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Binding modes of dihydroquinoxalinones in a homology model of bradykinin receptor 1

Sookhee N. Ha [a,*], Pat J. Hey [b], Rick W. Ransom [b], C. Meacham Harrell Jr. [b], Kathryn L. Murphy [b], Ray Chang [b], Tsing-Bau Chen [b], Dai-Shi Su [b], M. Kristine Markowitz [b], Mark G. Bock [b], Roger M. Freidinger [b], Fred J. Hess [b]

[a] *Basic Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA*
[b] *Department of Medicinal Chemistry and Neuroscience, Merck Research Laboratories, West Point, PA 19486, USA*

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Abstract

We report the first homology model of human bradykinin receptor B1 generated from the crystal structure of bovine rhodopsin as a template. Using an automated docking procedure, two B1 receptor antagonists of the dihydroquinoxalinone structural class were docked into the receptor model. Site-directed mutagenesis data of the amino acid residues in TM1, TM3, TM6, and TM7 were incorporated to place the compounds in the binding site of the homology model of the human B1 bradykinin receptor. The best pose in agreement with the mutation data was selected for detailed study of the receptor–antagonist interaction. To test the model, the calculated antagonist–receptor binding energy was correlated with the experimentally measured binding affinity (K_i) for nine dihydroquinoxalinone analogs. The model was used to gain insight into the molecular mechanism for receptor function and to optimize the dihydroquinoxalinone analogs.

Keywords: Bradykinin receptor; B1; TM; Bovine rhodopsin; Dihydroquinoxalinone; Homology model; Site-directed mutagenesis

Kinins are pro-inflammatory peptide agonists with eight to 10 amino acids. They are released in response to tissue injury, and elicit pain and inflammation. There are two types of kinin receptors, B1 and B2 [1]. The carboxypeptidase metabolites desArg^9-BK and desArg^{10}-KD act on the B1 receptor. The B1 receptor consists of 353 amino acids with seven helix transmembrane domain, which is a hallmark of GPCR. Many studies indicate that the B1 receptor is involved in inflammatory and pain response [2,3], hyperalgesia, and leukocyte infiltration through activation of a cytokine network [4–6]. Therefore, developing an antagonist against B1 holds great potential for treating pain and inflammation. After the discovery of the first class of peptide antagonists selective in B1 receptor in the late 1970s [7,8], modified analogs of this series have been reported with further improvements [9–14]. The prototype of this peptide compound is an analog of desArg^{10}kallidin with Phe9 changed to Leu [15]. However, the progress of developing non-peptide B1 receptor antagonists has been relatively slow and it is only recently that these efforts began to make significant progress. Recently, some B1 non-peptide receptor antagonists have been reported [16–18]. In preceding work, we reported a series of potent dihydroquinoxaline compounds which are non-peptidic and highly selective for the B1 receptor [19–21].

We describe here the first homology model of human bradykinin B1 receptor in conjunction with site-directed mutagenesis to investigate the interaction of the receptor with the potent dihydroquinoxalinone antagonists

* Corresponding author.
E-mail address: sookhee_ha@merck.com (S.N. Ha).

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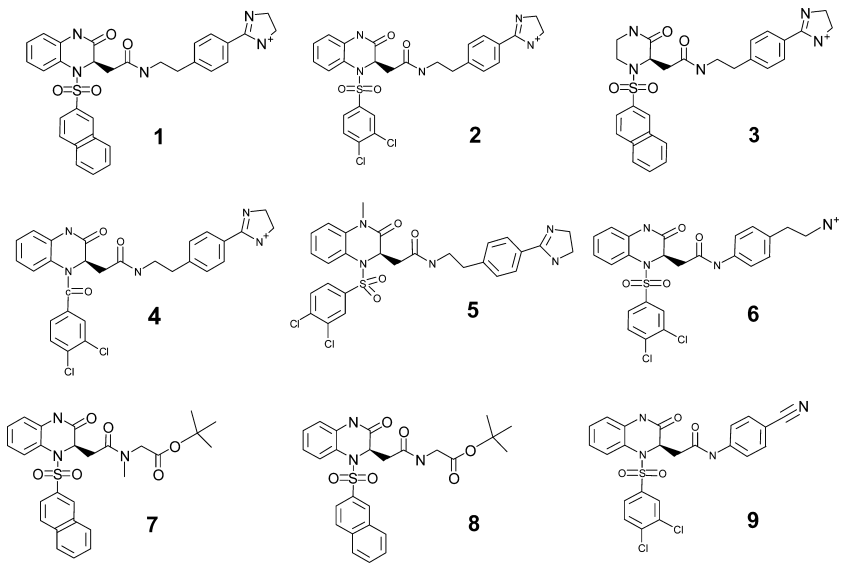

Fig. 1. Structure of the dihydroquinoxalinone compounds that were docked into the binding site of the bradykinin receptor B1 model.

