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New insights into the stereochemical requirements of the bradykinin B_2 receptor antagonists binding

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Abstract Bradykinin (BK) is a member of the kinin family, released in response to inflammation, trauma, burns, shock, allergy and some cardiovascular diseases, provoking vasodilatation and increased vascular permeability among other effects. Their actions are mediated through at least two G-protein coupled receptors, B_1 a receptor up-regulated during inflammation episodes or tissue trauma and B_2 that is constitutively expressed in a variety of cell types. The goal of the present work is to carry out a structure–activity study of BK B_2 antagonism, taking into account the stereochemical features of diverse non-peptide antagonists and the way these features translate into ligand anchoring points to complementary regions of the receptor, through the analysis of the respective ligand-receptor complex. For this purpose an atomistic model of the BK B_2 receptor was built by homology modeling and subsequently refined embedded in a lipid bilayer by means of a 600 ns molecular dynamics trajectory. The average structure from the last hundred nanoseconds of the molecular dynamics trajectory was energy minimized and used as model of the receptor for docking studies. For this purpose, a set of compounds with antagonistic profile, covering maximal diversity were selected from the literature. Specifically, the set of compounds include Fasitibant, FR173657, Anatibant, WIN64338, Bradyzide, CHEMBL442294, and JSM10292. Molecules were docked into the BK B_2 receptor model and the corresponding complexes analyzed to understand ligand-receptor interactions. The outcome of this study is summarized in a 3D pharmacophore that explains the observed structure–activity results and provides insight into the design of novel molecules with antagonistic profile. To prove the validity of the pharmacophore hypothesized a virtual screening process was also carried out. The pharmacophore was used as query to identify new hits using diverse databases of molecules. The results of this study revealed a set of new hits with structures not connected to the molecules used for pharmacophore development. A few of these structures were purchased and tested. The results of the binding studies show about a 33 % success rate with a correlation between the number of pharmacophore points fulfilled and their antagonistic potency. Some of these structures are disclosed in the present work.

Keywords Bradykinin B2 antagonism · Pharmacophore development · Virtual screening

Introduction

Kinins are a group of peptides ubiquitously produced by the action of kallikreins on circulating kininogens in response to inflammation, trauma, burns, shock, allergy and some cardiovascular diseases, provoking changes in blood pressure and vasodilation, increased vascular permeability, stimulation of sensory neurons, vascular and bronchial smooth muscle contraction, intestinal ion secretion, release of prostaglandins and cytokines, and the production of nitric oxide [1, 2]. Peptides of this group include bradykinin (BK), with sequence Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^9; the closely related kallidin (Lys-BK) and the metabolites of both, $desArg^9$-BK and Lys-$desArg^9$-BK. The pharmacological actions of kinins are

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mediated by at least two G-protein coupled receptors: B_1 and B_2. The former is up-regulated during inflammation episodes or tissue trauma whereas, the latter is constitutively expressed in a variety of cell types. Members of the kinin family bind to these receptors with diverse affinity. Thus, BK and Lys-BK exhibit much higher affinity to the B_2 receptor, whereas the desArg^9 metabolites bind only to the B_1 receptor, being Lys-desArg^9 a potent B_1 agonist. Due to their role in mediating pain and inflammation there has been a remarkable interest for identifying potent kinin antagonists for therapeutical intervention in the last years [3, 4].

Since the chemical synthesis of BK for the first time in the early 60 s [5] diverse analogs with agonistic activity have been reported, providing key information about the relevance of every residue for ligand activity. Analogs with antagonistic activity were not available until 1985, when Stewart and Vavrek replaced Pro^7 by an aromatic D-amino acid [6]. This led to the first generation of antagonists with the synthesis of potent analogs, including D-Arg-[Hyp^3, D-Phe^7]-BK (NPC-567) (Hyp = hydroxyproline); D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK (NPC-349) (Thi = thienylalanine); or D-Arg-[Hyp^3, D-Phe^7, Leu^8]-BK, among the most active compounds reported [7, 8]. Although this first generation of antagonists was useful to understand the involvement of BK in many pathophysiological processes, these compounds exhibit drawbacks that prevent them to be used for therapeutical intervention. Specifically, they exhibit low affinity for the B_2 receptor compared to BK itself and are not selective, showing higher affinity for B_2. Interestingly, removal of their C-terminal arginine by carboxypeptidases results in a decrease of affinity for the B_2 receptor, turning them selective B_1 antagonists.

A second generation of antagonists with improved pharmacological profile was designed on the basis that the C-terminus of BK adopts a β-turn when bound to the receptor, as had been suggested from spectroscopic and molecular modeling studies [9] and confirmed more recently, in solid state NMR experiments [10]. Thus, with the help of conformationally constrained unnatural amino acids, diverse analogs designed to mimic the secondary structural motif of BK at the C-terminus were synthesized. These studies resulted in the discovery of several potent antagonists, including icatibant (formerly known as HOE-140) with sequence D-Arg^0-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Tic = tetrahydroisoquinoline; Oic = octahydroindole carboxylic acid) [11] or NPC17731 (D-Arg-[Hyp^3, D-HypE(trans-proyl)^7, Oic^8]-BK) [12]. In a parallel effort, the search for the shortest peptide sequence retaining antagonistic activity led to conclude that adoption of a β-turn conformation at the C-terminus is a necessary condition for high affinity to the B_2 receptor, but not sufficient. This conclusion came from the analysis of the binding affinity of diverse cyclic peptides inspired on the C-terminus of icatibant. Thus for example compounds like the cyclo-(Gly-Thi-D-Tic-Oic-Arg) [13] or cyclo-(Pro-Orn-D-Tic-Oic-Arg) [14] show poor antagonistic affinity for the B_2 receptor. Accordingly, the affinity of icatibant and analogs was rationalized in terms of the interactions of the compound with the receptor, such that the β-turn at the C-terminus was thought to occupy a hydrophobic region on the orthosteric pocket, whereas the N-terminal arginine were thought to interact with the negatively charged residues Asp^{266} and Asp^{284}, putatively located at the mouth of the receptor [15]. As an indirect proof of concept, the high affinity peptide D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-cyclo[Dab^6-D-Tic^7-Oic^8-Arg^9] (Dab = diaminobutyric acid) (MEN11270) exhibits a cyclic structure at the C-terminus mimicking the β-turn secondary structure and preserves the N-terminal segment of icatibant [16].

The second generation of B_2 antagonists represented an improvement in regard to the first one. Thus, in addition to have designed antagonists with high affinity for the B_2 receptor, these compounds are highly selective and exhibit an improved pharmacokinetic profile due to their higher resistance to enzymatic degradation. However, they exhibit a limited oral bioavailability. Thus icatibant, the first B_2 antagonist to reach the market and currently used for the symptomatic treatment of acute attacks of hereditary angioedema in adults with C1-esterase-inhibitor deficiency, needs to be administered via subcutaneous injection [17]. In order to improve the oral bioavailability, research efforts were put forward to design non-peptide B_2 selective antagonists. This third generation of BK B_2 antagonists includes diverse molecules disclosed during the 90 s and the beginning of the twenty-first century [18, 19]. Specifically, WIN64338 developed at Sterling Winthrop was the first BK B_2 non-peptide antagonist disclosed (**4** in Fig. 1) [20]. Other compounds were disclosed in the following years, including a series of compounds developed by Fujisawa, like FR173657 (**2** in Fig. 1) [21]; bradyzide developed by Novartis (**5** in Fig. 1) [22]; anatibant developed by Fournier (**3** in Fig. 1) [23] or fasitibant developed by Menarini (**1** in Fig. 1) [24]. These compounds are high affinity BK B_2 selective antagonists with limited oral bioavailability. The drawback of these compounds regards their high molecular mass, ranging between 500 and 600. Aimed at finding compounds with lower molecular mass, scientists at Jerini carried out a medicinal chemistry optimization process, using the 8-benzyloxy-2-methyl-quinoline moiety, that is the common scaffold of several of the non-peptide antagonists listed above, as starting structure. Their study led to the design and synthesis of JSM10292 a potent B_2 antagonist with similar affinity and selectivity to the previous compounds, but with lower molecular mass [25].

