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Research Article

Phenylbutazone, a New Long-Acting Agent that can Improve the Peptide Pharmacokinetic Based on Serum Albumin as a Drug Carrier

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As a NPY-2 receptor agonist, $PYY_{24–36}$-Leu_{31} is reported to suppress appetite and has a potential in obesity treatment, but its short half-life limits the clinical application. The use of chemical modification to improve interactions with human serum albumin (HSA) is an effective strategy for prolonging the half-lives of peptide analogues. So based on the characteristics that phenylbutazone has a good combination with HSA, we selected a proper linker to link with $PYY_{24–36}$-Leu_{31} to create long-acting and highly biologically active $PYY_{24–36}$-Leu_{31} conjugates, and successfully find a novel, long-acting $PYY_{24–36}$-Leu_{31} conjugate 8 that, when dosed every other day in diet induce obese (DIO) mice for 2 weeks, results in a significant reduction in food intake and body weight and improvement in blood parameter and hepatic steatosis.

Key words: anorexia, HSA, obesity, $PYY_{24–36}$ conjugate, weight loss

Abbreviations: DIO, diet induce obese; DMAP, 4-dimethylaminopyridine; HSA, human serum albumin; OGTT, oral glucose tolerance test; $PYY_{24–36}$, $PYY_{24–36}$-Leu_{31}; TFA, trifluoroacetic acid.

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Obesity presents an increasing public health burden that is associated with several hyperlipemia, hypertension, osteoarthritis, type 2 diabetes, and other illnesses, and with reduced life expectancy. Current therapeutic options are limited, and the unmet medical need for new, effective treatments is high (1). Several neurological pathways contribute to food intake and energy homeostasis that moderate appetite, weight maintenance, and weight gain (2). One family of G protein-coupled receptors involved in the regulation of food intake is the NPY family (3–5). The observation that acetylation of $PYY_{24–36}$-Leu_{31}($PYY_{24–36}$) (6–8) increases NPY-2 receptor affinity while maintaining specificity against the NPY-1 and NPY-5 receptors suggests that further improvements may be obtained with more complex modifications of the N-terminal amino group of $PYY_{24–36}$ (9,10). But short half-life limits its clinical application (11). Albumin is emerging as a versatile protein carrier for drug targeting and for improving the pharmacokinetic profile of peptide or protein-based drugs (12). The use of chemical modification to improve covalent or non-covalent interactions with serum albumin is an effective strategy for prolonging the half-lives of peptide analogues (13,14). At present, the most common used long-term peptide modified agents are fatty chain, PEG, and bile acid. They are many successful case; acylation (attachment of a fatty acid side chain) facilitated non-covalent binding to serum albumin in the GLP-1 analogue liraglutide (15). This delayed both the absorption and clearance rates of the compound. D-Ala_8,Lys_{37}[2-[2-[2-maleimidopropionamido(ethoxy)-ethoxy]acetamide GLP-1(7–37) (CJC1131), a GLP-1 analogue with a reactive maleimide group at the carboxyl terminus, binds covalently to serum albumin following injection (14).

This subject is to find a new long-acting method, which can be widely used to improve peptides pharmacokinetic profile. Previous studies have found that an anti-inflammatory drug, phenylbutazone (16,17), has a strong combining ability with human serum albumin (HSA). So based on this characteristic, we carefully modified its proper group to link with $PYY_{24–36}$ (Figure 1) to create long-acting and highly biologically active $PYY_{24–36}$ conjugates.

So here we synthesized three modifying groups (the two known molecules—bile acid and fatty acid chain, and a new molecule–phenylbutazone) that allow for amidation with $PYY_{24–36}$ N-terminus to improve peptide duration of action *in vivo*. The structural properties (Table. 1), *in vitro* biological activity, and physicochemical characteristics of **1**–**9** were explored. The studies result in a novel, long-acting NPY2 receptor agonist **8** that, when dosed every other day in DIO mice, results in a significant reduction in food intake and body weight and improvement in blood parameter and hepatic steatosis.

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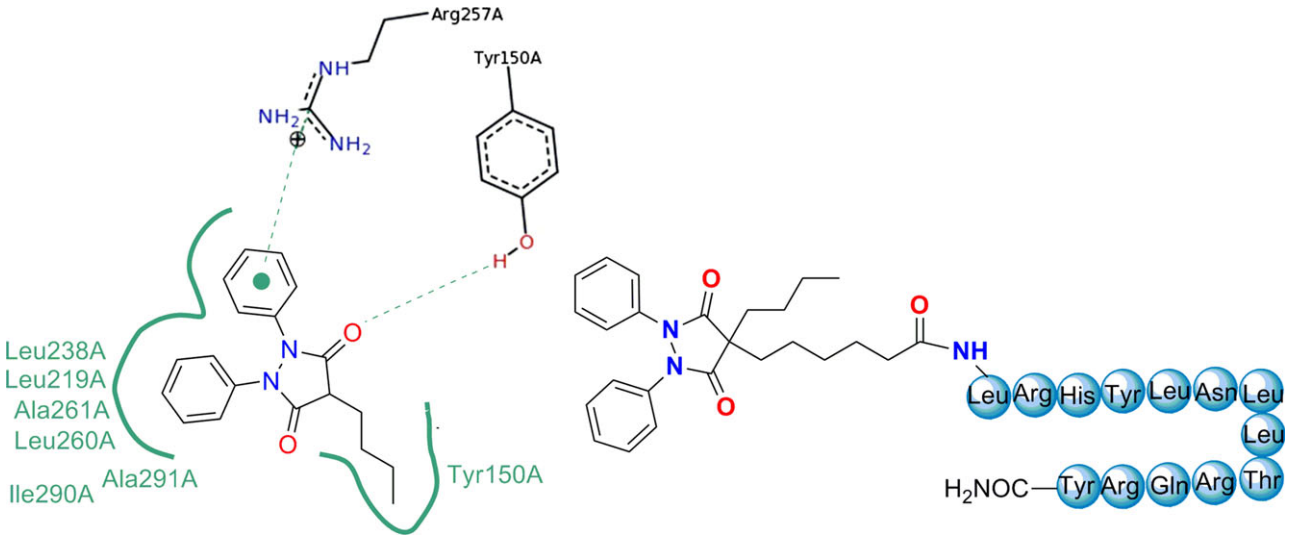


