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A Deep Hydrophobic Binding Cavity is the Main Interaction for Different Y_2R Antagonists

Kerstin Burkert,[a] Tristan Zellmann,[a] René Meier,[a] Anette Kaiser,[a] Jan Stichel,[a] Jens Meiler,[b] Gopi K. Mittapalli,[c] Edward Roberts,[c] and Annette G. Beck-Sickinger*[a]

Dedicated to Evamarie Hey-Hawkins on the occasion of her 60th birthday.

The neuropeptide Y_2 receptor (Y_2R) is involved in various pathophysiological processes such as epilepsy, mood disorders, angiogenesis, and tumor growth. Therefore, the Y_2R is an interesting target for drug development. A detailed understanding of the binding pocket could facilitate the development of highly selective antagonists to study the role of Y_2R in vitro and in vivo. In this study, several residues crucial to the interaction of BIIE0246 and SF-11 derivatives with Y_2R were investigated by signal transduction assays. Using the experimental results as constraints, the antagonists were docked into a comparative structural model of the Y_2R. Despite differences in size and structure, all three antagonists display a similar binding site, including a deep hydrophobic cavity formed by transmembrane helices (TM) 4, 5, and 6, as well as a hydrophobic patch at the top of TM2 and 7. Additionally, we suggest that the antagonists block $Q^{3.32}$, a position that has been shown to be crucial for binding of the amidated C terminus of NPY and thus for receptor activation.

Introduction

G protein-coupled receptors (GPCRs) form the largest group of cell surface receptors and play a central role in transmitting extracellular signals into signal cascades. Involved in various regulatory systems, they represent highly attractive targets for drug development as 30% of all pharmaceuticals on the market address GPCRs.[1] The neuropeptide Y_2 receptor (Y_2R) is a rhodopsin-like GPCR (class A) belonging to the neuropeptide Y (NPY) receptor family and couples to G_i and G_o proteins. In humans, this receptor family consists of four different receptor subtypes, Y_1R, Y_2R, Y_4R and Y_5R, which are expressed mainly in the central and peripheral nervous system. They are activated by the different peptide ligands neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP). The Y_2R is mainly activated by NPY and PYY and is the most abundant receptor subtype in the central nervous system.[2] It is predominantly expressed in neuronal tissue like thalamus, hypothalamus,[2a,3] amygdala, hippocampus,[4] as well as in the spleen, liver, gastrointestinal tract, fat tissue and blood vessels.[2b] The Y_2R plays a role in many pathological processes like epilepsy, pain and mood disorders.[2] Moreover, the Y_2R is overexpressed in glioblastoma and neuroblastoma, mediating tumor growth and vascularization.[5] Therefore, the Y_2 receptor is a highly interesting pharmacological target. So far, many different Y_2R-selective antagonists had been developed and tested,[6] including BIIE0246,[7] JNJ-5207787,[8] JNJ-31020028,[9] [^{3}H]UR-PLN196,[10] 136 (GSK), 141 (GSK), 149 (GSK),[11] CYM 9484, SF-11 and its derivatives,[12] ML072 and ML075.[13] BIIE0246 was the first potent Y_2R-selective antagonist[7] investigated in a range of in vivo studies.[5a,d,14] Intracerebroventricular administration of BIIE0246 resulted in decreased ethanol self-administration in mice with a history of ethanol dependency[15] and a reduced anxiolytic-like profile.[14a,16] In vitro, BIIE0246 decreased cell growth of neuroblastoma cell lines SK-N-BE(2) and SK-N-AS. In a xenograft model of these cell lines in mice, a decreased proliferation and an increased apoptosis were determined.[5d] In obese mice, administration of BIIE0246 resulted in suppressed tumor growth and angiogenesis for melanoma xenografts.[14b] Consequently, Y_2R antagonists could be very promising pharmacological tools for anticancer therapies.[5d] However, it has been shown that BIIE0246 also binds to dopamine, adrenergic, opioid, histamine, and serotonin receptors.[17] Furthermore, its disadvantages including its high molecular weight (896 Da), poor penetration of the blood–brain barrier and its insurmountable effect after a pre-incubation of 30 min limit the pharmacological potential of this compound.[17,18] Development of new selective Y_2R antagonists resulted in the compound SF-11 and its derivatives.[12] SF-11 was found to display improved selectivity and brain penetration in comparison with BIIE0246.[17] Structure–activity relationships (SAR) based on this small-molecule study resulted in the development of compound **40** and compound **46**.[12] As the thiourea functionality of compounds **40** and **46** is associat-

[a] K. Burkert, T. Zellmann, Dr. R. Meier, Dr. A. Kaiser, Dr. J. Stichel, Prof. Dr. A. G. Beck-Sickinger
Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Institute of Biochemistry, Brüderstr. 34, 04103 Leipzig (Germany)
E-mail: abeck-sickinger@uni-leipzig.de

[b] Prof. Dr. J. Meiler
Center for Structural Biology, Vanderbilt University, 465 21st Avenue South, Nashville, TN 37203 (USA)

[c] Dr. G. K. Mittapalli, Prof. Dr. E. Roberts
Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)

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ed with potential toxicity, derivatives using a carbamate functionality were synthesized. This change is well tolerated but the carbamate group proved to be unstable in rat plasma because of hydrolysis. That made the compound unsuitable for in vivo approaches.[6,12] We selected the antagonist BIIE0246 and the two SF-11 (CYM 5852) derivatives compound **40** (CYM 9691) and compound **46** (CYM 9624) to investigate the binding pocket on Y_2R in more detail and to understand the antagonistic activity of different non-peptidic compounds at the Y_2R (Figure 1).[12]

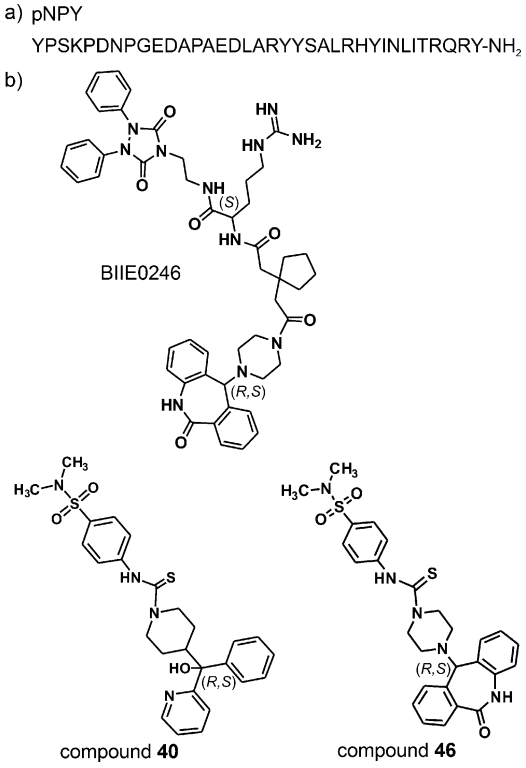

Figure 1. a) Amino acid sequence of the endogenous agonist NPY and b) structures of the non-peptidic antagonists BIIE0246, compound **40**, and compound **46**. The chiral carbon atoms and the configurations (*S*) and (*R*,*S*) are given, indicating that diastereomeric (BIIE0246) or enantiomeric (compounds **40** and **46**) mixtures of the antagonists were used.