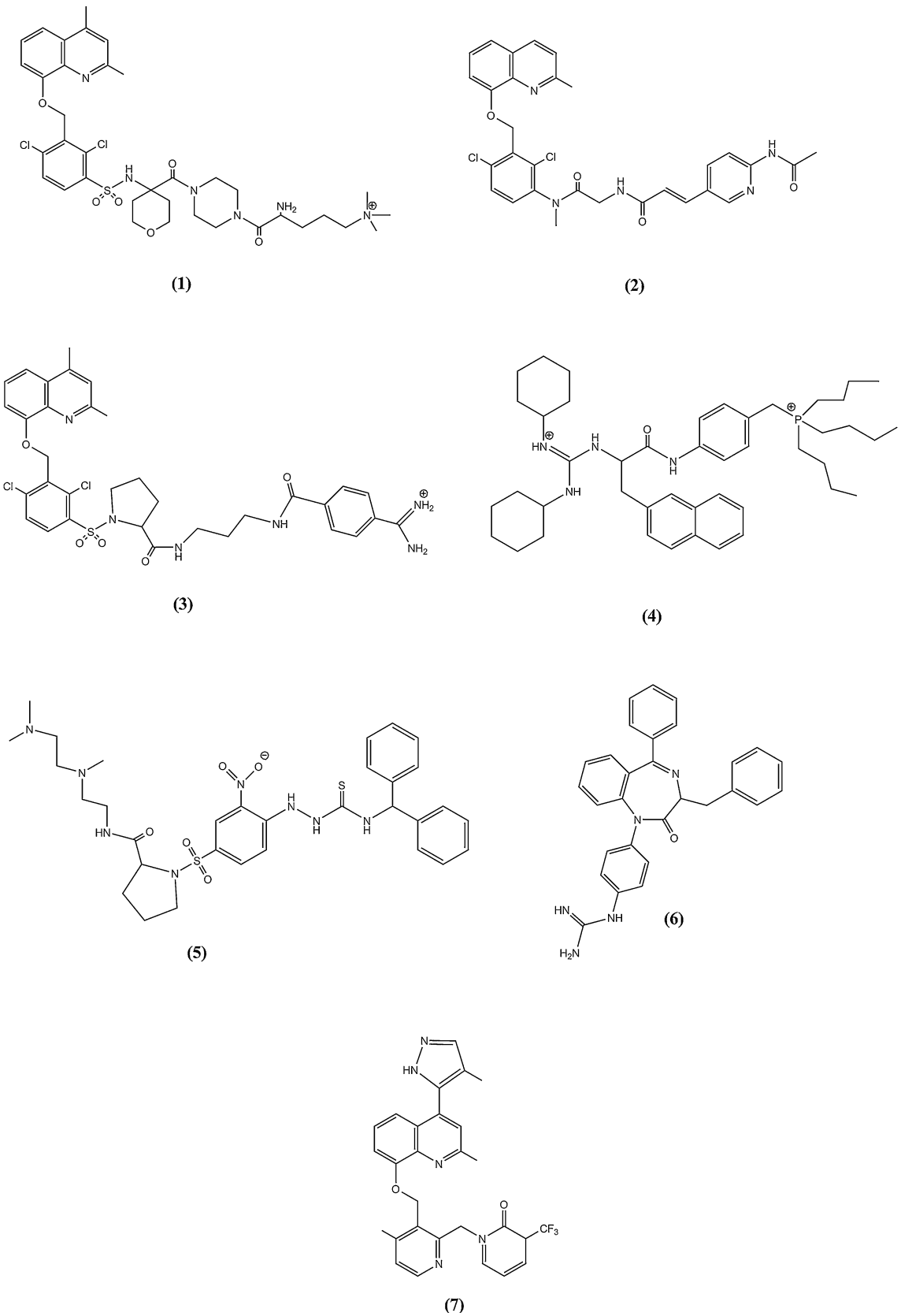

Fig. 1 Chemical structures of the B_2 antagonists studied in the present work. Fasitibant (**1**), FR173657 (**2**), Anatibant (**3**); WIN64338 (**4**); Bradyzide (**5**); CHEMBL442294 (**6**); JSM10292 (**7**)

The goal of the present work is to carry out a structure–activity study of BK B_2 antagonism, taking into account the stereochemical features of diverse non-peptide antagonists and the way these features translate into ligand anchoring points to complementary regions of the receptor, through the analysis of the respective ligand-receptor complex. For this purpose we selected a set of compounds from the literature covering maximal diversity. The compounds selected for the present study include Fasitibant (**1**) [24], FR173657 (**2**) [21], Anatibant (**3**) [23], WIN64338 (**4**) [20], Bradyzide (**5**) [22], CHEMBL442294 (**6**) [25], and JSM10292 (**7**) [26], shown in Fig. 1. Compounds were docked into a refined model of the BK B_2 receptor constructed by homology modeling, following the procedure explained in the methods section, and the complexes were further analyzed for their ligand-receptor interactions. The outcome of this study is summarized on a 3D pharmacophore that explains the observed structure–activity results and provides insight into the design of novel molecules with antagonistic profile.

Methods

Computational methods

A starting model of the human BK B_2 receptor was constructed by homology modeling using the chemokine CXCR4 receptor as template (pdb entry code 3ODU) [27]. The template was selected due to its proximity to BK B_2 in the GPCRs phylogenetic tree among those GPCRs whose crystallographic structure is known. The sequences of the two receptors were aligned, taking into account the conserved motifs found in all GPCRs, as well as the location of the disulfide bridges. These motifs, together with salt bridges are important factors in constraining the conformation of the extracellular and transmembrane domains of the B_2 receptor. From the aligned sequences a starting model of the receptor was constructed using the Modeller 9 version 8 (9v8) software [28]. Model validation was carried out using the Molecular operating Environment (MOE) program [29]. In a subsequent step the B_2 antagonist fasitibant (compound 7 in Fig. 1) was docked into the orthosteric site of the starting model using the GLIDE software [30]. The choice of this ligand was due to the abundant information available from site directed mutagenesis experiments [31]. Finally, the ligand-receptor complex was embedded in a lipid bilayer and refined using molecular dynamics. Specifically, the protein was embedded in a box consisting in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipids and water molecules previously equilibrated according to the procedure described elsewhere [32]. The box had an initial size of $10.3 \times 8.0 \times 10.2$ nm^3 (XYZ), organized in such a way that the bilayer plane was oriented on the XY plane. Before protein insertion, the box contained 256 lipids (corresponding to an area per lipid of 0.64 nm^2) and circa 17,000 water molecules. The protein was placed in the center of the box, and the overlapping molecules were removed. More specifically, all water molecules with oxygen atoms closer than 0.40 nm to a non-hydrogen atom of the protein, as well as all lipid molecules with at least one atom closer than 0.25 nm to a non-hydrogen atom of the protein were removed. This resulted in a final box containing 197 lipids and circa 16,000 water molecules. Removal of these atoms introduced small voids between the protein and water or lipid molecules that disappeared during the first part of the MD simulation, in which a progressive adjustment of the lipid bilayer and water molecules to the protein takes place. Next, 114 randomly selected water molecules were replaced by 58 sodium and 56 chloride ions, providing a neutral system with a concentration approximately 0.2 M on sodium chloride. This concentration is fairly similar to that found in biological organisms, although they exhibit different intra- and extra-cellular ion concentrations.

