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Site-directed mutagenesis at the human B_2 receptor and molecular modelling to define the pharmacophore of non-peptide bradykinin receptor antagonists

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Abstract

Combining site-directed mutagenesis with information obtained from molecular modelling of the bradykinin (BK) human B_2 receptor (hB_2R) as derived from the bovine rhodopsin crystal structure [Science 289 (2000) 739], we previously defined a putative binding mode for the non-peptide B_2 receptor antagonists, FR173657 and LF16-0687 [Can J Physiol Pharmacol 80 (2002) 303]. The present work is aimed to define the specific role of the quinoline moiety in the pharmacophore of these non-peptide antagonists. The effect of the mutations I110A, L114A (TM, transmembrane 3), W256A (TM6), F292A, Y295A and Y295F (TM7) was evaluated. None of the mutations affected the binding interaction of peptide ligands: the agonist BK and the peptide antagonist MEN 11270. The affinities in competing for [^{3}H]-BK binding and in blocking the BK-induced IP production by the non-peptide antagonists LF16-0687 and FR173657 at the wild type and mutant receptors were analysed. While the affinities of LF16-0687 and FR173657 were crucially decreased at the I110A, Y295A, and Y295F mutants, the W256A mutation affected the affinity of the LF16-0687 only. The important contribution of the quinoline moiety was shown by the inability of an analogue of LF16-0687, lacking this moiety, to affect BK binding at the wild type receptor. On the other hand, the benzamidine group did not interact with mutated residues, since LF16-0687 analogues without this group or with an oxidated benzamidine displayed pairwise loss of affinity on wild type and mutated receptors. Further differences between FR173657 and LF16-0687 were highlighted at the I110 and Y295 mutants when comparing binding (pK_i) and functional antagonist (pK_B) affinity. First, the I110A mutation similarly impaired their binding affinity (250-fold), but at a less extent the antagonist potency of FR173657 only. Second, both the hydroxyl and the phenyl moieties of the Y295 residue had a specific role in the LF16-0687 interaction with the receptor, as demonstrated at the Y295F and Y295A mutants, respectively, but not in that of FR173657. Present data identify a receptor binding pocket comprised among TM3, 6, and 7, which concerns the interaction of the non-peptide antagonists FR173657 and LF16-0687, but not that of the peptide agonist or antagonist. Results indicate the quinoline group as the involved pharmacophoric element, and that the studied residues are differently involved in the interaction. The analysis performed by means of the GRID software led us to propose different spatial orientations of the quinoline moieties and partially overlapping binding pockets for the two ligands: that of LF16-0687 is located in the lipophilic environment amongst I110 (TM3), W256 (TM6), and Y295 (TM7) residues, whereas that of FR173657 lies essentially between I110 and Y295.

Keywords: Binding site; G protein-coupled receptor; Molecular modelling; Non-peptide antagonists; Site-directed mutagenesis

1. Introduction

BK (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) is a vasoactive peptide which mediates vasodilation, increase in vascular permeability, smooth muscle contraction, recruitment of inflammatory cells, induction of pain and

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Abbreviations: TM, transmembrane; GPCR, G protein-coupled receptor; IP, inositol phosphate; hB_2R, human B_2 receptor; BK, bradykinin; CHO, Chinese Hamster ovary; DHFR, dihydrofolate reductase.

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hyperalgesia [3,4]. The effects of BK are mediated by the B_2 receptor which has been sequenced and belongs to the superfamily of GPCRs [5,6]. Because of its role in mediating pain and inflammation, a number of potent and selective non-peptide antagonists for the BK B_2 receptor have been identified in recent years [7]. By means of a site-directed mutagenesis approach it was previously shown that binding sites of peptide agonists and antagonists at the human and rat B_2 receptors are partially overlapping [8–10]. We recently defined a putative binding mode for non-peptide B_2 receptor antagonists [2], FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl]acrylamide) [11,12], and LF16-0687 (1-[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy]methyl]-phenyl]sulfonyl-*N*-[3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]propyl]-2(*S*)-pyrrolidinecarboxamide) [13]. We observed that these two ligands had a different binding behaviour when the W256 residue, located in TM6, was mutated into alanine: the affinity of FR173657 was not affected as much as that of LF16-0687. This observation led us to postulate a different orientation of the quinoline moiety of the two antagonists: that of FR173657 in a smaller lipophilic pocket between TM2 and the Y295 residue in TM7 and, in agreement with a recent study [14], that of LF16-0687 between the receptor residues W256 in TM6 and Y295 in TM7 [2].