Table 1
Corresponding values of the experimental B1 receptor binding affinity (K_i, in nM) and calculated interaction energy (kcal/mol) for dihydroquinoxalinone analogs

Compound	K_i (nM)	$-\log K_i$	Interaction energy
1	0.01	11.0	−39.6
2	0.03	10.5	−38.2
3	0.11	9.96	−35.5
4	0.61	9.21	−32.3
5	0.84	9.08	−31.1
6	2.6	8.59	−33.6
7	285	6.55	−30.9
8	308	6.51	−29.8
9	449	6.35	−28.8

discovered at Merck. The structures of the antagonists with their binding affinity to the B1 receptors ranging from sub-nanomolar to hundreds of nanomolar are shown in Fig. 1 and Table 1. To place the mutagenesis data in context, a molecular model of the human bradykinin B1 receptor has been generated based on the crystal structure of bovine rhodopsin [22].

Materials and methods

Mutation. Point mutations were introduced into the human B1 bradykinin receptor in pcDNA3 using Quick-Change reagents and protocol (Stratagene). Initially, additional nucleotides were mutated to provide for a silent restriction site, linked to the mutation of interest, which could be used for screening purposes. This strategy was discontinued due to the high efficiency of mutagenesis protocol. Mutations were identified by DNA sequence analysis using BigDye terminator cycle sequencing chemistry followed by analysis on an ABI 377 DNA sequencer. To eliminate any errors from PCR, the entire coding sequence of the human B1 receptor was sequenced. DNA encoding mutant B1 bradykinin receptor was transfected into either HEK293 cells by calcium phosphate or HEK293T cells by Lipofectamine2000. Membranes were prepared from transfected cells and the pharmacological profile of the mutant receptor was determined by competition of specific binding of either the peptide antagonist (^{3}H)des-Arg^{10},Leu^{9}kallidin or the agonist (^{3}H)des-Arg^{10}kallidin. Saturation binding with the appropriate radiolabeled peptide was performed so that a K_i value for each test compound could be determined using Graph-Pad Prism.

Homology model building of B1 receptor.[1] A model was generated with the homology module in MOE. A local energy minimization was carried out first for the regions of gap and insertion. A global minimization was followed to relieve the steric repulsion, while the backbones of the transmembrane helices constrained in a harmonic fashion so that the backbones of the TM region remained unchanged. The loops were further refined with the high temperature molecular dynamics simulations with Charmm and followed by simulated annealing.

Docking of the antagonists. A database of 100 different conformations of each of compound 2 and compound 4 was prepared and minimized. Each conformation was docked into the refined active site with the docking module Flog in MIX, Merck molecular modeling environment. Approximately 30 high-ranking binding conformations of each compound were visually inspected. Poses exhibiting severe steric clashes with the receptor were discounted, whereas those possessing reasonable van der Waals contacts and good polar interactions were considered as candidates. When each of the candidate poses of the docked ligands was compared, a consensus pose emerged for compound 2 and compound 4 that is consistent with the mutation data.

Interaction energy. The binding site was defined as 10 Å around the compound 2 docking pose in the B1 receptor site. Each compound was docked into the binding site in reference to the binding poses of the compounds 2 and 4, and followed by energy minimization of each compound. The compounds were allowed to move freely while the receptor held fixed during the minimization. The interaction energy between the compound and the receptor was isolated from the total energy with Nenergy routine in MIX.

[1] The coordinate of the model is available in the supplementary material.

Results

Homology modeling

An alignment of the amino acid sequences of the human B1 with the bovine rhodopsin sequence is shown in Fig. 2, with the regions of the transmembrane helices colored in blue. The B1 receptor and bovine rhodopsin belong to family A and share a series of highly conserved key residues shown in bold letters in Fig. 2. All the key residues in family A are conserved in the B1 receptor and bovine rhodopsin.