Sampling was carried out for 600 ns using the OPLS-AA force field with the GROMACS package 4.6 [33]. The refined model of the BK B_2 receptor was generated from the average structure of the last 100 ns of the molecular dynamics trajectory. The structure was subsequently minimized in a two-step process using the steepest descent method with a dielectric constant of 2. First, side chains are optimized with the backbone atoms constrained to be subsequently released in a second minimization. This structure was used for further docking studies using the GLIDE [30] software. Docking was carried out with a rigid receptor and with the ligand free to move. However due to the flexibility of the ligands several docking attempts were carried out using a set of unique conformations resulted from a previous thorough conformational analysis of the ligands. Poses were rank ordered using the XP scoring function of GLIDE. Final poses of the compounds were decided based on their ranking and fulfillment of site directed mutagenesis information available. Final poses were energy minimized using the steepest decent method with a dielectric constant of 2, using the OPLS-AA force field [33] to get a full relaxation of the ligand-receptor complexes.

Binding assays

B_2 antagonism assays were carried out following a protocol described elsewhere [34]. Specifically, compounds were tested on human recombinant bradykinin B_2 receptors expressed in CHO cells. Saturation isotherms were obtained with [^{3}H]-bradykinin (0.2 nM) incubated for

60 min at room temperature. Non-specific binding was evaluated by adding bradykinin at 1 μM. Antagonism of unlabeled compounds was measured as the percentage of inhibition of the binding of [^{3}H]-bradykinin at one concentration using NPC-567 as reference compound.

Results and discussion

Although the number of crystallographic structures of GPCRs available has increased steadily during the last few years, there are still challenges that hamper the availability of new ones, including their low-expression yields, low receptor stability after detergent extraction from native membranes, and high conformational heterogeneity. In the absence of a crystallographic structure of the BK B_2 receptor we proceeded to construct an atomistic model by homology modelling for the present study. Under these circumstances homology modeling remains one of the important techniques aimed at constructing 3D models of proteins, however in order for the models constructed to be as accurate as possible the procedure requires a careful choice of the template and a robust refinement procedure. This is important because from the analysis of the diverse known structures, although they share a common seven helix bundle, each structure exhibits specific features that might be relevant for ligand design [35]. The CXCR4 chemokine receptor (pdb entry code 3ODU) [27] was selected as template for the present study due to its proximity with BK B_2 in the phylogenetic tree of the class A family of GPCRs.

Figure 2 shows the alignment of the sequences of the CXCR4 and BK B_2 receptors carried out taking into account the conserved motifs found among GPCRs, as explained in the methods section. This procedure is crucial for the assignment of the transmembrane regions. This information is then given as input to the Modeller software that produces a rank order set of models based on a scoring function. The final model selected for the refinement process was the one with the least steric conflicts from those that incorporated all the specified constraints considered to be conserved among GPCRs.

Before proceeding to the refinement process, fasitibant was docked into the initial model. Due to its flexible structure several docking attempts were carried out using diverse conformations that were generated automatically as explained in the methods section. The final complex considered for refinement was selected based on the degree of fulfilment of diverse site-directed mutagenesis studies. Special attention was given to residues Trp^{86}, Ile^{110}, Trp^{256}, Asp^{266} and Tyr^{295} [31].

The complex fasitibant bound-receptor was embedded into a pre-equilibrated bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and water and subjected to a molecular dynamics simulation. Previous experience in GPCR homology modeling (data not shown), suggest that the presence of the ligand permits a faster equilibration of the system. Time evolution of the root mean square deviation (rmsd) of the alpha carbons of the protein, as well as those of its helical bundle subset is show in Fig. 3. Inspection of the Figure indicates that when all the alpha carbons of the protein are considered equilibration is reached after 300 ns, whereas when the helical bundle subset is used equilibration is reached about 50 ns earlier. These results support the choice made in the present work of using the last 100 ns of the refinement process for the generation of an atomistic model of the BK B_2 receptor. As mentioned in the methods section, the last 100 ns segment of the molecular dynamics trajectory was used to generate an average structure that was subsequently minimized in a two-step process using the steepest descent method with a distance dependent dielectric constant of 2. The orthosteric site of the BK B_2 receptor can be described as two hydrophobic pockets, a lager one formed between TM3, TM4, TM5 and TM6 including residues like Ile^{110}, Met^{165}, Leu^{201}, Trp^{256}, Phe^{259} and a smaller one formed between TM2, TM3 and TM7 including residues like Trp^{86} and Tyr^{295}. Interestingly, the aromatic side chains Trp^{86}, Trp^{256} and Tyr^{295} are coupled through quadrupole–quadrupole interactions. On the other hand, at the mouth of the site there are several polar residues including, Glu^{24}, Gln^{33}, Asp^{266}, Asp^{284} and Gln^{288}. Figure 4 shows the orthosteric pocket of the receptor. Below, we describe the bound conformation of the diverse antagonists used in this study obtained from docking studies on the refined model.

Fasitibant

Fasitibant (MEN16132) (**1,** in Fig. 1) is a potent antagonist of the BK B_2 receptor with a K_i of 0.09 nM [31]. The structure of fasitibant bound to the model receptor from the present docking study is shown in Fig. 5. In accordance with previous docking studies [31], the quaternary terminal amine interacts with Asp^{266} and Asp^{284} at the mouth of the receptor, whereas the hydrophobic 8-benzyloxy-2-methyl-quinoline moiety gets deep into the orthosteric pocket. The involvement of the two aspartates in ligand binding is supported by site-directed mutagenesis analysis [36]. Inspection of Fig. 5 shows the tetrahydropyranyl moiety sitting in a hydrophobic region on top of Trp^{86}, with the heterocycle oxygen and the hydrogen of the indole nitrogen of the tryptophan side chain close enough to exhibit a polar interaction. This result is consistent with the fact that binding of fasitibant to the Trp^{86}Ala mutant is about 1200 times lower [31] and can be explained on the basis that the quadrupole–quadrupole interaction between the two rings

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---------DYKDDDDAGAPEGISIYTSDNYTEEMGSGDYDSMKEPCFRE
MFSPWKISMFLSVREDSVPTTASFSADMLNVTLQGPTLNGTFAQSKCPQV
          .  :*: .. .       * * :  : :    :. * : |
ENANFNKIFLPTIYSIIFLTGIVGNGLVILVMGYQKKLRSMTDKYRLHLS
EWLGWLNTIQPPFLWVLFVLATLENIFVLSVFCLHKSSCTVAEIYLGNLA
*  .: : : *.:  ::*: . : * :* : *:  :*.  :::: *  :*:

VADLLFVITLPFWAVDAVAN--WYFGNFLCKAVHVIYTVNLYSSVWILAF
AADLILACGLPFWAITISNNFDWLFGETLCRVVNAIISMNLYSSICFLML
.***:.  *****:    *  * **: **:.*:.* ::*****: :* :

ISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSE
VSIDRYLALVKTMSMGRMRGVRWAKLYSLVIWGCTLLLSSPMLVFRTMKE
:*:*****:*:: .  * * :   *:  : :*  :***: * ::* .:.*

ADDR----YICDRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYCIIISK
YSDEGHNVTACVISYPSLIWEVFTNMLLNVVGFLLPLSVITFCTMQIMQV
 .*.      *   **. :* *. ::   :**::**  **  *   *:.