The three different antagonists strongly differ in size and structure. Investigating the possible binding site, previous studies[19] revealed a multitude of candidate residues ($Y^{2.64}$, $L^{4.60}$, $L^{5.46}$, $Q^{6.55}$, and $L^{6.51}$) located mostly within the transmembrane domain as being relevant to BIIE0246 binding.[19] It has been postulated that BIIE0246 has a deep hydrophobic binding site in Y_2R, overlapping with that of NPY. Using site-directed mutagenesis and signal transduction assays, we were able to find further residues relevant for the antagonistic effect of the investigated compounds. Using these experimental results as constraints, we docked all three antagonists into the previously published comparative Y_2R model[20] and present the first structural models of the Y_2R-bound antagonists compound **40** and compound **46**, as well as a refined model of BIIE0246. These results could help to develop further improved Y_2R-selective antagonists to investigate the role of Y_2R in vivo and characterize the pharmacological potential of Y_2R antagonists. Highly selective, drug-like Y_2R antagonists with high potency could represent new approaches in treating Y_2R-mediated cancers.

Results

Understanding the activation and binding mechanism of agonists and antagonists with regard to their receptors can facilitate the development of new, highly potent and selective compounds. We thus sought to understand the binding and mode of action of these compounds.

Before the antagonist binding site was investigated by signal transduction assays, preliminary studies were conducted. To exclude a potential toxic effect of BIIE0246, compound **40** and compound **46** in the signal transduction assays, cell viability assays were performed (Supporting Information (SI) Table SI) and showed no toxic effect under inositol phosphate (IP) accumulation assay conditions. Furthermore, it could be demonstrated that BIIE0246, compound **40** and compound **46** (1 μM) exhibited antagonistic activity only on Y_2R in comparison with Y_1R, Y_4R, and Y_5R (SI Figure SI, Table SII). In competition binding assays, competitive behavior against [^{125}I]PYY was determined for all three tested antagonists (SI Figure SII, Table SIII).

Next, the binding pockets of BIIE0246, compound **40** and compound **46** (Figure 1 b) were investigated. We systematically mutated residues within the TM-cavity of Y_2R. First, the membrane localization of these receptor mutants was verified with fluorescence microscopy (Figure 2), signal transduction assays were subsequently performed to assess the impact of each mutant on agonist and antagonist potency. To examine whether a tested residue (Figure 3 a) is important for NPY, antagonist or both, the EC_{50} values of the NPY curves and the associated EC_{50} ratios were compared between wild-type receptor and receptor mutants. An increased EC_{50} value for the NPY curve of a receptor mutant serves as evidence for the importance of the residue in activating Y_2R by NPY.[20] In contrast, the EC_{50} ratio is defined as shift between the NPY- and NPY/antagonist-curve and represents the antagonistic activity. The EC_{50} ratio of a receptor mutant in relation to the wild-type receptor can be decreased, increased or similar, and can therefore be used to evaluate the importance of the mutated residue for the antagonist. This method was previously described by the research group of Jacobson.[21] Shift calculations were carried out similar to K_i calculations, as reported by different groups.[19,22]

For the different antagonists, we observed that BIIE0246 is the strongest antagonist, as displayed by the highest EC_{50} ratio of 2020 (Figure 3 b). Compound **40** and compound **46** showed comparably weaker antagonistic effects on Y_2R (EC_{50} ratio of 53 or 67, respectively). This correlates well with the published rank order of affinity (IC_{50} BIIE0246: 3.3 nM, compound **40**: 98 nM, compound **46**: 67 nM).[7,12] To investigate the binding pocket in more detail, several residues in TM2, 3, 4, 5, 6, and 7 were tested by IP accumulation assays.

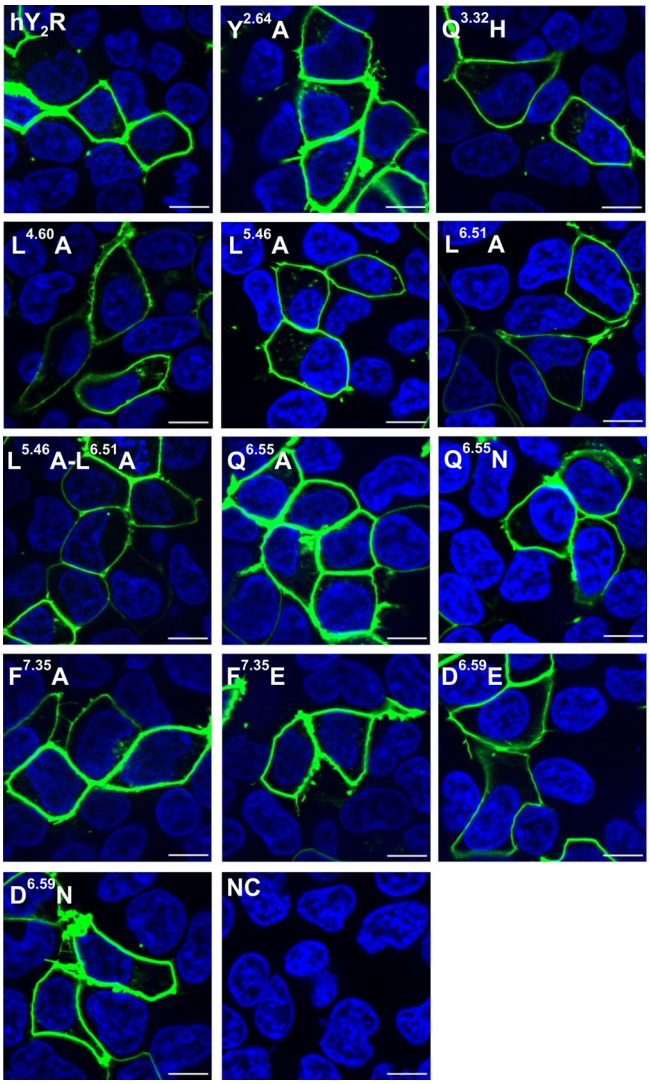

Figure 2. Membrane localization of hY_2R wild-type and receptor mutants after transfection into HEK293 cells. All receptors were C-terminally coupled to enhanced YFP (green), and nuclei were stained with Hoechst 33342 (blue). Membrane localization was observed by fluorescence microscopy. Representative images from two independent experiments are shown. All receptor mutants were localized in the cell membrane comparably to the wild-type receptor. NC: negative control, non-transfected cells; scale bars: 10 μm.

BIIE0246, compound 40 and compound 46 are bound deep in a hydrophobic binding pocket

$L^{5.46}$ (L227) and $L^{6.51}$ (L284) are located in a deep, hydrophobic pocket formed by transmembrane helices 5 and 6 (Figure 3c). Based on earlier studies concerning the binding mode of BIIE0246[19b] and NPY/PYY,[19c,20] we hypothesized that this pocket might be also relevant for the antagonistic effect of compound **40** and compound **46**. In signal transduction assays, a mutation of this hydrophobic pocket affects receptor activity by NPY. Mutation of $L^{6.51}$ to alanine results in 83-fold decreased potency, whereas $L^{5.46}$A showed only a 6-fold decreased NPY activity (Table 1, Figure 3c). Co-application of the antagonists to these Y_2R mutants resulted in EC_{50} ratios ($EC_{50+antagonist}/EC_{50-antagonist}$) that are highly decreased relative to the wild-type receptor, which means a huge loss of antagonistic activity. This was observed especially for compound **40** and compound **46** (EC_{50} ratios of compound **40** for hY_2R: 53; $L^{5.46}$A: 3; $L^{6.51}$A: 3; EC_{50} ratios of compound **46** for hY_2R: 67; $L^{5.46}$A: 5; $L^{6.51}$A: 14), indicating significant contribution of this hydrophobic binding pocket to the interaction with these antagonists. The antagonistic activity of BIIE0246 was also reduced (EC_{50} ratios of BIIE0246 for hY_2R: 2020; $L^{5.46}$A: 187; $L^{6.51}$A: >450).

However, the signal transduction curve of NPY/BIIE0246 with 10^{-6} M was not in saturation for $L^{6.51}$A. A lower concentration of antagonist (10^{-7} M) was tested (Table 2). Compared with the wild-type receptor under the same conditions, a clearly decreased antagonistic activity was observed for BIIE0246 (EC_{50} ratios of BIIE0246 for hY_2R: 150; $L^{6.51}$A: 26). For this antagonist, however, residual antagonistic activity was observed, probably mediated by an additional binding site.