After refining the TM regions of the receptor model, the loop refinement was carried out. The simulated annealing created 10 homology models with different loop conformations. To select what we view as the most appropriate model, we took into account the size of the crevice between the transmembrane helices and the extracellular loops that is available for ligand binding.

Docking of the compounds

Docking of compounds 2 and 4 into the receptor model was carried out. All binding poses with high ranking scores [23] were visually inspected in the B1 receptor model and from these we selected the pose that agrees with the mutation and SAR data tabulated in Table 1. Fig. 3 presents a docking pose of compound 2 in the binding site of the B1 receptor viewed with a wide angle. The compound is positioned close to the extracellular region while interacting with the transmembrane helices TM1, TM3, TM6, and TM7. It is positioned as a V shape almost perpendicular to the direction of the horizontal plane of the lipid bilayer. As shown in Fig. 4, the dihydroquinoxalinone group is located deep into the pocket between TM3 and TM7 in the crevice surrounded by residues Phe302, Ile113, and Trp103, with the dichlorobenzene ring in the hydrophobic pocket surrounded by Ile97, Trp98, and Trp103 in TM1. The imidazole ring occupies the room created by the third extracellular loop between TM6 and TM7 interacting with Glu291 closely and with Asp273 from a somewhat longer distance. Compounds 2 and 4 differ in the carbonyl vs. sulfate group at the dihydroquinoxalinone ring. In our model, Compound 4 is shifted slightly towards TM3, TM1, TM7, and away from TM6, with the dihydroquinoxalinone ring being positioned deeper into the hydrophobic pocket surrounded by residues Phe302, Ile113, and Trp103. The dichlorobenzene ring is positioned closer to Ile97 and Trp98 at the entrance of the hydrophobic pocket. In addition, the carbonyl oxygen of compound 4 is moved away from Gln295 in TM6. The overlay of compounds 2 and 4 at the binding site of the B1 receptor is shown in Fig. 5 and the Connolly surface defines the shape of the binding crevice.

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    EC1                                     TM1
Br  MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMF
B1  MASSWPPLELQSSNQSQLFPQNATACDNAPEAWDLLHRVLPTFII
                       IC1      TM2              40
Br  LLIMLGFPINFLTLYVTVQHKKLRTPLNYILLNLAVADLFMVFGG
B1  SICFFGLLGNLFVLLVFLLPRRQLNVAEIYLANLAASDLVFVLGL
    EC2                    TM3                  80
Br  FTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIER
B1  PFWAENIWNQFNWPFGALLCRVINGVIKANLFISIFLVVAISQDR
    IC2              TM4                 EC3 120
Br  YVVVCKPM-SNFRFGENHAIMGVAFTWVMALACAAPPLVGWS-LL
B1  YRVLVHPMASGRQQRRRQARVTCVLIWVVGGLLSIPTFLLRSIQA
                           TM5                  160
Br  IPEGM-QCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFC
B1  VPDL-NITAC-I-LPH-ERYAWHFARIVELNILGFLLPLAAIVFF
        IC3                TM6                  196
Br  YGQLVFTVKEAAA-SAT--TQKAEKEVTRMVIIMVIAFLICWLPY
B1  NYHILASLRTREEVSRTRVRGPKDSKTTALILTLVVAFLVCWAPY
              EC4              TM7                236
Br  AGVAFYIFTHQGS--------DFGPIFMTIPAFFAKTSAVYNPVI
B1  HFFAFLEFLFQVQAVRGCFWEDFIDLGLQLANFFAFTNSSLNPVI
    IC4                                         296
Br  YIMMNKQFRNCMVTTLC-CG-KN--P-STTVSKTE
B1  YVFVGRLFRTKVWELYKQCTPKSLAPISSSHRKEI 311
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Fig. 2. Alignment of amino acid sequences of bovine rhodopsin (Br) and the human B1 receptor. TM helices are colored with blue background, extracellular loops are with gray. TM denotes transmembrane helices, EC extra cellular loops, and IC intracellular loops. Highly conserved residues are marked in bold letter and the cysteins of the disulfide bond are colored with yellow background. "-" depicts the gap.

