Supporting Information

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

Naoki Nishizawa, Ayumu Niida, Yasushi Masuda, Satoshi Kumano, Kotaro Yokoyama, Hideki Hirabayashi, Nobuyuki Amano, Tetsuya Ohtaki, Taiji Asami*

Pharmaceutical Research Division, Takeda Pharmaceutical Company, Ltd.,
Fujisawa 251-8555, Japan
taiji.asami@takeda.com

Contents

EXPERIMENTAL SECTION

Instruments and Materials. Manual 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide syntheses were conducted consisting of Fmoc cleavage with 20% piperidine/*N*,*N*-dimethylformamide (DMF) (20 min) and an Fmoc amino acid condensation reaction using *N*,*N*'-diisopropylcarbodiimide (DIPCDI)/*N*-1-hydroxy-7-azabenzotriazole (HOAt) (4 eq.), or by the ABI 433A automated peptide synthesizer (Applied Biosystems, Foster City, CA, USA) [as per the Fmoc/DCC/*N*-hydroxybenzotriazole (HOBt) 0.25-mmol protocol]. All final compounds were purified to ≥ 95% homogeneity by reversed-phase (RP)-HPLC analysis with a photodiode array detector across a wavelength range of 190–500 nm; the absence of co-eluting impurities (heterogeneous peaks) was confirmed by liquid chromatography-mass spectrometry (LC/MS) analysis. The identity of the peptides was confirmed by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis on a Bruker autoflex speed system (Billerica, MA, USA). The purity, retention time, and molecular weight of each peptide are summarized in Table S1. Commercially available amino acid derivatives and resins were purchased from Novabiochem (Billerica, MA, USA), Watanabe Chemical Industries (Hiroshima, Japan), Peptide Institute (Osaka, Japan), Bachem (Bubendorf, Switzerland), AnaSpec (Fremont, CA, USA), Chem-Impex International (Wood Dale, IL, USA), and American Peptide Company (Sunnyvale, CA, USA), whereas other reagents, such as coupling and deprotection reagents, were purchased from Wako Pure Chemical Industries (Osaka, Japan), Novabiochem, Watanabe Chemical Industries, and Nacalai Tesque (Kyoto, Japan).

General Procedure for Synthesis of PYY Analogues. All peptides were synthesized in the same manner as the following synthesis procedure for benzoyl-[Cha^{27,28,36},Aib^{31}]PYY(25–36) (**1**). Using commercially available Sieber Amide resin (347 mg, 0.25 mmol) as a starting material and an ABI433A peptide synthesizer (DCC/HOBt 0.25 mmol protocol), amino acids were successively condensed to give H-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Pbf)-His(triphenylmethyl, Trt)-Cha-Cha-Asn(Trt)-Leu-Aib-Thr(*tert*-butyl, *t*Bu)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Cha-Sieber Amide Resin (1.1292 g, 0.232 mmol/g). Then, 401.6 mg (0.1 mmol) of the obtained resin was weighed, washed with DMF and, after swelling, treated with benzoic acid (48.8 mg, 0.4 mmol), DIPCDI (63.6 μL, 0.4 mmol), and 0.5 M HOAt/DMF (0.8 mL, 0.4 mmol) in DMF for 90 min to benzoylate the N-terminus. The resin was washed with DMF and methanol, and then dried to give benzoyl-Arg(Pbf)-His(Trt)-Cha-Cha-Asn(Trt)-Leu-Aib-Thr(*t*Bu)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Cha-Sieber Amide Resin (452.0 mg, 0.1 mmol). Next, TFA:thioanisole:*m*-cresol:H_2O:1,2-ethanedithiol:triisopropylsilane (80:5:5:5:2.5:2.5) (3 mL) was added to the entire amount of the obtained resin and the mixture was stirred at ambient

temperature for 90 min, after which diethyl ether was added to the reaction solution to allow precipitation of a white powder. An operation to remove the ether by decantation after centrifugation of the suspension was repeated twice to remove the acid and scavenger material. The residue was extracted with aqueous acetic acid solution and purified by preparative HPLC using Daisopak-SP100-5-ODS-P (20 × 250 mm I.D.) (OSAKA SODA, Osaka, Japan) to give 84.1 mg of a white powder; mass spectrum: MALDI-TOF (α-cyano-4-hydroxycinnaminic acid, monoisotopic) $[M+H]^+$ 1727.92 (Calcd 1728.05). Elution time on RP-HPLC: 8.19 min. Elution conditions: Phenomenex Kinetex® 1.7 μm XB-C18 column (100 × 2.1 mm I.D.) (Torrance, CA, USA), linear density gradient elution with eluents A/B = 95/5–45/55 (10 min), using 0.1% TFA in water as eluent A and 0.1% TFA-containing acetonitrile as eluent B; flow rate: 0.5 mL/min.

Establishment of Cloned Human Y2R-expressing CHO Cells. The entire coding region of the human *NPY2R* cDNA was amplified by polymerase chain reaction (PCR) from human brain cDNA (Takara Bio, Shiga, Japan). The DNA sequence of the PCR product was confirmed to represent human Y2R and finally cloned into expression vector pAKKO-111H for the expression of Y2R in CHO cells. This expression vector was transfected into CHO (*dhfr*-) cells and CHO cells stably expressing human Y2R were established as described previously.[1]

Membrane Preparation from CHO Cells Expressing Human Y2R. The affinity of the synthesized peptides for human Y2R was determined by a competitive binding experiment with membranes of CHO cells expressing cloned human Y2R. The membrane was prepared as described previously.[1] The CHO cells were detached from the culture dish with PBS-ethylenediaminetetraacetic acid (EDTA). The cells were recovered by centrifugation at 1000 rpm for 10 min. The cell pellets obtained were homogenized in ice-cold homogenizing buffer [10 mM $NaHCO_3$, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL E-64] with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 700 × *g* for 15 min at 4 °C and then the supernatant obtained was ultracentrifuged at 10000 × *g* for 60 min. The resultant pellet was suspended in suspending buffer (50 mM Tris, 5 mM $MgCl_2$, 150 mM NaCl, 0.5 mM PMSF, 10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL E-64, 0.03% NaN_3, pH 7.4). The protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Receptor Binding Assay for Human Y2R. First, 2 μL test compound was incubated in a 96-well plate with 100 μL of the membrane diluted with assay buffer (50 mM Tris, 5 mM $MgCl_2$, 150 mM NaCl, 0.03% NaN_3, pH 7.4) to 0.5 μg protein/mL and 100 μL of 400 pM ^{125}I-PYY (NEX341, PerkinElmer, Waltham, MA, USA). After incubation at room

temperature for 60 min, the reaction mixture was filtered through a UniFilter-96 GF/C (PerkinElmer) presoaked in polyethylenimine (PEI) solution (20 mM Tris, 0.3% PEI, pH 7.4). The filter was washed three times with ice-cold filtration buffer (50 mM Tris, 5 mM $MgCl_2$, 150 mM NaCl, 0.03% NaN_3, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, pH 7.4). The UniFilter-96 GF/C was then dried; 15 μL of the liquid scintillator MicroScint O (PerkinElmer) was next added to each well and the radioactivity was measured using TopCount (PerkinElmer). The data obtained were analyzed using Prism (GraphPad Software, La Jolla, CA, USA) to calculate the IC_{50} value.

[^{35}S]GTPγS Binding Assay. The agonist activity of the compounds for human Y2R was evaluated using a [^{35}S]GTPγS binding assay. First, 2 μL test compound was incubated in a 96-well plate with 100 μL of the membrane diluted with assay buffer (50 mM Tris, 5 mM $MgCl_2$, 150 mM NaCl, 1 μL GDP, 0.03% NaN_3, 0.1% bovine serum albumin, pH 7.4) to 1 μg protein/mL and 100 μL of 1 nM [^{35}S]GTPγS (NEG030H, PerkinElmer). After incubation at room temperature for 120 min, the reaction mixture was filtered through a UniFilter-96 GF/C, washed, dried, and the radioactivity was measured as for the receptor binding assay. The data obtained were analyzed using Prism to calculate the EC_{50} value.

Food Intake Assay. Male C57BL/6J mice (8–9-week-old) were divided into groups (n = 5) based on the body weight and food intake. Compounds were dissolved in 50% dimethyl sulfoxide/H_2O and administered subcutaneously by infusion using an osmotic pump (DURECT Corporation, Cupertino, CA, USA) for 3 days. The body weight and food intake were measured.