In the present study, site-directed mutagenesis at the hB_2R was used to investigate in more detail the receptor binding site involved in the interaction with the above antagonists, and to compare them to the binding properties of the potent and selective B_2 receptor antagonist of peptidic nature MEN 11270 [15,16]. The binding affinities of FR173657 and LF16-0687 in competing for [^{3}H]-BK and their antagonist potency in blocking the BK-induced IP production were investigated at the wild type and mutant hB_2R. The newly studied mutants were I110A and L114A (TM3), F292A (TM7), and the binding and antagonist affinities were compared with the W256A (TM6), Y295A and Y295F (TM7) mutants. The importance of the quinoline moiety and its participation in the interaction with the investigated residues have also been verified by means of modified derivatives of LF16-0687.

2. Materials and methods

2.1. Stable receptor expression in CHO cells

Large scale preparation of vector DNA for transfection experiments was carried out using a Qiagen maxi-preparation column (Qiagen). Wild type and mutated hB_2R cDNAs in pmCMVβSV1dhfr were introduced by lipofection into DHFR-deficient CHO DUKX-B11 cells. Stable DHFR$^+$ transformants were selected in nucleoside-free α-modified minimum essential medium containing 5% dialysed foetal bovine serum; 12–14 days after transfection, more than 100 individual DHFR$^+$ clones stably expressing the hB_2R were pooled, and cultured in Iscove's modified Dulbecco's medium with 2 mM L-glutamine and foetal bovine serum (10%). The cells were subcultured by using 0.25% trypsin and 1 mM ethylenediaminetetraacetate to detach them, and then cultured in 175 cm^2 flasks and maintained in a humidified atmosphere at 37° with 5% CO_2.

2.2. Membrane preparation

Cells at confluence were harvested by incubating at 37° with *N*-[2-hydroxy-ethyl]piperazine-*N*′-[2-ethanesulphonic acid] (10 mM), ethylenediaminetetraacetate 1 mM, in Hanks buffered salt solution (pH 7.4) containing a cocktail of peptidase inhibitors: 1,10-phenanthroline (1 mM), ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (1 mM), captopril, leupeptin, soybean trypsin inhibitor, D,L-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (1 μM each), chymostatin (3.3 μM), phenylmethyl-sulphonyl fluoride (0.1 mM), and bacitracin (140 μg mL^{-1}). Cells were then washed in *N-tris*[hydroxymethyl]methyl-2-aminoethanesulphonic acid (10 mM, pH 7.4, at 4°), containing the above described peptidase inhibitors cocktail, and homogenized with a Polytron (PT 3000, Kinematica), set at 15,000 rpm for 30 s. Homogenate was centrifuged at 45,000 *g* for 45 min (4°). The pellet was resuspended to obtain 7.5 mg mL^{-1} membrane protein concentration and was frozen immediately in 1 mL aliquots by immersion in liquid nitrogen, and then stored at −80° until use. The protein concentration was determined by the method of Bradford [17] using a Bio-Rad kit. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

2.3. Radioligand binding

The buffer used for binding experiments was *N-tris*-[hydroxymethyl]methyl-2-aminoethanesulphonic acid (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 μg mL^{-1}), and bovine serum albumin (1 g L^{-1}). Binding assay was performed at room temperature in a final volume of 0.5 mL, and an incubation time of 60 min was used. The chosen radioligand concentration was comparable with the calculated K_D value (0.1–0.2 nM [^{3}H]-BK). At this concentration the bound was less than 10% of the total added radioligand concentration. In preliminary experiments we observed that the specific binding of [^{3}H]-BK was directly proportional to membrane protein concentration (data not shown), and a concentration of 80–100 μg mL^{-1} was chosen. The specific binding represented approximately 70–80% of the [^{3}H]-BK total binding. Competing ligands were tested in a wide range of concentrations (1 pM–10 μM). Non-specific

binding was defined as the amount of labelled ligand bound in the presence of 1 μM BK. Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard Instrument Co), pre-soaked for at least 2 hr in polyethylenimine 0.6%, and using a MicroMate 96 Cell Harvester (Packard Instrument Co). The tubes and filters were then washed five times with 0.5 mL aliquots of Tris buffer (50 mM, pH 7.4, 4°). Filters were dried and soaked in Microscint 40 (Packard Instrument Co), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Co).

2.4. Inositol phosphate measurements

Cells grown on 24-well plates were labelled with *myo*-[1,2-^{3}H] inositol (0.5 mL per well, 1 μCi mL^{-1}) for 24 hr in Iscove'modified Dulbecco's medium and Ham's F12 medium (1:1) containing dialysed foetal bovine serum (1%) and L-glutamine (2 mM). After a 15 min preincubation period at 37° in IP buffer consisting of PBS Ca^{2+}/Mg^{2+} free (135 mM), HEPES (20 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), EGTA (1 mM), glucose (11.1 mM), BSA 0.05%, and LiCl (25 mM) (IP buffer) cells were incubated for 30 min at 37° in 0.5 mL of IP buffer added with different concentrations of agonist. Antagonists were added 15 min prior to stimulation with the agonist. Each experiment was carried out in triplicate. The reaction was stopped by 1 mL of ice-cold mixture of methanol and HCl 0.1 N (1:1, v/v), and samples were applied to a Bio-Rad AG1X8 column. The columns were washed twice with 6 mL of ammonium formate (0.06 M) in sodium tetraborate (0.005 M) to remove free inositol. After these washing steps, the total [^{3}H]-IP was eluted twice with 3 mL aliquots of ammonium formate (1.2 M) in formic acid (0.1 M). The radioactivity in the eluates was determined by a liquid β-scintillation counter (2200 CA, Packard).