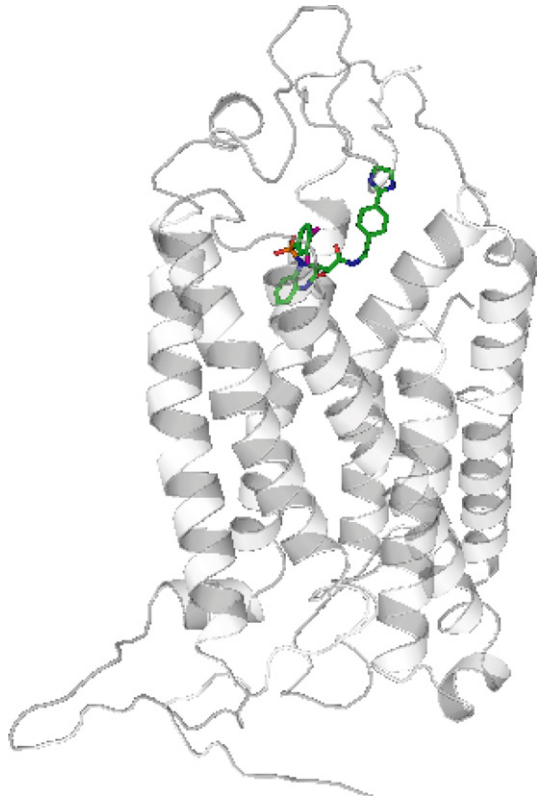

Fig. 3. Overall view of compound 2 docked into the B1 receptor model. The compound 2 is interacting mainly with TM3, TM6, and TM7.

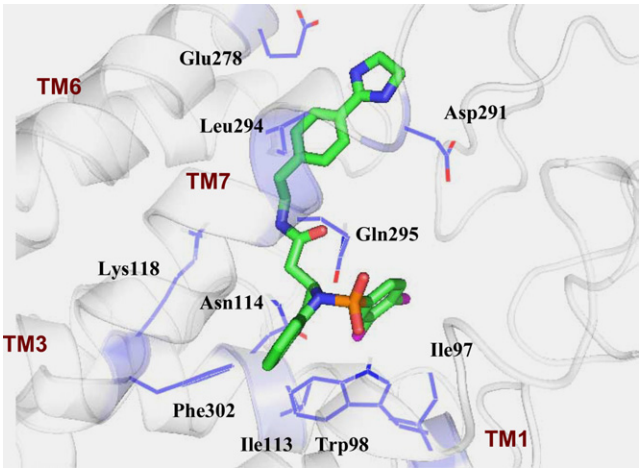

Fig. 4. Binding site of the homology model of B1 receptor with compound 2.

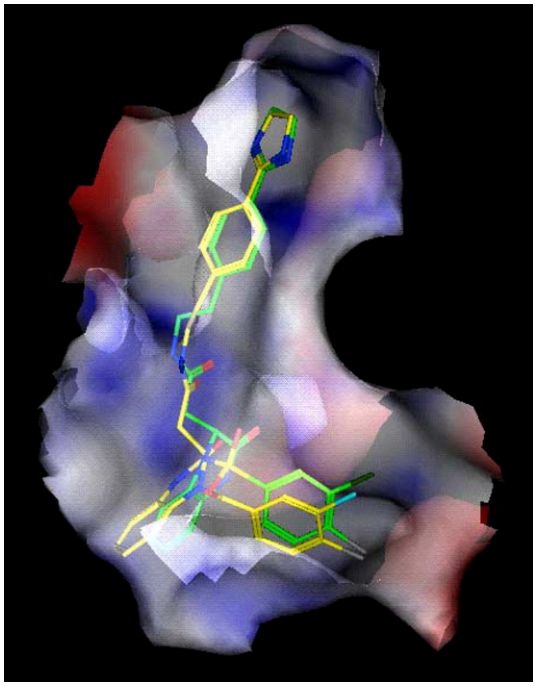

Fig. 5. Docked antagonists at the binding site of the B1 receptor model. Compound 2 is shown in green and compound 4 in yellow.

Interaction energies between the B1 receptor and the dihydroquinoxalinone analogs were calculated and correlated with their binding affinity (K_i) in Fig. 6. It is shown that the binding mode yielded a reasonable correlation between interaction energy and $-\log K_i$, although it is qualitative, this trend supports the current docking mode.

