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Preparation of Fluorescent Nonpeptidic Neuropeptide Y Receptor Ligands: Analogues of the Quinazoline-type Anti-obesity Y_5 Antagonist CGP 71683A

As part of a programme to develop fluorescence-based methods for the study of the interactions between G-protein coupled receptors (GPCRs) and their ligands the preparation of low molecular weight fluorescence-labelled neuropeptide Y (NPY) Y_5 antagonists is reported. The naphthylsulfonyl group in the potent quinazoline-type NPY Y_5 receptor antagonist CGP 71683A was replaced with a dansyl, nitrobenzoxadiazole (NBD) or acridine-9-carbonyl group. In radioligand binding studies on human Y_5 receptor expressing HEC-1B cells the substances labelled with acridine (K_i 311 nM) and NBD (K_i > 1000 nM) proved to be moderately active or inactive, respectively. By contrast, a K_i value of 49 nM was found for the dansyl analogue compared to 2 nM for CGP 71683A. No binding to Y_1 receptors (SK-N-MC cells, displacement of [^{3}H]propionyl-NPY) was detected for the new compounds at concentrations $\leq$ 1 μM.

Keywords: Neuropeptide Y; Y_5 receptor antagonist; Fluorescence labelling; Quinazoline; CGP 71683A

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Introduction

Fluorescent probes offer attractive alternatives to the application of radioligands, e.g., to detect receptors in cells and tissues, and to study drug-receptor interactions on soluble or cell-bound targets. As part of a programme to develop fluorescence-based methods for the study of ligand receptor interactions at G-protein coupled receptors (GPCRs) [1] histamine and neuropeptide Y (NPY) receptors were selected as models to design fluorescent probes. Previously, by using cyanine5-labelled NPY (cy5-NPY) we demonstrated that the affinity of agonists and antagonists can be determined in a very convenient manner by flow cytometry under equilibrium conditions [1]. In contrast to the labelled peptide cy5-NPY that binds to all NPY receptors the use of selective fluorescent ligands should be of advantage to detect receptor subtypes in tissue preparations and on cells. Usually, the decrease in affinity induced by incorporation of bulky fluorophores into low molecular weight ligands is much more pronounced than in case of peptides. Nevertheless, as demonstrated very recently for histamine H_1 and H_2 receptor ligands, the combination of pharmacophores with suitable fluorescence dyes conferring additional affinity may result in highly potent antagonists [2, 3]. Here, we report on a first approach to prepare fluorescent low molecular weight NPY antagonists.

Among the NPY receptors (for nomenclature see IUPHAR recommendations [4]) especially the Y_1 and Y_5 receptors have been considered interesting drug targets, e.g. for the development of new anti-obesity drugs. In search for low molecular weight fluorescent Y_5 receptor [5] ligands we started from the quinazoline derivative CGP 71683A [6, 7] (Scheme 1) which is a highly potent and probably the most intensively studied NPY Y_5 receptor antagonist. Numerous structural modifications of the arylsulfonamide moiety were shown to be tolerated without loss of the Y_5 antagonistic activity. Therefore, we used the quinazolinylaminomethylcyclohexylmethylamine scaffold to design several fluorescent Y_5 receptor ligands.

Chemistry

The reference compound **6 a** as well as the fluorescent analogues **6 b**–**d** were prepared according to standard procedures as shown in Scheme 1. The building block *trans*-1,4-bis(aminomethyl)cyclohexane (**2**) [8] was synthesized from *N*-Boc-protected tranexamic acid [9] *via* formation of the amide **1** by activation with 1,1′-carbonyl-

