

Supporting Information

Guanidine–Acylguanidine Bioisosteric Approach in the Design of Radioligands: Synthesis of a Tritium-Labeled N^G -Propionylargininamide ($[^3\text{H}]\text{-UR-MK114}$) as a Highly Potent and Selective Neuropeptide Y Y_1 Receptor Antagonist

Max Keller,[†] Nathalie Pop,[†] Christoph Hutzler,[†] Annette G. Beck-Sickinger,[‡] Günther Bernhardt,[†] Armin Buschauer^{†*}

[†] Dept. Pharmaceutical/Medicinal Chemistry II, Faculty of Chemistry and Pharmacy, University of Regensburg, Universitätsstr. 31, D-93053 Regensburg, Germany; [‡] Institute of Biochemistry, University of Leipzig, Brüderstr. 34, D-04103 Leipzig, Germany

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1 Experimental details and analytical data for compounds 1-7, 8a and 9-13

General. Chemicals and solvents were purchased from commercial suppliers (Merck, Darmstadt, Germany; Sigma, München, Germany) and used without further purification unless otherwise stated. *D*-ornithine hydrochloride was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Petroleum ether (40-60 °C) was distilled before use. DMF was stored over 3 Å molecular sieves. Anhydrous reactions were run under an atmosphere of dry nitrogen or argon. Thin layer chromatography was performed on Merck silica gel 60 F₂₅₄ TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on an Avance 300 (¹H: 300 MHz) and an Avance 600 (¹H: 600 MHz, ¹³C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Elemental analysis (C, H, N) was performed in-house on a Heraeus Elementar Vario EL III. Mass spectrometry analysis (MS) was performed on a Finnigan ThermoQuest TSQ 7000 (ES-MS), a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS) and Finnigan MAT 95 (LSIMS, HRMS). Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected. Lyophilization was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Eurospher-100 C18, 250 × 32 mm, 5 µm; Knauer, Berlin, Germany) at a flow rate of 38 mL/min. Analytical HPLC analysis of non-labeled compounds was performed on a system from Thermo Separation Products composed of a SN400 controller, a P4000 pump, an AS3000 autosampler, a Spectra Focus UV-VIS detector and a RP-column (Eurospher-100 C18, 250 × 4.0 mm, 5 µm; Knauer, Berlin, Germany) at a flow rate of 0.8 mL/min. Tritiated compounds were chromatographically analysed and purified on a RP-column (Agilent Scalar C18, 250 × 4.6 mm, 5 µm) either with a Shimadzu system C-R4A (pump LC-6A, UV-VIS-spectrophotometric detector SPD-6AV, system controller SCL-6A, Chromatopac C-R4AX) or on a Waters system (two pumps 510, pump control module, 486 UV detector, Packard Radiomatic Flow-1 beta Series A-500 detector, flow rate of liquid scintillator (Quicksint 212; Zinsser Analytic, Frankfurt, Germany): 4 mL/min).

(R)-N^δ-Benzylloxycarbonylornithine (1).^{1, 2} Benzylloxycarbonyl chloride (45% in toluene, 48.5 mL, 130.46 mmol, 1.1 eq) was added to an ice-cold solution of *D*-ornithine hydrochloride (20 g, 118.6 mmol, 1 eq), potassium carbonate (16.4 g, 118.6 mmol, 1 eq) and copper(II)sulfate pentahydrate (14.8 g, 59.3 mmol, 0.5 eq) in aqueous NaOH (0.5 N, 237 mL, 118.6 mmol, 1 eq) over a period of 15 min. The ice-bath was removed and the reaction was left under stirring at rt overnight. The resulting solid was filtered off, washed twice with ice-cold water (50 mL), ice-cold MeOH (50 mL) and water (50 mL). Lyophilization yielded a blue-violet powder which was suspended together with Tritriplex III (22.1 g, 59.3 mmol, 0.5 eq) in water (200 mL) under vigorous stirring and heated to 100 °C for 60 min. After cooling to 4 °C the solid was separated, washed with ice-cold water (3 × 40 mL) and dried (white solid, 26.8 g, 83%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.35-1.61 (m, 3H, CH-CH₂-CH₂), 1.69 (m, 1H, CH-CH₂-CH₂), 2.97 (m, 2H, CH₂-NH), 3.09 (m, 1H, CH^α), 5.0 (s, 2H, CH₂-Ph), 7.3-7.4 (m, 5H, Ph); MS (ESI, H₂O/MeOH + 10 mM NH₄OAc): *m/z* 267 [M + H]⁺; C₁₃H₁₈N₂O₄ (266.3)

(R)-N^δ-Benzylloxycarbonyl-N^α-(9-fluorenylmethoxycarbonyl)ornithine (2).³ A solution of 9-fluorenylmethyl succinimidyl carbonate (FmocOSu) (26.35 g, 78.1 mmol, 1 eq) in 1,4-dioxane (200 mL) was added to a solution of **1** (20.8 g, 78.1 mmol, 1 eq) and sodium carbonate (8.3 g, 78.1 mmol, 1 eq) in a mixture of water and 1,4-dioxane (2.5/1 v/v, 350 mL) over a period of 60 min and the mixture was stirred at rt overnight. The volume was reduced to 50% *in vacuo*, the product was precipitated by the addition of 1 M aq. hydrochloric acid (160 mL) and filtered off. Washing with hydrochloric acid (0.1%, 50 mL) and ice-cold water (50 mL) afforded the product as a white solid (36.6 g, 75 mmol, 96%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.4-1.68 (m, 3H, CH-CH₂-CH₂), 1.73 (m, 1H, CH-CH₂-CH₂), 3.0 (m, 2H, CH₂-NH), 3.91 (m, 1H, CH^α), 4.17-4.31 (m, 3H, CH-CH₂ from Fmoc), 5.01 (s,

2H, $\text{CH}_2\text{-Ph}$), 7.26-7.45 (m, 10H, Ph, Fmoc, $\text{CH}_2\text{-NH}$), 7.60 (d, 1H, $^3\text{J} = 7.95$ Hz, CO-NH-CH), 7.73 (d, 2H, $^3\text{J} = 7.37$ Hz, Fmoc), 7.89 (d, 2H, $^3\text{J} = 7.45$ Hz, Fmoc), 12.76 (bs, 1H, COOH); MS (ESI, $\text{H}_2\text{O}/\text{MeOH} + 10$ mM NH_4OAc): m/z 506 [M + NH_4]⁺, 489 [M + H]⁺; $\text{C}_{28}\text{H}_{28}\text{N}_2\text{O}_6$ (488.5)