Figure 1: Phenylbutazone visually dock with HSA.

Table 1: Sequence and experimental data for $PYY_{24–36}$-Leu_{31} conjugates

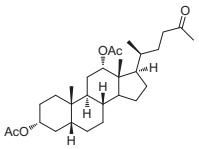
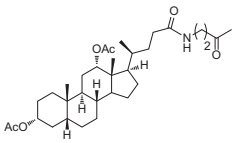
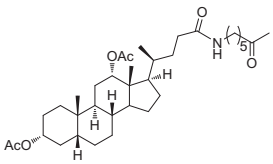
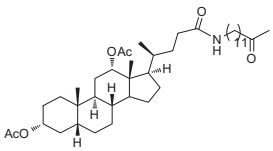
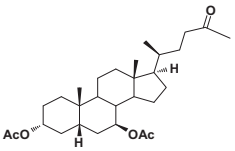
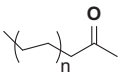
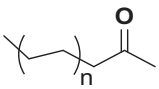
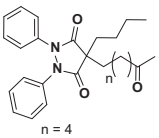
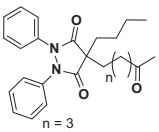
Name	R	Molecular mass	
		Calculated	Found
1		1081.8[M + 2H]$^{2+}$ 721.5[M + 3H]$^{3+}$	1081.4[M + 2H]$^{2+}$ 721.5[M + 3H]$^{3+}$
2		555.9 [M + 4H]$^{4+}$	555.1[M + 4H]$^{4+}$
3		1159.1[M + 2H]$^{2+}$ 773.3[M + 3H]$^{3+}$	1159.4[M + 2H]$^{2+}$ 773.4[M + 3H]$^{3+}$
4		1201.3[M + 2H]$^{2+}$ 801.4[M + 3H]$^{3+}$	1201.3[M + 2H]$^{2+}$ 801.2[M + 3H]$^{3+}$
5		1102.9[M + 2H]$^{2+}$	1103.7[M + 2H]$^{2+}$

Table 1: continued

Name	R	Molecular mass	
		Calculated	Found
		735.6[M + 3H]$^{3+}$	735.6[M + 3H]$^{3+}$
6	 n = 5	964.7[M + 3H]$^{3+}$ 643.5[M + 4H]$^{4+}$	963.9[M + 3H]$^{3+}$ 643.2[M + 4H]$^{4+}$
7	 n = 7	992.7[M + 2H]$^{2+}$ 662.2[M + 3H]$^{3+}$	992.4[M + 2H]$^{2+}$ 662[M + 3H]$^{3+}$
8	 n = 4	717.5[M + 3H]$^{3+}$ 538.4[M + 4H]$^{4+}$	717.4[M + 3H]$^{3+}$ 538.3[M + 4H]$^{4+}$
9	 n = 3	712.9[M + 3H]$^{3+}$ 534.9[M + 4H]$^{4+}$	712.7[M + 3H]$^{3+}$ 534.7[M + 4H]$^{4+}$

Methods and Materials

Materials

Peptide $PYY_{24–36}$-Leu_{31} and analogues were synthesized using a CEM microwave peptide synthesizer. Fmoc Rink Amide MBHA resin and Fmoc-protected amino acids were obtained from GL Biochem (Shanghai, China). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other reagents, unless indicated, were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The HPLC analysis was performed on a Shimadzu 2010C HPLC system (Nakagyo-ku, Kyoto, Japan). For purification, Shimadzu LC-10 preparative RP-HPLC system was used. The ESI-MS spectra of the peptides were obtained with Waters ACQUITY UPLC Systems with Mass (Waters, Milford, MA, USA). C57BL/6J and ICR mice (male, 8 weeks old) were purchased from the comparative medical center of Yangzhou University (Jiangsu, China). All animal experimental protocols adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85–23, revised 1986). The structure of HSA crystalline complex (ID: 2BXP) was searched in PDB database and resolved by LIGANDSCOUT V2.02 program (Inteligand GmbH, Vienna, Austria). ^{1}H NMR spectra were recorded on a 300 MHz; chemical shifts are given in ppm (d) relative to TMS as internal standard; coupling constants (J) are in hertz (Hz); and signals are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet, etc.).