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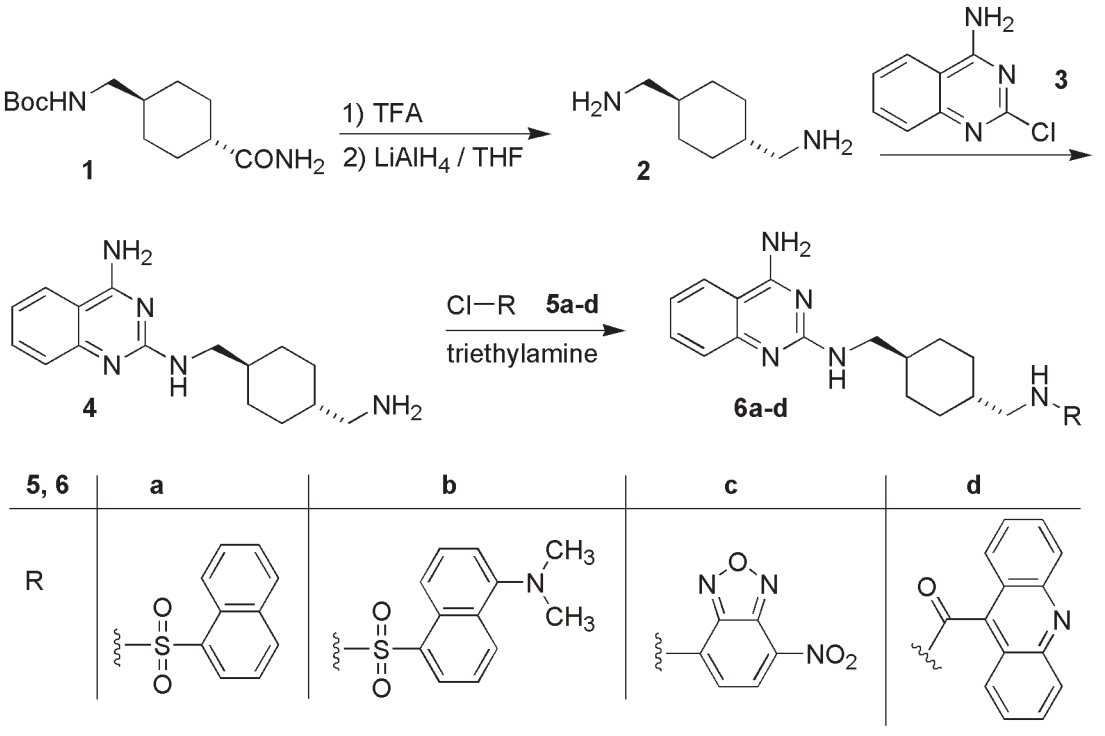

Scheme 1. Synthesis of the quinazoline-type NPY Y_5 antagonists.

diimidazole (CDI) and subsequent ammonolysis, followed by deprotection with trifluoroacetic acid (TFA) and reduction with $LiAlH_4$. The diamine **2** was allowed to react with 4-amino-2-chloroquinazoline (**3**) [10] to give the amine **4** which was coupled with naphthalene-1-sulfonyl chloride (**5 a**), 5-dimethylaminonaphthalene-1-sulfonyl chloride (**5 b**), 4-chloro-7-nitrobenzo[2,1,3]oxadiazole (**5 c**) or acridine-9-carbonyl chloride (**5 d**) to give **6 a**–**d**.

Pharmacology

The NPY Y_5 receptor affinities of the compounds **6 a**–**d** were determined by radioligand binding studies (displacement of [^{3}H]propionyl-NPY) on human endometrial carcinoma cells stably transfected with the human Y_5-receptor (hY_5-HEC-1B cells) [11]. Additionally, the compounds **6 b**–**d** were investigated for Y_1 receptor binding

Table 1. NPY receptor binding data of compounds **6 a**–**d** and longest wavelength absorption and fluorescence emission maxima of the fluorescence-labelled ligands.

Substance No.	λ_{max} Ex[a] (nm)	λ_{max} Em[a] (nm)	Y_5 receptor binding[b] K_i [nM]	Y_1 receptor binding[c] K_i [nM]
6 a[d] (CGP 71683A)			2.06 ± 0.25	
6 b	340	482	49.05 ± 9.43	⩾ 1000
6 c	483	537	>1000	⩾ 1000
6 d	359	432	311 ± 127	⩾ 1000

[a] Determined with solutions of the compounds in a buffer (NaCl 120 mM, KCl 5 mM, $MgCl_2$ 2 mM, $CaCl_2$ 1.5 mM, Hepes 25 mM, glucose 10 mM, adjusted to pH 7.4 with NaOH).

[b] determined from the displacement of [^{3}H]propionyl-NPY (0.8 nM) from human Y_5 receptors stably expressed in HEC-1B cells [11].

[c] Investigated on SK-N-MC cells using [^{3}H]propionyl-NPY (1 nM) as the radioligand according to the procedure described elsewhere.

[d] Reported: IC_{50} 2.9 nM [12].