(R)- $N^{\delta}\text{-Benzylloxycarbonyl-N-(4-tert-butoxybenzyl)-N}^{\alpha}\text{-(9-fluorenylmethoxycarbonyl)ornithinamide (3).}$ The carboxylic acid **2** (37 g, 75.7 mmol, 1 eq) was activated with carbonyldiimidazole (15.4 g, 94.7 mmol, 1.3 eq) in THF (650 mL) for 60 min followed by the addition of **9** (13.6 g, 75.7 mmol, 1 eq) in THF (100 mL). Stirring was continued under argon at rt for 20 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (1000 mL) under warming. The solution was washed with 0.1 M aq. KHSO_4 /brine 8/1 (180 mL), water/brine 8/1 (180 mL), 5% aq. Na_2CO_3 /brine 15/1 (160 mL), water/brine 3/1 (200 mL) and brine (150 mL). The solvent was removed under reduced pressure after drying over Na_2SO_4 and filtration. Subsequent purification with column chromatography (eluent: EtOAc/PE 1/1 to EtOAc) afforded the product as a white solid (30.6 g, 47.2 mmol, 62%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.24 (s, 9H, *t*-Bu), 1.34-1.74 (m, 4H, $\text{CH-CH}_2\text{-CH}_2$), 3.0 (m, 2H, $\text{CH}_2\text{-NH}$), 4.05 (m, 1H, CH^{α}), 4.17-4.32 (m, 5H, CH-CH_2 from Fmoc, $\text{CH}_2\text{-Ar-O-t-Bu}$), 5.01 (s, 2H, $\text{CH}_2\text{-Ph}$), 6.88 (d, 2H, $^3\text{J} = 8.30$ Hz, AA'BB'), 7.14 (d, 2H, $^3\text{J} = 8.42$ Hz, AA'BB'), 7.26-7.45 (m, 10H, Ph, Fmoc, $\text{CH}_2\text{-CH}_2\text{-NH}$), 7.56 (d, 1H, $^3\text{J} = 8.18$ Hz, CO-NH-CH), 7.74 (d, 2H, $^3\text{J} = 7.24$ Hz, Fmoc), 7.89 (d, 2H, $^3\text{J} = 7.45$ Hz, Fmoc), 8.36 (t, 1H, $^3\text{J} = 5.66$, CH-CO-NH-CH_2); MS (ESI, $\text{CH}_2\text{Cl}_2/\text{MeOH} + 10$ mM NH_4OAc): m/z 650 [M + H]⁺, 667 [M + NH_4]⁺; $\text{C}_{39}\text{H}_{43}\text{N}_3\text{O}_6$ (649.8)

(R)- $N^{\delta}\text{-Benzylloxycarbonyl-N-(4-tert-butoxybenzyl)ornithinamide (4).}$ A solution of **3** (30.6 g, 47.1 mmol,) in CH_2Cl_2 (1200 mL), DMF (100 mL) and diethylamine (150 mL) was stirred at rt for 4 h. The volatiles were removed under reduced pressure and the residue was taken up in CH_2Cl_2 (400 mL). The solution was filtered from a quite small amount of insoluble material and the filtrate was subjected to column chromatography (eluent: EtOAc to EtOAc/MeOH 5/1). The product was obtained as a yellow oil (15.3 g, 35.8 mmol, 76%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.26 (s, 9H, *t*-Bu), 1.3-1.63 (m, 4H, $\text{CH-CH}_2\text{-CH}_2$), 2.98 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 3.15 (m, 1H, CH^{α}), 4.23 (d, 2H, $^3\text{J} = 5.87$ Hz, $\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 5.01 (s, 2H, $\text{CH}_2\text{-Ph}$), 6.9 (d, 2H, $^3\text{J} = 8.45$ Hz, AA'BB'), 7.02 (bs, 2H, NH_2), 7.15 (d, 2H, $^3\text{J} = 8.47$ Hz, AA'BB'), 7.25-7.4 (m, 5H, Ph), 7.64 (t, 1H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 8.26 (t, 1H, $^3\text{J} = 5.93$, CH-CO-NH-CH_2); MS (ESI, $\text{CH}_2\text{Cl}_2/\text{MeOH} + 10$ mM NH_4OAc): m/z 428 [M + H]⁺, 855 [2M + H]⁺; $\text{C}_{24}\text{H}_{33}\text{N}_3\text{O}_4$ (427.5)

(R)- $N^{\delta}\text{-Benzylloxycarbonyl-N-(4-tert-butoxybenzyl)-N}^{\alpha}\text{-(2,2-diphenylacetyl)ornithinamide (5).}$ A solution of **10** (14.4 g, 46.5 mmol, 1.3 eq) in 1,2-dimethoxyethane (100 mL) and THF (40 mL) was added to a solution of **4** (15.3 g, 35.8 mmol, 1 eq) and NEt_3 (3.6 g, 35.8 mmol, 1 eq) in 1,2-dimethoxyethane (130 mL). The reaction mixture was concentrated under reduced pressure to give a volume of about 80 mL after stirring at 35 °C for 20 h. Water was added (220 mL) and the pH was adjusted to 2.5-3 with KHSO_4 . The precipitate was separated by filtration, washed with water (60 mL), dried *in vacuo*, dissolved in $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ 3/1 (150 mL) and purified by column chromatography (eluent: $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ 3/1 to EtOAc) affording **5** as a white solid (18.8 g, 30.3 mmol, 84%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.38 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CH}_2$), 1.5-1.76 (m, 2H, CH-CH_2), 2.98 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 4.24 (m, 2H, $\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 4.34 (m, 1H, CH^{α}), 5.02 (s, 2H, $\text{CH}_2\text{-Ph}$), 5.08 (s, 1H, CH-(Ph)_2), 6.87, (d, 2H, $^3\text{J} = 8.45$ Hz, AA'BB'), 7.12 (d, 2H, $^3\text{J} = 8.48$ Hz, AA'BB'), 7.1-7.4 (m, 15H, Ph), 8.43 (t, 1H, $^3\text{J} = 5.78$, CH-CO-NH-CH_2), 8.49 (d, 1H, $^3\text{J} = 8.03$, CO-NH-CH); MS (ESI, $\text{CH}_2\text{Cl}_2/\text{MeOH} + 10$ mM NH_4OAc): m/z 622 [M + H]⁺, 639 [M + NH_4]⁺, 660 [M + K]⁺; $\text{C}_{38}\text{H}_{43}\text{N}_3\text{O}_5$ (621.8)