Peptide $PYY_{24–36}$-leu_{31} and derivatives synthesis and characterization

$PYY_{24–36}$-Leu_{31} H-LRHWLNLLTRQRW-NH_2 were synthesized using standard solid-phase methodology on a Rink amide MBHA resin with a microwave synthesizer (CEM, Matthews, NC, USA). Microwave irradiation was applied in coupling and deprotection steps at 2450 MHz continuously. The final protected peptidyl-resin was deprotected with 20% piperidine (v/v) in dimethylformamide to remove Fmoc (18,19).

The N-Terminal peptide modification

To the above peptidyl-resin, five equivalents of each small molecule were manually added into CEM vessel. And the coupling procedures were carried on for 30 min with microwave irradiation at 2450 MHz. Upon completion of the synthesis, peptides derivatives were cleaved from the solid support with removal of side chain protecting groups by treatment with aqueous trifluoroacetic acid (TFA). Peptides were purified using a Shimadzu LC-10 preparative RP-HPLC and a C18 RP column (5, 340, 28 mm) with gradient elution using (A) water with 0.1% TFA and (B) methanol with 0.1% TFA as the mobile phase. The gradient was developed from 20% B to 80% B over 60 min at a flow rate of 5.0 mL/min, with UV detection at 214 nm. The purity of peptides administered to the mice was over 90%. HPLC analysis was performed on a Shimadzu 2010C HPLC system with gradient elution using (A) water with 0.1% TFA and (B) acetonitrile with 0.1% TFA as the mobile phase. The gradient was developed from 20% B to 80% B over 20 min at a flow rate of 1.0 mL/min, with UV detection at 214 nm.

Small molecule chemical synthesis

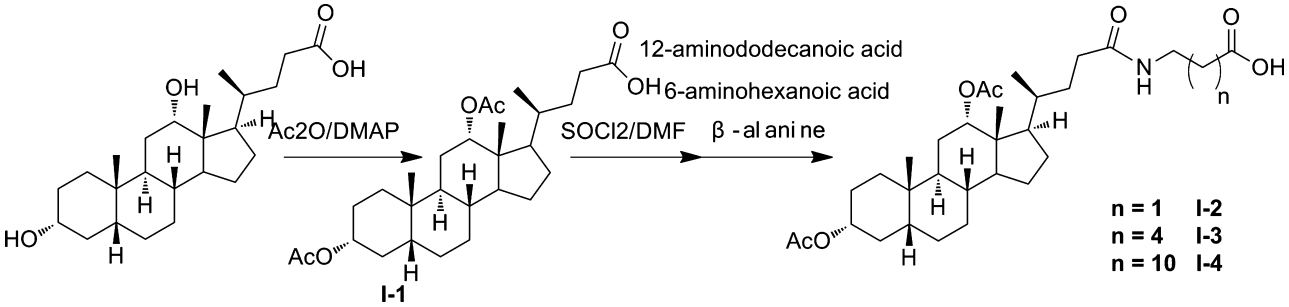
General procedure I for the preparation of I-1, I-2, I-3, I-4

Deoxycholic acid (1 g), acetic anhydride (0.524 mL), and DMAP in catalytic amount were dissolved in mixture solvent of pyridine (10 mL) and DCM (10 mL) (Scheme 1). It was stirred for 20 h at RT. The reaction was monitored by TLC and added 1N HCl for termination. Then, the mixture was extracted with DCM (3*30 mL). The combined organic layer was washed with water (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to afford light yellow solid. The product was directly put into use for next step. To the **I-1** in dichloromethane was added dichlorosulfoxide (2equiv) and DMF three drops. The solution was stirred for 3.5 h and concentrated under reduced pressure. The oil was dissolved in 10 mL THF and waited for use.

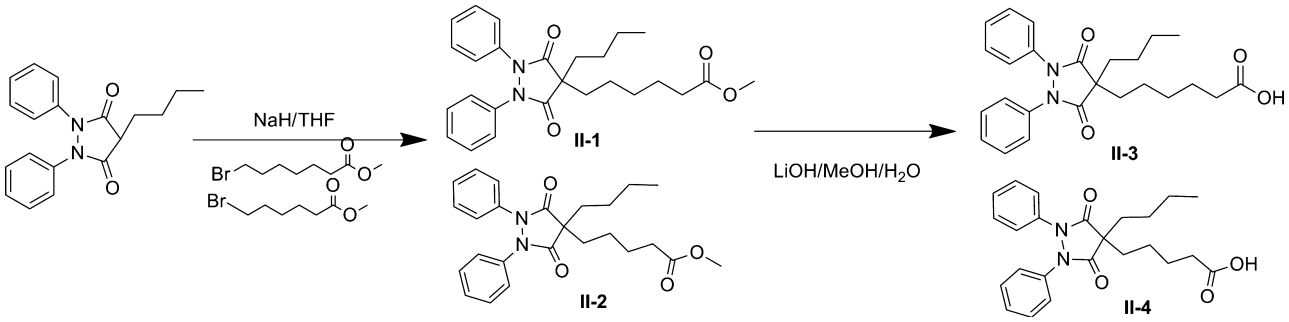
To a solution of β-alanine, 6-aminocaproic acid or 12-aminolauric acid (1.5equiv) in 1N aqueous sodium hydroxide respectively, added dropwise the above THF solution at 0 °C, and warmed to RT. After stirring for 4 h, the reaction mixture was concentrated in vacuo and H_2O was added. The mixture was extracted with diethyl ether. The combined organic extracts were washed with brine (15 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography (PE: EtOAc, 1:2→1:1) to furnish the desired product **I-2**, **I-3**, **I-4**. They were directly used to amide with peptide.