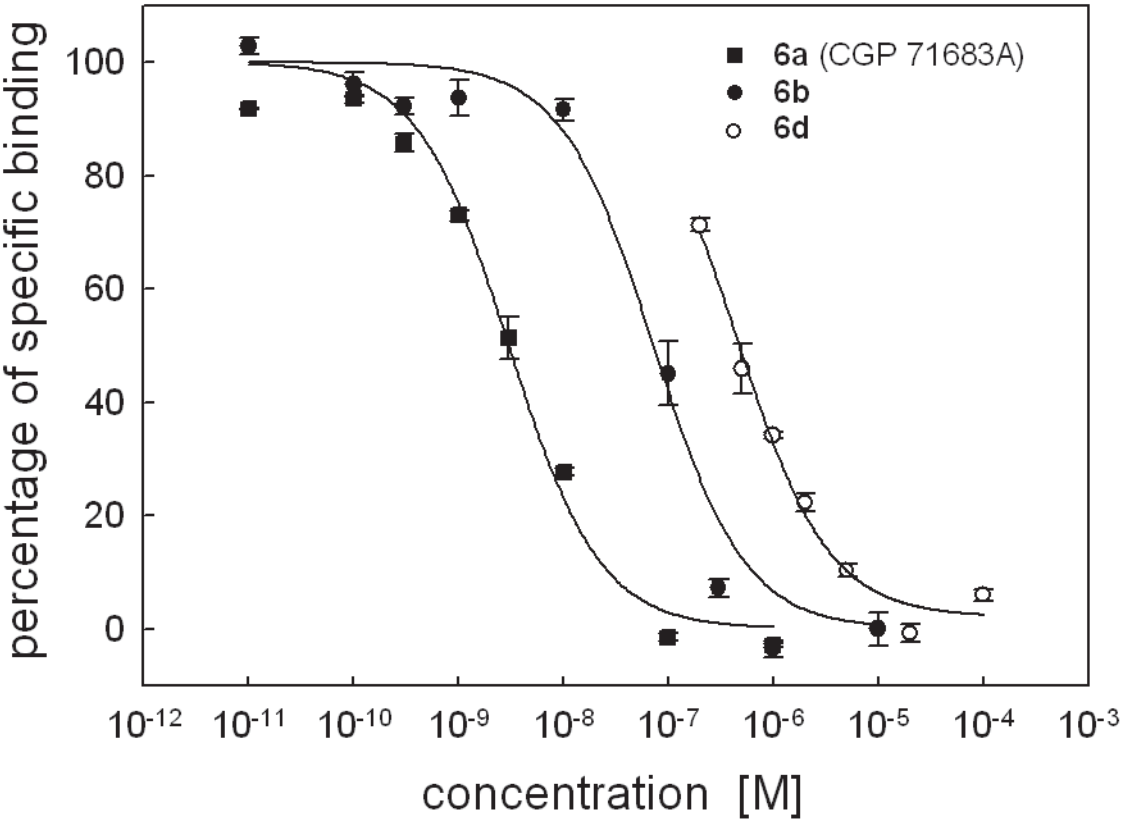

Figure 1. Y_5 receptor binding of compounds **6 a** (CGP 71683A), **6 b** and **6 d** on stably hY_5-transfected HEC-1B cells [11] (displacement of [^{3}H]propionyl-NPY, 0.8 nM); mean ± SEM, experiments performed in triplicate).

(radioligand: [^{3}H]propionyl-NPY) on human neuroblastoma (SK-N-MC) cells. The data are summarized in Table 1 and displacement curves for **6 a**, **b**, and **d** are shown in Figure 1.

Results and discussion

The 2,4-diaminoquinazoline motif was the scaffold of some early moderately active Y_5 antagonists with slight Y_1/Y_5 selectivity [12]. Structural modifications led to the discovery of numerous quinazoline-type Y_5 antagonists with different substitution patterns [7, 12–15]. A considerable increase in Y_5 receptor affinity and selectivity was achieved by the combination of the diaminoquinazoline moiety with a *N*-(cyclohexylmethyl)arylsulfonamide substructure as in compound CGP 71683A [6, 7]. As the naphthalene-1-sulfonyl residue is not essential, we selected this portion of the molecule for structural modification to obtain several fluorescent analogues of CGP 71683A. The congeners **6 b** and **6d** proved to be selective for Y_5 compared to Y_1 receptors, whereas **6 c** showed only very minor binding to both NPY receptors. According to a ligand-receptor interaction model published by Islam et al. [16] the sulfonamide-NH of Y_5 antagonists related to CGP 71683A may form a hydrogen bond with histidine-398 in transmembrane domain 6 of the receptor protein and the terminal aromatic rings are suggested to increase the affinity by hydrophobic interactions. In agreement with this model the NBD-labelled compound **6 c**, which is lacking an amide group, is only very weakly binding to Y_5 receptors. By contrast, K_i values in the nanomolar range were determined for the amides **6 b** and **6 d**. Although the activity of **6 a** (K_i 2 nM) was not achieved, the dansyl analogue **6b**, a very close congener of **6 a**, showed rather high Y_5 receptor affinity with a K_i value of 49 nM. Compound **6 d**, having a more bulky acridine-9-carbonyl group instead of the naphthalene-1-sulfonyl group in CGP 71683A, was about 150-fold less active than the reference compound.