Furthermore, a residue in transmembrane domain 4 was suggested to be part of the hydrophobic binding site for all three antagonists. $L^{4.60}$ (L183) was replaced by alanine and displayed a strongly reduced antagonistic effect for BIIE0246 (EC_{50} ratios of BIIE0246 for hY_2R: 2020; $L^{4.60}$A: 76), compounds **40** and **46** (EC_{50} ratios of compound **40** for hY_2R: 53; $L^{4.60}$A: 10; EC_{50} ratios of compound **46** for: hY_2R: 67; $L^{4.60}$A: 9) (Table 1, Figure 3d). NPY was not influenced and retained wild-type activity.

A residue close to the hydrophobic binding site is $Q^{3.32}$ (Q130). This position was recently shown to be important for the interaction with the amidated C terminus of NPY, and potential hydrophilic interactions with antagonists were probed.[20] Previously published data showed that a replacement by alanine or leucine results in visible impairment of membrane export of these receptor mutants, while $Q^{3.32}$H was localized at the membrane and showed increased binding affinity for BIIE0246.[19c] Therefore, we replaced $Q^{3.32}$ by histidine, which should retain hydrogen bonding properties and thus antagonistic activity. For NPY a 100-fold reduced activity was determined (Table 1, Figure 3d). At a BIIE0246 concentration of 10^{-6} M, an EC_{50} value of NPY/BIIE0246 could not be calculated as saturation was not reached. Therefore, the concentration of BIIE0246 was decreased (10^{-7} M) and tested on wild-type receptor and $Q^{3.32}$H (Table 2). No loss of antagonistic activity could be determined for BIIE0246 (EC_{50} ratios of BIIE0246 for hY_2R: 150; $Q^{3.32}$H: >300). Also for compound **40** no effect on activity was determined (Table 1, Figure 3d). Only for compound **46** a slightly reduced antagonistic activity was observed.

Transmembrane helix 6 exhibits different roles for Y_2R antagonists

Based on the size and flexibility of BIIE0246, large distances can be spanned within the binding pocket. Thus, we hypothesized that the guanidinyl moiety of BIIE0246 might interact with $D^{6.59}$ (D292) on top of TM6, similar to the binding mode of the endogenous agonist.[20,23] The guanidinyl group is present in BIIE0246, but not in compounds **40** and **46** (Figure 1b).

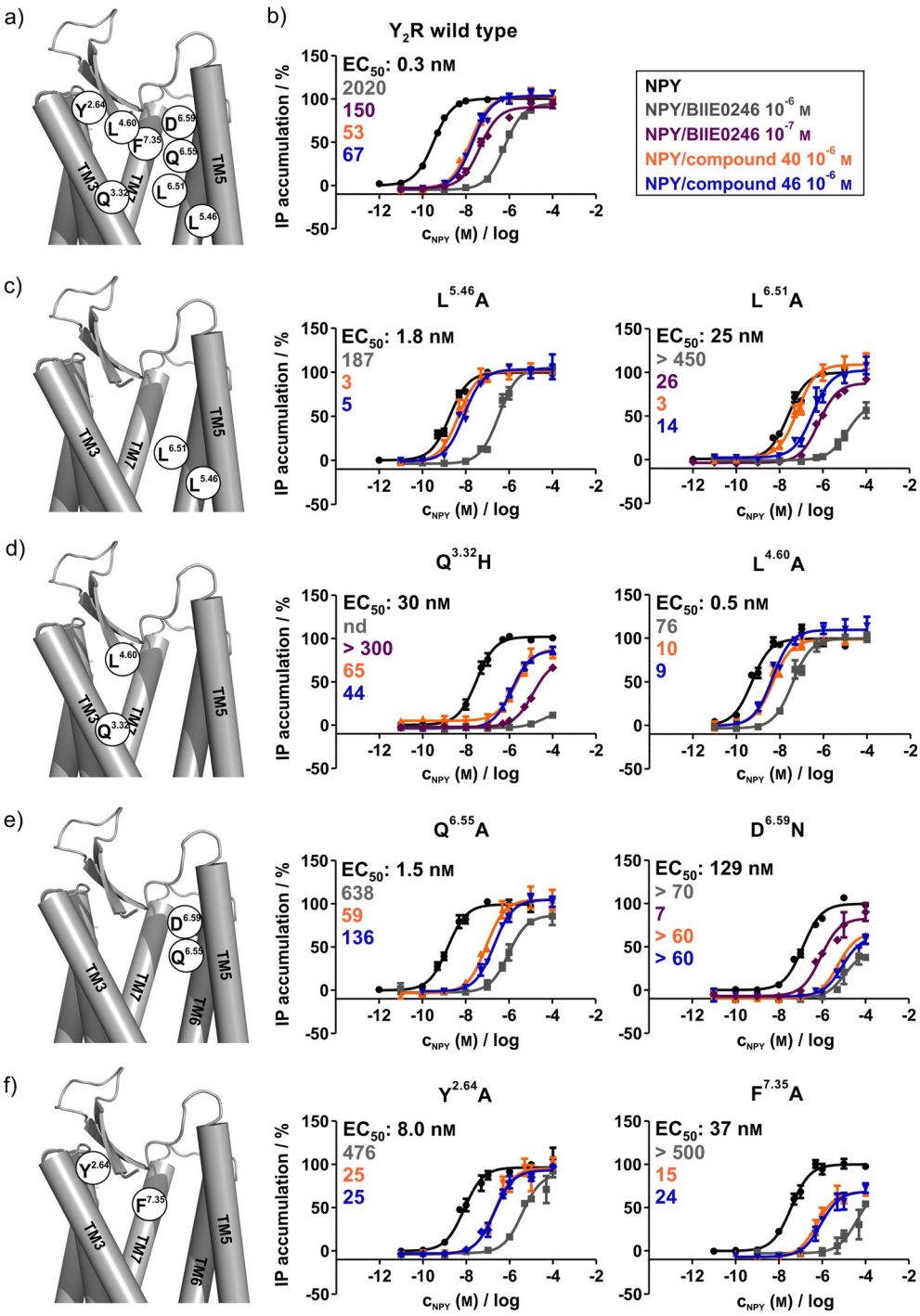

Figure 3. Investigation of the binding pocket for three different antagonists at hY_2R. a) Positions of residues that were investigated for the antagonist binding site in hY_2R are marked. b) EC_{50} values of NPY (black) and EC_{50} ratios for all three antagonists (colored) at the wild-type receptor are given at upper left corner of each plot. For measurements of the antagonist, increasing concentrations of NPY and a fixed concentration (1 μM) of antagonist (or 0.1 μM BIIE0246) for each concentration were used. BIIE0246 was the most potent antagonist, with the highest EC_{50} shift. Compounds **40** and **46** displayed decreased EC_{50} shifts relative to BIIE0246. All tested antagonists showed competitive behavior when co-applied with NPY. c)–f) Receptor mutants were tested with NPY and antagonists, and EC_{50} values of NPY and EC_{50} ratios were compared with the wild-type receptor. Decreased EC_{50} ratios combined with an increased EC_{50} value of NPY indicate residues important for NPY and for antagonist activity. A decreased EC_{50} ratio combined with no effect of the EC_{50} of NPY indicates an impact on the antagonistic effect only. TM: transmembrane helix (TM4 is hidden); nd: not determinable.