General procedure II for the preparation of II-3, II-4

Methyl 6-bromohexanoic (2.6 g, 11.7 mmol) or methyl 5-bromopentanoic (2.45 g, 11.7 mmol) combined with butazodine (3 g, 9.7 mmol) were dissolved in 20 mL THF. A total of 60% sodium hydride (0.58 g, 14.6 mmol) was added to the solution above and refluxed for 1.5 h and add ice water to quench the reaction (Scheme 2). Then, the mixture was extracted with DCM (3*30 mL). The



Scheme 1: Synthetic route for deoxycholic acid derivatives.



Scheme 2: Synthetic route for phenylbutazone derivatives.

combined organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to be purified by silica gel chromatography (PE:EtOAc, 1:2→1:1). The oil product was dissolved in the mixtures of 10 mL methanol and THF. Lithium hydroxide (0.32 g, 6.53 mmol) in 10 mL water was added to the above solution, which was stirring for 8 h at RT. The reaction was monitored by TLC and added with 1N HCl to pH 5~6. Then, the mixture was extracted with EA (3*30 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel using PE:EtOAc (1:4) as eluent to obtain light yellow oil.

Synthesis of 6-(4-butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl)hexanoic acid (**II-3**): Yield: 43.1%; MS (ESI) m/z: 423.6 $(M + H)^+$;1H-NMR (CDCl3, 300 MHz): δ(ppm) 0.94 (t, 3H, -CH_3), 1.14–1.60(m, 10H), 2.22(t, 2H, -$CH_2(CH_2)4CH_2COO$), 2.39(t, 2H, -$CH_2(CH_2)2CH_3$), 4.09 (t, 2H, -$CH_2(CH_2)4COO$), 5.1(S, 2H, PhCH_2-), 7.03–7.43(m, 15H, 4 Ar-H).

Synthesis of 6-(4-butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl) pentanoic acid (**II-4**) Yield: 46%; MS (ESI) m/z: 411.7 $(M + H)^+$; 1H-NMR (CDCl3, 300 MHz): δ(ppm) 0.95 (t, 3H, -CH3), 1.12–1.60(m, 8H), 2.25(t, 2H, -$CH_2(CH_2)3CH_2COO$), 2.41(t, 2H, -$CH_2(CH_2)2CH_3$), 4.10 (t, 2H, -$CH_2(CH_2)4COO$), 5.13(s, 2H, PhCH_2-), 7.04–7.5(m, 15H, 4 Ar-H).

Compound stability in rat plasma

The stability of these conjugates was assessed in rat plasma collected from adult male SD rats, as previously described (20). The plasma was stored at −80 °C until needed. *In vitro* stability of **1**–**9** and $PYY_{24–36}$ were measured using an initial concentration of 1000 ng/mL of each peptide in rat plasma at 37 °C. An amount of 100 μL of plasma was removed from the incubations at 0, 1, 2, 4, 6, 8, 12, and 24 h time-points and subjected to solid-phase extraction on a Waters Oasis HLB 96-well plate (Milford). An amount of 20 μL of extract was injected onto the LC–MS/MS system. Peptides were detected by multiple reactions monitoring (MRM) using electrospray ionization mass spectrometry (ESI-MS) on an Applied Biosystems Sciex API-4000 instrument (Foster City, CA, USA) fitted with a TurboIonSpray source. RPLC separation was performed on a Waters Acquity UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm) equilibrated in buffer A (water, 0.2% formic acid), and elution was with a linear gradient of

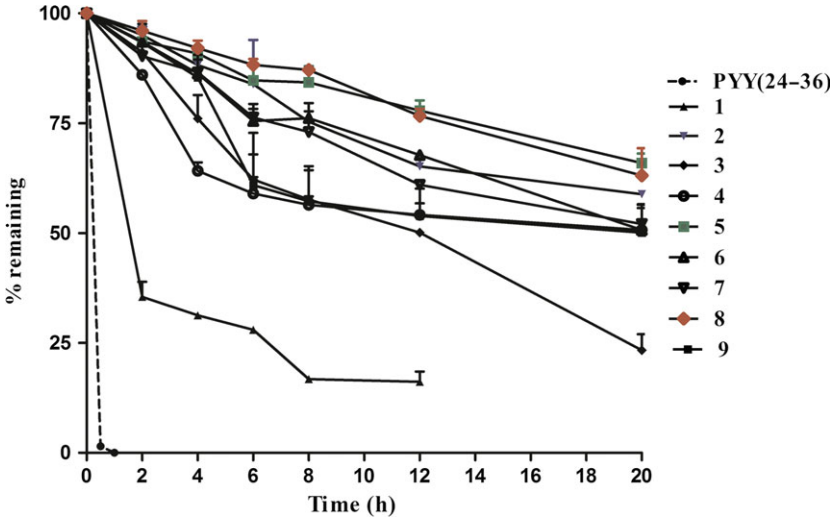


Figure 2: Degradation of $PYY_{24–36}$ and its conjugates (**1**–**9**). Data are expressed as the mean ± SD.