(R)- $N\text{-(4-tert-Butoxybenzyl)-N}^{\alpha}\text{-(2,2-diphenylacetyl)ornithinamide (6).}$ ⁴ To a suspension of **5** (18.5 g, 29.75 mmol, 1 eq) in MeOH (400 mL) were added ammonium formate (22.5 g, 357 mmol, 12 eq) and 10% Pd/C catalyst (2.7 g). The evolution of gas was monitored with a bubble counter. Compound **5** had

been completely dissolved after 4 h. The reaction mixture was left under stirring at rt overnight. The catalyst was removed by filtration, the filtrate was concentrated under reduced pressure and the residue was taken up in CHCl_3 (600 mL). The organic solution was treated with saturated aq. Na_2CO_3 (150 mL, 100 mL) and brine (100 mL), then dried over Na_2SO_4 and filtered. Removal of the solvent yielded an oil which turned to a hardened foam during drying *in vacuo* (12.3 g, 25.2 mmol, 85%). $^1\text{H-NMR}$ (300 MHz, DMSO-d₆): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.21-1.36 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CH}_2$), 1.48-1.77 (m, 2H, $\text{CH}\text{-CH}_2$), 3.29 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-NH}_2$), 4.21 (m, 2H, $\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 4.3 (m, 1H, CH^α), 5.12 (s, 1H, $\text{CH}\text{-}(\text{Ph})_2$), 6.86 (d, 2H, $^3\text{J} = 8.49$ Hz, AA'BB'), 7.09 (d, 2H, $^3\text{J} = 8.48$ Hz, AA'BB'), 7.16-7.34 (m, 10H, Ph), 8.47 (m, 1H, CO-NH-CH₂), 8.55 (d, 1H, $^3\text{J} = 8.03$, CO-NH-CH); MS (ESI, $\text{CH}_2\text{Cl}_2\text{/MeOH} + 10$ mM NH_4OAc): *m/z* 488 [M + H]⁺; $\text{C}_{30}\text{H}_{37}\text{N}_3\text{O}_3$ (487.6)

(R)-*N*-(4-*tert*-Butoxybenzyl)-*N*^ω-*tert*-butoxycarbonyl-*N*^α-(2,2-diphenylacetyl)argininamide (7). Compound **6** (3.5 g, 7.18 mmol, 1 eq) and *N*-*tert*-butoxycarbonyl-*N*^ω-benzyloxycarbonyl-1*H*-pyrazol-1-carboxamidine (**13**) (2.97 g, 8.6 mmol, 1.2 eq) were dissolved in CH_2Cl_2 (50 mL) and NEt_3 (0.36 g, 0.5 mL, 3.59 mmol, 0.5 eq) was added. The reaction mixture was stirred at rt overnight and then subjected to column chromatography (eluent: $\text{CH}_2\text{Cl}_2\text{/EtOAc}$ 20/1 to 3/1, column was packed with 0.5% NEt_3). The eluate was evaporated to dryness *in vacuo* yielding a hardened foam which was dissolved in MeOH (HPLC grade, 65 mL) in a two-necked round bottom flask equipped with a three-way valve (N_2 , H_2). A 10% palladium-on-charcoal catalyst (0.4 g) was added and a slow stream of hydrogen was led through a glass tube into the vigorously stirred suspension. After depletion of the starting material (3.5 h; control by TLC) the catalyst was removed using a PTFE-filter (25 mm, 0.2 μm). The filtrate was concentrated to give an oil and water (50 mL) was added. Lyophilization afforded the product as a white solid (3.83 g, 6.08 mmol, 84%). mp > 85 °C (decomp.); $^1\text{H-NMR}$ (600 MHz, DMSO-d₆, COSY, HSQC): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.36 (s, 9H, *t*-Bu), 1.4 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-CH}_2$), 1.55 (m, 1H, $\text{CH}\text{-CH}_2$), 1.68 (m, 1H, $\text{CH}\text{-CH}_2$), 3.06 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 4.21 (dd, 1H, $^2\text{J} = 15.28$ Hz, $^3\text{J} = 5.96$ Hz, $\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 4.25 (dd, 1H, $^2\text{J} = 15.28$ Hz, $^3\text{J} = 5.96$ Hz, $\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 4.34 (m, 1H, CH^α), 5.13 (s, 1H, $\text{CH}\text{-}(\text{Ph})_2$), 6.87 (d, 2H, $^3\text{J} = 8.48$ Hz, AA'BB'), 7.1 (d, 2H, $^3\text{J} = 8.49$ Hz, AA'BB'), 7.2 (m, 2H, Ph), 7.25-7.32 (m, 8H, Ph), 8.43 (t, 1H, $^3\text{J} = 5.65$ Hz, CO-NH-CH₂), 8.49 (d, 1H, $^3\text{J} = 8.03$, CO-NH-CH); $^{13}\text{C-NMR}$ (150 MHz, DMSO-d₆, HSQC, HMBC): δ (ppm) 25.4 ($\text{CH}_2\text{-CH}_2\text{-CH}_2$), 28.3 ($\text{C-(CH}_3)_3$), 28.5 ($\text{C-(CH}_3)_3$), 29.6 (CH-CH_2), 39.6 ($\text{CH}_2\text{-CH}_2\text{-NH}$), 41.5 ($\text{CH}_2\text{-C}_6\text{H}_4\text{-O-t-Bu}$), 52.5 (CH^α), 55.9 ($\text{CH-}(\text{Ph})_2$), 75.9 ($\text{C-(CH}_3)_3$), 77.7 ($\text{C-(CH}_3)_3$), 123.5 (aryl, AA'BB'), 126.48 (Ph), 126.52 (Ph), 127.6 (Ar AA'BB'), 128.1 (Ph), 128.2 (Ph), 128.4 (Ph), 128.6 (Ph), 133.7 ($\text{CH}_2\text{-CC}_4\text{H}_4\text{C-O-t-Bu}$), 140.3 ($\text{CH-(CC}_5\text{H}_5)_2$), 140.5 ($\text{CH-(CC}_5\text{H}_5)_2$), 153.8 ($\text{CH}_2\text{-CC}_4\text{H}_4\text{C-O-t-Bu}$), 161.1 (CO-O-t-Bu), 163.1 (guanidine), 170.9 (CO-NH-CH), 171.3 (CO-NH-CH_2); Anal. ($\text{C}_{36}\text{H}_{47}\text{N}_5\text{O}_5$) calcd. C 68.66, H 7.52, N 11.12, found C 68.41, H 7.74, N 11.05; HRMS: (FAB⁺, MeOH/Glycerin): *m/z* calcd. for $[\text{C}_{36}\text{H}_{47}\text{N}_5\text{O}_5 + \text{H}]^+$ 630.3655, found: 630.3646; $\text{C}_{36}\text{H}_{47}\text{N}_5\text{O}_5$ (629.8)

(R)-*N*^α-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*^ω-propanoylargininamide (8a). Compounds **7** (100 mg, 159 μmol , 1 eq) and **11a** (32.5 mg, 191 μmol , 1.2 eq) were dissolved in anhydrous acetonitrile (4 mL), NEt_3 (8 mg, 11 μL , 79 mmol, 0.5 eq) was added and the reaction mixture was stirred under argon at rt overnight. TFA (2 mL) and two drops of water were added and stirring was continued for 4 h. CH_2Cl_2 (20 mL) was added and the solution was concentrated under reduced pressure. Addition of CH_2Cl_2 (30 mL) and evaporation under reduced pressure was repeated twice. The residue (yellow oil) was taken up in a mixture of acetonitrile and 0.1% aq. TFA (1/1 v/v, 5 mL), filtered (0.2 μm) and injected into the preparative HPLC system (6 injections). Eluent: mixtures of acetonitrile (A) and 0.1% aq. TFA (B). Gradient: A/B 20/80 to 50/50 in 30 min. Acetonitrile was removed under reduced pressure from the eluate containing the product ($t_R \approx 24$ min). After lyophilisation of the remaining aqueous solution the product was obtained as TFA salt as a white fluffy solid (71.6 mg, 111 μmol , 70%).