Because the replacement by alanine resulted in a dramatic loss of activity for NPY (>600-fold),[23] more suitable mutations, $D^{6.59}N$ and $D^{6.59}E$, were used. With regard to NPY, we observed that a removal of the negative charge at this position led to 430-fold ($D^{6.59}N$) and 26-fold decreased potency in response to a slightly larger side chain ($D^{6.59}E$), respectively (Table 1, SI Table SIV). Interestingly, BIIE0246 exhibited a 2-fold decreased EC_{50} ratio for $D^{6.59}E$ (Table 1). For $D^{6.59}N$, the NPY/BIIE0246 curve was

Table 1. Data from IP accumulation assays on transiently transfected COS-7 cells treated with NPY and in the presence of antagonists (10^{-6} M), for receptor wild-type and receptor mutants.

Receptor	NPY		NPY/BIIE0246			NPY/compd **40**			NPY/compd **46**		
	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	*X*-fold WT[b]	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	EC_{50} ratio[c]	*X*-fold WT EC_{50} ratio[d]	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	EC_{50} ratio[c]	*X*-fold WT EC_{50} ratio[d]	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	EC_{50} ratio[c]	*X*-fold WT EC_{50} ratio[d]
hY_2R (WT)	0.3 (9.50 ± 0.03)	1	606 (6.22 ± 0.05)	2020	1	16 (7.80 ± 0.06)	53	1	20 (7.69 ± 0.06)	67	1
$Y^{2.64}A$	8.0 (8.10 ± 0.09)	27	3811 (5.42 ± 0.08)	476	0.24	196 (6.71 ± 0.12)	25	0.47	202 (6.69 ± 0.08)	25	0.37
$Q^{3.32}H$	30 (7.52 ± 0.06)	100	nd	nd	nd	1934 (5.71 ± 0.11)	65	1.23	1312 (5.88 ± 0.05)	44	0.66
$L^{4.60}A$	0.5 (9.29 ± 0.09)	1	38 (7.42 ± 0.07)	76	0.04	4.8 (8.32 ± 0.08)	10	0.19	4.6 (8.34 ± 0.09)	9	0.13
$L^{5.46}A$	1.8 (8.74 ± 0.05)	6	337 (6.47 ± 0.06)	187	0.09	4.6 (8.34 ± 0.09)	3	0.06	8.3 (8.08 ± 0.08)	5	0.07
$L^{6.51}A$	25 (7.60 ± 0.05)	83	> 12000	> 450	(0.22)	67 (7.18 ± 0.08)	3	0.06	338 (6.47 ± 0.10)	14	0.21
$Q^{6.55}A$	1.5 (8.82 ± 0.06)	5	958 (6.02 ± 0.09)	638	0.32	89 (7.05 ± 0.10)	59	1.11	204 (6.69 ± 0.06)	136	2.03
$F^{7.35}A$	37 (7.43 ± 0.07)	123	> 20000	> 500	(0.25)	555 (6.26 ± 0.10)	15	0.28	874 (6.06 ± 0.10)	24	0.36
$D^{6.59}N$	129 (6.89 ± 0.05)	430	> 10000	> 70	(0.04)	> 8000	> 60	(1.13)	> 8000	> 60	(0.90)

[a] EC_{50} values and pEC_{50} values after stimulation with NPY alone or parallel administration of 10^{-6} M antagonist. All curves were normalized to the top and bottom values of the associated NPY curve. The pEC_{50} value ± SEM ($n \geq 2$) corresponds to the negative decadic logarithm of the EC_{50} value. Nonlinear regression (curve fitting) was performed with GraphPad Prism 5.0. [b] *X*-fold over wild-type ($EC_{50\,(mutant)}/EC_{50\,(WT)}$) represents the influence of the respective residue on NPY, with Hill slope set to 1. [c] EC_{50} ratio between NPY and NPY/antagonist curves for Y_2R and receptor mutants. Determined using $EC_{50\,(NPY+antagonist)}/EC_{50\,(NPY)}$, with Hill slope set to 1. To evaluate if a residue is important for the antagonist, EC_{50} ratios were compared between wild-type and receptor mutant. [d] *X*-fold over wild-type EC_{50} ratio (EC_{50} ratio (mutant)/EC_{50} ratio (WT)); nd: EC_{50} value and therefore EC_{50} shift was not determinable, no saturation of the concentration–response curve was observed up to 10^{-4} M NPY.

Table 2. Data of IP accumulation assays on transiently transfected COS-7 cells treated with NPY and a decreased antagonist concentration of 10^{-7} M, for receptor wild-type and receptor mutants.

Receptor	NPY		NPY/BIIE0246		
	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	*X*-fold WT[b]	EC_{50} [nM] ($pEC_{50} \pm SEM$)[a]	EC_{50} ratio[c]	*X*-fold WT EC_{50} ratio[d]
hY_2R (WT)	0.3 (9.50 ± 0.03)	1	45 (7.35 ± 0.07)	150	1
$Q^{3.32}H$	30 (7.52 ± 0.06)	100	> 10000	> 300	(2.00)
$L^{6.51}A$	25 (7.60 ± 0.05)	83	648 (6.19 ± 0.04)	26	0.17
$D^{6.59}N$	129 (6.89 ± 0.05)	430	864 (6.06 ± 0.11)	7	0.05

[a] EC_{50} values and pEC_{50} values after stimulation with NPY alone or parallel administration of 10^{-7} M antagonist. All curves were normalized to the top and bottom values of the associated NPY curve. The pEC_{50} value ± SEM ($n \geq 2$) corresponds to the negative decadic logarithm of the EC_{50} value. Nonlinear regression (curve fitting) was performed with GraphPad Prism 5.0. [b] *X*-fold over wild-type ($EC_{50\,(mutant)}/EC_{50\,(WT)}$) represents the influence of the respective residue on NPY, with Hill slope set to 1. [c] EC_{50} ratio between NPY and NPY/antagonist curves for Y_2R and receptor mutants. Determined using $EC_{50\,(NPY+antagonist)}/EC_{50\,(NPY)}$, with Hill slope set to 1. To evaluate if a residue is important for the antagonist, EC_{50} ratios were compared between wild-type and receptor mutant. [d] *X*-fold over wild-type EC_{50} ratio (EC_{50} ratio (mutant)/EC_{50} ratio (WT)).

not able to reach saturation up to an NPY concentration of 10^{-4} M. Nevertheless, an apparent leftward shift of the NPY/BIIE0246 curve was observed (Figure 3e), demonstrating a dramatic loss of antagonistic activity for BIIE0246. Thus, a reduced BIIE0246 concentration (10^{-7} M) was tested (Table 2) and displayed a strongly reduced antagonistic effect for $D^{6.59}N$ (EC_{50} ratios of BIIE0246 for hY_2R: 150; $D^{6.59}N$: 7). Moreover, the mutations of $D^{6.59}$ showed no effect on the antagonistic activity of compounds **40** and **46**, the EC_{50} ratio for hY_2R, $D^{6.59}E$ and $D^{6.59}N$ were comparable (Figure 3e).

One helix turn below $D^{6.59}$, $Q^{6.55}$ (Q288) has also been suggested to be involved in the binding of BIIE0246.[19c,d] We mutated $Q^{6.55}$ to alanine or asparagine to investigate the influence of size and polarity at this position. Neither mutation exhibited any change in activity for NPY (Table 1, Figure 3e, SI Table SIV), but both showed a decreased EC_{50} ratio and therefore, a loss

of antagonistic activity for BIIE0246. Regarding compounds **40** and **46**, no change was found after a replacement by alanine ($Q^{6.55}$A): the EC_{50} ratio was either similar to Y_2R (compound **40**) or slightly increased (compound **46**). In contrast, mutation of the same position to asparagine ($Q^{6.55}$N) resulted in a decrease in EC_{50} ratio for all three antagonists (Table 1, SI Table SIV). These results suggest $Q^{6.55}$ as a possible interaction partner for all three tested antagonists, but not for the endogenous ligand NPY.