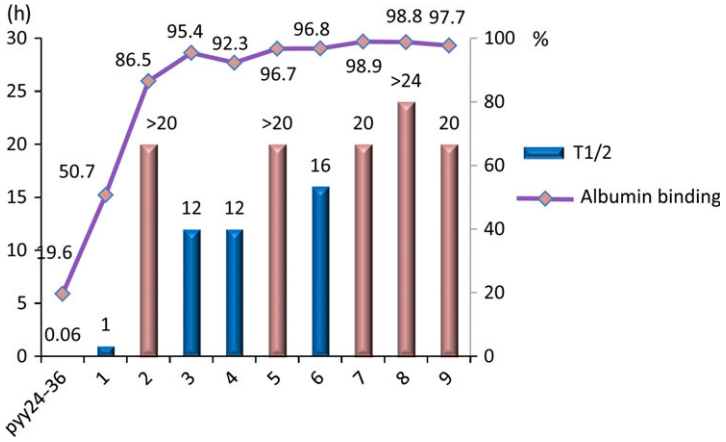


Figure 3: Correlation between the plasma stability and albumin binding of $PYY_{24–36}$ conjugates (**1**–**9**).

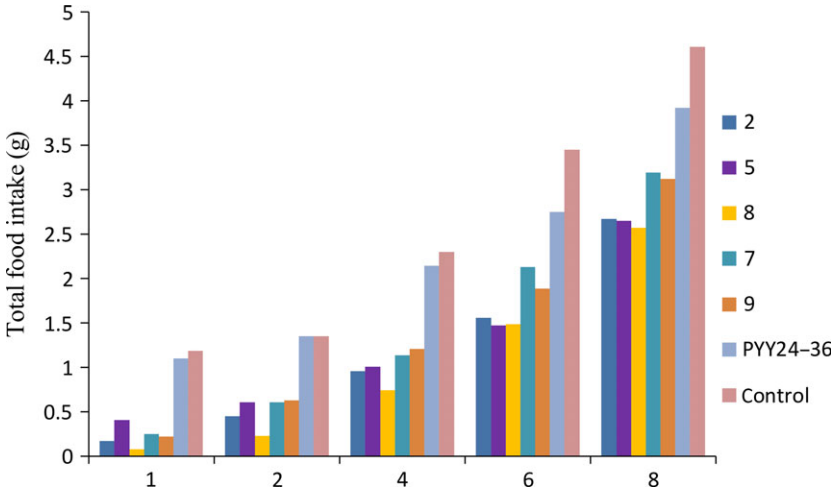


Figure 4: Acute effect on cumulative food intake for 8 h after an overnight fast in ICR mice, i.p. administration of $PYY_{24–36}$ or its conjugates all at 1 μmol/kg.

5–95% buffer B (acetonitrile, 0.2% formic acid) over 4 min at a flow rate of 0.3 mL/min.

Albumin binding

The albumin-binding characteristics of the $PYY_{24–36}$ conjugates **1**–**9** were investigated using an albumin-binding assay using albumin-conjugated Sepharose resin (15). NHS-activated Sepharose 4 fast flow (5 mL, wet resin volume, Amersham Bioscience, Uppsala, Sweden) and human serum albumin (HSA; 33 mg in 10 mM PBS, pH7.4) were mixed and allowed to react by gentle shaking at room temperature for 4.5 h. The albumin-conjugated resin was then recovered by centrifugation (278 × *g*, 5 min) and followed by PBS washing. The HSA content in the resin used was 6–7 mg/mL of wet resin. An albumin-free control resin was prepared by inactivating the resin with hydrolysis of the NHS-active ester. $PYY_{24–36}$ or conjugates **1**–**9** (1 μmol/mL in PBS, 50 mL) were mixed with HSA resin and incubated for 3 h at room temperature. The resin and supernatant were separated by centrifugation (278 × *g*, 10 min), and unbound peptide contents were determined using a Micro BCA Protein assay kit (PIERCE, Rockford, IL, USA). The non-specific absorptions of the peptides on albumin-free resin were determined using NHS-inactivated resin in a similar manner.

Acute feeding studies in lean ICR mice

Forty-two lean male ICR mice (20–25 g) were maintained under controlled conditions of temperature (21–23 °C) and 12 h light/12 h dark cycle. Mice were housed in pairs in cages with water and food (standard chow pellet diet) continuously available. The mice were fasted overnight with water available during the dark phase, and on the morning of study, mice (n = 6/group) were distributed by weight into treatment groups and dosed intraperitoneally with $PYY_{24–36}$, **2**, **5**, **7**, **8**, **9** in USP saline (1 μmol/kg) in the early light phase (9–10 h). Control groups were dosed with 0.9% saline. After injection, animals were returned to their home cages containing a preweighted amount of food that was reweighted at 0.5, 1, 2, 4, 8 h after injection.

Two-week study in lean ICR mice

In a follow-up experiment, the mice were dosed above peptides intraperitoneally every other day for 2 weeks. Body weight gain, food, and water intake of the mice were recorded every days during the experiment.

Effect on body weight of every other day dosing for 2 weeks in DIO mice

Male C57BL/6 mice (Qinglong Farms, Inc., Nanjing, China) 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 five standard deviations greater than mice fed a standard laboratory diet (10% calories from fat). A treatment group comprised six mice per treatment group with body weights of approximately 44 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. 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 0.9% saline intraperitoneally every other day before the dark phase, and body weight and food consumption were measured. On the final day, and body weight was measured.