Pharmacokinetics of PYY Analogues in Mice. Peptides were administered intravenously or subcutaneously to C57BL/6J mice at a dose of 1 mg/kg in fed animals. After administration, blood samples were collected at 5, 10, 15, 30 min, and at 1, 3, 6, 8, and 24 h and centrifuged to obtain the plasma fraction. The plasma samples were deproteinized followed by centrifugation and the supernatants were analyzed by LC/MC/MS to determine the plasma concentration of the peptides. The pharmacokinetics parameters were calculated by the moment analysis method. The plasma concentration 5 min after injection (C_{5min}), area under the concentration-time curve from time zero to 24 h (AUC_{0-24h}), mean residence time (MRT), Vd_{ss}, and CL_{total} for each mouse after intravenous administration, were obtained. The maximum plasma concentration (C_{max}), time to maximum plasma concentration (t_{max}), AUC_{0-24h}, mean residence time (MRT), and BA for each mouse after subcutaneous administration, were also obtained.

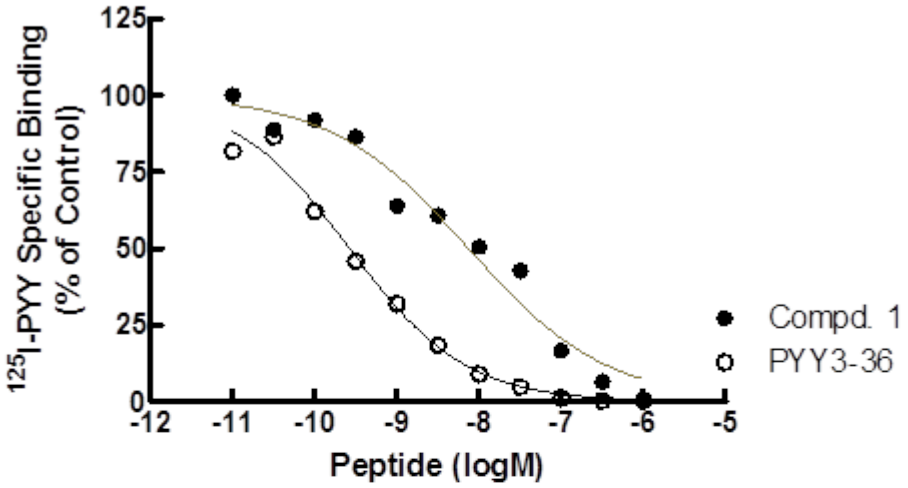
REFERENCES

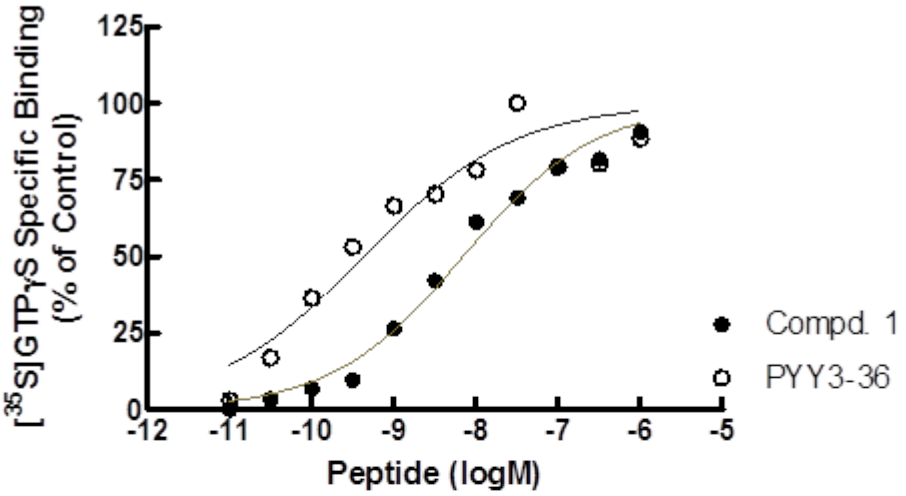
(1) Masuda, Y.; Sugo, T.; Kikuchi, T.; Kawata, A.; Satoh, M.; Fujisawa, Y.; Itoh, Y.; Wakimasu, M.; Ohtaki, T. Receptor binding and antagonist properties of a novel endothelin receptor antagonist, TAK-044 {*cyclo*[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl]disodium salt}, in human endothelin$_A$ and endothelin$_B$ receptors. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 675−685.

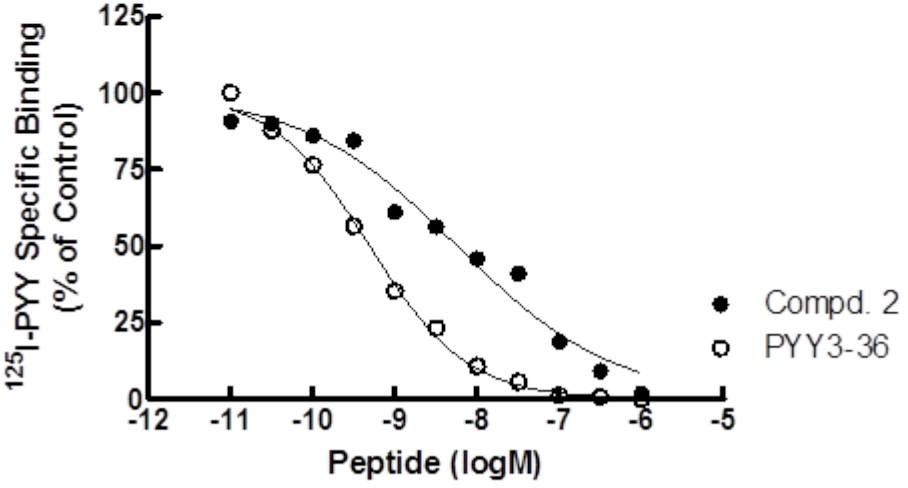
Table S1. Chemical Data for PYY Analogues

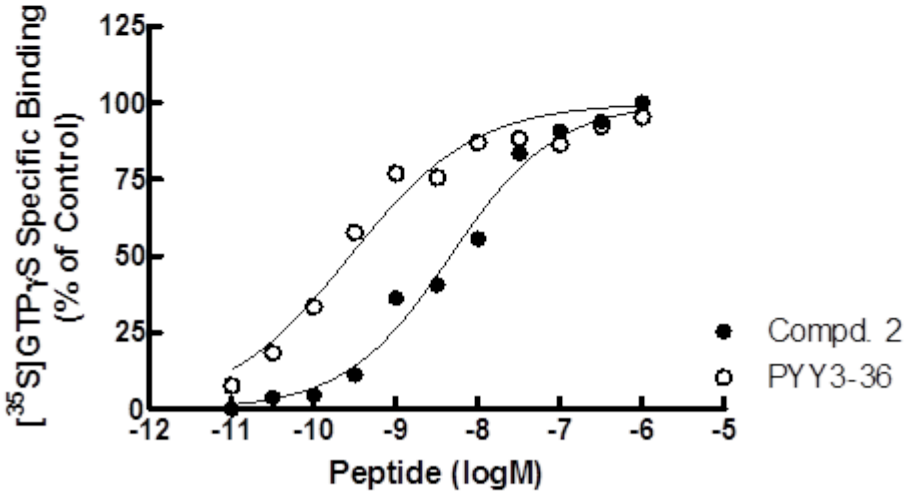
		RP-HPLC[a]		mass (Da)[b]	
compound		t_{ret} (min)	purity (%)	calculated	observed
1	benzoyl-[Cha^{27,28,36},Aib^{31}]PYY(25-36)	8.19	99.9	1728.05	1727.92
2	benzoyl-[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(25-36)	9.46	95.4	1676.05	1676.08
3	isobutanoyl-[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(25-36)	9.43	100.0	1642.06	1642.07
4	Ac-[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(24-36)	9.74	99.2	1727.12	1727.17
5	Ac-[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(23-36)	9.20	95.1	1814.15	1814.24
6	Ac-[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(22-36)	9.27	98.6	1885.19	1885.32
7	[Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(22-36)	8.24	96.0	1843.18	1843.25
8	[Cha^{27,28,36},Aib^{31}]PYY(22-36)	7.55	96.6	1895.18	1895.35
9	Ac-[D-Leu^{24},Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(23-36)	8.96	96.9	1814.15	1814.32
10	Ac-[D-Ser^{23},D-Leu^{24},Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(23-36)	8.88	98.3	1814.15	1814.39
11	Ac-[D-Ser^{23},Abu^{26},Cha^{27,28,36},Aib^{31}]PYY(23-36)	9.22	98.1	1814.15	1814.39
12	Ac-[MeAla^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	7.25	99.5	1838.12	1838.46
13	Ac-[D-MeAla^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	7.59	100.0	1838.12	1838.12
14	Ac-[Sar^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	6.88	99.2	1824.11	1824.09
15	Ac-[Aib^{24,31},Cha^{27,28,36}]PYY(23-36)	7.84	99.5	1838.12	1838.12
16	Ac-[Gly^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	6.94	99.3	1810.09	1810.13
17	Ac-[D-Pro^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	7.03	99.7	1850.12	1850.18
18	Ac-[D-Hyp^{24},Cha^{27,28,36},Aib^{31}]PYY(23-36)	6.83	99.0	1866.12	1866.24