2.5. Data analysis

All values in the text, tables or figures are mean and 95% confidence limits (cl), or mean ± SE mean of the given number of experiments.

Competition binding data and concentration–response curves for IP production were fitted with the GraphPad Prism 3.0 program in order to determine the −log of the inhibitory affinity constant (pK_i) for competition experiments, or the agonist molar concentration producing 50% of maximal effect (EC_{50}) for IP production bioassay.

In functional experiments (IP production) responses to BK either in the absence or presence of antagonist were normalized towards the maximal effect of control BK.

The apparent affinity of antagonists was expressed as apparent pK_B calculated from the equation: $pK_B = \log [CR - 1] - \log [\text{antagonist concentration}]$ where CR is the ratio of equiactive concentrations of agonist in the presence and absence of antagonist. Estimates of $\log [CR - 1]$ were plotted against the log of antagonist concentration, and the linearity and slope determined (Schild plot) [18].

2.6. Molecular modelling for receptor and pharmacophore definition

The hB_2R model was obtained as previously described [2]. Briefly, the starting point was the crystallographic structure of bovine rhodopsin receptor [1]. Residues were mutated according to the proper hB_2R sequence [5], and their side chains positions refined. Then an analysis was performed on receptor aminoacid residues within the TM segments, and presumed facing inward, by means of the GRID software [19] with water, hydrophobic, and amphiphilic probes, thus defining lipophilic pockets: a larger one among the TM helices 3, 4, 5, and 6, and a smaller one among the TM helices 2, 3, and 7. On this basis, candidate hB_2R residues with possible involvement in non-peptide ligand binding were targeted for site-directed mutagenesis, initially changing the residues to alanine [2]. The conformation of the 1-[[2,4-dichloro-3-[(X-quinolin-8-yl)oxy]methyl]-phenyl] moiety, in which X = 2-methyl for FR173657 and X = 2,4-dimethyl for LF16-0687, has been modelled *ab initio* with Spartan Pro (Wavefunction).

2.7. Materials

[^{3}H]-BK (specific activity 90 Ci mmol^{-1}) and *myo*-[1,2-^{3}H] inositol (specific activity 74.7 Ci mmol^{-1}) were provided by Perkin Elmer New England Nuclear. BK was obtained from Neosystem, Thiorphan from Bachem, D,L-2-mercaptomethyl-3-guanidoethylthiopropanoic acid was from Calbiochem. All salts used were purchased from Merck. Other materials were obtained from Sigma. B_2 receptor antagonists were synthesized in Menarini Ricerche. Non-peptide ligands were dissolved in dimethylsulphoxide up to 100 μM. All compounds were stored at −25°.

3. Results

3.1. Binding affinity and activity at the wild type and mutant hB_2Rs

Competition experiments at the [^{3}H]-BK binding site at the wild type or mutant hB_2Rs did not reveal significant differences in affinity for the endogenous ligand BK or the peptide antagonist MEN 11270 (Table 1).

Functional studies with CHO cells expressing either wild type or mutant B_2 receptors were carried out, and no differences in basal levels of IP formation were found (data not shown). BK produced a concentration-dependent IP accumulation in all mutant receptors and no differences

Table 1
Binding and activation properties of the wild type and mutant hB_2Rs

hB_2R	TM	[^{3}H]-BK binding, pK_i (95% cl)		IP accumulation, pEC_{50} nM (95% cl) BK
		BK	MEN 11270	
Wild type		9.3 (9.2–9.4)	9.1 (8.9–9.2)	9.1 (8.9–9.2)
I110A	3	9.8 (9.7–9.8)	9.2 (9.1–9.2)	9.2 (9.2–9.3)
L114A	3	9.5 (9.4–9.6)	9.6 (9.5–9.6)	9.2 (9.1–9.2)
W256A	6	9.6 (9.4–9.8)	9.2 (9.1–9.2)	8.9 (8.7–9.2)
F292A	7	9.4 (9.3–9.5)	9.6 (9.5–9.6)	8.9 (8.6–9.1)
Y295A	7	9.2 (9.1–9.2)	8.7 (8.6–8.9)	8.4 (7.9–8.9)
Y295F	7	9.5 (9.4–9.6)	8.7 (8.6–8.9)	9.0 (8.7–9.4)

The binding affinity (pK_i) of peptide ligands (the agonist BK and the antagonist MEN 11270) was measured in competition experiment performed at the [^{3}H]-BK binding site to membrane preparation of CHO cells (see Section 2) stably expressing the wild type or mutant hB_2Rs. Data result from the analysis of 3–4 independent experiments, each one performed in duplicate. pEC_{50} values refer to BK-induced IP production on whole cells (see Section 2). Data result from the analysis of 3 experiments, each one performed in triplicate. TM: transmembrane helix.