HPLC purity (210 nm): > 99% (chromatogram: Figure 7, supporting information); mp 166-168 °C; $^1\text{H-NMR}$ (600 MHz, DMSO-d₆, $^1\text{H-COSY}$, HSQC): δ (ppm) 1.04 (t, 3H, CH_3 , $^3\text{J} = 7.42$ Hz), 1.39-1.48

(m, 2H, $\text{CH}_2\text{-CH}_2\text{-CH}_2$), 1.54 (m, 1H, $\text{CH}\text{-CH}_2$), 1.67 (m, 1H, $\text{CH}\text{-CH}_2$), 2.43 (q, 2H, $\text{CH}_2\text{-CH}_3$, $^3\text{J} = 7.40$ Hz), 3.22 (m, 2H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 4.12 (dd, 1H, $^2\text{J} = 14.82$ Hz, $^3\text{J} = 5.78$ Hz, $\text{CH}_2\text{-C}_6\text{H}_4\text{-OH}$), 4.16 (dd, 1H, $^2\text{J} = 14.81$ Hz, $^3\text{J} = 5.85$ Hz, $\text{CH}_2\text{-C}_6\text{H}_4\text{-OH}$), 4.33 (m, 1H, CH^α), 5.12 (s, 1H, $\text{CH}(\text{Ph})_2$), 6.6 (d, 2H, $^3\text{J} = 8.49$ Hz, AA'BB'), 6.99 (d, 2H, $^3\text{J} = 8.49$ Hz, AA'BB'), 7.21 (m, 2H, Ph), 7.26-7.30 (m, 8H, Ph), 8.34 (t, 1H, $^3\text{J} = 5.81$ Hz, CO-NH-CH₂), 8.46 (d, 1H, $^3\text{J} = 8.10$, CO-NH-CH), 8.67 (bs, 2H, NH₂), 9.16 (t, 1H, $\text{CH}_2\text{-CH}_2\text{-NH}$), 9.28 (s, 1H, NH-CO-CH₂-CH₃), 11.7 (s, 1H, ArOH); ^{13}C -NMR (150 MHz, DMSO-d₆, HSQC, HMBC): δ (ppm) 8.2 (CH_3), 24.3 ($\text{CH}_2\text{-CH}_2\text{-CH}_2$), 29.3 ($\text{CH}\text{-CH}_2$), 29.5 ($\text{CH}_2\text{-CH}_3$), 40.4 ($\text{CH}_2\text{-CH}_2\text{-NH}$), 41.6 ($\text{CH}_2\text{-C}_6\text{H}_4\text{-OH}$), 52.3 (CH^α), 55.9 ($\text{CH}(\text{Ph})_2$), 115.0 (Ar AA'BB'), 126.5 (Ph), 126.6 (Ph), 128.1 (Ph), 128.2 (Ph), 128.4 (Ar AA'BB'), 128.46 (Ph), 128.48 (Ph), 129.1 ($\text{CH}_2\text{-CC}_4\text{H}_4\text{C-OH}$), 140.3 (CH-(CC₅H₅)₂), 140.5 (CH-(CC₅H₅)₂), 152.9 (guanidine), 156.2 (CH₂-CC₄H₄C-OH), 170.9 (CO-NH), 171.0 (CO-NH), 175.9 (COCH₂CH₃); HRMS: (FAB⁺, MeOH/glycerol): *m/z* calcd. for [C₃₀H₃₅N₅O₄ + H]⁺ 530.2767, found: 530.2774; C₃₀H₃₅N₅O₄ × TFA (643.6)

4-*tert*-Butoxybenzylamine (9).⁵ A solution of 4-*tert*-butoxybenzonitrile (50 g, 285.4 mmol, 1 eq) in anhydrous diethyl ether (200 mL) was dropped to a mechanically stirred suspension of LiAlH₄ (25 g, 658.8 mmol, 2.3 eq) in anhydrous diethyl ether (400 mL) over a period of 3 h. The reaction mixture was refluxed for additional 60 min, cooled to 0 °C and carefully quenched by the dropwise addition of water (20 mL), 15% aq. NaOH (17 mL) and again water (50 mL). Inorganic material was filtered off and washed twice with diethyl ether. The combined filtrate and washings were concentrated to about 150 mL followed by the addition of water (850 mL) and 1 M aq. KHSO₄ (about 300 mL) to adjust the pH value to 2-3. The aqueous phase was separated, washed with diethyl ether (180 mL) and alkalified with 2 N aq. NaOH (200 mL). The product was extracted with CH₂Cl₂ (three times 450 mL). The extracts were pooled, washed with brine (300 mL) and dried over Na₂SO₄. After filtration and removal of the solvent in high vacuo the product was obtained as a yellowish oil (50.1 g, 299.2 mmol, 98%). ^1H -NMR (300 MHz, CDCl₃): δ (ppm) 1.31 (s, 9H, *t*-Bu), 2.03 (s, 2H, NH₂), 3.8 (s, 2H, CH₂), 6.93 (d, 2H, $^3\text{J} = 8.51$, AA'BB'), 7.18 (d, 2H, $^3\text{J} = 8.64$, AA'BB'); MS (EI): *m/z* 122.0 [M - C(CH₃)₃]⁺, 123.0 [M - C(CH₃)₃ + H]⁺, 164 [M - ·CH₃]⁺, 179 (M⁺); C₁₁H₁₇NO (179.3)

Succinimidyl 2,2-diphenylacetate (10).^{6,7} DCC (40.8 g, 197.9 mmol, 1.05 eq) was added to a stirred solution of diphenylacetic acid (40 g, 188.5 mmol, 1 eq) and *N*-hydroxysuccinimide (22.8 g, 197.9 mmol, 1.05 eq) in anhydrous THF (800 mL). After stirring at rt overnight the white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 2/1 to 1/2) yielded the product as a white semi-crystalline solid (54.8 g, 177.2 mmol, 94%). ^1H -NMR (300 MHz, CDCl₃): δ (ppm) 2.79 (s, 4H, CH₂), 5.36 (s, 1H, CH), 7.26-7.4 (m, 10H, Ph); MS (CI, NH₃): *m/z* 327 [M + NH₄]⁺; C₁₈H₁₅NO₄ (309.3)

