, 6, 8, and 24 h after injection. Parameters were calculated from the mean (*n* = 3) plasma concentration–time profile. The pharmacokinetic data from the intravenous administration of **1** were determined in a previous study.¹¹ MRT, mean residence time; MAT, mean absorption time; BA, bioavailability.

h), whereas **1** possessed a long MRT (3.17 h) for a short-length peptide, indicating that **1** is slowly adsorbed into the blood vessels and remains in circulation for a longer period. Indeed, the MATs of **1** and **18** after subcutaneous administration were 2.72 and 0.14 h, respectively. Compound **1** showed a low BA (5.9%) when injected subcutaneously, whereas **18** showed a slight increase in BA (18.6%).

Finally, the antiobesity effect of the peptides was studied using 29-week-old DIO C57BL/6J mice, which were fed a high-fat diet (58% fat; D12331, Research Diets, New Brunswick, NJ, U.S.A.) from 5-week-old on to gain the body weight of 45–50 g (Figure 1). The peptides were continuously administered using osmotic pumps at 1 mg/(kg·day) for 7 days. Compound **18** and the positive control PYY_{3–36} reduced food intake, particularly for the first 4 days (Figure 1b). As expected for an anorectic effect, the body weight declined continuously after peptide administration (Figure 1a). The percentage of body weight loss at day 7 by PYY_{3–36} and **18** was 8.3% and 7.4%, respectively. The antiobesity effect after continuous subcutaneous administration of **18** was thus comparable to that of PYY_{3–36} in DIO mice.

We designed and synthesized short-length PYY_{3–36} analogues, and the lead peptide (**1**) showed anorectic activity comparable to that of PYY_{3–36}.¹¹ However, the subcutaneous continuous administration of **1** did not affect the food intake and body weight of DIO mice, in contrast to PYY_{3–36}, and we assumed that the discrepancy in the *in vivo* activities of **1** between the intraperitoneal and subcutaneous administration was caused by its relatively long MAT, indicating slow absorption from subcutaneous tissues. The low BA and long MAT of **1** were considered to result from its highly hydrophobic amino acid sequence. Furthermore, **1** had lower affinity and less potent agonist activity for Y2R than PYY_{3–36}. These results suggest that more potent analogues could be obtained by increasing affinity for Y2R, as well as a higher

Figure 1. Antiobesity effect of **18** and peptide YY (PYY)_{3–36} in diet-induced obese (DIO) mice. (a) Changes in body weight and (b) cumulative food intake. Peptides at 0.3 mg/(kg·day) were continuously administered using osmotic pumps for 7 days in DIO mice (*n* = 6). **, *p* < 0.01 vs vehicle by Student's *t* test; \$\$, *p* < 0.01 vs vehicle by Aspin-Welch test.

absorption rate from subcutaneous tissues. Amino acid substitutions at positions 23 and 24 provided valuable information about the preference for D-/L-configuration: L- for position 23 and D- for position 24 for further optimization. Among peptide analogues containing amino acid substitutions at position 24, the substitution with D-Hyp²⁴ (**18**) led to an improvement in binding affinity and agonist activity for Y2R, which were comparable to those of PYY_{3–36}. The hydrophilic profile of **18** apparently decreased MAT and improved BA compared to **1**. Continuous subcutaneous administration of **18**, which had PYY_{3–36}-comparable Y2R agonist activity, decreased food intake to a similar extent as PYY_{3–36}. The antiobesity efficacies of **18** and PYY_{3–36} after continuous and subcutaneous administration in DIO mice were similar. The present study suggests that short-length PYY analogues without any modification, such as PEGylation, fatty acylation, or long-chain alkylation, have a potential to become an alternative option of effective therapeutic agents for obesity in a sustained-release formulation. Bolus subcutaneous administration of **18** at 300 nmol/kg showed moderate anorectic activity (23% food intake inhibition), less robust than that of PYY_{3–36} (53%) in lean mice, although **18** was more potent than **1** (20% food intake inhibition at 1000 nmol/kg). It is conceivable that more practical analogues for continuous and repeated dosing could be obtained by further improving the pharmacokinetic profiles in the blood.

■ EXPERIMENTAL PROCEDURES

All peptides were synthesized by a standard method for 9-fluorenylmethoxycarbonyl-based solid phase peptide synthesis. N-Terminal acylation of peptides was performed by carboxylic acid activation by *N,N'*-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole, or by acetylation with acetic anhydride. After final cleavage with trifluoroacetic acid (TFA)–*m*-cresol–thioanisole–H₂O–ethanedithiol–triisopropylsilane (80:5:5:5:2.5:2.5), purification was performed using reversed-phase high-performance liquid chromatography (RP-HPLC) in a linear gradient of H₂O/acetonitrile containing 0.1% (v/v) TFA. The purity of each peptide was confirmed by analytical RP-HPLC, and the peptides were characterized using matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry. The final compounds were purified to a homogeneity of ≥95% in RP-HPLC analysis with UV detection at 210 nm.

The binding affinity of synthesized peptides for human Y2R was evaluated by a receptor binding assay with membranes obtained from Chinese hamster ovary (CHO) cells expressing cloned human Y2R. The agonist activity of peptide analogues was also examined by a [³⁵S]GTPγS binding assay with the membranes of human Y2R-expressing CHO cells. Peptides with better in vitro profiles were further evaluated for their anorectic activity by continuous subcutaneous administration to C57BL/6J mice using an osmotic pump (DURECT, Cupertino, CA, U.S.A.) at 0.3 or 1 mg/(kg·day) for 3 days. The results are summarized in Table 1.

■ ABBREVIATIONS

Amino acid abbreviations and peptide designations follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature.¹² Amino acid symbols denote the L-configuration unless otherwise indicated. Abu, 2-aminobutylic acid; Aib, 2-aminoisobutylic acid; Cha, 3-cyclohexylalanine; Hyp, *trans*-4-hydroxyproline; MeAla, *N*-methylalanine; RP-HPLC, reversed-phase high-performance liquid chromatography; Sar, sarcosine, *N*-methylglycine.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00047.

Experimental Section, Table of chemical data for PYY analogues, and figures showing binding affinity and agonist activity of PYY analogues, and plasma concentrations of 1 and 18 (PDF)

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Notes

The authors declare no competing financial interest.

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