Many attempts have been made to use fluorescent ligands as an alternative to radioligands for the study of receptors. This approach is hampered especially when receptors are to be investigated in their natural environ-

ment, i.e. in membranes or in whole cells as in case of G-protein coupled receptors (GPCR), due to a low signal-to-noise ratio resulting from autofluorescence. This problem can be overcome by the use of fluorophores emitting light of long wavelengths as, for instance, cyanine dyes such as cy5. In this respect, the dyes used for the preparation of the fluorescent Y_5 antagonists **6 b–d** are far from being optimal. On the other hand, the bulk of cyanine dyes may limit their value as labelling reagents for small molecules. As an alternative, regardless of fluorescence at shorter wavelengths, a shift in the emission spectrum on binding of the ligand or high intensity of the emitted light compared to autofluorescence could be sufficient to solve the aforementioned problem of detection. Generally, fluorescent GPCR antagonists should be superior to labelled agonists, as the latter may be internalised after receptor binding and activation resulting in apparently non-displaceable binding of the ligand. Also for NPY receptors agonist-induced β-arrestin interaction and receptor internalisation was demonstrated [17–19]. The dansyl group is known to emit fluorescence light with high intensity and it is a close structural analogue of the naphthalene-1-sulfonyl group present in CGP 71683A. The first results obtained with compound **6 b** in binding studies are promising with respect to the development of highly potent and selective fluorescent probes for Y_5 receptors. The optimization of the fluorescence properties and the investigation of the applicability of such compounds to study Y_5 receptors in tissues and on whole cells is subject of ongoing work.

Conclusion

As demonstrated previously by using cy5-NPY, the flow cytometric determination of receptor affinity represents an interesting alternative to conventional radioligand binding studies. Availability of suitable fluorescent probes provided, this technique may also offer perspectives to develop multiparametric fluorimetric methods to determine both affinity and functional data.

The results on Y_5 antagonists presented in this paper as well as data presented in the literature on other classes of compounds, e.g. recent publications on histamine receptor antagonists [2, 3], demonstrate that, in principle, the development of low molecular weight fluorescence-labelled ligands of GPCRs is possible. However, compared to the labelling of peptides or proteins the affinity of the resulting fluorescent probes is much more affected by the bulk of the fluorophores. Thus, the spectrum of appropriate dyes is restricted to structures conferring additional receptor affinity. As predictions are nearly impossible, the combination of pharmacophore and fluorophore must be optimized in each individual case.

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Experimental

Melting points (m.p.; are uncorrected) were determined on a Büchi 530 apparatus (Uster, Switzerland). Elemental analyses (indicated by elemental symbols) were performed at the analytical department of the faculty of chemistry and pharmacy, University of Regensburg. The structures were confirmed by ^{1}H-NMR spectra using a Bruker WM 250 (250 MHz; δ in ppm relative to TMS as internal standard; J in Hz) (Bruker, Karlsruhe, Germany) and mass spectroscopy (EI-MS: Varian MAT 112S. CI-MS and $^+$FAB-MS: Varian MAT 95. $^+$FAB-MS: xenon, MeOH/glycerol. CI-MS: desorption chemical ionization (DCI), ammonia) (Varian, Darmstadt, Germany). Excitation/emission spectra were recorded with a Kontron UV-VIS spectrometer (Kontron, Neufahrn, Germany) and a Perkin-Elmer LS-50B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). Chromatographic separations on a preparative scale were carried out on a Chromatotron model 8294 (Harrison Research, Muttenz, Switzerland) using glass rotors with 4 mm layers of silica gel 60 PF_{254} containing gypsum (Merck, Darmstadt, Germany) or on a preparative HPLC system from Knauer (Wellchrom preparative pump K-1800; UV-detector K-2000, precolumn Eurospher 100-C18, column Eurospher 100-C18 B962170 (5 µm, 250 × 40 mm)) (Knauer, Berlin, Germany).