Mutations in the third transmembrane helix

The chimeric receptor of B1/B2 in the third membrane decreased affinity for BK was proposed to be

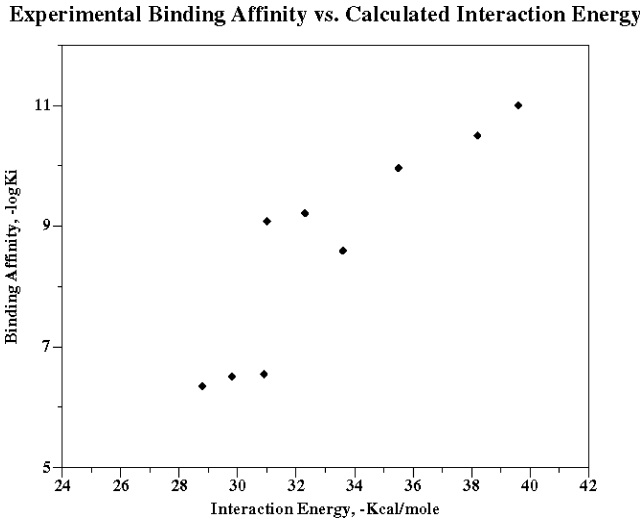

Fig. 6. Correlation between experimental binding affinity ($-\log K_i$) vs calculated binding energy (kcal/mol) for the dihydroquinoxalinone analogs, $R^2 = 0.79$.

due to the residue Lys118 in the B1 receptor for the agomists [24–26].

However, we found that binding affinities of compounds 2 and 4 do not decrease upon mutation of Lys118 to Ser or to Ala. In fact, a 5-fold increase in the binding affinity of compound 4 has been observed by changing K118 to Ser, and a 10-fold increase upon mutation of Lys118 to Ala (see Table 2). A more modest, if any, increase in the binding affinity of compound 2 was observed for these mutations. The data indicate that the role of key residues in the B1 receptor binding pocket differs in the binding of peptide agonist and the dihydroquinoxilinone antagonists.

Table 2
Site-directed mutagenesis data of compound 2 and compound 4

	Compound 2	Compound 4
wt	0.034 nM	0.6 nM
K118A	0.013	0.14
K118S	0.019	0.12
Y266A	0.037	12
Y266F	0.041	0.61
H267Q	0.0095	0.64
A270T	0.044	0.90
E273A	0.0078	4.0
D291A	0.049	12
Q295A	0.89	0.41
N298S	0.038	0.77
N114A	0.51	66
N114S	0.29	53
L294A	0.03	0.83
F302A	0.11	8.2
I97S	0.16	0.68
W98A	0.48	7.4
W98G	0.034	2.7
W98R	0.048	2.0
I113F	0.13	5.8
I113S	0.099	2.3

The binding mode for the two compounds is slightly different because of the difference in the group attached to the dihydroquinoxalinone ring. Lys118 is closer to the nitrogen of the amide linker of compound 4 providing an unfavorable interaction. Mutation of Lys118 to serine or alanine is predicted to relieve these steric and electrostatic clashes. The B1 homology model was constructed from an inactive form of bovine rhodopsin. Furthermore, as would be expected, the SAR data, mutagenesis data, and the binding modes of the B1 receptor antagonists suggest that the activated form of the receptor, stabilized by agonists, differs from the inactive form that is stabilized by antagonist binding. As the modeling poses of compounds 2 and 4 show, and the mutation data indicate, Lys118 is in close proximity to the non-peptide compounds but this residue's role in peptide agonist binding is very distinct from its role in influencing binding of the dihydroquinoxaline antagonists.

Mutation of Asn114 to Ala results in a more than 10-fold decrease in the binding affinity of both compounds 2 and 4 to the mutant receptor. Similarly, mutation of Asn114 to Ser results in 8-fold decrease in binding affinity of both compounds to the receptor. The binding poses of compounds 2 and 4 indicate that the nitrogen of the fused ring of dihydroquinoxalinone acts as a hydrogen bond donor to the oxygen of the Asn114 side chain. This finding is in good agreement with the structure activity data for compound 2 and compound 5. Compound 2 binds to the receptor with binding affinity of 0.03 nM when a free hydrogen bond at the nitrogen of the dihydroquinoxalinone is available, however, compound 5 with the methyl group attached to the same nitrogen binds to the receptor with a reduced binding affinity of 0.84 nM (see Table 2). Mutation data, together with structure activity data and binding mode in the B1 receptor model, led us to believe that Asn114 is important for binding of this series of compounds to the B1 receptor.