LSHS--GSGSKGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEI
LRNNEMQKFKEIQTERRATVLVLVVLLLFIICWLPFQISTFLDTLHRLGI
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IKQGCEFENTVHKWISITEALAFFHCCLNPILYAFLGAKFKTS-------
LSS-CQDERIIDVITQIASFMAYSNSCLNPLVYVIVGKRFRKKSWEVYQG
:.. *: *. :.   .*:. :*: :.****::*.::* :*:..

-AQHALTSGRPLEVLFQ------------------------
VCQKGGCRSEPIQMENSMGTLRTSISVERQIHKLQDWAGSRQ
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Fig. 2 Sequence alignment of human BK B_2 (*bottom*) and CXC4 receptors. Transmembrane segments are inserted in boxes and sequence identities are colored in *blue*

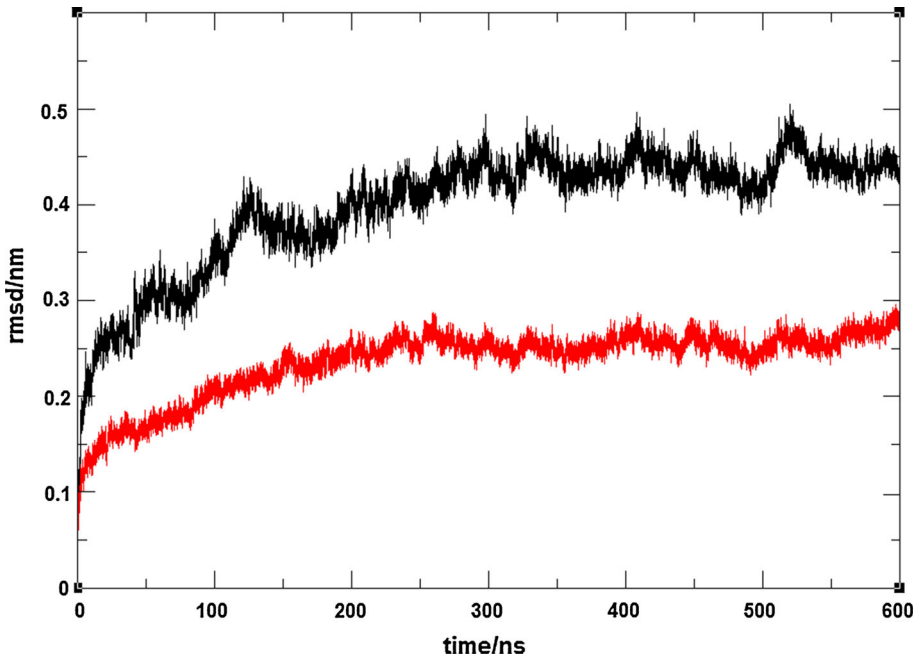


Fig. 3 Time evolution of the root mean square deviation (rmsd) of the bradykinin B_2 receptor during the refinement process. In *black* is the rmsd of the alpha carbons of the protein and in *red* the rmsd of the alpha carbons of the helix bundle subset

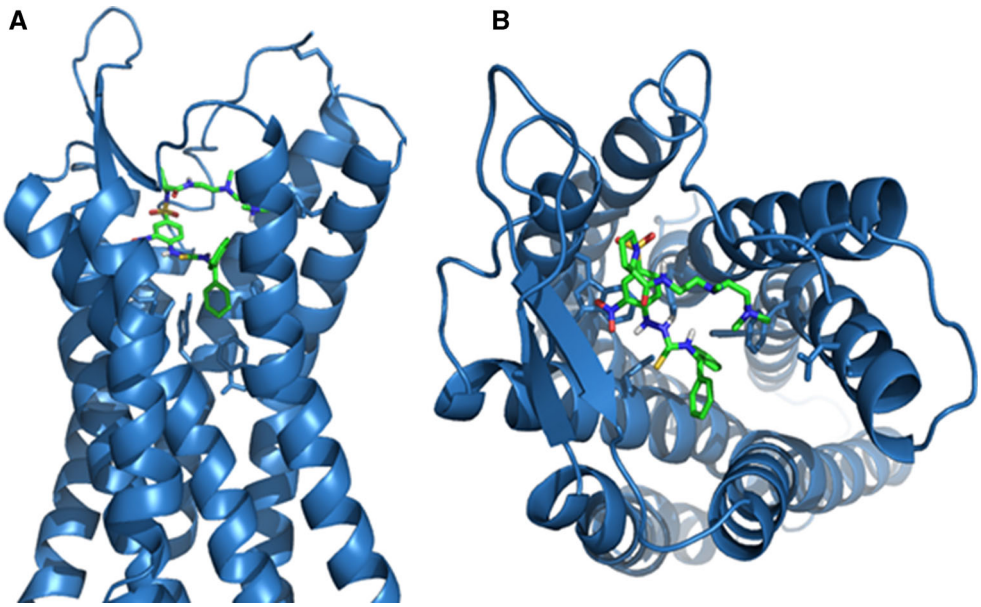


Fig. 4 (**A**) Lateral view of the orthosteric binding pocket of the human BK B_2 receptor with fasitibant bound; (**B**) Same as (**A**) viewed from the extracellular side (*top*)

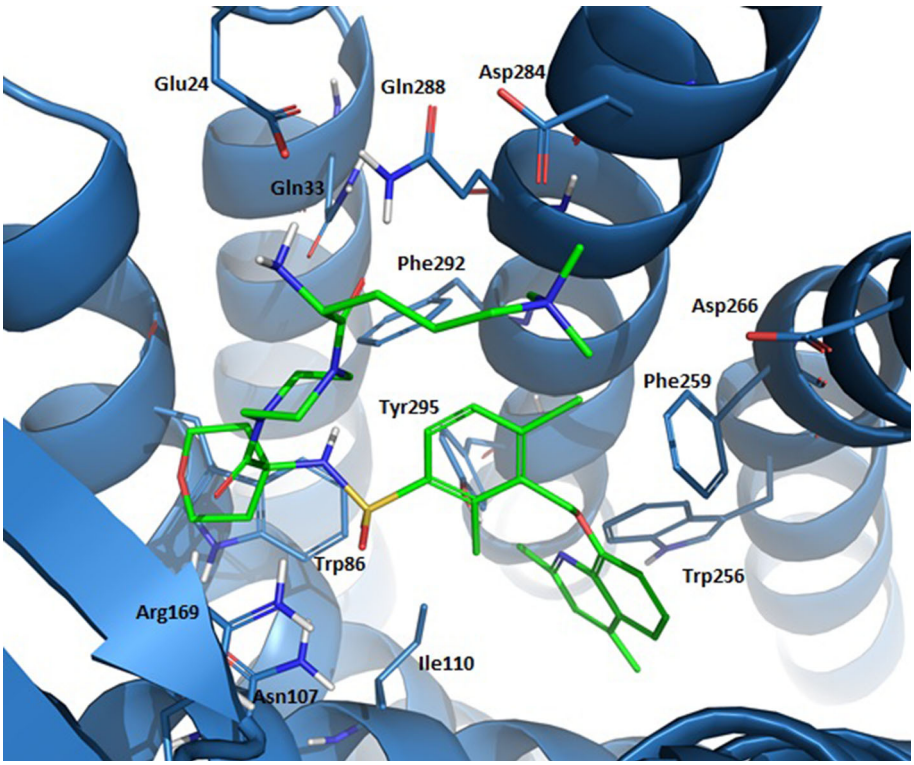


Fig. 5 Pictorial view of the proposed binding mode of fasitibant to the BK B_2 receptor