Table 2
Binding and antagonist affinity of the non-peptide ligands FR173657 and LF16-0687 at the wild type and mutant hB_2Rs

hB_2R	TM	[^{3}H]-BK binding, pK_i (95% cl)		BK induced IP accumulation, pK_B ± SEM	
		FR173657	LF16-0687	FR173657	LF16-0687
Wild type		7.8 (7.6–7.9)	9.2 (9.2–9.3)	8.7 ± 0.03	8.8 ± 0.03
I110A	3	5.4 (5.4–5.5)*	6.7 (6.6–6.7)*	7.2 ± 0.3*	6.4 ± 0.2*
L114A	3	8.4 (8.3–8.5)	9 (9–9.1)	8.9 ± 0.1	8.9 ± 0.1
W256A	6	7.6 (7.5–7.7)	8.2 (8.1–8.3)*	8.4 ± 0.4	7.9 ± 0.2*
F292A	7	8.4 (8.3–8.4)	9.2 (9.2–9.3)	n.d.	n.d.
Y295A	7	<6*	7.3 (7.2–7.4)*	6.3 ± 0.2*	6.8 ± 0.1*
Y295F	7	<6*	7.8 (7.7–7.8)*	5.9 ± 0.1*	8.1 ± 0.2*

Binding affinity, expressed as pK_i, was calculated from ligand competition curves at the [^{3}H]-BK binding to membrane preparation of CHO cells (see Section 2) stably expressing the wild type or mutant hB_2Rs. Reported values are obtained from the analysis of 3–4 independent experiments, each one performed in duplicate. Concentration–response curves to BK in IP production were performed on whole cells (see Section 2) in the absence and in the presence of the antagonist, added with a preincubation period of 15 min. Antagonist affinity is expressed as apparent pK_B calculated from the equation $pK_B = \log [CR - 1] - \log$ [antagonist concentration], where CR is the ratio of equiactive concentrations (EC_{50}) of agonist calculated from curves performed in the presence or absence of antagonist. pK_B values are obtained from 3 to 4 independent experiments, each one performed in triplicate. TM: transmembrane helix; n.d.: not determined.

*$P < 0.05$ vs. wild type.

in BK potency (evaluated as pEC_{50}, Table 1) were measured. The only exception was the Y295A mutant, where BK potency was reduced by 8-fold (Table 1).

Non-peptide antagonists competed for [^{3}H]-BK binding site at the wild type hB_2R with affinity values (pK_i) significantly different, FR173657 affinity being 25-fold lower than that of LF16-0687 (Table 2, Fig. 1). On the other hand, in functional experiments the concentration–response curve to BK was similarly shifted to the right by the two non-peptide antagonists when used at different concentrations (Fig. 2A and C). The Schild plot analysis (Fig. 2B and D) was consistent with a competitive antagonism the slopes being -0.97 ± 0.08 and -1.08 ± 0.03 for LF16-0687 and FR173657, respectively, and their affinity, calculated as apparent pK_B being 8.8 ± 0.03 (N = 9) and 8.7 ± 0.03 (N = 9) (Table 2).

The binding affinity and antagonist potency of FR173657 and LF16-0687 were evaluated at mutant hB_2Rs. L114A and F292A mutations did not reduce the affinity of both the non-peptide antagonists FR173657 and LF16-0687 (Table 2). The mutation W256A caused a 10-fold decrease in binding affinity, as compared to the wild type receptor, of the only LF16-0687 whereas no significant differences were obtained with FR173657 (Table 2). In the IP production assay both LF16-0687 and FR173657 at 100 nM concentration shifted to the right the concentration–response curve to BK. The FR173657

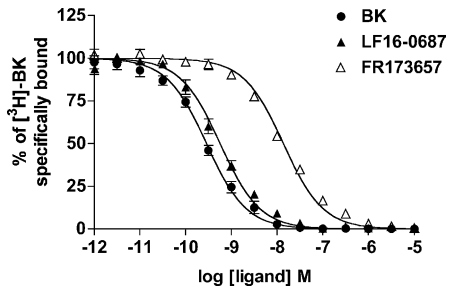


Fig. 1. Homologous and heterologous competition binding curves for BK and non-peptide antagonists FR173657 and LF16-0687. Experiments were performed in membrane preparations from stably transfected CHO cells using tritiated BK as radioligand, as described in Section 2. Values are the means ± SEM from 3–4 independent experiments.