Succinimidyl propanoate (11a).⁸ DCC (3 g, 14.85 mmol, 1.1 eq) was added to a stirred ice-cold solution of propanoic acid (1 g, 1 mL, 13.5 mmol, 1 eq) and *N*-hydroxysuccinimide (1.71 g, 14.85 mmol, 1.1 eq) in anhydrous THF (30 mL). The ice-bath was removed after 2 h and stirring was continued at rt overnight. The white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 3/1 to 1/1) yielded the product as a white solid (1.4 g, 8.2 mmol, 60%). ^1H -NMR (300 MHz, CDCl₃): δ (ppm) 1.27 (t, 3H, $^3\text{J} = 7.51$, CH₃), 2.63 (q, 2H, $^3\text{J} = 7.52$, CH₂-CH₃), 2.82 (s, 4H, CH₂-CH₂); MS (CI, NH₃): *m/z* 189 [M + NH₄]⁺; C₇H₉NO₄ (171.2)

N-Benzylloxycarbonyl-1*H*-pyrazole-1-carboxamidine (12).^{4,9} Concentrated hydrochloric acid (76 mL) was added dropwise to a stirred suspension of aminoguanidine carbonate (80 g, 587.8 mmol, 1.05 eq) in water (120 mL) resulting in a clear solution of aminoguanidine hydrochloride which was added to a gently warmed (45 °C) suspension of 1,1,3,3-tetramethoxypropane (91.9 g, 559.8 mmol, 1 eq) in water (120 mL) over a period of 60 min. The obtained darkly colored solution was stirred for additional 30

min. Since crystallization of *1H*-pyrazole-1-carboxamidine hydrochloride could not be initiated by the treatment with acetone (as described^{4, 9}) or acetonitrile, all solvents were removed by evaporation under reduced pressure and drying *in vacuo*. The darkly colored residue (only soluble in water under warming) was suspended in MeOH/CH₂Cl₂ 1/1 (1000 mL) and NEt₃ (178 mg, 244 μ L, 1763 mmol, 3 eq) was added. The mixture was cooled to 0 °C, benzyloxycarbonyl chloride (45% in toluene, 559.8 mmol, 218.5 mL, 1.0 eq) was added over a period of 2.5 h and the reaction was left under stirring at rt overnight. All volatiles were removed under reduced pressure. To the residue ethyl acetate (1300 mL) and water (400 mL) were added. The two phases were mixed vigorously and then separated. The darkly colored aqueous phase was treated with ethyl acetate (200 mL), the organic layers were combined and washed with saturated aqueous NaHCO₃ solution (300 mL), water (300 mL) and brine (300 mL). After drying over Na₂SO₄ and filtration the solvent was removed *in vacuo* and the product was roughly purified by column chromatography (eluent: CH₂Cl₂/EA 3/1). The product was crystallized from CH₂Cl₂ (35.8 g, 146.7 mmol, 25%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 5.13 (s, 2H, CH₂-Ph), 6.58 (dd, 1H, ³J = 1.61 Hz, 2.76 Hz, pyrazole), 7.32-7.42 (m, 5H, Ph), 7.92 (dd, 1H, ³J = 1.52 Hz, ⁴J = 0.60 Hz, pyrazole), 8.45 (dd, 1H, ³J = 2.75 Hz, ⁴J = 0.48 Hz, pyrazole), 8.97 (bs, 2H, NH₂); MS (CI, NH₃): *m/z* 245 [M + H]⁺; C₁₂H₁₂N₄O₂ (244.2)

***N*-Benzylloxycarbonyl-*N'*-*tert*-butoxycarbonyl-*1H*-pyrazole-1-carboxamidine (13).**^{10, 11} A solution of **12** (35.5 g, 145.3 mmol, 1 eq) and di-*tert*-butyl dicarbonate (76 g, 348 mmol, 2.4 eq) in chloroform (150 mL) was treated with DMAP (3.6 g, 29.1 mmol, 0.2 eq) and stirred overnight. Chloroform (250 mL) was added and the reaction mixture was washed with 0.5 M aq. KHSO₄ (100 mL), water (100 mL) and brine (100 mL). The solution was dried over Na₂SO₄, filtered, and the volume was reduced to about 100 mL under reduced pressure. The residue was subjected to column chromatography (eluent: CH₂Cl₂/EtOAc 20/1 to 5/1) to isolate the twofold Boc-protected intermediate as a yellow oil which was treated with Mg(ClO₄)₂ (6.2 g, 27.8 mmol, 0.2 eq) in THF (500 mL) at 55 °C for 3 h. The solvent was removed *in vacuo* and the residue was dissolved in CH₂Cl₂ (1000 mL). The organic solution was washed with water (200 mL) and brine (200 mL), then dried over Na₂SO₄ and filtered. The filtrate was concentrated. Purification with column chromatography (eluent: PE/EtOAc 5/1 to 1/1, column packed with 0.5% NEt₃) afforded the product as a highly viscous yellow oil (26.7 g, 77.5 mmol, 53%). ¹H-NMR (300 MHz, DMSO-d₆): δ (ppm) 1.44 (s, 9H, *t*-Bu) 5.14 (s, 2H, CH₂-Ph), 6.60 (dd, 1H, ³J = 1.61 Hz, 2.76 Hz, pyrazole), 7.32-7.44 (m, 5H, Ph), 7.88 (dd, 1H, ³J = 1.58 Hz, ⁴J = 0.53 Hz, pyrazole), 8.37 (dd, 1H, ³J = 2.79 Hz, ⁴J = 0.53 Hz, pyrazole); MS (CI, NH₃): *m/z* 345 [M + H]⁺, 245 [M - Boc + H]⁺; C₁₇H₂₀N₄O₄ (344.4)

2 Purity control of **8a**

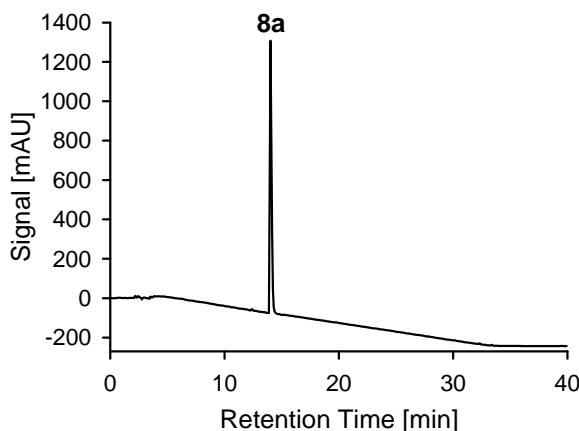


Figure 7. HPLC purity control of **8a**. Eluent: mixtures of acetonitrile (A) and 0.05% aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, detection: 210 nm, injection: 100 μ L of a 50 μ M solution of **8a** in a mixture of acetonitrile and 0.05% aq. TFA (20/80) prepared from a 5 mM solution in acetonitrile, t_R = 14.0 min, k = 4.2.