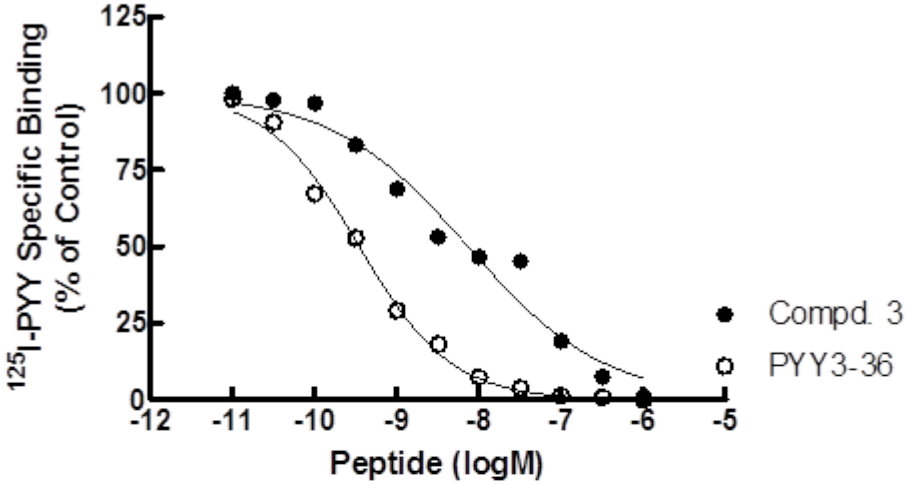
[a]Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using Phenomenex Kinetex 1.7 µm XB-C18 column (100 × 2.1 mm) at a wavelength of 210 nm. Peptides were eluted with a linear gradient 5-55% acetonitrile in water containing 0.1% TFA over 10 min (0.5 mL/min). [b]Molecular weights of peptides were determined by autoflex speed MALDI-TOF mass spectrometer.