The building blocks **2** [8] (CAS no. 10029-07-9), **3** [10] (CAS no. 59870-43-8), **4** [7] (hydrochloride: CAS no. 192323-10-7), and **5 d** [20] as well as the Y_5 antagonist **6 a** [7] (hydrochloride: CAS no. 192321-23-6) were prepared according to or by analogy with the methods described in the literature. The derivatization reagents **5 a–c** are commercially available. The spectral and analytical data of synthesized compounds are in agreement with the structures shown in Scheme 1.

trans-4-[[(tert-Butoxycarbonyl)amino]methyl]cyclohexanecarboxylic amide (**1**)

A solution of 1,1′-carbonyldiimidazole (2.0 g, 12.3 mmol) and *trans*-4-[[(*tert*-butoxycarbonyl)amino]methyl]cyclohexanecarboxylic acid [9] (2.27 g, 8.8 mmol) in 30 mL of anhydrous THF was stirred at room temperature for 1 h. After cooling to 0 °C, the mixture was gassed with dry NH_3 at 0 °C for 20 min and then stirred at room temperature for 5 h. The precipitate was filtered off and washed with THF to obtain 2.23 g (98 %) of **1** as colourless crystals, m.p. 195 °C (dec.). – Analysis $C_{13}H_{24}N_2O_3$ (256.34): calcd. C 60.91 H 9.44 N 10.93; found C 60.64 H 9.39 N 11.21. – EI-MS: m/z (%) = 256 (<1, $M^{\bullet+}$), 57 (100, $[C_4H_9]^+$). – ^{1}H-NMR (DMSO-d_6): δ (ppm) = 7.14 (s, 1 H), 6.78 (t, J = 5.8, 1 H), 6.62 (s, 1 H), 2.74 (t, J = 6.3, 2 H), 1.97 (m, 1 H), 1.70 (m, 4 H), 1.36 (s, 9 H), 1.24 (m, 2 H), 0.82 (m, 2 H).

trans-1,4-Cyclohexanebis(methylamine) (**2**)

Compound **1** (4.5 g, 17.6 mmol) was stirred with 10 mL of trifluoroacetic acid at room temperature for 48 h. After removal of the volatiles *in vacuo* the residue crystallized from EtOAc to give the trifluoroacetate of *trans*-4-(aminomethyl)cyclohexane-

carboxylic amide in quantitative yield. M.p. 77–78 °C; EI-MS: m/z (%) = 157 (<1, $M^{\bullet+}$), 139 (39), 126 (18), 30 (100). Lithium aluminium hydride (2.0 g) was added portionwise to the suspension of *trans*-4-(aminomethyl)cyclohexanecarboxylic amide (4.5 g, 11.8 mmol) in 60 mL of anhydrous THF at room temperature. The mixture was heated under reflux for 5 h. After cooling THF was removed by evaporation under reduced pressure, and the residue was treated with 100 mL of $CHCl_3$. Hydrolysis was carried out by dropwise addition of water. The solid was filtered off, the filtrate was dried over $NaSO_4$ and evaporated to give 1.3 g (78 %) of **2** as colourless oil which was used without purification for further reactions. $C_8H_{18}N_2$ (142.2); CI-MS: m/z (%) = 143 (100, $[M + H]^+$), 160 (15, $[M + NH_4]^+$).

4-Amino-2-[trans-(4-aminomethylcyclohexyl)methylamino]-quinazoline (**4**)

The solution of 4-amino-2-chloroquinazoline (**3**) [10] (200 mg, 1.1 mmol) and **2** (176 mg, 1.2 mmol) in 200 mL of methanol was heated under reflux for 16 h. After concentration *in vacuo* the mixture was chromatographed (Chromatotron, $CHCl_3$: MeOH, 1:1, ammonia atmosphere) to obtain 150 mg (48 %) of **4** as pale yellow solid, m.p. 91 °C ([7]: mp. of the monohydrochloride: 189–192 °C). – Analysis: $C_{16}H_{23}N_5 \times H_2O$ (303.41), calcd. C 63.34, H 8.31, N 23.08; found C 63.29, H 8.38, N 23.39. – CI-MS: m/z (%) = 286 (13, $[M + H]^+$), 171 (93), 144 (35), 116 (100).