3 Detailed experimental protocol for the preparation of the radioligand **8b**

(*R*)-*N*^a-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*^a-(2,3-³H]-propanoyl)argininamide (**8b**).

(1) Synthesis of **8b** from 185 MBq of succinimidyl [2,3-³H]-propanoate (**11b**) (a_s = 3.48 TBq/mmol, 185 MBq in 5 mL of toluene, GE Healthcare, München, Germany, formerly Amersham Biosciences).

Compound **7** (1.34 mg, 2.13 μ mol, 40 eq) was dissolved in anhydrous acetonitrile (30 μ L) yielding a concentration of 70.9 mM, and NEt₃ was diluted in acetonitrile to a concentration of 0.55 μ L NEt₃/22.5 μ L. Compound **7** (70.9 mM in acetonitrile, 15 μ L, 1.064 μ mol, 20 eq) and NEt₃ (7.5 μ L of the above described solution in acetonitrile, 1.33 μ mol, 25 eq) were added to **11b** (9.3 μ g, 0.053 μ mol, 1 eq) in toluene (5 mL), and the solvent was removed with a slight stream of nitrogen (blown through a PTFE filter, 13 mm, 0.2 μ m, nylon) over a period of 2 h. The 5-mL glass vial was rinsed three times with acetonitrile (100 μ L each), the washings were combined in a 1.5-mL Eppendorf reaction vessel (screw top) and **7** (70.9 mM in acetonitrile, 15 μ L, 1.064 μ mol, 20 eq) as well as NEt₃ (15 μ L of the above described solution in acetonitrile, 2.66 μ mol, 50 eq) were added. The reaction mixture was concentrated to about 80 μ L in a vacuum concentrator and stirred with a magnetic micro stirrer at 35 °C overnight. TFA (100 μ L) was added and the reaction mixture was allowed to stand under stirring at 60 °C for 2 h. MeOH (400 μ L) was added and the volume was reduced to about 20 μ L in a vacuum concentrator (at 40 °C). The residue was diluted with a mixture of acetonitrile and 0.05% aq. TFA (25/75 v/v, 300 μ L) and the product was isolated with a Shimadzu HPLC system (4 injections). Eluent: mixtures of acetonitrile plus 0.04% TFA (A) and 0.05% aq. TFA (B), gradient: 0 to 22 min: A/B 25/75 to 53/47, 22 to 24 min: 53/47 to 95/5, 24 to 29 min: 95/5. The product was eluted at 18.9 min and collected in a 1.5-mL Eppendorf reaction vessel (screw top), which was put into the vacuum concentrator between the injections. The combined product fractions were evaporated to dryness in a vacuum concentrator (about 5 h), the residue was dissolved in ethanol (500 μ L), transferred into the clean 5-mL Amersham glass vial and combined with the washings (160 μ L and 140 μ L).

Quantification: A five point calibration was performed with **8a** (0.5, 0.75, 1.0, 1.25 and 1.5 μ M, inj. vol.: 100 μ L). Eluent: mixtures of acetonitrile plus 0.04% TFA (A) and 0.05% aq. TFA (B), gradient: 0 to 17 min: A/B 20/80 to 50/50, 17 to 19 min: 50/50 to 90/10, 19 to 24 min: 90/10 (t_R = 18.7 min). The solutions for injection were prepared in a mixture of acetonitrile and 0.05% aq. TFA (20/80) less than 3 min prior to the injection. All standard solutions were prepared separately from a 100 μ M solution of **8a**.

in a mixture of acetonitrile and 0.05% aq. TFA (20/80), which was freshly prepared for each standard from a 10 mM solution of **8a** in DMSO. 6.6 μ L of the ethanolic solution (total volume: 800 μ L) were diluted with 103.4 μ L of a mixture of acetonitrile and 0.05% aq. TFA (20/80) in duplicate and 100 μ L of each solution were analyzed by HPLC. The molarity of the ethanolic solution of **8b** was calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. Yield: 9.6 μ g, 0.018 μ mol, 34%.

Determination of the specific activity: four aliquots (1 μ L) of the ethanolic solution were diluted with 499 μ L of acetonitrile and 10 μ L of the 1:500-dilutions were counted in 3 mL of Rothiszint eco plus (Roth, Karlsruhe, Germany) in a LS 6500 liquid scintillation counter (Beckmann-Coulter, München; Germany). Calculated specific activity: 3.6 TBq/mmol (97.3 Ci/mmol). The activity concentration was adjusted to 18.5 MBq/mL (0.5 mCi/mL) by the addition of ethanol (3311 μ L) with a TFA additive (100 μ M) to the remaining solution yielding a concentration of 4.4 μ M. The radioligand **8b** was stored at -20 °C.

(2) Synthesis of **8b** from 74 MBq of succinimidyl [2,3-³H]-propanoate (**11b**) (a_s = 2.22 TBq/mmol, 185 MBq in 1 mL of ethyl acetate, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA).

Compound **7** (0.84 mg, 1.33 μ mol, 40 eq) was dissolved in anhydrous acetonitrile (60 μ L) yielding a concentration of 22.2 mM, and NEt₃ was diluted in acetonitrile to a concentration of 0.18 μ L NEt₃ / 10 μ L. Compound **7** (22.2 mM in acetonitrile, 20 μ L, 0.44 μ mol, 13.3 eq) was added to **11b** (5.84 μ g, 0.033 μ mol, 1 eq, 74 MBq, specific activity: 2.22 TBq/mmol) in ethyl acetate (0.4 mL) in a 1.5-mL Eppendorf reaction vessel (screw top). The solvent was removed in a vacuum concentrator (40 °C) over a period of 50 min. **7** (22.2 mM in acetonitrile, 40 μ L, 0.89 μ mol, 26.7 eq) and NEt₃ (10 μ L of the above described solution in acetonitrile, 1.33 μ mol, 40 eq) were added. The reaction mixture was vigorously blended (vortexer) for 1 min, briefly centrifuged and stirred with a magnetic micro stirrer at rt overnight. For deprotection TFA (60 μ L) was added and stirring was continued for 3 h at 40 °C. For HPLC analysis 2 μ L of the “deprotection mixture” were diluted (1:20) with 38 μ L of acetonitrile/0.05% aq. TFA (50/50), and 2 μ L of this solution were diluted (1:50) with 98 μ L of acetonitrile/0.05% aq. TFA (20/80). This solution was completely injected into the Waters HPLC-system (Figure 8). The residual “deprotection mixture” (\approx 98 μ L) was diluted with acetonitrile/0.05% aq. TFA (10/90, 260 μ L) and combined with the remaining 1:20-diluted “deprotection mixture” (\approx 38 μ L). The product was isolated with a Waters HPLC system (3 injections) using the conditions specified for the analysis of the “deprotection mixture”. For this purpose radiometric detection was not performed (Figure 8). The product was eluted at 25.4 to 26.1 min and collected in a 1.5-mL Eppendorf reaction vessel (screw top), which was put into the vacuum concentrator between the injections. The combined product fractions were evaporated to dryness in a vacuum concentrator (about 5 h), the residue was dissolved in 200 μ L of ethanol containing TFA (100 μ M) and transferred to a clean 5-mL Amersham glass vial together with the washings (3 \times 100 μ L).