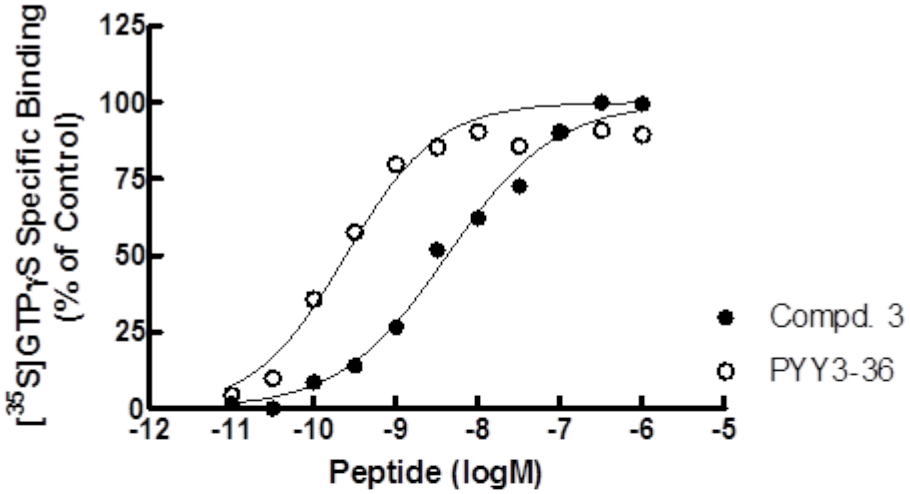

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 1
PYY3-36

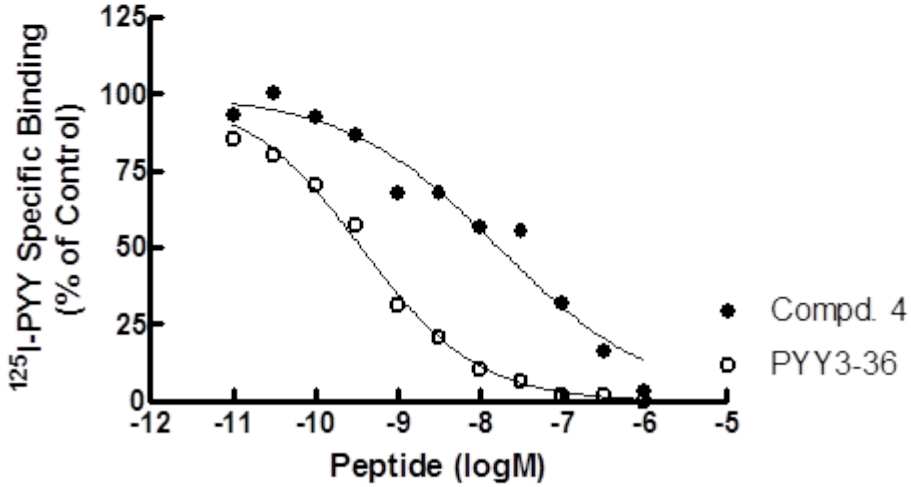

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 1
PYY3-36

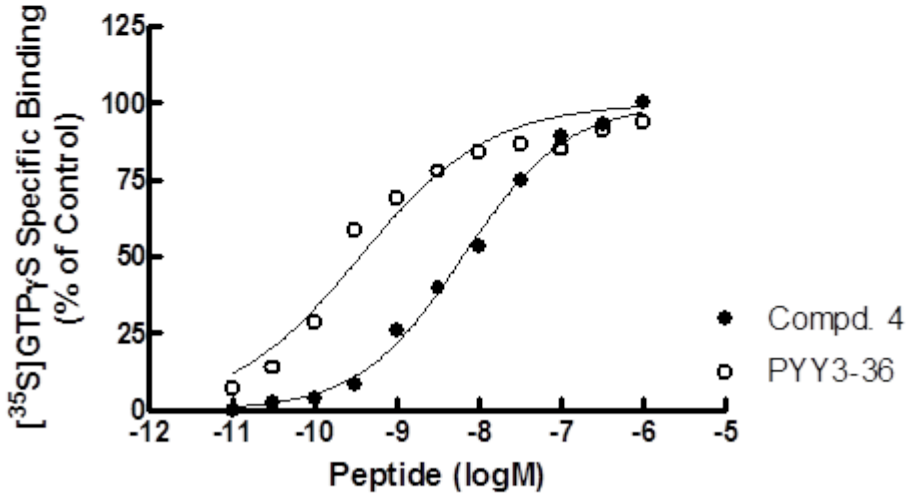

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 2
PYY3-36

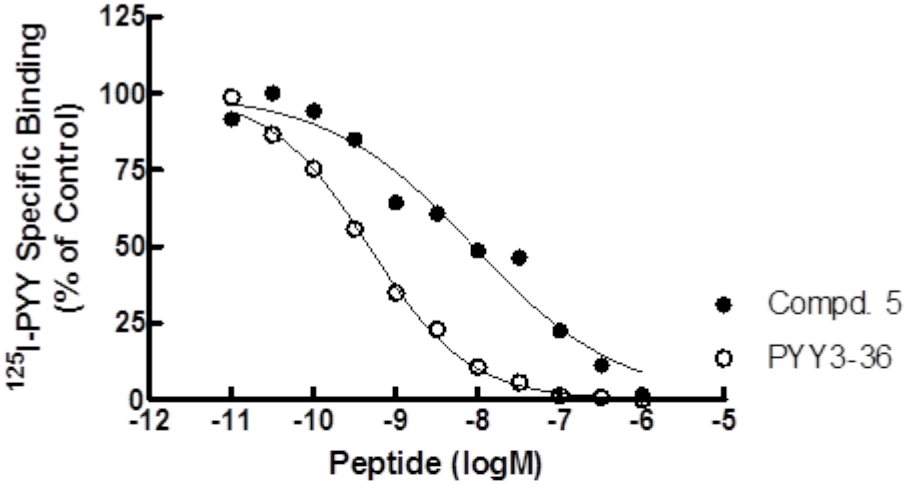

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 2
PYY3-36

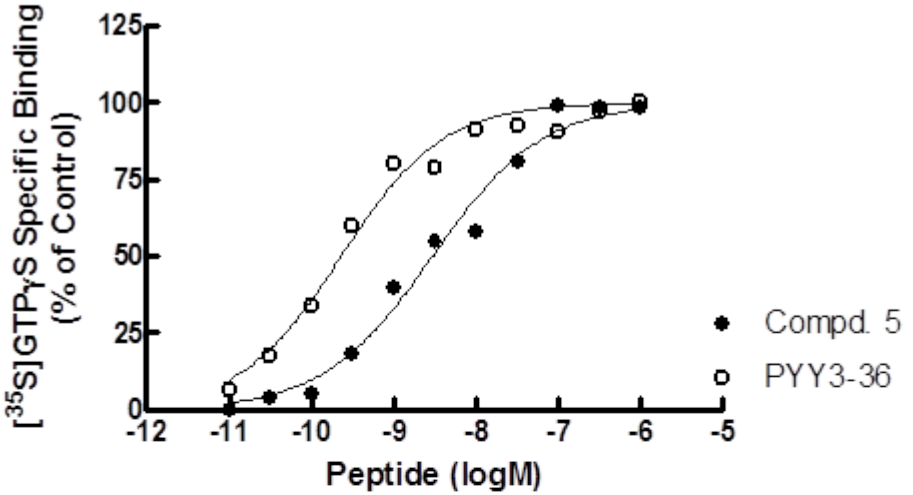

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 3
PYY3-36

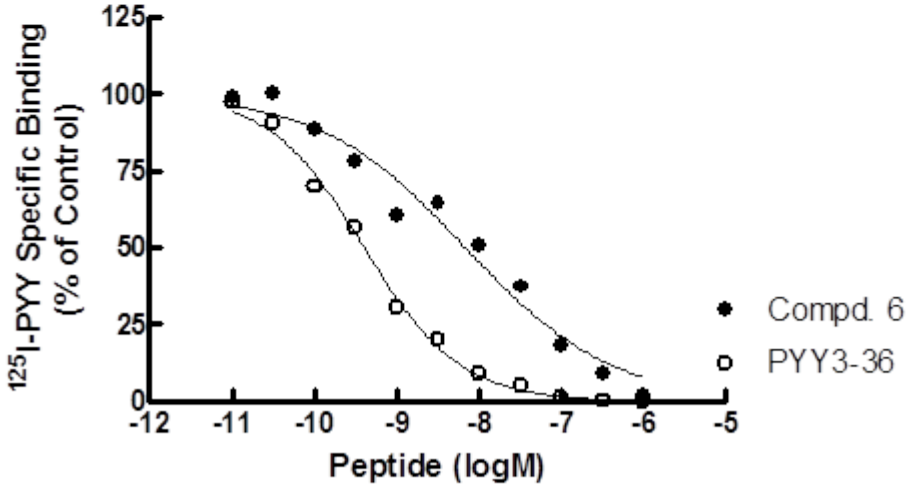

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 3
PYY3-36

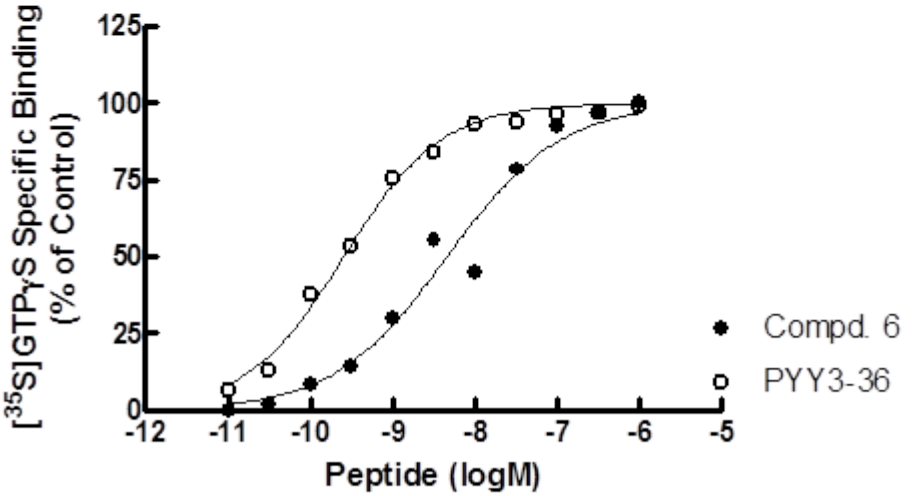

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 4
PYY3-36