Oral glucose tolerance test of 8 in DIO mice

Mice were fasted for 16 h with free access to water before basal blood glucose was measured (t = −30 min). Compound **8** (1 μmol/kg) or $PYY_{24–36}$-Leu_{31} (1 μmol/kg) and 0.9% saline were then intraperitoneally injected, and an oral glucose load of 2 g/kg was administered to all animals. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min postglucose load using blood glucose monitor (SanNuo; Changsha Company, Changsha, China).

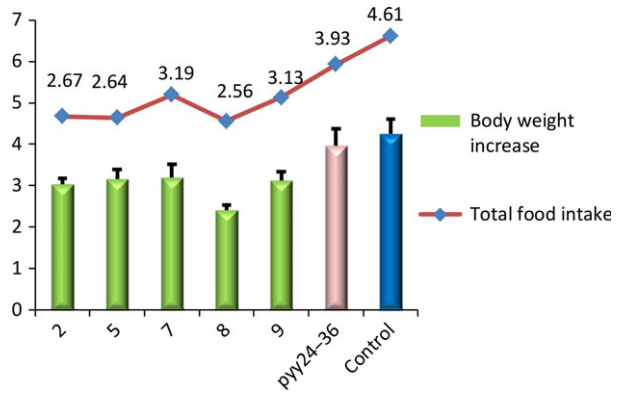


Figure 5: Body weight increase of 2 weeks (bar graph) and total food intake at day 0 (line chart) in ICR lean mice.

Blood parameters

Blood was collected after a 6-h fast from tail veins using Microvette tubes and stored at room temperature for 2 h. After 15 min of centrifugation at 3000 × *g* and 4 °C, plasma was stored at –80 °C. Plasma adiponectin and leptin were measured by Elisa kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All assays were performed according to the manufacturers' instructions.

Statistical analysis

The statistical analyses were performed using GRAPHPAD PRISM version 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and EXCEL 2010 (Microsoft Corp., Richmond, WA, USA). The results are shown as mean ± SEM and compared using the unpaired Student's *t*-test. Groups of data were considered to be significantly different if $p < 0.05$.

Results and Discussion

Stability of the $PYY_{24–36}$ conjugates in rat plasma

Nine conjugates (**1**–**9**) were incubated with plasma taken from SD rats over 24 h and compared with $PYY_{24–36}$. Solid-phase extraction was conduct at each time-point, and the extract was analyzed using LC–MS/MS to examine plasma stability (Figure 2). $PYY_{24–36}$ possessed a half-life of less than 0.5 h at 37 °C. All $PYY_{24–36}$ conjugates showed better stability than $PYY_{24–36}$. The half-lives of

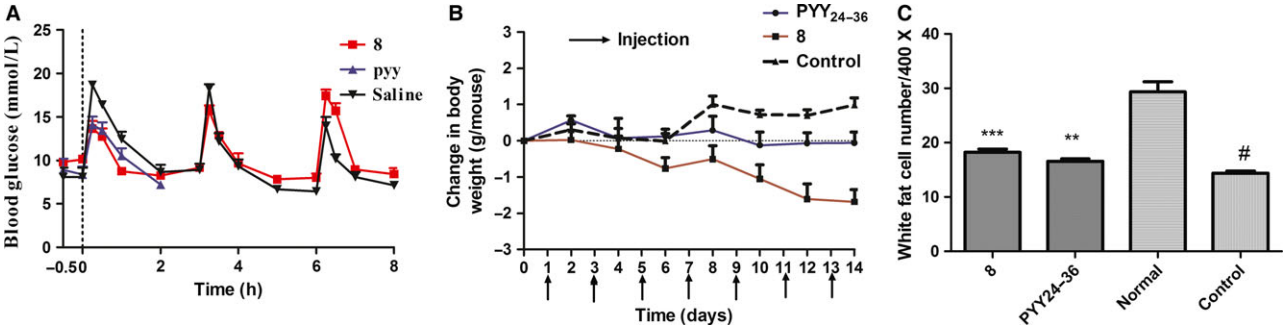


Figure 6: Two-week body weight loss studies in C57BL/6 DIO mice with $PYY_{24–36}$ and **8** (A–C): (A) Oral glucose tolerance at day 14. (B) Dynamic body weight change for 2 weeks. (C) Epididymis cell number. **$p < 0.01$; ***$p < 0.001$ compared with control group; $^{\#}p < 0.001$ compared with normal group.