Quantification: A five point calibration was performed with **8a** (0.3, 0.5, 0.75, 1.0 and 1.5 μ M, inj. vol.: 100 μ L). Eluent: mixtures of acetonitrile + 0.05% TFA (A) and 0.05% aq. TFA (B), gradient for the 1.5 μ M standard: 0 to 30 min: A/B 20/80 to 90/10, 30 to 38 min: 90/10 (retention time of **8a**: 16.7 min), gradient for the other standards: 0 to 12 min: A/B 30/70 to 50/50, 12 to 12.5 min: 50/50 to 90/10, 12.5 to 18 min: 90/10 (retention time of **8a**: 12.85 min). The solutions for injection were prepared in acetonitrile/ 0.05% aq. TFA (20/80) less than 3 min prior to the injection. All standard solutions were prepared from a 40 μ M solution of **8a** (in acetonitrile/0.05% aq. TFA (20/80)), which was freshly made for each standard solution from a 2 mM stock solution of **8a** in acetonitrile/water 80/20. Two aliquots (4.0 μ L) of the ethanolic solution (total volume: 500 μ L) of the product were diluted with 100 μ L of acetonitrile/0.05% aq. TFA (20/80), and 100 μ L were analyzed by HPLC. Whereas one sample was used for quantification of the product by UV detection the second sample was additionally monitored radiometrically to determine radiochemical purity (Figure 1). The molarity of the ethanolic solution of **8b** was calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. Yield: 5.86 μ g, 0.011 μ mol, 33%.

Determination of the specific activity: 1.5 μ L of the ethanolic solution were diluted with 448.5 μ L of a mixture of acetonitrile and water (65/35) in triplicate, and 9 μ L of the 1:300-dilutions were counted in Rothiszint™ eco plus (3 mL). Calculated specific activity: 1.9 TBq/mmol (51 Ci/mmol). The activity concentration was adjusted to 9.25 MBq/mL (0.25 mCi/mL) by addition of ethanol (1696.5 μ L) containing TFA (100 μ M) to the residual solution yielding a molarity of 4.9 μ M. The radioligand **8b** was stored at -20 °C.

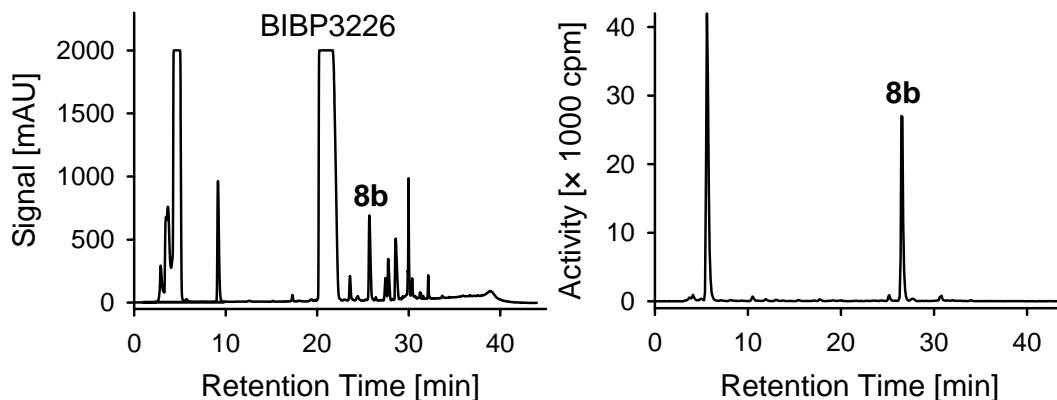


Figure 8. HPLC analysis of the deprotection mixture containing the non-purified radioligand **8b**. Eluent: mixtures of acetonitrile + 0.05% TFA (A) and 0.05% aq. TFA (B); gradient: 0 to 26 min: A/B 20/80 to 47/53, 26 to 27 min: 47/53 to 90/10, 27 to 35 min: 90/10. Left: UV detection (220 nm), $t_R = 25.8$ min, followed by radiometric detection (right panel): $t_R = 26.4$ min.

4 Pharmacology: experimental protocols

General. Unless otherwise noted, all chemicals (analytical grade) were obtained from commercial suppliers. Millipore water was used throughout. Dy-635-pNPY was synthesized as described previously.¹² $[K^4]$ -hPP was synthesized in the laboratory of Prof. Beck-Sickinger (University of Leipzig, Germany) and labeled with the cyanine dye Cy5 in our laboratory.¹³ Porcine NPY (pNPY) was prepared in-house in the laboratory of Dr. C. Cabrele. The fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in anhydrous DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to a concentration of 20%. The buffer for the Y₁R binding studies of **8b** on SK-N-MC cells, MCF-7 cells and tissue sections was prepared by the addition of BSA (1% for binding on cells, 0.2% for binding on tissue sections) and bacitracin (50 μ g · L⁻¹) to a buffer (pH 7.4) consisting of HEPES (10 mM), NaCl (150 mM), KCl (5 mM), CaCl₂·2H₂O (2.5 mM), KH₂PO₄ (1.2 mM), Mg₂SO₄·7H₂O (1.2 mM), and NaHCO₃ (25 mM). This HEPES buffer without the additives was used to wash the cells and tissue sections previous to and after radioligand binding. The loading buffer (pH 7.4) for the determination of intracellular Ca²⁺ mobilization in HEL cells was prepared by dissolving NaCl (120 mM), KCl (5 mM), MgCl₂·6H₂O (2 mM), CaCl₂·2H₂O (1.5 mM), HEPES (25 mM), and glucose (10 mM). The lysis solution for the radioligand binding assays consisted of urea (8 M), acetic acid (3 M) and Triton-X-100 (1%) in water. Stock solutions of **8a** (10 mM in DMSO, 2 mM in acetonitrile/water 80/20), BIBP3226 (10 mM in DMSO) and pNPY (1 mM in DMSO) were stored at -20 °C. Tritium counting was done in 3 mL of Rothiszint eco plus with a Beckman LS 6500 beta-counter. Animals were taken from the livestock breeding of the University of Regensburg.