Hydrophobic side chains on top of transmembrane helices 2 and 7 interact with all three antagonists

Located at the top of TM2, $Y^{2.64}$ (Y110) has been shown to be crucial for the binding of PYY to Y_2R and for binding of BIIE0246.[19a] We could confirm this finding in our studies and further investigated a possible impact on the antagonists compound **40** and compound **46**. After replacement of $Y^{2.64}$ by alanine, a 27-fold loss in potency was observed for NPY (Table 1, Figure 3 f). With regard to all three investigated antagonists, a slight decrease in EC_{50} ratio of about 2–4-fold could be observed, suggesting this position as a common site of interaction. Located close to $Y^{2.64}$, $F^{7.35}$ (F307) is also highly conserved in all neuropeptide Y receptor subtypes. To investigate the role of this residue for the Y_2R, $F^{7.35}$ was mutated to alanine and glutamate (Table 1, SI Table SIV). Both mutants showed a dramatic loss of NPY activity ($F^{7.35}$A: 123-fold, $F^{7.35}$E: 60-fold). For BIIE0246, compound **40**, and **46**, only a threefold decreased EC_{50} ratio was determined for $F^{7.35}$A (Table 1, Figure 3 f), suggesting only a slight loss of antagonistic effect for all derivatives. Interestingly, for compounds **40** and **46** a non-competitive behavior was observed. NPY was not able to reach efficacies similar to the antagonist curves for the wild-type receptor (Figure 3 f). In contrast, a replacement by glutamate indicated a clear decrease in antagonistic activity for BIIE0246. For compounds **40** and **46**, only a 2-fold reduction of the EC_{50} ratio was determined. These results suggest that $F^{7.35}$ has an influence on NPY activity as well as on antagonistic activity.

Docking of BIIE0246, compound 40 and compound 46 in a comparative Y_2R model

Based on mutagenesis data and previous binding data,[19] we were able to dock BIIE0246, compounds **40** and **46** into the comparative structural model of Y_2R[20] and present the binding modes of all three antagonists for the first time.

Compounds **40** and **46** were derived from antagonist SF-11 as previously described.[12] They share a thiourea and a dimethylsulfonamide group. Compound **40** has a diphenylcarbinol group and compound **46** a dibenzoazepinone (Figure 1). BIIE0246 possesses a guanidinyl group, mimicking the R^{35} in the C terminus of NPY. In compounds **40** and **46**, this guanidyl group is not present. Notably, all three antagonists possess one (compounds **40** and **46**) or two (BIIE0246, where the arginine moiety is always *S*-configured) stereocenters and were synthesized as enantiomeric or diastereomeric mixtures, indicated in Figure 1 b. The generated results of the signal transduction assays were used for docking BIIE0246, compounds **40** and **46** into a comparative structure model of Y_2R[20] with ROSETTA as described more thoroughly in the experimental section. The presented models for the antagonists show a likely configuration for binding antagonists (*R* configuration) with different sizes and moieties in Y_2R, based on all experimental evidence available.[19]

The binding pocket of the endogenous ligand NPY on Y_2R is given in Figure 4 a.[20] According to this, NPY interacts with the following residues: $D^{6.59}$–R^{33}, $Q^{3.32}$–Q^{34} and the amidated C terminus ($CONH_2$), $I^{4.71}$–L^{24}, $I^{4.77}$–L^{24} and I^{28}.[20,23] Based on mutagenesis data shown above, the binding mode for the different antagonists is suggested in Figure 4 b–d. The dibenzoazepinone or the diphenylcarbinol group of BIIE0246, compound **40** and compound **46** interact with the deep hydrophobic binding site $L^{5.46}$ and $L^{6.51}$ in transmembrane helices 5 and 6 as well as transmembrane 4 $L^{4.60}$. Furthermore, we suggest that $Q^{6.55}$ interacts with the thiourea group of compound **40** and compound **46**, and with the oxygen close to the piperazinidyl of BIIE0246 by electrostatic interactions. For NPY, mutation of $Q^{6.55}$ to alanine or asparagine does not result in any change in activity. Hydrophobic interactions between $Y^{2.64}$ and $F^{7.35}$ with the methyl groups of compound **40** and compound **46** are suggested as well as π-stacking interactions with the *N*-phenyl groups of BIIE0246. Via hydrogen bonds, $Y^{2.64}$ also interacts with the sulfonamide group present in compound **40** and compound **46**. BIIE0246 is the only antagonist that interacts with $D^{6.59}$, an important residue also for the endogenous ligand NPY.[23]

Discussion

The neuropeptide Y receptor family has a strong therapeutic potential. Especially the Y_2R has been suggested as therapeutic target for neuroblastoma or psychiatric diseases.[6] Besides, highly selective non-peptidic antagonists could help to understand diverse physiological processes. In this work, the binding pocket of the Y_2R selective antagonists BIIE0246, compound **40** and compound **46** were investigated using IP signal transduction assays. The three tested antagonists differ in size, functional groups and polarity (Figure 1 b). Based on our previous results, the antagonists were docked into a comparative structural model of hY_2R.[20] Previously published results were used to guide some of the mutational experiments.[19]

As the original publication relating to BIIE0246 presents the compound as a diastereomeric mixture according to its systematic name, we assume that further studies used the same diastereomeric compound. This is challenging for docking approaches as it influences the conformational space that needs to be covered to a great extent, especially in case of the dibenzoazepinone group that is shared by both BIIE0246 and compound **46**. Also compounds **40** and **46** possess a stereo center. In case of compound **40**, *R* and *S* configurations of the diphenylcarbinol group are nearly alike with regards to steric demands and hydrophobic properties while only the hydrogen bond accepting nitrogen changes its position. Importantly, experimental data shows that all three ligands share the same