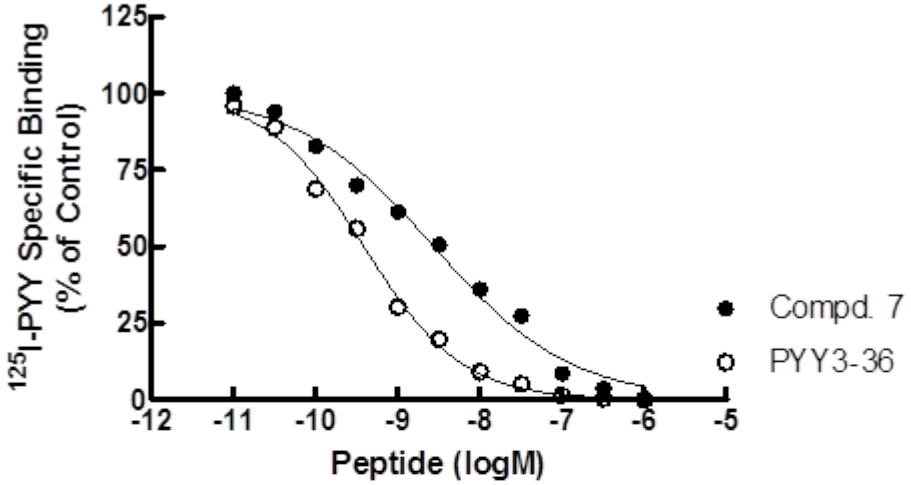

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 4
PYY3-36

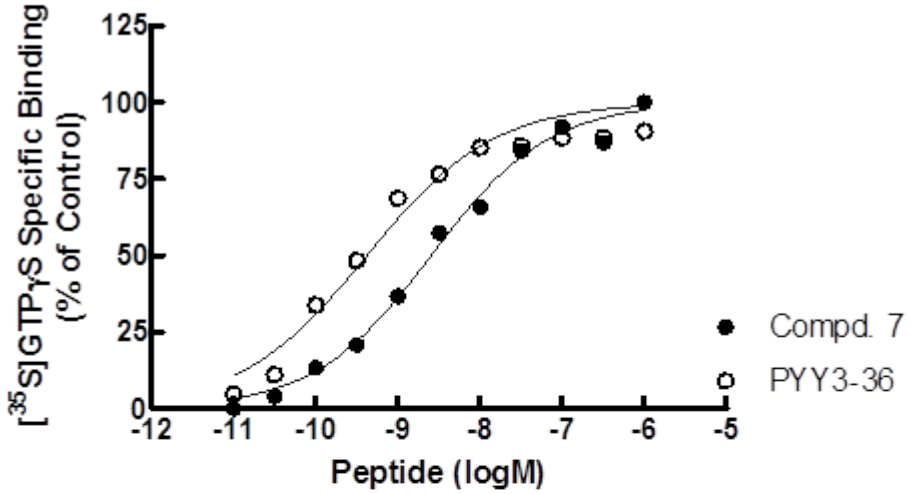

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 5
PYY3-36

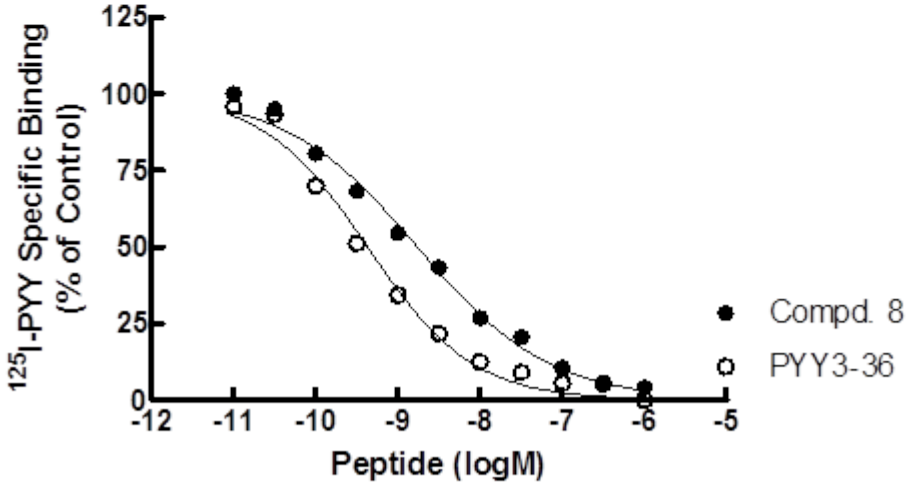

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 5
PYY3-36

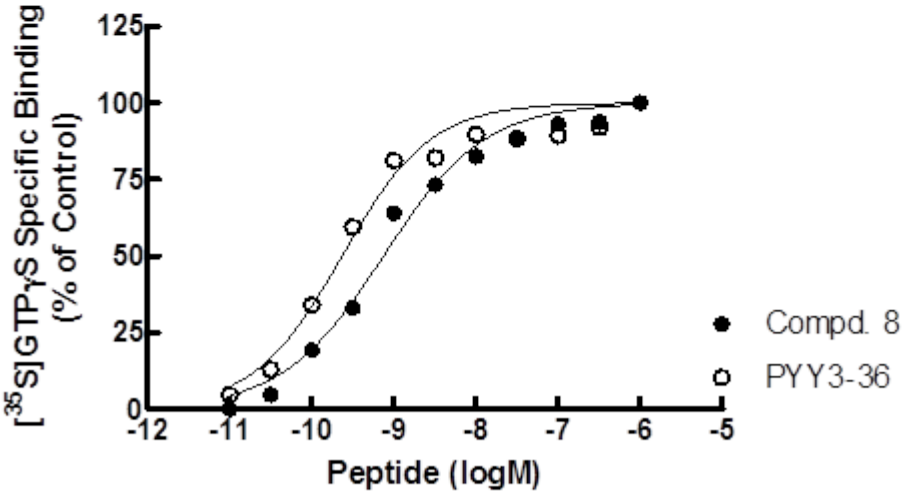

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 6
PYY3-36

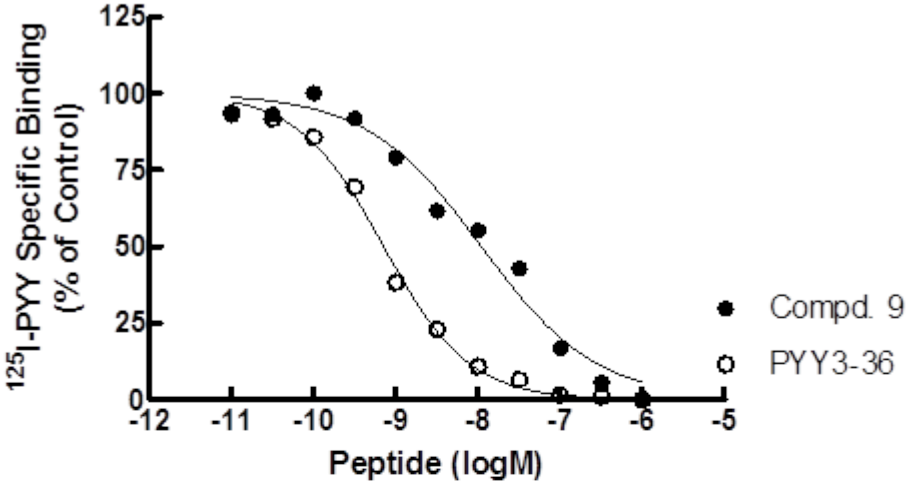

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 6
PYY3-36

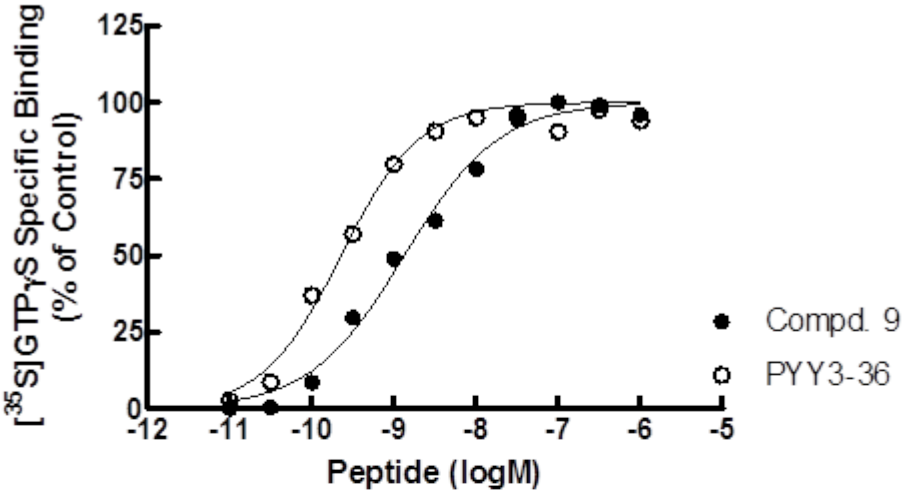

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 7
PYY3-36

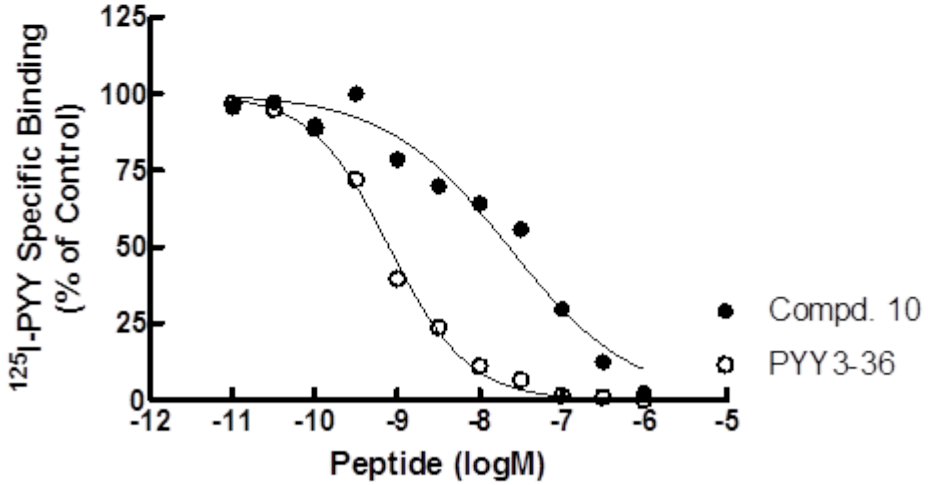

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 7
PYY3-36

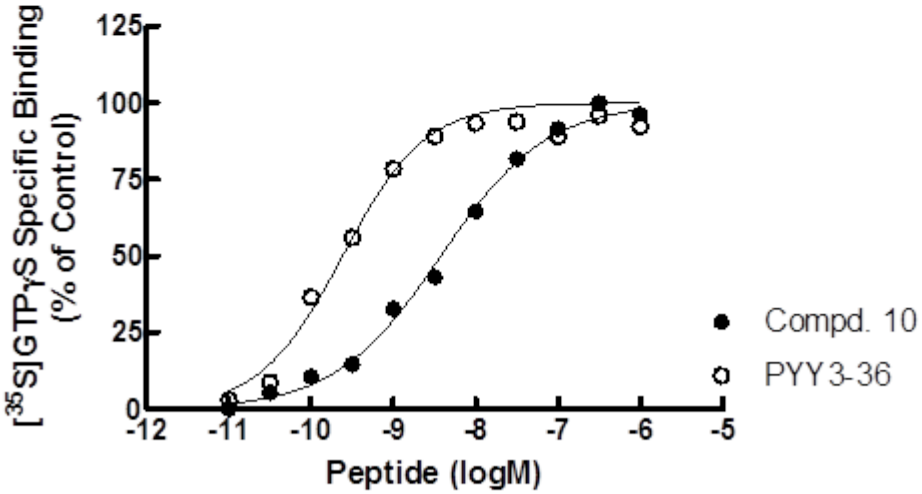