Mutation of the sixth transmembrane helix

Previous mutagenesis studies of the rat and human B2 receptor identified several residues in TM6 that are critical for high affinity binding of BK [27–29]. These studies indicated that the most critical residues are Phe261, Leu104, Val108, and Ile112 in TM3. We examined the effect of mutating the corresponding residues in the B1 receptor. Mutation of Tyr266 Phe did not significantly alter B1 receptor affinity for compound 2 or compound 4. In Fig. 5, one can see that Tyr266 is believed to be located around the linker between the dihydroquinoxaline group and the imidazole ring of the compound, the nature of interaction between Tyr266 and the compounds is hydrophobic and non-specific, therefore any hydrophobic residue at this position would be expected to be sufficient for binding these compounds.

The B1 receptor mutant, Ala270Thr, had no loss in binding affinity for either compound 2 or compound 4. This result is consistent with the binding mode of the compounds shown in Fig. 5, in that Ala270 is located near the binding site, however, the side chain points away, rather than towards, the compound. Mutation of His267Gln, not located in close proximity to the compounds, does not have any impact on the binding affinity of the compounds.

Amino acid residues in the seventh transmembrane helix

A study using an approach of B1/B2 chimeric receptors coupled with site-directed mutagenesis identified a few residues in the seventh transmembrane helix as playing a role in ligand binding [30]. We observed a marked decrease in binding affinity of compound 4 to the mutant Phe302Ala B1 receptor and modest decrease in affinity of compound 2. In our model, the benzene ring of the dihydroquinoxaline group occupies the hydrophobic crevice created by Phe302, Ile113, Trp103, and Phe299. Replacing the aromatic ring of Phe302 with a smaller aliphatic residue, Ala, will reduce π stacking interactions with the benzene ring of the dihydroquinoxaline. This interaction would occur to a greater extent with compound 4 than compound 2 because the ring of compound 4 is more optimally situated in the crevice, and thus be more sensitive to the Phe302Ala mutation. Indeed, the affinity of compound 4 is reduced by approximately 10-fold by this mutation, whereas the affinity of compound 2 is not affected. Further evidence that the aromatic ring interacts with Phe302 and occupies the hydrophobic pocket is also shown in the binding activities of two compounds 1 and 7 (see Table 1 and Fig. 1). The binding affinity is decreased 10-fold when the aromatic ring is not present in compound 7 compared to compound 1 with the aromatic ring at the dihydroquinoxaline.

For the second site specific mutation characterized, Leu294Ala in B1 receptor, we observed only a slight trend toward decreased affinity for compound 2 and compound 4. Therefore, Leu294 plays a minor role in the ability of the B1 receptor to bind the dihydroquinoxaline antagonists. From the docking model, Leu294 interacts non-specifically with the benzene ring adjacent to the imidazole. Change from a large hydrophobic group to smaller hydrophobic does not appear to alter the affinity of the receptor for compound 2 and compound 4.

In the mutation in the B1 receptor, Gln295Ala, there is no discernable impact of the mutation on compound 4 binding to the mutant receptor. In contrast, there was a more than 10-fold decrease in binding affinity of compound 2 to the mutant receptor. Comparing this

mutation result to the docking modes of the two compounds suggests that the sulfate oxygen actively participates in hydrogen boding with the Gln295 whereas the carbonyl oxygen is not positioned suitably to make a hydrogen bond with the side chain of Gln295. It is also well explained that the binding affinity of compound 2 is 20-fold higher than that of compound 4. As mentioned before, compound 4 is slightly tilted towards TM1, therefore, this compound is affected by the mutation of Lys118 positively, whilst compound 2 is affected negatively by the mutation of Gln295 in TM7. The difference in conformations arising from a subtle structural difference between these two compounds (sulfate vs. carbonyl) plays an important role in how they may fit into the binding pocket of the receptor, as is demonstrated by the differential effects of mutants in TM3 and TM7 on the binding of the two compounds.