Naphthalene-1-sulfonic acid {trans-4-[(4-aminoquinazolin-2-ylamino)methyl]cyclohexylmethyl}amide (**6 a**)

Naphthalene-1-sulfonyl chloride (**5 a**, 260 mg, 1.15 mmol) was added to the solution of **4** (300 mg, 1.05 mmol) and 200 µL of Et_3N in a mixture of $CHCl_3$ (20 mL) and DMSO (5 mL) at room temperature under N_2 atmosphere. The reaction mixture was stirred at room temperature in the dark overnight. TLC indicated complete disappearance of **4**. After evaporation of the solvents *in vacuo*, the residue was purified twice by chromatography (Chromatotron, $CHCl_3$: MeOH, 95:5) to give 410 mg (82 %) of **6 a** as a white solid, m.p. 99 °C ([7]: m.p. of the hydrochloride: 155–164 °C). – Analysis: $C_{26}H_{29}N_5O_2S \cdot 1.5\,H_2O$ (502.6), calcd. C 62.13, H 6.42, N 13.93; found C 62.31, H 6.50, N 13.96. – FAB-MS: m/z (%) = 476 (100, $[M + H]^+$). – ^{1}H-NMR (CD_3OD): δ = 8.70 (d, J = 8.7, 1 H), 8.16 (dd, J = 7.1 and 1.2, 1 H), 8.08 (d, J = 8.3, 1 H), 7.95 (dd, J = 7.9 and 1.2, 1 H), 7.84 (dd, J = 8.3 and 1.2, 1 H), 7.69–7.48 (m, 4 H), 7.33 (d, J = 8.3, 1 H), 7.08 (m, 1 H), 3.15 (d, J = 6.7, 2 H), 2.64 (d, J = 6.7, 2 H), 1.75–1.53 (m, 4 H), 1.46–1.10 (m, 2 H), 0.85–0.55 (m, 4 H).

5-(Dimethylamino)naphthalene-1-sulfonic acid {trans-4-[(4-aminoquinazolin-2-ylamino)methyl]cyclohexylmethyl}amide (**6 b**)

Dansyl chloride (**5 b**, 30 mg, 0.11 mmol) was added to the solution of **4** (421 mg, 0.073 mmol) and 40 µL of Et_3N in a mixture of 2 mL of $CHCl_3$ and 0.5 mL of DMSO. The mixture was stirred at room temperature in the dark for 10 h, then another 29 mg of dansyl chloride was added. The mixture was stirred for 20 h. After evaporation of the solvent *in vacuo* the residue was purified twice with a Chromatotron ($CHCl_3$: MeOH, 3:1) to give 30 mg (79 %) of **6 b**, as hygroscopic pale green solid. HR-$^+$FAB-MS: $C_{28}H_{35}N_6O_2S$ ($M + H^+$), calcd.: 519.2524, found: 519.2517; $^+$FAB-MS: m/z (%) = 519 (100, $[M + H]^+$). – ^{1}H-NMR(DMSO-d_6): δ (ppm) = 8.43 (d, J = 8.7, 1 H), 8.29 (d, J = 8.5, 1 H), 8.06 (d, J = 7.1, 1 H), 7.94 (d, J = 7.9, 1 H), 7.87 (t, J = 5.5, 1 H), 7.65–7.40 (m, 4 H), 7.35–7.10 (m, 3 H), 6.99 (t, J = 7.3, 1 H), 6.80–6.20 (br, 1 H), 3.08 (t, J = 7.1, 2 H), 2.80 (s, 6 H), 2.59 (t, J = 7.1, 2 H), 1.75–1.50 (m, 4 H), 1.48–1.05 (m, 2 H), 0.85–0.55 (m, 4 H).