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 8
PYY3-36

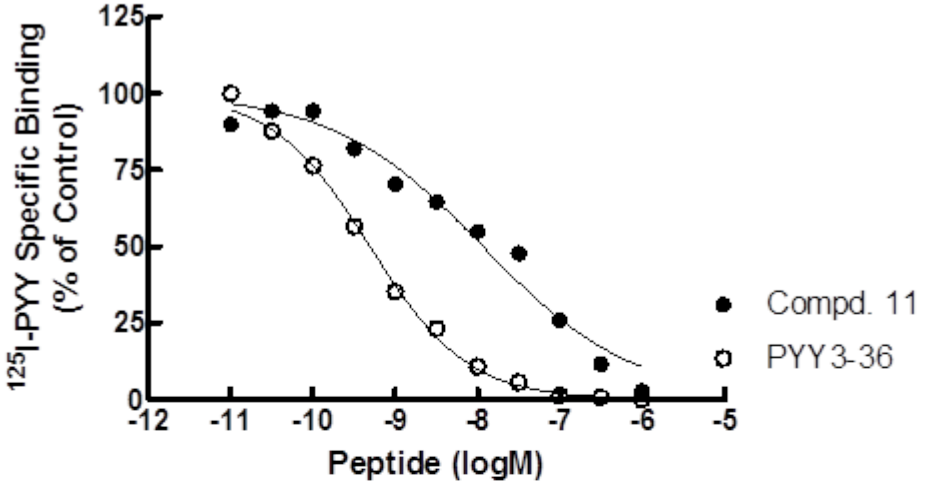

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 8
PYY3-36

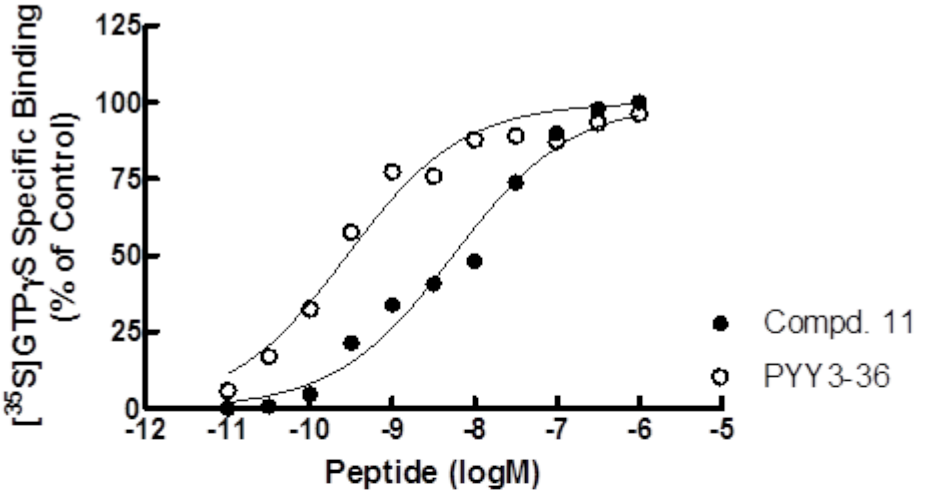

125I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 9
PYY3-36

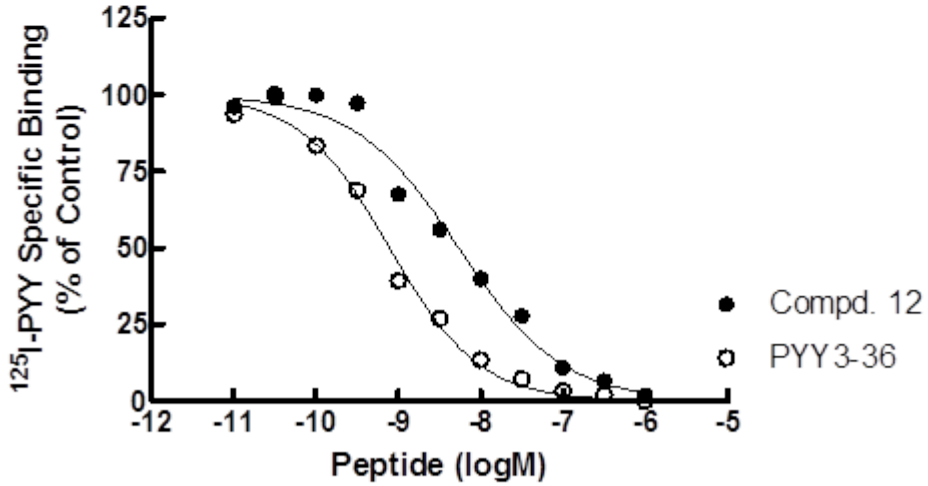

[35S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 9
PYY3-36

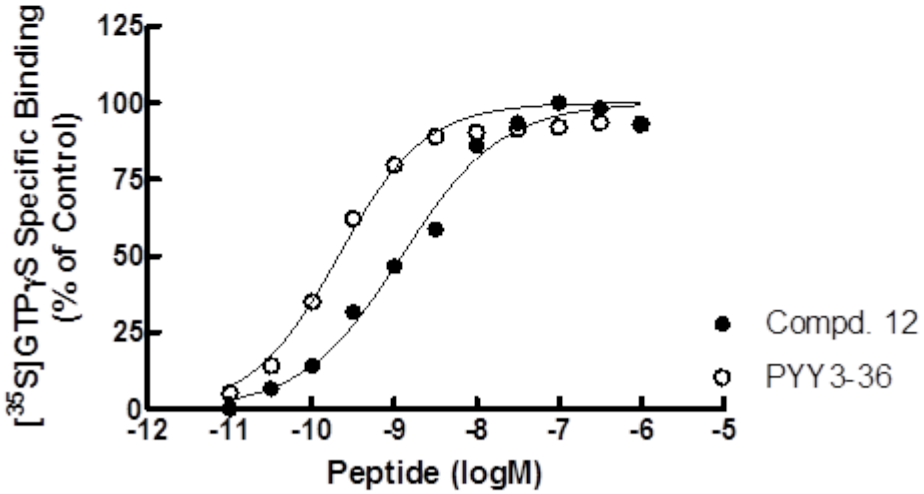

125I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 10
PYY3-36

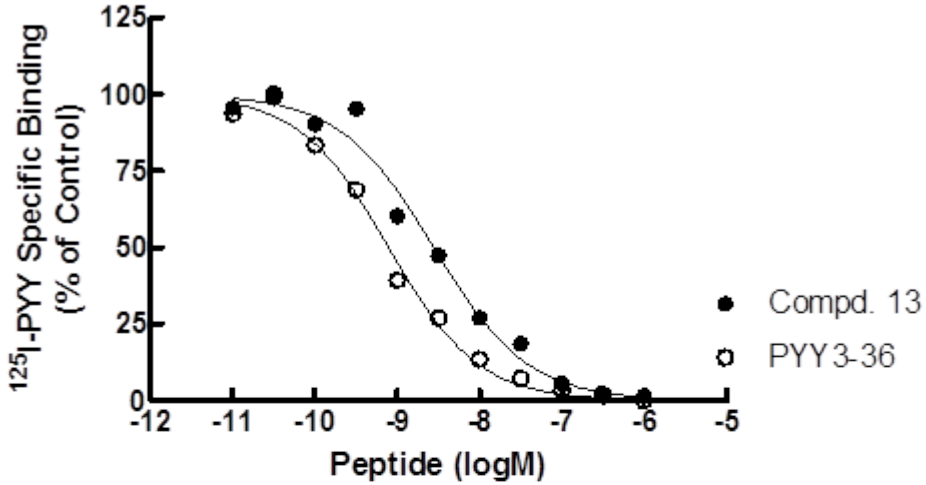

[35S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 10
PYY3-36

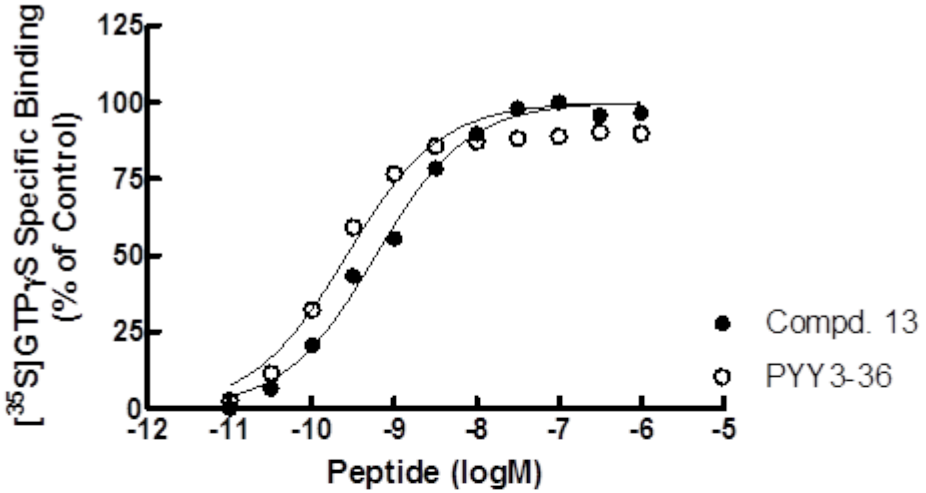

125I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 11
PYY3-36