The mutation, Asn298Ser, does not show much change in binding affinity towards the antagonist. The side chain of Asn298 is in close contact with the antagonists, making a hydrogen bond with the oxygen of the amide group. It is assumed that because both serine and asparagine are able to lend a hydrogen to the oxygen of the amide linker of the antagonists, the mutation of Asn to Ser will not alter the binding affinity of the antagonists to the wild type or mutant receptors. The nitrogen of the amide linker of this antagonist series does not appear to have any favorable polar contact with the receptor. Structure activity data shown in Table 1 indicate that compound 8 and compound 7 exhibit similar binding affinity to B1 wild type receptor. The structures of the two compounds only differ at the nitrogen of the amide bond linker, in that a methyl group is attached to the nitrogen of the amide in compound 7. If the amide nitrogen were hydrogen bonded to the receptor, the binding affinity of both compounds would presumably be different.

The mutations of Glu273Ala and Asp291Ala show decrease in activity of compound 4 and compound 2. The structure–activity relationship of two compounds 9 and 6 agrees with this finding that compound 9 with its partially negative nitrile group is repulsive to both acid residues and has lower affinity (449 nM) to B1 receptor than compound 6 (2.6 nM) with its partially positive amine which can interact favorably with the acids. These mutant receptors appear to show a great decrease in affinity for antagonist relative to agonist, a pattern opposite to that exhibited for the Lys118Ala mutant [31].

Other mutations based on the human B1 receptor model

The initial phase of the mutagenesis analysis, described above, built upon information derived from previous mutagenesis studies with the B2 receptor and B1/B1 receptor chimers. The data from this portion of study were incorporated into the model of the human B1 receptor that was constructed using the bovine rhodopsin crystal structure as a template. The construction of the model led to the design of an additional set of mutants that were prepared and analyzed. Three mutations impacted B1 receptor affinity for the non peptidic antagonists, Ile97Phe in TM2, Ile113F in TM3, and Trp98Ala in the TM1. The impact of binding affinity from the mutation is mainly on compound 4. The mutations of I97F and I113F seemed to create a steric hindrance on the dichlorophenyl ring of compound 4, which is optimally situated differently within the hydrophobic pocket due to the carbonyl group that is planar to thedichlorobenzene ring. Mutation of W98 in TM1 has an impact on both antagonists by presumably losing an aromatic π stacking interaction.

Discussion

To place the mutagenesis data in context, a homology model of the human B1 receptor was constructed from the crystal structure of bovine rhodopsin and binding modes of the B1 receptor antagonists have been determined. The binding modes of the dihydroquinoxalinone analogs at the binding site of the B1 receptor were further verified by the correlation of interaction energies of the receptor–antagonists to their binding affinities (K_i). Mutation data were also compared with the results of the mutation data of peptidic agonists and antagonists of the B1 and B2 receptors from the literature.

Mutations of Asp291 and Glu273 in the third extracellular loops, N114 in the third transmembrane helix, and W98 in TM1 cause a significant decrease in affinity for the non-peptidic antagonists, dihydroquinoxalinone analogs of compound 2 and compound 4. Mutations of Ile113 in TM3, Ile97 in TM1, and Phe302 TM7 cause a moderate decrease in affinity for compound 4 and interestingly mutation of Lys118 in TM3 resulted in 10-fold increase in binding affinity of compound 4. Mutation of Gln295 causes a decrease in binding affinity of compound 2. Taken together, these data indicate that the two non-peptidic antagonists compound 4 and compound 2 occupy the same binding pocket but there are slight variations in the amino acids that are important for their interaction within this binding pocket which presumably reflects conformational differences between these two highly similar compounds. Lys118 in TM3 is known as a critical amino acid for agonist binding in the B1 receptor but is not necessary for antagonist binding. We postulate that the antagonists studied herein bind to an inactive form of the receptor, furthermore the current homology model of B1 receptor is based on an inactive form of bovine rhodopsin. The binding poses of the antagonists are in good agreement with the structure–activity relationship of the

dihydroquinoxalinone analogs. The model was refined iteratively by integration of the mutagenesis data into the docking modes of the dihydroquinoxalinone analogs at the binding site of the receptor. Together with mutation data, the binding model was used to optimize this series of compounds and to gain insight into the molecular mechanism for receptor function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.03.142.

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