and a hydrogen bond are lost in the mutant. Analyzing other functional groups of the ligand, one of the oxygens of the sulfonamide appears forming a hydrogen bond with Arg^{169}; one of the chlorines of the dichlorobenzyloxy moiety forms a polar interaction with Arg^{169} and Asn^{107}, whereas the other sits in a hydrophobic environment flanked by Phe^{259}, Phe^{292} and possibly with Tyr^{295}. Unfortunately, there is no mutagenesis data available about the involvement of Arg^{169} or Phe^{259} in the affinity of the ligand to give support to these interactions. At the molecule ending, the quinoline moiety sits in the vicinity of Ile^{110}, Trp^{256} and Tyr^{295} interacting with the latter two through quadrupole–quadrupole interactions. Moreover, the interaction with Tyr^{295} is enhanced by the presence of a hydrogen bond between the quinoline amine group of the ligand and the hydroxyl group of Tyr^{295}. These results are

consistent with diverse site directed mutagenesis data available. Thus, the mutant $Ile^{110}Ala$ reduces 300 times the affinity of fasitibant and furthermore, the mutation $Tyr^{295}Phe$ reduces the affinity 100 times, whereas the mutation $Tyr^{295}Ala$ 755 times, suggesting a dual role as aromatic/hydrophobic residue and as hydrogen bond donor/acceptor for Tyr^{295} [31].

FRI73657

This Fujisawa compound (**2** in Fig. 1) is a potent B_2 antagonist with an IC_{50} of 1.4 nM at the human receptor [21]. This compound is the result of an extensive medicinal chemistry program aimed at designing B_2 selective antagonists from a hit found in a screening program for angiotensin II AT_1 antagonists. As can be seen the compound shares with fasitibant the 8-benzyloxy-2-methyl-quinoline moiety and consequently, it could be thought that the compound binds in a similar manner, however site-directed mutagenesis studies suggest that the bound conformation is different. Thus, for example the affinity of FR173657 is not affected by the mutation of Asp^{266} and Asp^{284} [36] or Ser^{111} and Trp^{256} [37], suggesting that these residues do not act as anchoring points in the bound conformation of the compound. In contrast, Trp^{86} and Tyr^{295} must be actively involved since their mutation to alanine decreases the affinity of the compound about 500 times [31]. Bearing these results in mind, several docking attempts were carried out, obtaining diverse alternative poses. Analysis of the results in view of the site-directed mutagenesis results available suggests that the ligand binds according to the ligand-receptor complex shown in Fig. 6. Specifically, the molecule adopts a L-shape with the dichlorobenzyloxyl moiety found on top of Trp^{86}, nicely interacting through a parallel π–π stacking in such a way that both chlorines in addition to sit in a hydrophobic environment, interact through a hydrogen bond with Asn^{107} and Gln^{288}, respectively. Unfortunately, there are not mutagenesis results of the role of these two residues, but structure activity studies of different analogs of FR173657 suggest that replacing the chlorines for methyl groups decreases the affinity about five times, supporting the existence of the hydrogen bond interaction [38]. The position of the dichlorobenzyloxyl permits to direct the quinoline moiety -similar as it is found in fasitibant- in the vicinity of residues Ile^{110} and Phe^{256} whose mutation is known to affect significantly ligand binding. An additional piece of information to support the position of the quinoline moiety comes from a CoMFA analysis described in reference 36. Specifically, the authors proposed constraints for favorable groups to improve the affinity of the ligand by extension of the molecule and these fit well with the positions of residues Trp^{256}, Phe^{259} and Asn^{198} in the present ligand-receptor complex. On the other side of the molecule, structure activity studies of diverse analogs suggest the importance of this part of the molecule for obtaining good antagonists at the human BK B_2 receptor. Analysis of ligand-receptor complex shown in Fig. 6 suggests that the amide groups interact with polar residues like Glu^{24} or Thr^{89} whereas the pyridine ring interacts with Phe^{94} and Tyr^{174} through quadrupole–quadrupole interactions. These results agree well with the structure–activity studies carried out on these compound series. Specifically, the introduction of a phenylurea moiety induces an increase in the affinity one order of magnitude in guinea pig ileum membrane preparations and nearly two orders in A-431 cells that express the human receptor due to the favorable interaction with the side chains of Phe^{94} and Tyr^{174}. Finally, Lys^{172} interacts with the nitrogen of the pyridine ring of the ligand.

Anatibant

Previously known as LF 16-0687 (**3** in Fig. 1), anatibant is a potent B_2 antagonist of Fournier with a IC_{50} of 0.67 nM at the human receptor [23]. The compound shares the 8-benzyloxy-2-methyl-quinoline moiety with the two antagonists described above, and in this case linked to a pyrrolidine sulfonamide with a 4-amidinofenil moiety as charged terminal group. Analysis of the different poses found in our docking studies in view of the site-directed mutagenesis results available suggest the molecule sits inside the orthostheric site as shown in Fig. 7. As can be seen the bound conformation of anatibant shows similarities with that of fasitibant. Indeed, the common 8-dichlorobenzyloxy-2-methyl-quinoline substructure sits in a similar manner inside the receptor. However the stereochemical differences on the other side of the molecules force them to exhibit differential poses as discussed below.

The dichlorobenzyloxyl moiety of anatibant binds in the same region of the receptor as fasitibant, consequently the other moieties attached to it and shared between the two molecules, including the quinoline and the sulfonamide, access similar regions of the receptor. Specifically, the sulfonyl groups exhibit hydrogen bonds with Asn^{107} and Arg^{169}, respectively. Moreover, the side chain of the latter shows an additional hydrogen bond with one of the chlorides of the dichlorophenoxyl moiety. On the other hand, the quinoline moiety like in fastibant sits close to Tyr^{295} and interacts through a hydrogen bond and the quadrupole–quadrupole interaction between the two aromatic rings. Moreover, our docking studies also show the quinolone moiety interacting with Ile^{110}, Trp^{256} and Phe^{259}. These results are supported by mutagenesis studies, since the mutation of Tyr^{295} to Phe^{295} reduces the affinity one order of magnitude and to two orders of magnitude when