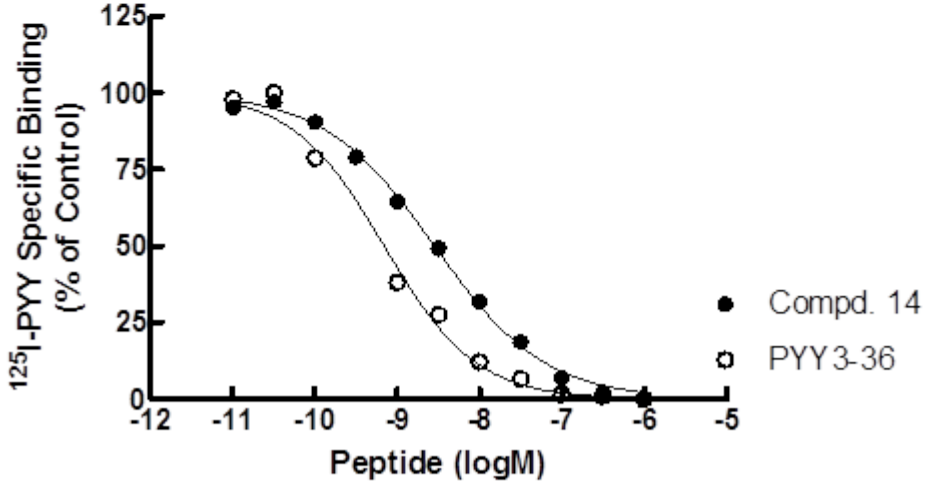

[35S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 11
PYY3-36

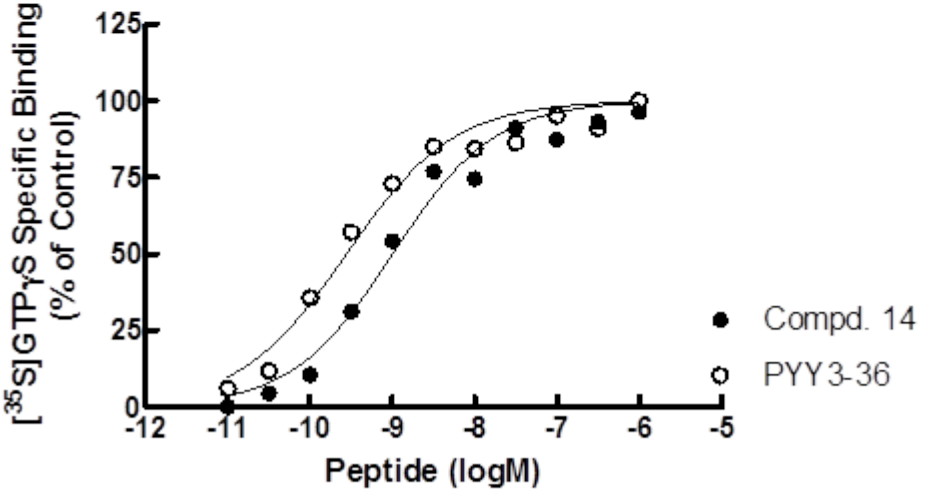

125I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 12
PYY3-36

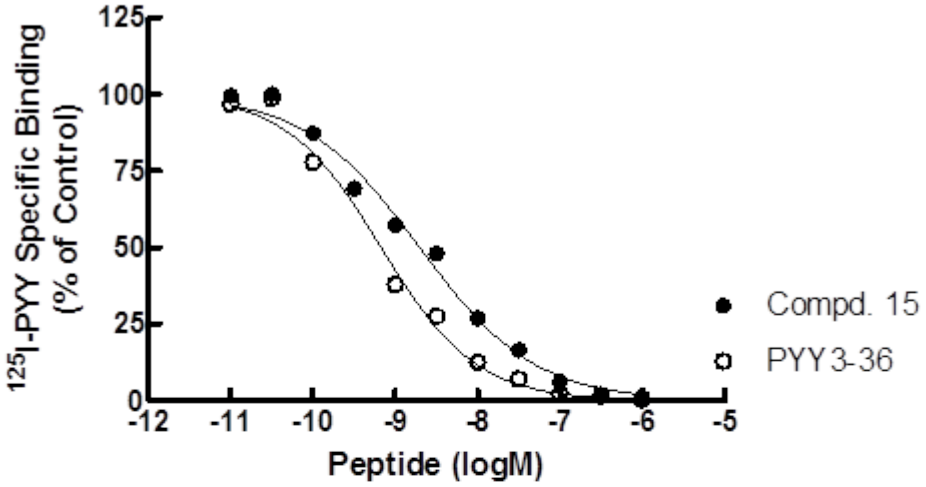

[35S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 12
PYY3-36

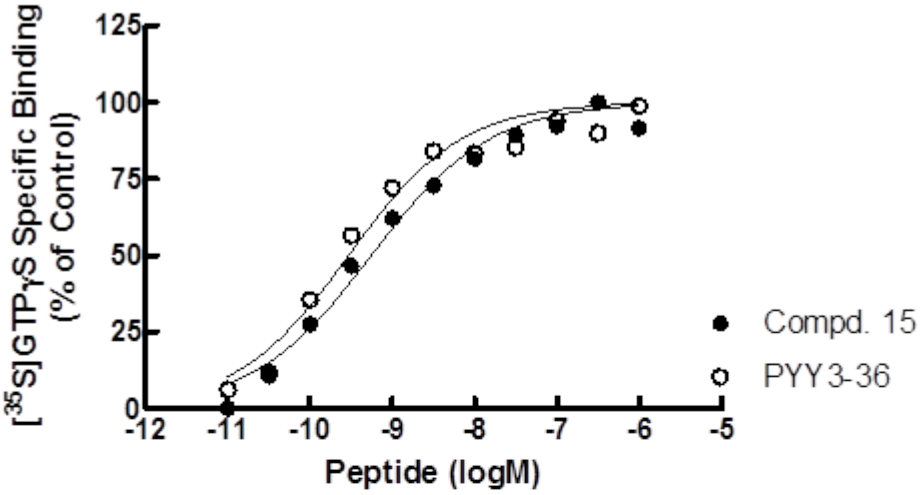

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 13
PYY3-36

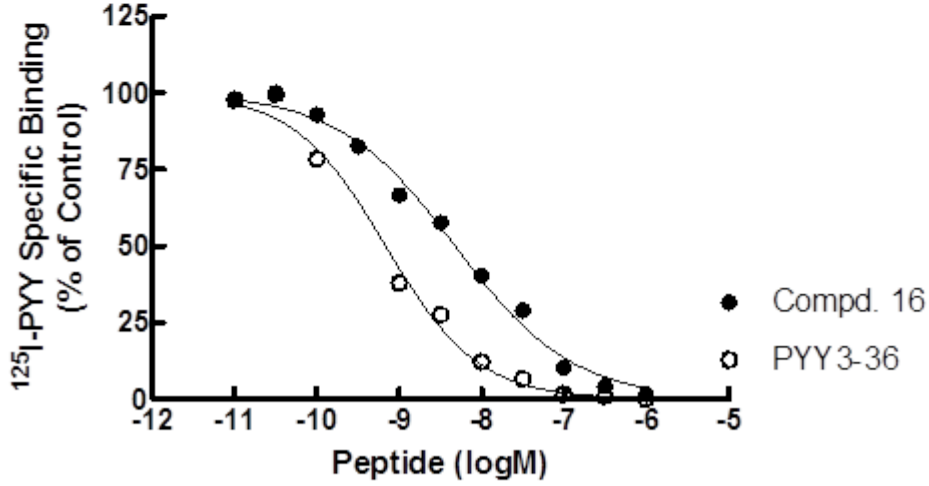

[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 13
PYY3-36

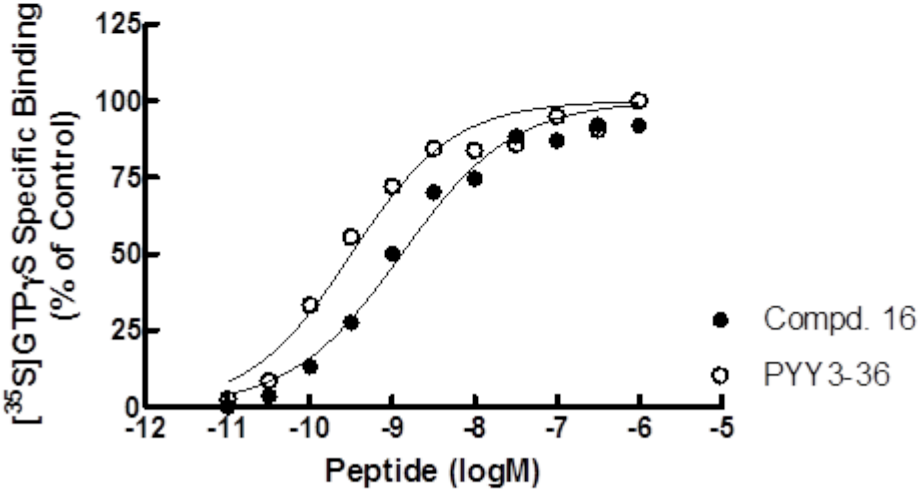

^{125}I-PYY Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 14
PYY3-36


[^{35}S]GTPγS Specific Binding
(% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 14
PYY3-36