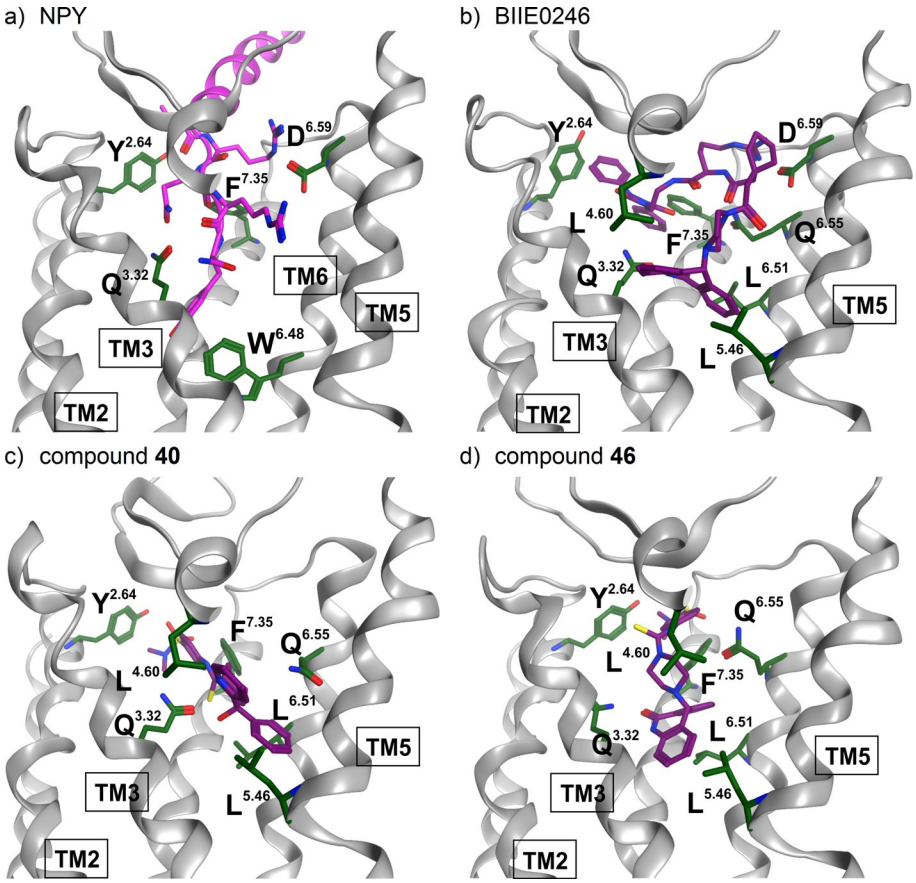

Figure 4. Comparison of the binding modes of NPY (pink) and the antagonists (purple) BIIE0246, compound **40** and compound **46** (*R* configuration). a) Comparative structure model of hY_2R with NPY.[20] b)–d) Suggested binding modes of three different antagonists. Docking of the antagonists was followed by filtering the docking results for closeness to experimentally evidenced residues involved in antagonist binding. In contrast to compounds **40** and **46**, BIIE0246 interacts with $D^{6.59}$, a residue which has been shown to be crucial for the binding of the endogenous agonist NPY. This interaction could explain the stronger antagonistic effect of BIIE0246. The amidated C terminus of NPY has been shown to interact with $Q^{3.32}$, which is blocked by all three antagonists. All antagonists share interactions with $Y^{2.64}$, $L^{4.60}$, $L^{5.46}$, $L^{6.51}$, $Q^{6.55}$, and $F^{7.35}$. TM: transmembrane helix (TM4 is hidden).

hydrophobic pocket ($L^{4.60}$, $L^{5.46}$, $L^{6.51}$). The only shared hydrophobic moiety between the three ligands is the diphenylcarbinol group of compound **40** and the dibenzoazepinone groups of BIIE0246 and compound **46**, respectively, making it very likely that this pocket binds those hydrophobic groups. Furthermore, it is suggested that $L^{6.51}$ could be more important for compound **40** than for BIIE0246 and compound **46**, based on a stronger loss of activity after an alanine replacement (Table 1). Moreover, BIIE0246 showed the highest influence on $L^{4.60}$ within the hydrophobic binding site (Table 1).

Besides hydrophobic properties of the ligand, binding also requires hydrogen bonding capabilities.[12] As the only viable donor and acceptor for hydrogen bonds close to the hydrophobic pocket is $Q^{3.32}$, the dibenzoazepinone groups of BIIE0246 and compound **46** have to bind in a specific orientation, in which they are nearly parallel to the membrane layer while the amide bond is turned toward $Q^{3.32}$. In *S* configurations, this orientation would result in clashes between the rest of the ligand and the transmembrane helices 5 and 6. In *R* configuration, the rest of the ligand is oriented nearly perpendicular to the membrane. As the *R* configurations provided the best possible conformations with regard to experimental data during initial docking with GOLD, only these enantiomers were included in docking with ROSETTA.

For BIIE0246, a partially overlapping binding site with NPY was assumed[19b–d] as well as a deep hydrophobic binding site.[19b] It was published that a replacement of $L^{6.51}$ by alanine leads to a 2-fold decreased pK_i value for BIIE0246. More interestingly, a replacement of $L^{5.46}$ by alanine resulted in a dramatic loss of binding affinity while the double mutant $L^{5.46}Q/L^{6.51}F$ exhibited a 680-fold decrease in binding affinity for BIIE0246.[19b] Moreover, $L^{4.60}A$ leads to a dramatically decreased binding affinity for BIIE0246.[19d] We were able to corroborate these data in the context of the impact of these positions on signal transduction. Furthermore, we found that the deep, hydrophobic binding pocket in human Y_2R is also the main region for antagonistic activity of compound **40** and compound **46** ($L^{4.60}$, $L^{5.46}$ and $L^{6.51}$). A replacement of $L^{5.46}$ and/or $L^{6.51}$ as well as $L^{4.60}$ by alanine led to a dramatic loss of antagonistic activity. Moreover, $L^{6.51}A$ showed a higher decrease in antagonistic activity for compound **40** than for compound **46** and BIIE0246, suggesting that $L^{6.51}$ is more important for compound **40** and its diphenylcarbinol group. The residual activity exhibited by BIIE0246 (EC_{50} ratio: >140) after simultaneous mutations of $L^{5.46}$ and

$L^{6.51}$ to alanine suggests that further sites besides this hydrophobic pocket contribute to the binding of BIIE0246, because some antagonistic activity is also present after this double mutation.

Regarding the same mutant, compounds **40** (EC_{50} ratio: 3) and **46** (EC_{50} ratio: 6) lost nearly the complete antagonistic activity (SI Table SIV). Thus, the contribution of the thiourea and sulfonamide groups is probably much weaker to binding.

As evidenced by the high impact of $L^{4.60}$A, $L^{5.46}$A, $L^{6.51}$A, the hydrophobic pocket should represent a common site for hydrophobic moieties of the three antagonists. BIIE0246 and compound **46** share a dibenzoazepinone group, while compound **40** bears a diphenylcarbinol group. Based on SAR studies that resulted in the development of the two latter compounds,[12] removal of hydrogen bond donors and acceptors in this region (for example by methylation, removal of the hydroxy moiety in case of the diphenylcarbinol group, or substitution of the amide bond by methyl groups in case of the dibenzoazepinone group) results in decreased binding. This suggests that besides simply occupying the hydrophobic pocket, the dibenzoazepinone and diphenylcarbinol contribute to bind the receptor via hydrogen bond interactions. Contrasting these results, a BIIE0246 variant where the dibenzoazepinone group is replaced by diphenylmethyl group only resulted in an increase of about 4-fold in IC_{50}.[24] Probably this is the case because BIIE0246 is larger in size and has more possible binding partners, counteracting a single missing interaction.

The selectivity of BIIE0246, compound **40** and compound **46** could be explained by residue $L^{5.46}$, which is a key player in the binding pocket of Y_2R. Within the NPY family, only Y_2R has a hydrophobic leucine at this position, while Y_1R, Y_4R and Y_5R have a hydrophilic glutamine. Therefore, they miss an important part of the deep hydrophobic binding pocket.[25]

$Q^{3.32}$ is in a position close to the hydrophobic pocket and has been reported to directly interact with the amidated C terminus of NPY.[20] For BIIE0246, a mutation of this residue to glutamate resulted in a 6-fold decrease in binding affinity.[19c] Besides increasing the polarity of the hydrophobic pocket, glutamate is not able to function as an hydrogen bond donor, which could explain this decrease. Therefore, a direct interaction between this position and BIIE0246, compound **40** and compound **46** is suggested. For compound **40**, a removal of the hydroxy group or changing the position of the nitrogen atom in the pyrinidyl ring or a replacement of the amide group in the dibenzoazepinone of compound **46** by two methylene groups led to a complete loss of antagonistic activity.[12] Concluding, this part of compound **40** and compound **46** as well as BIIE0246, interact with $Q^{3.32}$, which is suggested to bind with the amidated C terminus of NPY.[20] This interaction could contribute to increase the potential binding between antagonist and receptor. A replacement of $Q^{3.32}$ by histidine retaining hydrogen bonding properties had no effect on antagonistic activity of BIIE0246 and compound **40**, and showed only a slightly reduced activity for compound **46** (Table 1).