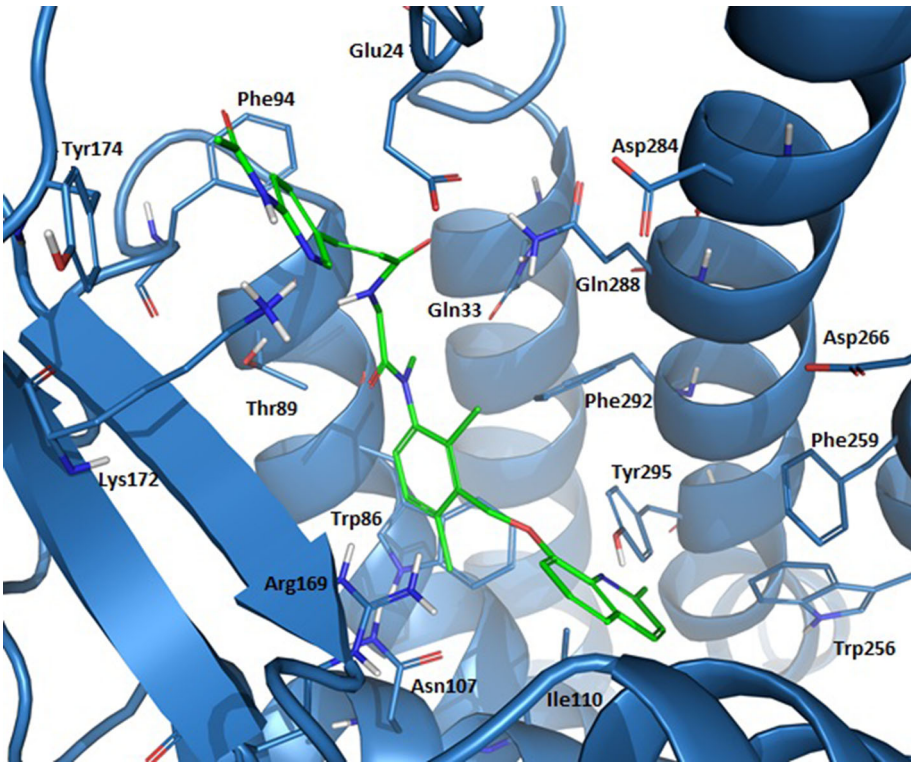


Fig. 6 Pictorial view of the proposed binding mode of FR173657 to the BK B_2 receptor

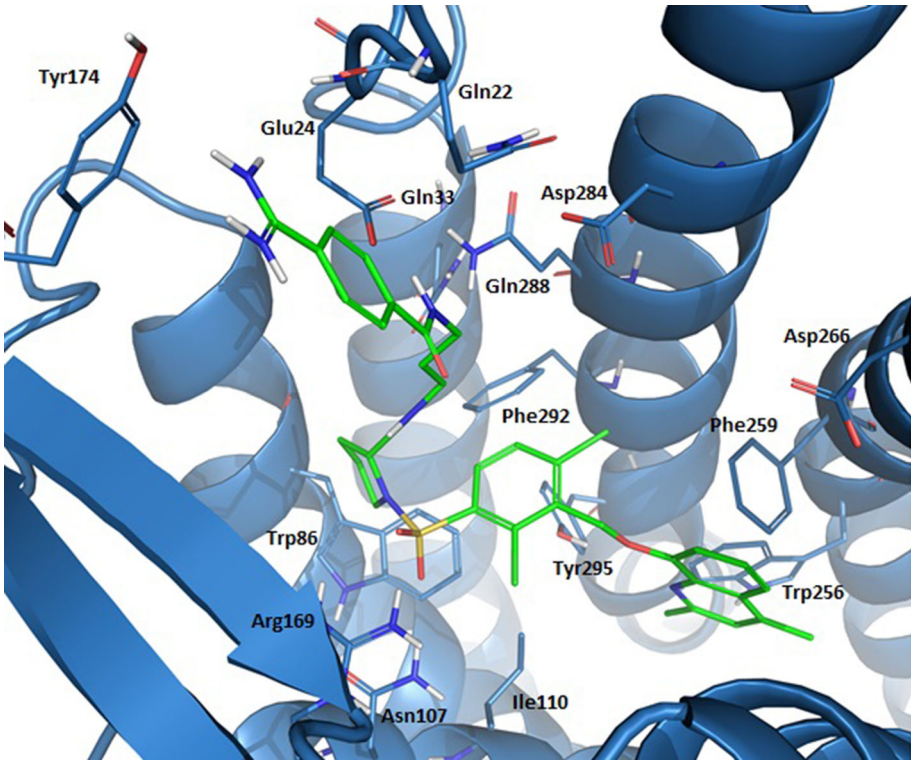


Fig. 7 Pictorial view of the proposed binding mode of anatibant to the BK B_2 receptor

mutated to Ala^{295} [39]. Similarly, the mutation of Trp^{256} to Ala^{256} decreases the affinity one order of magnitude. Moreover, this idea also explains the observed effect of the mutation $Asn^{297}Ala$ in close contact with Trp^{256} [39]. Unfortunately there are no results available on the mutation of Ile^{110} or Phe^{259} on the affinity of the ligand, but

according to the present modeling study it is expected one order of magnitude decrease as shown in fasitibant [31]. On the other side of the molecule anatibant and fasitibant exhibit differential stereochemical features that force them to bind in a differential way. This idea is supported by mutagenesis studies, since the mutation of Asp^{266} or Asp^{284} to alanine decreases dramatically the binding affinity of fasitibant but does not alter that of anatibant [36]. As it can be seen in Fig. 7 the pyrrolidine ring sits perpendicular to Trp^{86} interacting through a quadrupole–quadrupole interaction, being its position reinforced by a hydrogen bond with the side chain of Arg^{169}. The ring plays a fundamental role in distributing the rest of the molecule and this justifies the dramatic effect observed in the affinity of the compound when the residue is mutated to alanine [40]. From the pyrrolidine, the molecule extends towards the mouth of the receptor with the aminoiminophenyl moiety surrounded by polar residues including, Tyr^{174} of the second extracellular loop or Gln^{22} located at the N-terminus. Moreover, there are diverse polar residues that interact with the amide groups of the molecule, including Glu^{24}, Gln^{33} and Gln^{288}. The importance of the latter has been shown by mutagenesis studies [39].

WIN64338

This was the first non-peptide antagonist disclosed in the literature with a Ki of 64 nM in human IMR90 fibroblasts [20]. The antagonist was designed using a simple pharmacophore defined by two charged groups separated by a distance of about 10 Å—mimicking the distance between the two terminal arginines of BK in its bioactive conformation- linked by lipophilic groups [20]. From a hit obtained, subsequent medicinal chemistry efforts yielded the compound WIN64338 (**4** in Fig. 1). As can be seen the compound exhibits two charged groups at both ends surrounded by bulky hydrophobic groups, together with a naphtyl moiety. The results of our docking study of the compound onto the B_2 receptor model is shown in Fig. 8. WIN64338 is much shorter than the rest of the compounds described so far and is consequently, expected to cover a smaller region of the binding pocket. Although there are no reports on directed mutagenesis studies conducted with the compound, structure activity studies underline the importance of the two charges for high affinity. As can be seen, the positively charged phosphine group nicely sits surrounded by two negatively charged residues Asp^{266} and Asp^{284}, although the model suggest that is the latter that is actually involved in a charge–charge interaction. In regard to the dicyclohexylguanidinium moiety, of the bulky cyclohexyl groups, one sits in the proximity of Trp^{86}, whereas the other points toward the solvent in a region surrounded by Ala^{183} and Phe^{94} that provide a hydrophobic environment. On the other hand, the charged nitrogen of the guanidinium moiety exhibits a polar interaction with the carbonyl oxygen of the Cys^{184} backbone. Finally, the naphtyl group sits in the hydrophobic pocket in the proximity of Trp^{256}, Phe^{259} and Ile^{110}, similarly as does the quinoline moiety in the previous antagonists described *Bradyzide*.