^{125}I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 15
PYY3-36


[^{35}S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 15
PYY3-36


^{125}I-PYY Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 16
PYY3-36


[^{35}S]GTPγS Specific Binding (% of Control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
Peptide (logM)
Compd. 16
PYY3-36

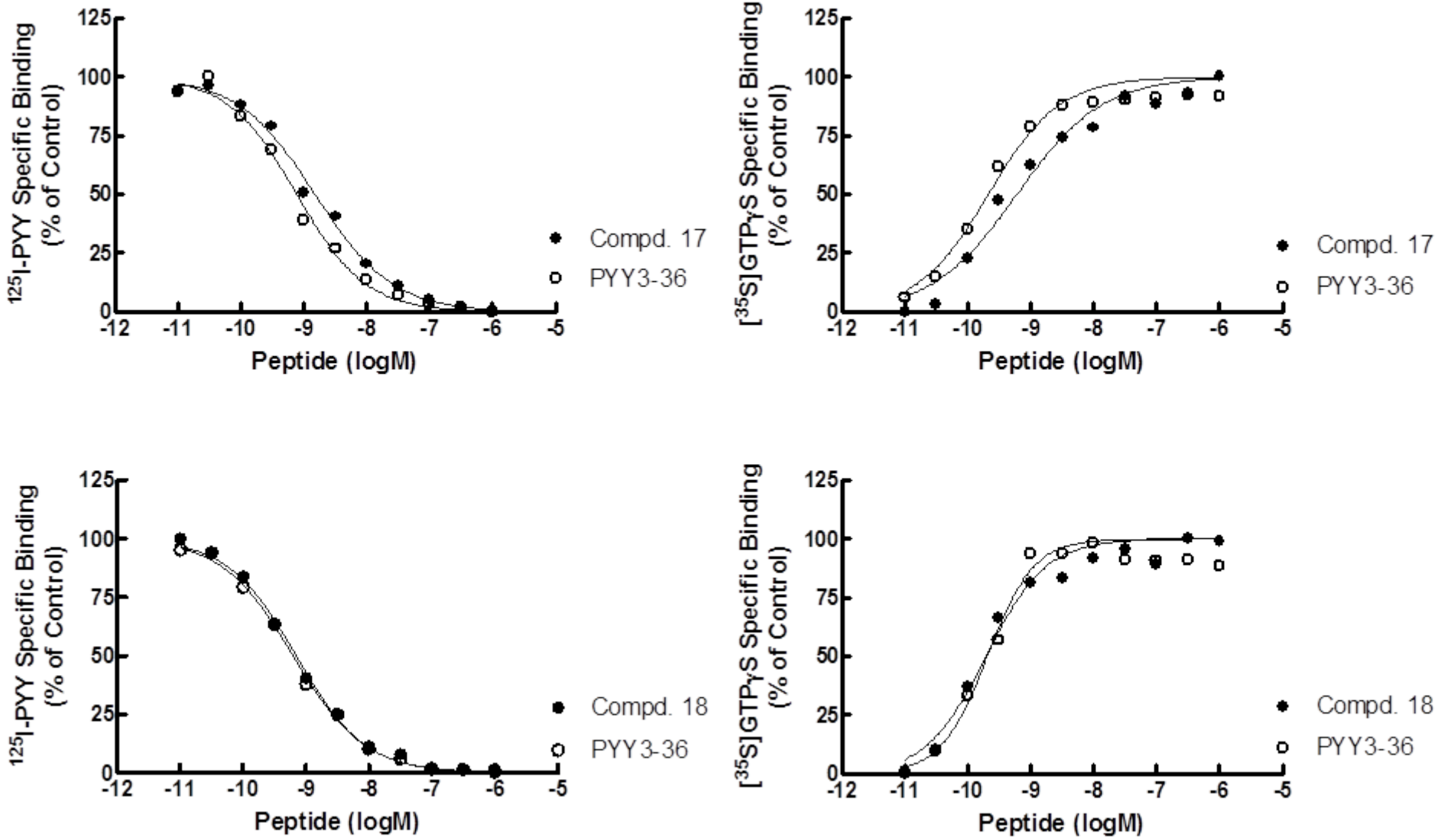


Figure S1. Binding affinity and agonist activity of PYY analogues. Binding affinity of all peptides (**1**−**18**) was evaluated by a receptor binding assay with membranes obtained from Chinese hamster ovary (CHO) cells expressing cloned human Y2R. Agonist activity of peptide analogues was also examined by [^{35}S]GTPγS binding assay with the membranes of human Y2R-expressing CHO cells.

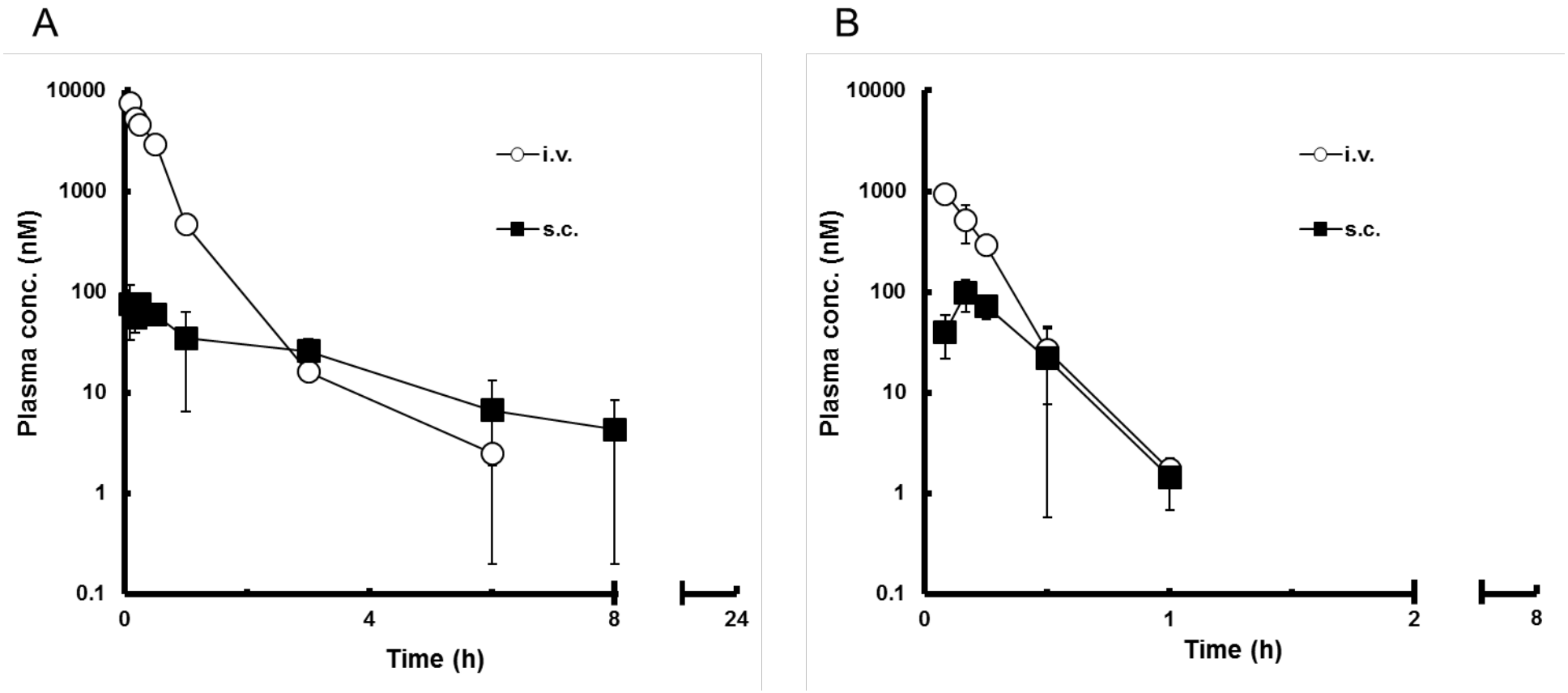


Figure S2. Plasma concentrations of **1** (A) and **18** (B) after intravenous and subcutaneous administration in mice. Peptides were administered intravenously or subcutaneously to C57BL/6J mice at 1 mg/kg. Blood samples were collected at 5, 10, 15, and 30 min, and at 1, 3, 6, 8, and 24 h after injection. Parameters were calculated from the mean (n = 3) plasma concentration-time profile.