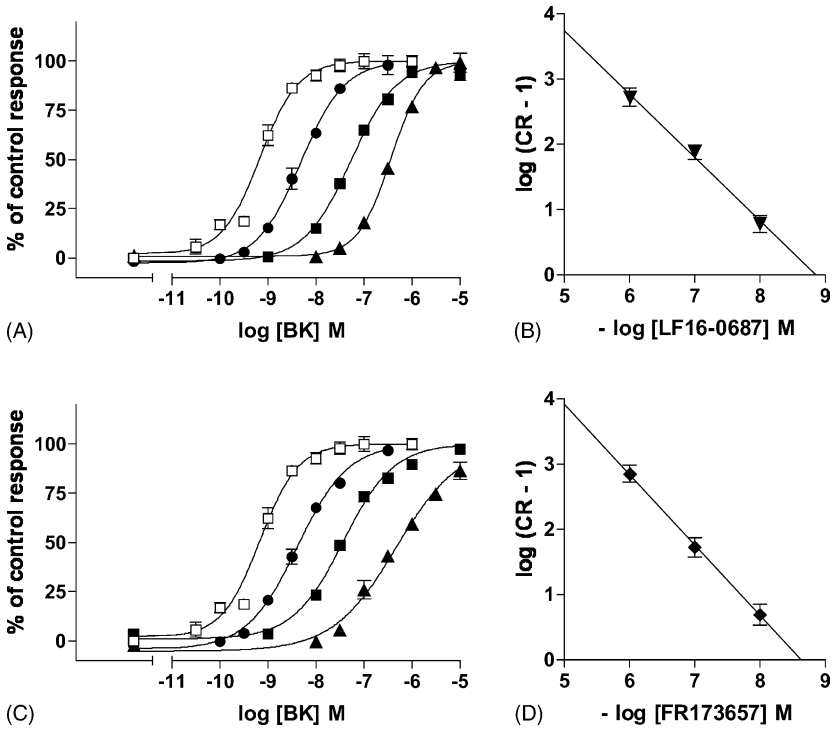


Fig. 2. Comparison of antagonist activity of LF16-0687 and FR173657 at the wild type hB_2R. Concentration–response curves to BK on IP production in the absence (control, □) and presence of different concentrations of the non-peptide antagonists: LF16-0687 (A) and FR173657 (C) were preincubated 15 min before the agonist at 10 nM (●), 100 nM (■) and 1000 nM (▲) concentration. Corresponding Schild plot of LF16-0687 (B) and FR173657 (D) the log (concentration ratio-1) was plotted against the indicated antagonist concentrations. The slope of the linear regression was not statistically different from the unity (see text). Values are the means ± SEM from three independent experiments each one performed in triplicate.

antagonist affinity, calculated as pK_B value, was unchanged as compared to that measured at the wild type receptor, but that of LF16-0687 was decreased by 10-fold (Table 2).

On the contrary, the I110A mutation severely impaired the binding affinity of both FR173657 and LF16-0687, with pK_i values decreased by 250- and 316-fold, respectively (Table 2). When both non-peptide antagonists were tested for their capability to inhibit BK-induced IP accumulation on the cells expressing the I110A mutant receptor, a rightward shift of the concentration–response curve to BK was significant only at 10 μM antagonists concentration. In these experimental conditions pK_B values of FR173657 and LF16-0687 were 7.2 and 6.4, respectively, resulting 40- and 400-fold less potent than at the wild type (Table 2).

A loss in affinity for both non-peptide antagonists resulted also from binding experiments performed at the Y295F and Y295A mutants. FR173657 1 μM concentration was able to inhibit by 21 ± 5% and 10 ± 2% the [^{3}H]-BK binding at the Y295A and Y295F mutant receptors, respectively. LF16-0687 inhibited the BK binding in a concentration-dependent manner and the calculated affinity values were reduced, as compared to the wild type receptor, by 25-fold at the Y295F (pK_i 7.8) and 80-fold at the Y295A mutant (pK_i 7.3) (Table 2). Similar results were obtained in IP production functional experiments: non-peptide antagonists concentrations necessary to obtain a rightward shift of the BK concentration–response curve at the Y295F and Y295A mutant receptors were higher than those at the wild type receptor. At 10 μM FR173657 similarly shifted (by 9-fold) the BK concentration–response curve at both mutants, the calculated pK_B values being 5.9 and 6.3 (Table 2). On the other hand LF16-0687 1 μM concentration shifted by 64-fold the BK concentration–response curve, as compared to the control, in cells expressing the Y295F mutant; whereas in cells expressing the Y295A mutant receptor LF16-0687, at a higher concentration (10 μM), produced a shift of only 22-fold thus yielding significantly reduced estimates of pK_B values (8.1 and 6.8, at Y295F and Y295A, respectively, Table 2).