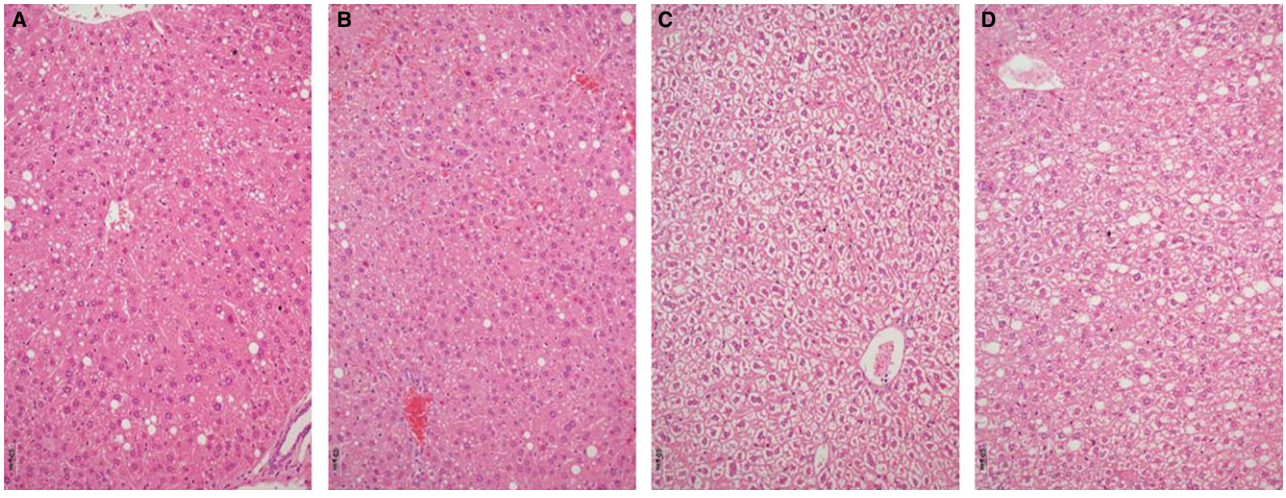


Figure 7: Chronic effect on liver lipid droplets of DIO mice (A–D): (A) $PYY_{24–36}$. (B) **8**. (C) normal. (D) DIO vehicle.

most of the conjugates were over 12 h, and especially, **8** exhibited the longest half-life of over 24 h (Figure 2). Notably, the plasma half-life values of **1**–**4** correlated with the alkyl chain length of these Ac-deoxycholic acid, and compound **2** was most stable (> 20 h). It might indicate that the medium alkyl chain length was most proper. Fat acid chain conjugates showed comparable results.

Binding to human serum albumin

We predicted that the increased albumin binding might lead to the increased metabolic stability, and this was investigated for **1**–**9**. With $PYY_{24–36}$ used as control, all the conjugates exerted improved albumin affinity. Albumin binding correlated well with plasma stability (Figure 3).

Effects of acute feeding studies and 2 weeks treatment in lean ICR mice

Peptide conjugates **2**, **5**, **7**, **8**, **9** with their half-lives higher than 20 h were chose for further pharmacology study. The total amount of food intake in the mice for each time was reduced (Figure 4), and the total consumption for 8 h was significantly less than that of the control (Figure 5). The results showed that all the tested compounds had appetite control effect and had a longer duration of action than $PYY_{24–36}$. In particular, the anorexia effect of **8** was relatively strong and stable, which is worthy of further development.

In addition, 2-week treatment was given to ICR mice to illustrate body weight change. As shown in Figure 5, most of the compounds exhibited good weight control compared with control (saline) and $PYY_{24–36}$. In particular, treatment of **8** increased body weight of only 2.4 ± 0.13 g, while control group increased body weight of 4.25 ± 0.36 g after 2 weeks.

Effect in DIO mice of every other day dosing on body weight and oral glucose tolerance test (OGTT)

Peptide **8** was further selected for weight loss and OGTT studies in DIO mice. Subcutaneous administration of **8** every other day resulted in a dose-dependent reduction in body weight (Figure 6B). The injections decreased body weight of DIO mice by 1.45 ± 0.2 g, respectively, compared to vehicle increased by 1.75 ± 0.12 g ($p < 0.01$, $n = 6$ per group) (Figure 6B). Food intake is reduced for the treated animals compared with the untreated control groups. The initial reduction and subsequent restoration of food intake are also observed with $PYY_{24–36}$ upon continuous dosing in DIO mice, which is consistent with result in ICR mice (data not shown). Oral glucose tolerance test was following performed for evaluating hypoglycemic (Figure 6A). Compared with control (0.9% saline), compound **8** exhibited moderate hypoglycemic effect unexpectedly after two round of injection glucose, while $PYY_{24–36}$ lost its potency after first turn, which means $PYY_{24–36}$ and its analogues may have blood glucose regulation effect (21).

Histological changes in the liver and brown fat tissue of DIO mice

As **8** treatment had pronounced weight loss effects in DIO mice, liver, white fat, and brown fat tissue were histologi-