Position 6.55 is highly conserved in class A GPCRs and belongs to the binding pocket for example in peptide binding receptors like human κ-opioid receptor (κOP) and human protein-activated receptor 1 (PAR1).[1b] After replacement of $Q^{6.55}$ by alanine or asparagine, a loss of antagonistic activity was observed for BIIE0246, hinting at a possible contribution of this residue to binding the molecule and confirming published competition assays.[19c] For compound **40** and compound **46**, a reduced EC_{50} ratio was detected only after a mutation to asparagine (SI Table SIV). As the asparagine moiety is smaller than glutamine but exhibits very similar physicochemical properties, this mutation would mainly result in a somewhat wider binding pocket. Consequently, the smaller compounds **40** and **46** could be bound weaker to the wider binding pocket in Y_2R and lose some of the antagonistic activity. In contrast, a mutation to alanine does not result in reduced EC_{50} ratios for compound **40** or compound **46**. As these antagonists are smaller in size than BIIE0246, they could bind relatively more flexible within the binding pocket. Therefore, a complete loss of the polar side chain of $Q^{6.55}$ could lead to a slight re-orientation of the antagonists within the binding pocket, with no impact on antagonistic activity. Alternatively, a mutation to alanine could result in a higher number of weakly bound water molecules at the site, which would be released during binding. This increase in entropy could offset the weaker interaction. Notably, both alanine and asparagine mutations at this positions had no effect on activation of the receptor by NPY.

$Y^{2.64}$ is conserved between neuropeptide Y receptor subtypes, except for Y_5R.[25] In accordance with previously published results,[19a] a replacement of $Y^{2.64}$ by alanine results in a 27-fold EC_{50} shift for NPY. The investigated antagonists all display a reduced antagonistic activity, suggesting an overlapping binding site for NPY and all three antagonists. Regarding the SF-11 derivatives, the dimethylsulfonamide group of compounds **40** and **46** is an important interaction partner in Y_2R. At this position, a replacement of the sulfonamide group by good hydrogen bond acceptors like ester, amide or dimethylamine groups were the only tolerated changes.[12] Therefore, we suggest that this area primarily interacts with $Y^{2.64}$ via hydrogen bonds.

$F^{7.35}$ is a position that has been reported to interact with ligands in several GPCR, including peptide binding GPCR.[1b] Whereas an impact on agonist binding could be shown for Y_4R,[26] this is not the case for the Y_1R agonist PYY nor antagonists (1229U91, J-104870).[3b,27] After a replacement of $F^{7.35}$ by alanine or glutamate, we observed a 60- or 123-fold loss of NPY activity for Y_2R (Table 1). As a bulky hydrophobic side chain, $F^{7.35}$ would have a central role in shaping the binding pocket for agonists and antagonists. Regarding the receptor mutants $F^{7.35}$A and $F^{7.35}$E, BIIE0246, compound **40** and compound **46** all display a similarly reduced antagonistic effect.

The main difference in the binding profile of the three antagonists is the effect of replacing $D^{6.59}$ by asparagine or glutamate. This position was shown to be the interaction partner of R^{33} in NPY[23] and the guanidinyl group of the Y_1R antagonist BIBP3226.[22] Besides, this residue is also relevant in the related NPFF receptor system. Small, non-peptidic compounds like AC-093, AC-099 and AC-970 interact via its guanidinyl group with $D^{6.59}$ in $NPFF_1R$ and AC-970 and AC-216 with $NPFF_2R$, respectively.[28] Among the small organic compounds investigated in

this study, only BIIE0246 possesses a positively charged group that would be likely to interact with $D^{6.59}$. After mutation of $D^{6.59}$ to asparagine or glutamate, we observed a loss of antagonistic activity only for BIIE0246, but not for compounds **40** and **46**. These results suggest that BIIE0246 interacts with $D^{6.59}$ by ionic interactions. Compared with compounds **40** and **46**, the additional blocking of a central interaction partner of the agonist NPY should lead to a higher antagonistic effect.

Our results are in agreement with previously published data investigating the binding of BIIE0246 and Y_2R.[19] A further relevant residue for BIIE0246 binding on Y_2R that has been identified is $H^{7.39}$,[19c, 19d], which faces into the antagonist binding pocket. $H^{7.39}$ is probably part of an extensive hydrogen bonding network involving $Q^{3.32}$.[19c] Contrary to previously published results, $Y^{3.30}$, $Q^{3.37}$ and $T^{3.40}$[19b–d] are not part of the binding pocket in our current model either due to their orientation or their distance to the pocket. We speculate that any changes in BIIE0246 antagonism exhibited after mutation of these residues may be caused by indirect effects, for example changes in overall receptor conformation.

New approaches in developing Y_2R selective, non-peptidic antagonists could therefore use the hydrophobic pocket using a dibenzoazepinone or a diphenylcarbinol group as central building block. $L^{6.51}$ contributes to the shape of this pocket, but also to that of the endogenous agonist NPY as demonstrated by the 65-fold EC_{50} shift after mutation to alanine. In the closely related PYY, this pocket has been reported to tolerate a wide range of hydrophobic substitutions such as cyclohexylalanyl, phenyl, biphenyl and naphthyl groups.[29] Based on this, it seems beneficial to use the same pocket for antagonist binding, possibly by extending the dibenzoazepinone or a diphenylcarbinol unit with more hydrophobic groups. Attached by a flexible linker, these groups could increase binding and specificity. Based on the SF-11 SAR data, keeping the dimethylsulfonamide moiety seems favorable. As the thiourea linker has been suggested to be toxic, it should be replaced by alternatives such as urea. Modification of this central building block could nevertheless be useful and could be performed by substitutions at the piperidinyl ring. As evidenced by our data, an interaction with the highly exposed $D^{6.59}$ could strongly increase binding. Positively charged groups would be an option, but generally decrease the bioavailability. Alternatives should aim to provide strong hydrogen bond donor functions and could include acylated guanidyl variants.[10] In BIIE0246, such a masking of the positive charges has been shown to result in derivatives that are still active antagonists.[10]

Conclusions

In this article, the selective antagonists BIIE0246, compound **40** and compound **46** were docked into a Y_2R comparative structure model. Their binding sites overlap with that of the endogenous agonist NPY. We suggest that all antagonists share the same deep, hydrophobic binding pocket ($L^{4.60}$, $L^{5.46}$, $L^{6.51}$) and interact with TM2 and 7 ($Y^{2.64}$, $F^{7.35}$). The main difference is the interaction with $D^{6.59}$, which is only bound by BIIE0246. All antagonists block $Q^{3.32}$, which is a central interaction partner for the amidated C terminus of NPY. Blocking this residue results in the antagonistic activity exhibited by BIIE0246, compound **40** and compound **46**. These results open up the possibility to develop new selective, small non-peptidic antagonists with low molecular weight. These could be used to investigate in vitro and in vivo approaches of elucidating the physiological role of Y_2R, as well as for Y_2R-targeting therapy in the future.

Experimental Section

Chemistry

Materials: N^{α}-9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and 4-(2,4-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink amide) resin were purchased from Iris Biotech (Marktredwitz, Germany). Thiocresol and thioanisole were obtained from Fluka (Buchs, Switzerland), acetonitrile from VWR (Darmstadt, Germany), *N*,*N*′-dimethylformamide from Biosolve (Valkenswaard, The Netherlands), diethyl ether and ethanol (EtOH) from Scharlau (Barcelona, Spain) and trifluoroacetic acid from Sigma–Aldrich (Taufkirchen, Germany). MALDI-ToF (Ultraflex III MALDI-ToF/ToF) and ESI-HCT (High-capacity ion trap ESI-MS) were purchased from Bruker Daltonics (Billerica, MA, USA).

Peptide NPY and antagonists: Porcine NPY (YPS KPD NPG EDA PAE DLA RYY SAL RHY INL ITR QRY-NH_2) was synthesized by automated solid-phase peptide synthesis using an Fmoc/*t*Bu strategy as previously described.[26] A Rink amide resin (15 μmol scale) was used to generate C-terminally amidated NPY. Purity of NPY was determined by two different analytical reversed-phase HPLC systems, showed purity of $>95\,\%$, as well as MALDI-ToF and ESI-HCT. Compound **40** (CHEMBL2030179, CYM 9691) and compound **46** (CHEMBL2030185, CYM 9624) were synthesized as described previously.[12] BIIE0246 was purchased from TOCRIS bioscience/R&D Systems (purity $>98\,\%$). All antagonists were diastereomeric (BIIE0246) or enantiomeric (compound **40** and compound **46**) mixtures, in a ratio 1:1 (*R* and *S* configurations) based on the syntheses.