In a further set of binding experiments the affinity of LF16-0687 was compared to that of some derivatives whose structures have been reported in Table 3 together with their affinity values (pK_i). At the wild type receptor the compound with a neutral amide in place of the positive charged amidine group (MEN 13307) was 40-fold less potent than LF16-0687. The compound lacking the *p*-amidine-benzoic acid moiety (MEN 14506) was 25-fold less potent, while the one lacking the *p*-amidine-benzoxy-dipropylendiamide (MEN 14620) was 500-folds less potent, and the compound lacking the 2,4-dimethyl-quinolin-8-yl-oxy group (MEN 14614) was 15,000-fold less potent than LF16-0687 itself (Table 3). The affinity

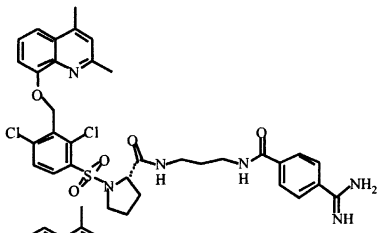
Table 3
Binding affinities at the wild type and mutant hB_2Rs of LFI6-0687 and derivatives

hB_2R	TM	pK_i (95% cl)				
		LF16-0687	MEN 13307	MEN 14506	MEN 14620	MEN 14614
Wild type		9.2 (9.2–9.3)	7.6 (7.5–7.8)	7.8 (7.7–8.0)	6.5 (6.4–6.6)	<5
I110A	3	6.7* (6.6–6.7)	<5*	<5*	<5*	n.d.
L114A	3	9 (9–9.1)	7.8 (7.7–7.9)	7.8 (7.5–8.0)	6.3 (6.3–6.5)	n.d.
W256A	6	8.2* (8.1–8.3)	6.4* (6.3–6.6)	6.8* (6.7–6.8)	<5*	n.d.
F292A	7	9.2 (9.2–9.3)	7.4 (7.3–7.6)	7.9 (7.8–8.0)	6.6 (6.5–6.7)	n.d.
Y295A	7	7.3* (7.2–7.4)	5.6* (5.4–5.9)	6.0* (5.8–6.1)	<5*	n.d.
Y295F	7	7.8* (7.7-7.8)	5.5* (5.3-5.7)	5.9* (5.8-6.1)	<5*	n.d.

Competition binding experiments were performed at the [^{3}H]-BK binding to membrane preparation of CHO cells (see Section 2) stably expressing the wild type or mutant hB_2Rs. Affinity values are reported as pK_i obtained from the analysis of 3–4 independent experiments, each one performed in duplicate; TM: transmembrane helix; n.d.: not determined; $^*P < 0.05$ vs. wild type.

Structure of ligands

LF16-0687



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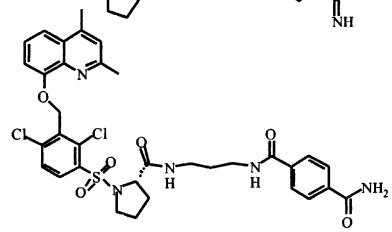

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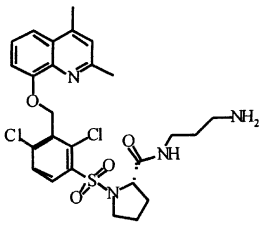

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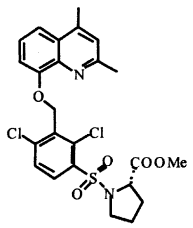
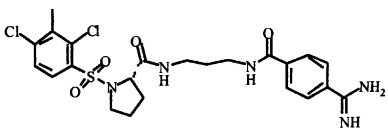


MEN 14614



values of the above LF16-0687 derivatives were compared also at the investigated mutant receptors: results reported in Table 3 highlight that receptor mutations which impaired the LF16-0687 affinity all impaired the binding affinity of MEN 13307, MEN 14506, MEN 14620, and MEN 14614.

3.2. Molecular modelling and the non-peptide antagonists FR173657 and LF16-0687

The hB_2R model previously obtained placed the residues L114 and I110 (on TM3) in close proximity of W256 (TM6) and Y295 (TM7). Since the present experimental