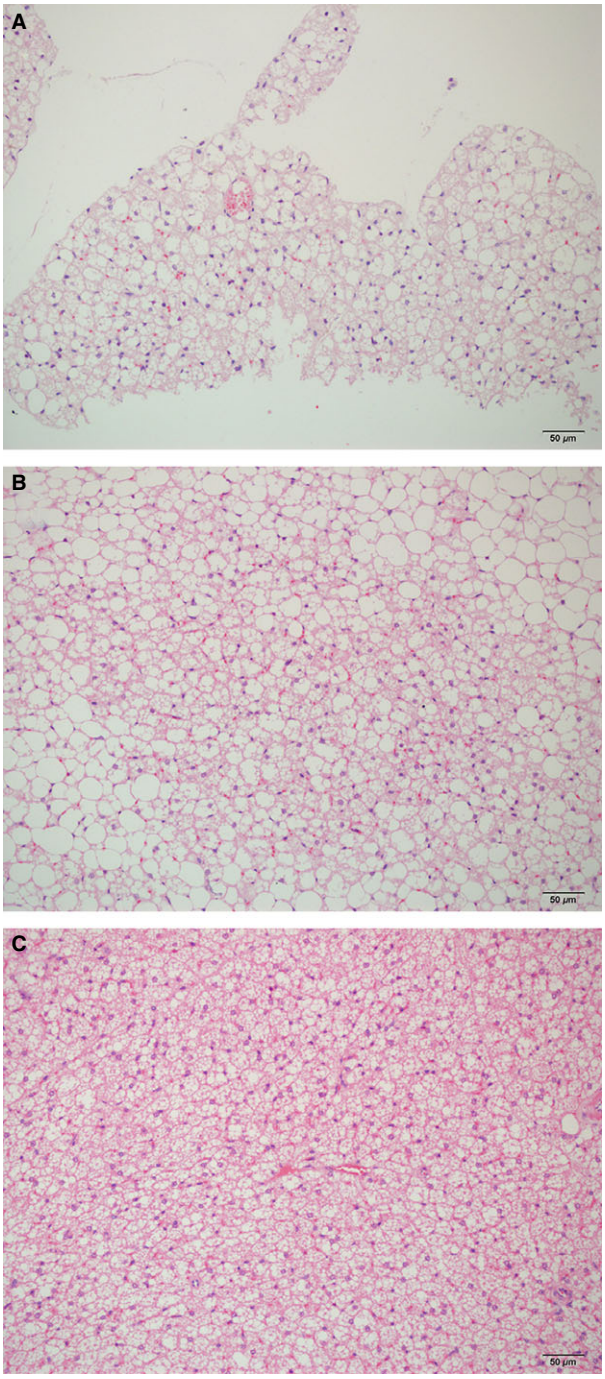


Figure 8: Chronic effect on liver brown fat cell of DIO mice (brown fat cells were not seen in DIO mice) (A–C): (A) $PYY_{24–36}$. (B) **8**. (C) normal.

Plasma parameters	Normal	**8**	$PYY_{24–36}$	DIO
Insulin (ng/mL)	7.96 ± 0.13	9.210 ± 0.35	10.94 ± 0.23	12.56 ± 0.25
Leptin (ng/mL)	6.33 ± 0.15	5.85 ± 0.27	5.33 ± 0.18	4.37 ± 0.11
Adiponectin (μg/mL)	4.78 ± 0.15	5.01 ± 0.25	5.17 ± 0.26	4.57 ± 0.12

Table 2: Chronic treatment with **8** and $PYY_{24–36}$ in DIO mice: plasma determinations at the end of the chronic study

Data are means ± SEM. $n = 6$, *$p < 0.05$ **8** and $PYY_{24–36}$ versus vehicle.

cally assessed in mice from each of the four experimental groups. Liver cells of untreated DIO control mice appeared to be full of lipid droplets (Figure 7), while the **8** treatment group showed a little and small lipid droplets. The body weight changes were associated with an increase in white adipose (WAT) cell number in the same area (18.24 ± 1.58 for **8**, 16.62 ± 1.19 for $PYY_{24–36}$, and 14.4 ± 1.26 for controls, 29.4 ± 5.83 for normal $p < 0.01$) (Figure 6C). Brown adipose tissue can speed up the metabolism and promote white fat consumption. It helps to promote obesity treatment. In contrast to no image of brown fat cell seen in control DIO mice, mice treated with **8** showed a significant increase in brown fat cell number. Images of representative liver and brown fat for each group are shown (Figure 7 and 8).

Chronic treatment improves metabolism

Several other metabolic parameters in plasma were also improved by chronic treatment with **8** (Table. 2). Leptin and adiponectin are important factors of body mass index, insulin resistance, and glucose metabolism. Increases in adiponectin and leptin and decreases in insulin levels correlated with the decreased adiposity observed at the end of the study in each treatment group.

Conclusion

In this study, to develop long-acting and highly biologically active $PYY_{24–36}$ derivatives, we modified the carboxylic groups of peptide by amidation with small molecules and obtained a series of $PYY_{24–36}$ conjugates. Besides two known small molecules bile acid and fatty chain that commonly used in prolong peptide half-life, phenylbutazone was firstly used based on its characteristic that it can strongly bind to HSA (22). By visually resolved with HSA (Figure 1), we know that the phenylbutazone structure is very conservative. So we decided to add a linker to it and connect it with $PYY_{24–36}$ by amidation.

The results show that phenylbutazone-$PYY_{24–36}$ conjugate **8** is most potent and has a significant weight reduction in DIO mice. Other improvements are also accompanied by weight loss. Other effects of compound **8** are better than $PYY_{24–36}$, but not that much. That may because the metabolism improvement is a slow process and may need longer time of treatment. In conclusion, anorexia effect, energy expenditure, and its duration time may all possible affect its potential of weight loss.

Therefore, compound **8** may be considered as a promising candidate for obesity treatment. It also has a certain hypoglycemic activity (Figure 6) (21), which may provide a new way to find hypoglycemic peptide. In addition, even phenylbutazone has long been known as anti-inflammatory drug, but as a new type of long-acting chemical agent, it can be easily modified with other peptides through amidation to improve their pharmacokinetic profile, which may provide a new strategy to prolong peptides lifetime.

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Conflict of Interest

All authors report no conflict of interest.

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