Bradyzide (**5**, in Fig. 1) is a potent, rat-selective B_2 antagonist that causes a long lasting reversal of inflammatory hyperalgesia [22]. The compound is the result of a lead optimization from a hit discovered by random screening. Interestingly, the compound exhibits high affinity for the rat B_2 receptor (0.5 nM) expressed in NG108-15 cell membranes, but much lower in human BK B_2 expressed in Cos-7 cells (772 nM). The result of the docking study of this compound onto the BK B_2 receptor is shown in Fig. 9. There are no mutagenesis studies available that can help to understand the effect of diverse mutations on the binding of bradyzide to the BK B_2 receptor, but there is information about the effect of diverse chemical substitutions on the molecule [41] that can be analyzed through view of the complex model. Thus, the terminal charged amino nitrogen located at the end of the diaminoalkyl chain in bradyzide sits at the mouth of the receptor, interacting with Asp^{266} in TM6 and Asp^{284} in TM7, although the model actually suggests that is the latter that is involved in a charge–charge interaction. Structure–activity studies support this result by since elimination of this chain decreases the affinity about 100 times [41]. Furthermore, these studies also point to the basicity of the nitrogen as important feature to get better affinities, confirming the role of the terminal nitrogen in a charge–charge interaction. In the model the sulfone group provides an anchoring point to ligand through a hydrogen bond with Gln^{33} in TM1. Further down the ligand, the aromatic ring of the nitrobenzene moiety interacts with Trp^{86} in TM2 with the nitro group interacting with Asn^{107} via a hydrogen bond. The sulfur of the thiosemicarbazyl moiety establishes a hydrogen bond with Asn^{107} as has been previously suggested and though a hydrophobic interaction with Ile^{110}; finally, the phenyl groups close to the thiosemicarbazyl moiety sit well in the aromatic region Trp^{256}, Phe^{259}, Tyr^{295}.

CHEMBL442294

This compound (**6**, in Fig. 1) was the most active compound of a series of benzodiazepines designed to mimic the β–turn adopted by BK in its bioactive form [25]. This peptidomimetic exhibits a binding affinity for the BK B_2 receptor in the micromolar range, result that is consistent with the low affinity exhibited by a series of cyclic peptides designed to mimic the C-terminus of BK [13, 14]. These

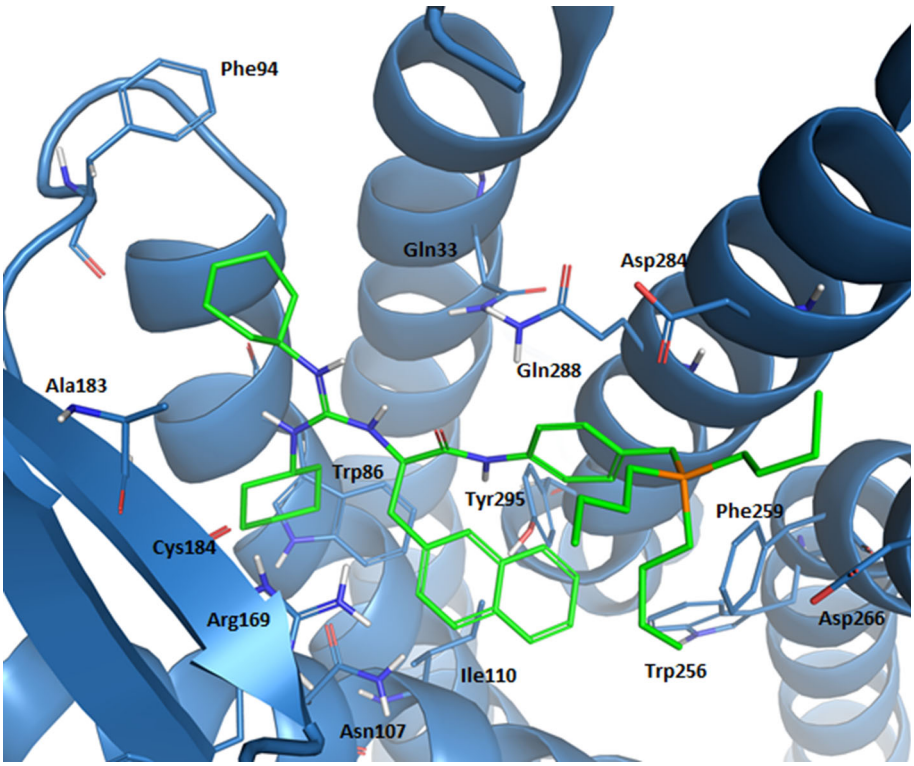


Fig. 8 Pictorial view of the proposed binding mode of WIN64338 to the BK B_2 receptor

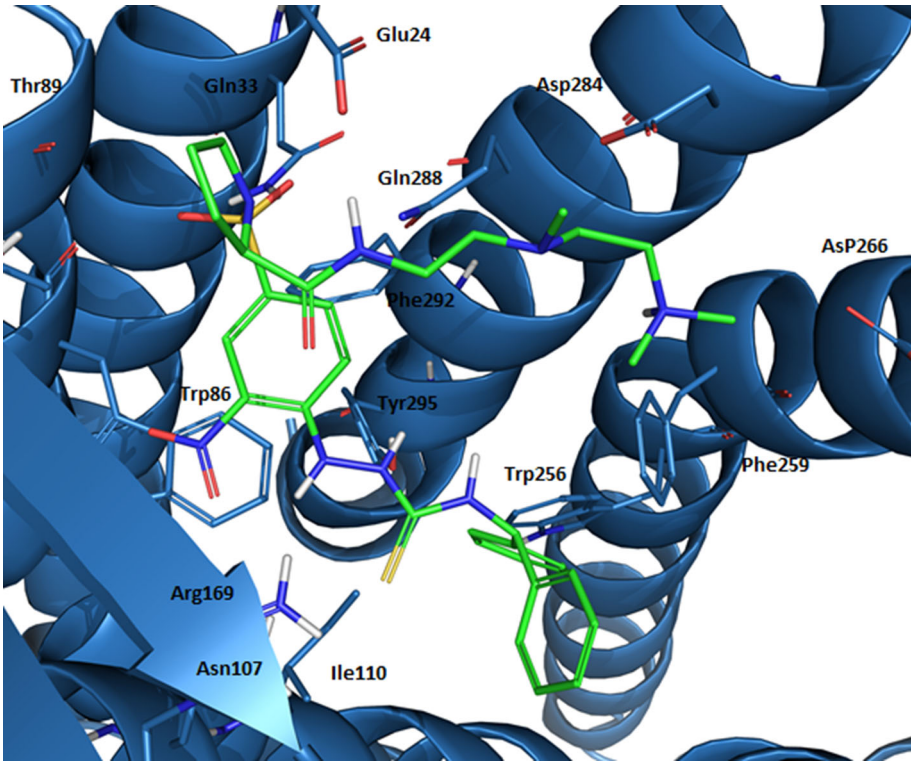


Fig. 9 Pictorial view of the proposed binding mode of Bradyzide to the BK B_2 receptor

results indicate that mimicking the C-terminus of the peptide is necessary condition to get good binding affinity, but not sufficient. Docking of the molecule into the receptor generated diverse poses that were analyzed in order to understand the features of the ligand-receptor interaction. Figure 10 shows the ligand-receptor complex