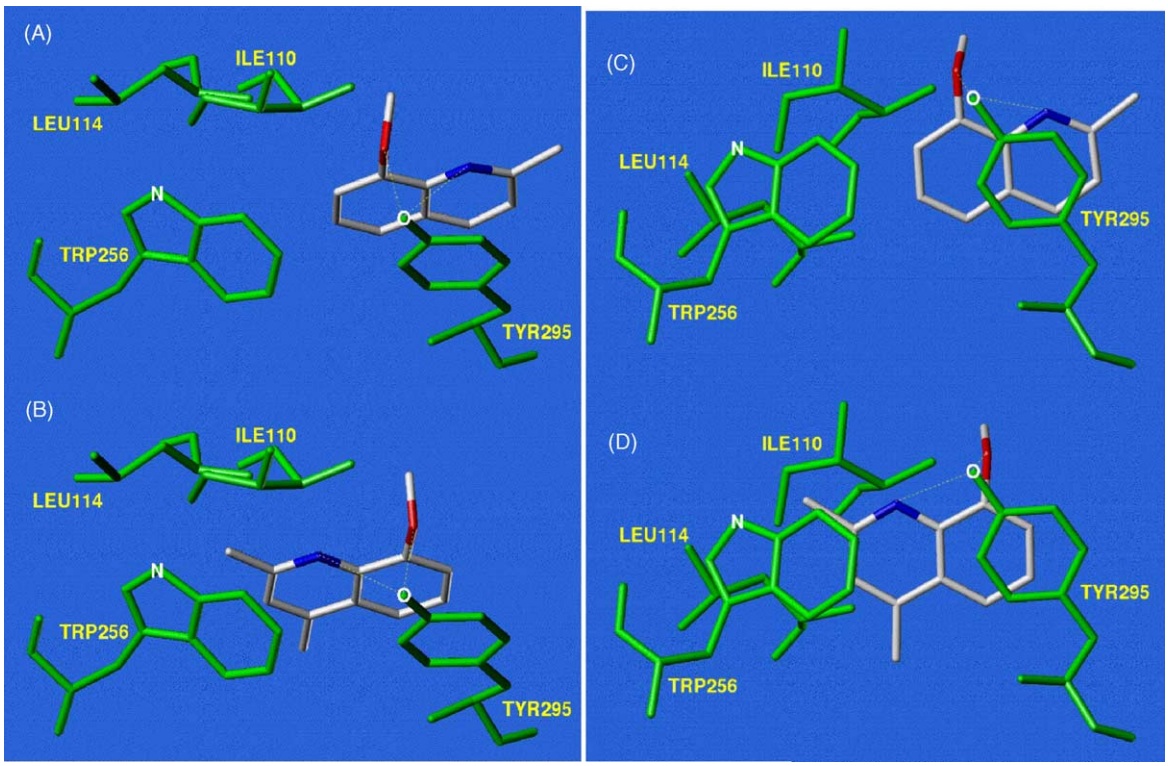


Fig. 3. Model of the quinoline pharmacophore groups of FR173657 and LF16-0687 docked to their recognition site in the wild-type hB_2R. Left panels represent the models viewed from the extracellular side, and right panels the side views of the same binding modes. Transmembrane helices have been deleted to aid visualization. In the suggested binding modes the quinoline moiety of FR173657 (panels A and C) would interact with I110 (TM3) and Y295 (TM7) residues, whereas that of LF16-0687 (panels B and D) would interact with W256 (TM6) as well. The suggested electrostatic/hydrogen bond interactions with the –OH group of Y295 are indicated by dotted lines. For the sake of simplicity receptor residue side-chains are shown in the same positions for both ligands: we can clearly expect that upon binding these side-chains will find their best arrangement in respect to optimal ligand interaction.

results indicated that I110 residue is part of the binding site of the two non-peptide antagonists but not L114, we modified in the TM3 position the receptor model. Therefore, TM3 was shifted one helix turn towards the intracellular side thus placing I110 in front of W256 and L114 out of the binding site for the quinoline moieties. The resulting quinoline pharmacophore group arrangements for the two ligands are depicted in Fig. 3. The suggested model considers electrostatic/hydrogen bond interactions of the quinoline nitrogen and the proximal ether oxygen of the two antagonists with the hydroxyl function of Y295 receptor residue in TM7. In order to allow the quinoline ring of LF16-0687 to have a lipophilic interaction with both W256 and I110 receptor residues, we oriented it differently from that of FR173657.

4. Discussion

Data presented in this study highlight a difference between the binding sites of non-peptide ligands and BK at the hB_2R. In fact, the investigated point-mutations, all located in the middle of TMs structure, neither affected the binding of the peptide hormone BK nor its capability to trigger receptor activation. The fact that BK can bind normally and activate the signal transduction indicates that the overall structure of the considered mutant hB_2Rs is not perturbed. Possible interaction points for BK [8–10] had been previously identified which are located superficially in the receptor structure: a close proximity of the N-terminus of BK to the second and third extracellular loop, an interaction of the C-terminus of BK with acidic residues in the fourth extracellular loop, and a further interaction with residues at top of TM3, 4, and 6. In the present study, we have included also the peptide antagonist MEN 11270 [15,16] whose affinity is not modified by the studied mutations. Contrary to peptidic ligands, our results highlight that receptor residues comprised among TM3 (I110), 6 (W256), and 7 (Y295) selectively impair the binding affinity of the two non-peptide ligands FR173657 and LF16-0687. It is worth mentioning that the non-peptide ligands conserved their antagonist profile at all mutated receptors. This observation, together with the observed homogeneous basal activity (IP assay) of the mutants vs. the wild type hB_2R, indicates that none of the studied mutations altered the receptor conformational state into a constitutively activated one [20]. Additionally, we performed an analysis of binding affinity of modified derivatives of LF16-0687 (MEN 13307, MEN 14506, MEN 14620, and MEN 14614, structures in Table 3) which

overall indicates firstly the essential role of the quinoline group in the design of this class of antagonists, as demonstrated by the dramatic loss of affinity (>10^4-fold) of MEN 14614, and secondly the important role of the positive charge of benzamidine, as demonstrated by the drop in affinity (40-fold) observed with MEN 13307, which maintains an antagonist profile (pK_B 7.5, data not shown). The fact that all the LF16-0687 derivatives maintaining the quinoline moiety in their structure (Table 3), undergo an analogous drop in affinity, as LF16-0687 itself, at the W256A, I110A, Y295A and Y295F mutants, indicates that the dominant structural part they share, i.e. the quinoline group, interacts with the mutated receptors residues.