Biology

Materials: Wizard plus Mini or Midi DNA purification system kit were purchased from Promega (Mannheim, Germany). PfuTurbo DNA polymerase (2.5 U μL^{-1}) was obtained from Agilent Technologies (Santa Clara, USA). *Escherichia coli* DH5α (Library Efficiency DH5α™ Competent Cells) were obtained from Invitrogen (Carlsbad, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich and hygromycin B from Invivogen (Toulouse, France). Cell culture media was purchased from Lonza (Basel, Switzerland) including Dulbecco's modified Eagle's medium (DMEM, with 4.5 g L^{-1} glucose and L-glutamine), Ham's F12 and Dulbecco's phosphate-buffered saline (DPBS). Fetal calf serum (FCS) was purchased from Biochrome GmbH (Berlin, Germany), Opti-MEM from Life Technologies (Basel, Switzerland) and Hoechst 33342 from Sigma–Aldrich. Lipofectamine™ transfection 2000 Reagent was obtained from Invitrogen, Metafectene PRO from Biontex Laboratories GmbH (Munich, Germany), and eight-well micro-slides from ibidi GmbH (Martinsried, Germany). Myo[2-^{3}H]inositol and scintillation cocktail Optiphase HiSafe were obtained from PerkinElmer (Boston, USA), ammonium formate (NH_4HCO_2) from Paul Lohmann GmbH (Emmerthal, Germany), ethylenediamine tetraacetic acid (EDTA) from Sigma–Aldrich, formic acid (HCOOH) from AppliChem GmbH (Darmstadt, Germany), sodium tetraborate ($Na_2B_4O_7$), sodium formate (HCOONa) and lithium chloride (LiCl) from Merck (Darmstadt,

Germany) and sodium hydroxide from Grüssing GmbH (Filsum, Germany).

Plasmids: The receptor construct hY_2R-eYFP pVitro2 was generated as described by Böhme et al.[30] The pVITRO2-hygro-2mcs vector was bought from Invivogen (Toulouse, France). $G_{\alpha\Delta6qi4myr}$ pcDNA3 was kindly provided by E. Kostenis.[31] Receptor positions were designated according to nomenclature of Ballesteros and Weinstein.[32] All receptor constructs were C-terminally fused to enhanced yellow fluorescent protein (eYFP) by a linker GCGGATCCACCGGTC on the DNA level.

Preparation of hY2R mutants: The wild-type receptor construct hY_2R-eYFP pVitro2 was used as a template to generate receptor mutants using site-directed mutagenesis (QuikChange™ mutagenesis, Stratagene) using PfuTurbo DNA polymerase. The success of the mutation was confirmed by sequencing of the complete receptor coding sequence of the plasmids. Wild-type receptor (hY_2R-eYFP pVitro2) and receptor mutants were transformed into chemically competent *Escherichia coli* (*E. coli*) DH5α. The plasmid DNA was isolated using a Wizard plus Mini or Midi DNA purification system kit.

Cell culture: HEK293 cells (human embryonic kidney) and COS-7 cells (African green monkey) were cultivated in 75 cm^2 flask by 37 °C, 5% CO_2 and 95% humidity (standard conditions). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, with 4.5 g L^{-1} glucose and L-glutamine) and Ham's F12 (1:1), 15% (*v*/*v*) heat-inactivated FCS, COS-7 cells in DMEM supplied with 10% (*v*/*v*) heat-inactivated FCS.

Fluorescence microscopy: Membrane localization of Y_2R variants was verified in HEK293 cells. Cells were seeded in eight-well microslides and transiently transfected with plasmid DNA (500 ng) using Lipofectamine™ 2000 transfection reagent. After overnight cultivation, cells were starved for 30 min in Opti-MEM. The nuclei were stained with Hoechst 33342 and cells were analyzed using AxioVert Observer Z1 (YFP: Filter Set 46, DAPI: Filter Set 49, ApoTome, 63×/1.40 oil objective) and edited by ZEN2012 microscopy- and imaging software (ZEISS, Jena, Germany). As negative control untransfected cells were used.

IP signal transduction assay with antagonists: To determine the EC_{50} values of NPY, NPY/antagonist curves and the EC_{50} ratio from concentration–response curves, an inositol phosphate (IP) accumulation assay was used, as previously described in Witte et al.[33] Briefly, COS-7 cells were transiently co-transfected (Metafectene PRO) with wild-type receptor or mutated receptor plasmid DNA (320 ng per well) and chimeric G protein ($G_{\alpha\Delta6qi4myr}$)[31] plasmid DNA (80 ng per well) to redirect $G_{\alpha i}$ signaling to phospholipase C pathway.[31] Cells were labeled with 2 μCi mL^{-1} Myo[2-^{3}H]inositol for 16–20 h. For the NPY curves, the cells were stimulated with peptide solutions in concentration ranges of 10^{-12} to 10^{-4} M. For the antagonist curves, the cells were co-incubated with an increasing concentration of NPY and 1 μM (10^{-6} M) antagonist (BIIE0246, compound **40** or compound **46**) or 0.1 μM (10^{-7} M) BIIE0246. All antagonists were initially dissolved in dimethyl sulfoxide (DMSO), resulting in a final concentration of 0.1% (*v*/*v*). After the cells were incubated for 60 min, the medium was aspirated, the cells were lysed subsequently. The accumulated radioactive IP derivatives were isolated by anion exchange chromatography (method and buffer conditions as published previously in Els et al.[34] with minor changes: 5 mL regeneration buffer and H_2O for regeneration of the columns were used; $IP/IP_2/IP_3$ elution buffer: 1 M ammoniumformate + 0.1 M formic acid) and quantified by liquid scintillation counting. Concentration response-curves were analyzed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). All curves were normalized to the top (100%) and bottom (0%) values of the associated NPY curve. The row means total function was used to summarize concentration–response curves of different experiments to one single concentration–response curve. The EC_{50} and $pEC_{50} \pm SEM$ values were examined using nonlinear regression (curve fit). The EC_{50} ratio was determined using row means total NPY and NPY/antagonist curves ($EC_{50(NPY+antagonist)}/EC_{50(NPY)}$), setting the Hill slope $n_H = 1$. All experiments were performed in duplicates of at least two independent experiments.

Docking of antagonists in a comparative Y2R model: A structural model of Y_2R was constructed as described by Kaiser et al.[20] Prospective docking of *R* and *S* enantiomers of BIIE0246, compound **40** and compound **46** was performed with GOLD 5.2.1.[35] As the central cavity has been shown to bind the C terminus of NPY as well, the same region was defined as a putative binding site of the competitive antagonists. Docking was performed using 1000 genetic algorithm runs, CHEMPLP scoring and clustering to enhance the generation of diverse solutions. Results were analyzed using MOE (Chemical Computing Group, Montreal, Canada) interaction fingerprinting. To account for the large conformational flexibility caused by the high number of rotatable bonds in BIIE0246, the docking results were only filtered using experimental results and known contacts. To confirm the initial docking results, docking the compounds was also carried out using RosettaLigand.[36] Initially, ligand conformations were generated by OpenEye Omega.[37] After the calculation of partial charges, these results were used to create parameter files for use with ROSETTA. Docking was performed by defining the same general area used in the GOLD docking while allowing for 5 Å of translational movement and 360° of rotational movement. After generation of 50 000 docking poses per ligand, these poses were clustered using the select_best_unique_ligand_poses application. The output was filtered for viable solutions on the basis of experimental results.

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Keywords: antagonists · binding models · G protein-coupled receptors · neuropeptide Y

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