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.^{14, 15} HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the fura Ca²⁺-assay. MCF-7 cells were maintained in MEM (Sigma), supplemented with 5% FCS (Biochrom AG, Berlin, Germany). The

CHO cells transfected with the Y₂R, G_{αq/15} and aequorin were cultured as previously described.¹² CHO cells transfected with the Y₄R, G_{αq/15} and aequorin were cultured under the same conditions. HEC-1B cells were cultured as previously described.¹⁶

Radioligand binding assay. The radioligand binding assay was essentially performed as described¹⁴. Cells were seeded in 24-well plates 2 or 3 days prior to the experiment. On the day of the experiment confluence of the cells was at least 70%. The culture medium was removed, the cells were washed once with buffer (500 µL), and covered with binding buffer (200 µL). Binding buffer (25 µL) and binding buffer (25 µL) with **8b** (10-fold concentrated) was added for total binding. For non-specific binding and displacement of **8b** binding buffer (25 µL) with the competing agent (pNPY, **14** (BIBP 3226) or **8a**, 10-fold concentrated) and binding buffer (25 µL) with **8b** (10-fold concentrated) was added. During incubation at room temperature (22 – 25 °C) the plates were gently shaken. After incubation (saturation experiments and competition with **14** or **8a**: 20 min; competition with pNPY: 2 h) the binding buffer was removed, the cells were washed twice with buffer (500 µL, 4 °C, ≤ 30 s), covered with lysis solution (200 µL) and the plates were gently shaken for at least 30 min. The lysis solution was transferred into 6-mL scintillation vials filled with scintillator and the dishes were washed once with lysis solution (100 µL). In case of the investigation of the dissociation kinetics the cells were incubated with binding buffer (250 µL) containing BIBP 3226 (100-fold higher concentrated than the previously used radioligand) after the incubation with radioligand (6 nM) and subsequent washing (twice 500 µL, 4 °C). After different periods of time the cells were washed again twice with buffer (500 µL, 4 °C) followed by the addition of lysis solution. Assays were performed in triplicate.

Fura-2 assay on HEL cells (Schild analysis). The fura assay was performed with HEL cells as previously described¹⁷ using an LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). Concentration-response curves of pNPY were constructed in the presence of **8a** at different concentrations. The signal intensities were related to the maximal intensity obtained from a pNPY effect curve in the absence of **8a**.

Autoradiography. Subcutaneous MCF-7 tumors were established in female NMRI (nu/nu) mice bearing an estrogen depot¹⁸ by subcutaneous injection of a cell suspension in culture medium without FCS (4 Mio cells/50 µL, 171st in vitro passage). The tumor and the rat brain (male Wistar rat, 10 month) were taken from the animals (brain: 24 h prior to experiment, tumor: 48 h prior to experiment), immediately frozen in Tissue-Tek with the help of dry ice, and stored at -18 °C (brain) and at -78 °C (tumor). Cryosections (12 µm) were obtained at -16 °C with a 2800 Frigocut E freezing microtome (Reichert-Jung/Leica, Germany). Two adjacent sections were mounted on a microscopic slide (Superfrost Plus, 75 × 25 × 1 mm), put 1 min into a chamber of 100% humidity and then carefully covered with binding buffer or fixed for 20 s in an alcoholic formaldehyde fixative (40 mL of 37% formaldehyde, 360 mL of 95% ethanol and 0.2 g calcium acetate¹⁹). The binding buffer was removed (after a period of less than 60 min under cooling) by putting the slides uprightly on a paper towel (≈ 1 min). For total binding the sections were covered with binding buffer (about 800 to 1000 µL for one slide) containing **8b** (3 nM) and for non-specific binding with binding buffer containing **8b** (3 nM), **8a** (30 nM) and pNPY (300 nM)^a. The sections were incubated at room temperature (22 - 25 °C) for a period of 8 min (tumor) or 30 min (brain). After incubation the binding buffer was removed, the slides were immersed three times into buffer split to 3 vessels (4 °C, 10 s) and finally rinsed with distilled water (4 °C, 3 s). The slides were put uprightly on a paper towel for 1 min and then dried in horizontal position in a desiccator over P₄O₁₀. The slides were set in close contact with a tritium sensitive screen (PerkinElmer, 192 × 125 mm) using an X-ray film cassette and stored in a dark room for 10 d. The autoradiographic image was generated from the tritium screen using a phosphorimager (Cyclone Storage Phosphor System, Packard).

The fixed sections were stained with haematoxylin and eosin (brain) or using the Masson-Goldner (Jerusalem's modification) staining procedure (tumor): H & E: rinsing (demineralized H₂O), Mayer's

haemalum solution (Merck) 1:3 diluted in water (11 min), rinsing (tap water), 1% aq. acetic acid (3 × immersion), running tap water (10 min), rinsing (H_2O_{demin}), eosin standard solution (2 min), running H_2O_{demin} (5 min); Masson-Goldner (Jerusalem's modification): Weigert's iron-haematein (45 s), rinsing (H_2O_{demin}), running tap water (10 min), differentiation with 200 mL of H_2O_{demin} + 20 mL of 2 M aq. hydrochloric acid (15 s), running tap water (10 min), rinsing (H_2O_{demin}), 0.5% aq. phosphotungstic acid (15 s), running H_2O_{demin} (10 min), acid fuchsine-Ponceau (30 s), 1% aq. acetic acid (3 × immersion), phosphoric acid-Orange G (10 s), 1% aq. acetic acid (3 × immersion), 0.2% light green (3.5 min), 1% aq. acetic acid (3 × immersion), 96% aq. ethanol (2 × 3 min), 100% ethanol (2 × 3 min), 100% xylene (3 min). Entellan (Merck) was used for covering.

(^a **8b** was displaced with pNPY in combination with **8a** to determine non-specific binding in tissue structures inaccessible to pNPY.)

Flow Cytometric Binding Assays.

Flow cytometric binding studies at the Y_2R (CHO cells transfected with the Y_2R , $G\alpha_{q5}$ and aequorin)²⁰ and at the Y_5R (expressed by HEC-1B cells transfected with the Y_5R)^{12, 21} were performed as previously described. The cell density in loading buffer was 1 Mio/mL (Y_2R) or 0.5 Mio/mL (Y_5R). For Y_2R binding studies Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY (10 nM).

Flow cytometric binding studies at the Y_4R (expressed by CHO cells transfected with the Y_4R , $G\alpha_{q5}$ and aequorin)¹³ were performed as reported²² with the following variations: Cy5-[K⁴]-hPP¹³ (5 nM) was used instead of S0586-[K⁴]-hPP (10 nM). The cell density in loading buffer was 10⁶/mL. Non-specific binding was determined in the presence of hPP (100 nM). The stock solution of Cy5-[K⁴]-hPP was prepared in 10 mM HCl + 0.1% BSA (500 nM).

All flow cytometric assays were performed in triplicate on a FACSCaliburTM flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm). **8a** was tested at a concentration of 1 μ M and 10 μ M (for Y_2R binding also 30 μ M). Whereas no displacement of the fluorescent ligands was observed in case of the Y_2R and Y_4R by **8a** (10 μ M), displacement at the Y_5R was around 50%.

5 References

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