It should be noted that the binding affinity and antagonist potency of LF16-0687 at the wild type hB_2R are not significantly different from each other (pK_i 9.2, pK_B 8.8), whereas the antagonist potency of FR173657 is about 10-fold higher than its measured binding affinity (pK_i 7.8, pK_B 8.7). This discrepancy occurs despite the remarkable structural overlapping between the two non-peptide antagonists. We do not have presently a satisfactory explanation to account for this observation. Discrepancies in apparent affinity values have been already disclosed in other peptide GPCRs when agonist affinities were compared by means of heterologous vs. homologous competition binding experiments [21–23]. Several explanations have been advanced including (1) presence of receptor subtypes, (2) multiple binding sites on the same receptor, and (3) putative receptor conformers [24]. Recent data [25,26] indicate that receptor interaction with intracellular proteins (pre-coupling with different G-proteins or proteins involved in receptor desensitization) can be responsible for variable agonist binding affinities measured at certain peptide GPCRs, thus providing evidence that an interchanging between different molecular receptor phenotypes, or conformers, can be responsible for the agonists complex pharmacology. However, no example of this type has been thus far reported for non-peptide antagonists. Notably, the results of our mutational analysis indicate that the binding site of the two antagonists at the hB_2R is largely overlapping, yet several data indicate both qualitative and quantitative differences in their binding mode (see below). The possibility that different antagonist bound receptor conformers may account for the observed differences of FR173657 remains speculative at the present. In particular, discrepancies were highlighted by the I110A mutation, which similarly and drastically hampers the binding and antagonist potency of LF16-0687 (250–300-fold), whereas FR173657 binding affinity drops more (250-fold) than its antagonist affinity (30-fold) compared to the wild type receptor. Thus, the difference observed at the wild type receptor with the only FR173657 is preserved by this mutation and even magnified, which *per se* may suggest that the ligand-inactive receptor conformation complex is less impaired by the I110A mutation. A further difference between the non-peptide antagonist ligands was evidenced by experiments at the Y295A and Y295F mutants: FR173657 affinity was severely impaired at a similar extent by the two aminoacidic substitutions, whereas the affinity of LF16-0687 was differently affected. In particular LF16-0687 affinity is decreased by 10–25-fold at Y295F mutant and 100-fold at Y295A. These data may suggest that both the hydroxyl and phenyl moieties of the Y295 residue are involved in the receptor recognition site of this ligand. Last, both binding and functional experiments show that the affinity of LF16-0687, but not of FR173657, is slightly decreased (10-fold) at the W256A mutant, thus suggesting a participation of this residue in the binding site of the only LF16-0687. It can be hypothesized that the inability of the FR173657 compound to interact with the W256 residue could account for its lower affinity. We clearly cannot exclude the possibility that the ligand–receptor interactions could have been affected by other mutations, besides those cited in the present study.

Several biophysical and mutagenesis studies provided evidence that the activation of GPCRs involves rearrangements of TM3, 6 and 7 [27]. In this perspective it is intriguing that some of the residues which are differently involved in the receptor interaction with FR173657 and LF16-0687, as W256 in TM6, and Y295 in TM7, have been suggested as part of a network of residues which controls the balance between active and inactive conformations of the hB_2R [14].

The differences raised with mutagenesis data and the analysis performed by the GRID software for the best placements of the quinoline in the TM region of the hB_2R let us to suggest a different orientation of the quinoline moieties of FR173657 and LF16-0687 (as depicted in Fig. 3). Data suggest that I110 (but not L114) should be close to both W256 and Y295 residues, and that these residues make part of a binding crevice for LF16-0687 and FR173657. Although we cannot exclude the participation of other receptor residues in the receptor recognition site for these antagonists, we can state that they exploit a binding pocket which is at least partially overlapping. Alternatively both ligands could bind to the same area but with different interaction with residues, as it has been shown for homologous receptors [28]. In the present study we propose that the quinoline pharmaphoric element of the non-peptide antagonists might interact in a lipophilic pocket comprised among TM3, 6 and 7, with different orientations: that of LF16-0687 amongst the I110, W256, and Y295 residue and the quinoline of FR173657 between I110 and Y295.

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