N^2-{trans-4-[(7-Nitrobenzo[2,1,3]oxadiazol-4-ylamino)methyl]-cyclohexylmethyl}quinazoline-2,4-diamine (**6 c**)

NBD-chloride (**5 c**, 20.6 mg, 0.103 mmol) was added to the solution of **4** (26.7 mg, 0.093 mmol) in a mixture of $CHCl_3$ (2 mL), DMSO (1 mL) and Et_3N (50 µL). The mixture was stirred at room temperature in the dark for 24 h. After evaporation *in vacuo* the residue was chromatographed twice (Chromatotron, EtOAc: MeOH, 10:1) to give 15 mg (36 %) of **6 c** as a brown solid, m.p. 65 °C. – HR-$^+$FAB-MS: $C_{22}H_{25}N_8O_3$ ($M + H)^+$, calcd. 449.20496, found: 449.20303. – $^+$FAB-MS: m/z (%) = 449 (100, $[M + H]^+$). – ^{1}H-NMR (acetone-d_6): δ = 8.51 (d, J = 8.7, 1 H), 8.40 (br, 1 H), 7.91 (d, J = 8.3, 1 H), 7.51 (m, 1 H), 7.32 (d, J = 8.3, 1 H), 7.04 (m, 1 H), 6.65 (br, 2 H), 6.49 (d, J = 8.7, 1 H), 5.80 (br, 1 H), 3.60–3.50 (m, 2 H), 3.35–3.27 (m, 2 H), 2.03–1.85 (m, 4 H), 1.76–1.36 (m, 2 H), 1.18–1.04 (m, 2 H).

Acridine-9-carboxylic acid {trans-4-[(4-aminoquinazolin-2-ylamino)methyl]cyclohexylmethyl}amide (**6 d**)

The mixture of 130 mg (0.58 mmol) of acridine-9-carboxylic acid and 3 mL of thionyl chloride was heated under reflux for 3 h. After removal of the volatiles under reduced pressure, the residue was dried *in vacuo* to give acridine-9-carboxylic acid chloride (**5 d**) as a yellow powder which was dissolved in 5 mL of abs. $CHCl_3$ and added to a solution of **4** (140 mg, 0.49 mmol) and 200 µL of diisopropylethylamine in 10 mL of abs. $CHCl_3$ at 0 °C (N_2 atmosphere). The mixture was stirred at room temperature overnight, then 10 mL of $CHCl_3$ and 5 mL of water were added, the organic layer was washed with 5 mL of water and dried over sodium sulfate. After removal of the solvent the residue was purified with HPLC to give 105 mg (24.5 %) of **6 d**, as yellow crystals, m.p. 81 °C. – Analysis: $C_{30}H_{30}N_6O \cdot 3\,CF_3COOH \cdot 2\,H_2O$ (868.70), calcd. C 49.77, H 4.29, N 9.67; found C 50.16, H 4.28, N 9.65. – Chromatographic HPLC separation on analytical scale: Eurospher 100-C18 B971185 [Knauer], precolumn: 100-C18 B952163 [Knauer], eluent: MeOH/0.1 % TFA (50/50), flow rate: 1 mL/min, UV: 254 nm, EX: 360 nm, EM: 460 nm. Retention time of **6 c**: 10.6 min. Preparative HPLC: Eurospher 100-C18 B962170 [Knauer], precolumn: 100-C18 [Knauer]; eluent: MeOH/0.1 % TFA (50/50), flow rate: 60 mL/min, UV: 254 nm. Retention time of **6 c**: 11.5 min. – $^+$FAB-MS: m/z (%) = 491 (100, $[M + H]^+$). – ^{1}H-NMR (CD_3OD): δ = 12.51 and 11.60 (br, 1 H), 9.05 (t, J = 5.6, 1 H), 9.00–8.50 (br, 2 H), 8.21 (d, J = 8.7, 2 H), 8.19 (d, J = 7.9, 1 H), 7.99 (d, J = 8.7, 2 H), 7.91 (dd, J = 6.7 and 1.2, 2 H), 7.80 (t, J = 7.7, 1 H), 7.69 (dd, J = 6.7 and 1.2, 2 H), 7.55–7.30 (m, 2 H), 3.46–3.20 (m, 4 H), 1.90 (s, 4 H), 1.64 (s, 2 H), 1.05 (m, 4 H).

Receptor binding data

SK-N-MC and HEC-1B cells were obtained from the American Type Culture Collection (Rockeville, MD, USA). The HEC-1B cells were stably transfected with the human Y_5 receptor as described previously [11]. The cultivation of the cells and the performance of the radioligand binding studies using [^{3}H]propionyl-NPY (Amersham, Braunschweig, Germany) as the radioligand on Y_1 (SK-N-MC cells) and Y_5 receptors (hY_5-HEC-1B cells) followed the procedures described elsewhere in detail [11, 21]. K_i values were calculated from IC_{50} values according to the Cheng-Prusoff equation [22]. The experiments were done in triplicate.

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