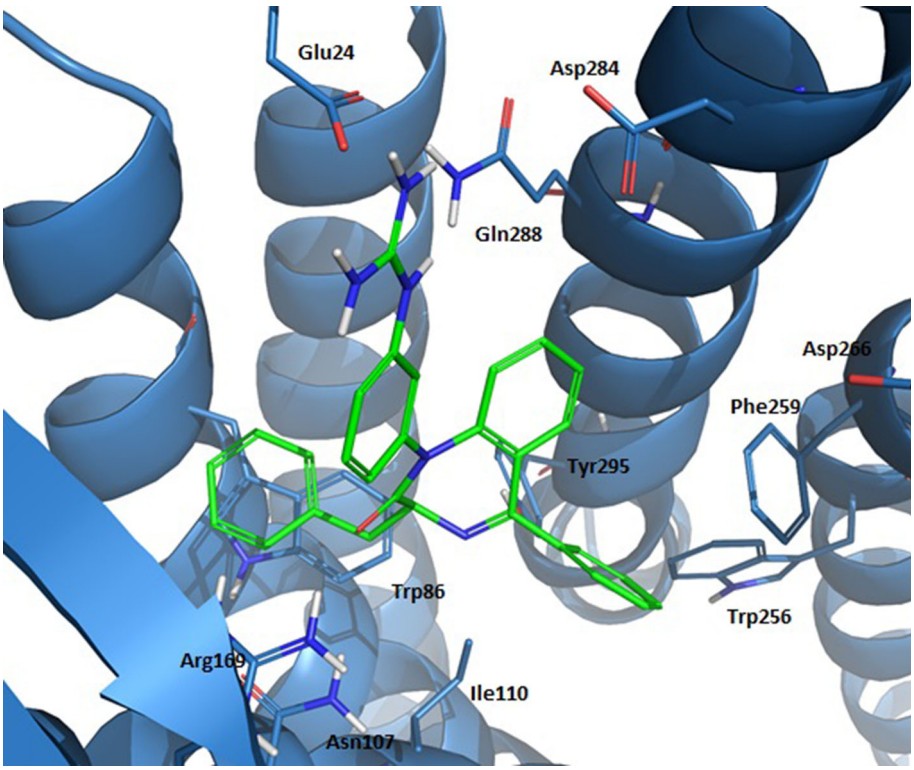


Fig. 10 Pictorial view of the proposed binding mode of CHEMBL442294 to the BK B_2 receptor

with the best docking score. As can be seen the ligand adopts a pose covering a number of residues identified in the docking in previous ligands. In this case, the guanidinium moiety binds to Asp^{284} and Gln^{288} in such a way that allows the interaction of an aromatic ring with Trp^{86} and another with Trp^{256} and Phe^{259} in TM6. In addition, the ligand exhibits a hydrogen bond between the carbonyl of the diazepine ring and Arg^{169}.

JSM10292

This compound (**7**, in Fig. 1) is the result of an optimization process aimed to find low molecular mass non-peptide B_2 antagonists based on the structures of previously disclosed compounds [26]. Specifically, inspection of the structure of diverse antagonist including FR173657 [21], compound 8d [38], anatibant [23] and fasitibant [31] shows that these molecules share a 8-benzyloxy-2-methyl-quinoline as common structural feature. Thus, the authors used this moiety as starting structure to follow a medicinal chemistry approach, leading to the compound JSM10292 that exhibits an IC_{50} of 8.7 nM in the human BK B_2 receptor, expressed in HEK293 cells [26]. Although the molecule shares a common substructure with those molecules used for its design, JSM10292 binds in different way. Analysis of different poses obtained during the docking process and analyzed according with the mutagenesis results available [42], it was selected as putative bound conformation the one shown in Fig. 11. Indeed, the pyrazole substituent to the quinoline group sits close to Phe^{259} with one of the pyrazole nitrogens acting as proton acceptor in a hydrogen bond interaction with Thr^{263}. Although there are not mutagenesis results on the role of the latter, the results underline the important role of Phe^{259} acting as anchoring point of the bound conformation. The nitrogen of the quinolone ring and the oxygen atom of the benzyloxy moiety interact via a hydrogen bond with Tyr^{295}; the nitrogen of the pyridine interacts with the side chain of Arg^{169}, whereas the aromatic ring and its methyl group sit close to Ile^{110}; the carbonyl oxygen of the trifluoropyridone moiety also interacts with Arg^{169} and the pyridone ring interacts with Trp^{86} whereas the trifluromethyl group interacts with Asn^{107}.

The bound conformation of JSM10292 found in the present study differs slightly of the one described in reference 40. The two models actually differ in the conformation of the ligands. In our model the ligand gets an extended conformation, whereas in their model the ligand adopts a conformation the trifluoropyridone ring folds back towards the pyridine ring. As result of the different conformation, the trifluromoiety interacts in the present model with Asn^{107} whereas in the other model gets close to Ser^{111}. In fact, there are not mutagenesis results of the mutation of serine to alanine; however the replacement to lysine provokes a great loss of affinity [42]. However, due to the size of the lysine side chain in both models a steric

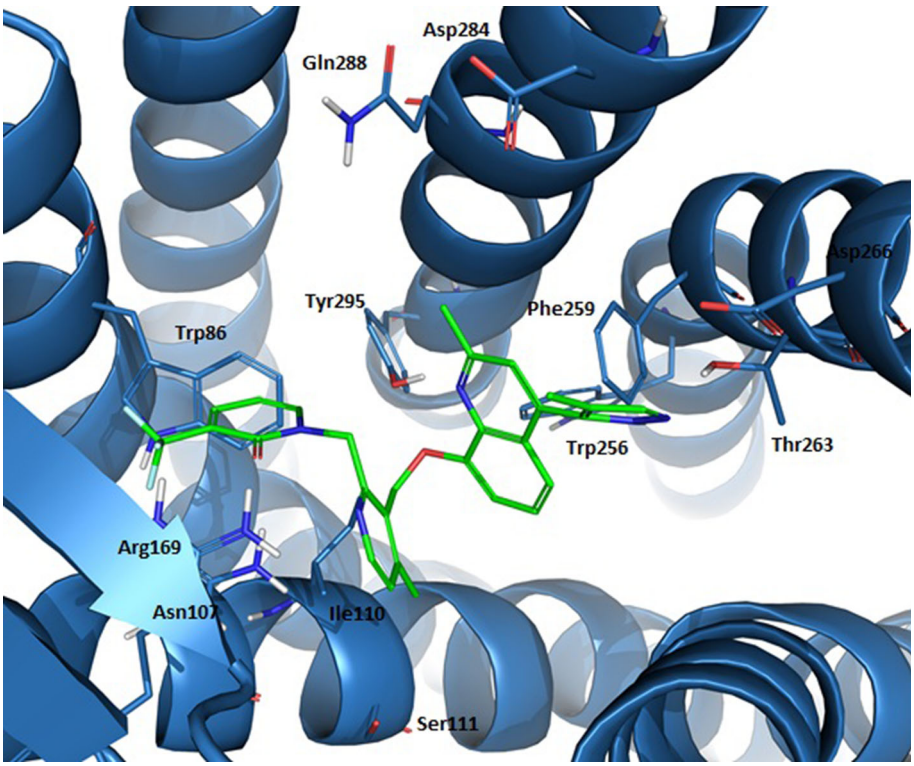


Fig. 11 Pictorial view of the proposed binding mode of JSM10292 to the BK B_2 receptor

hindrance is generated to perturb the binding of the ligand and consequently does not allow discriminating between the two models.

Comparison of the complex ligand-receptor of the different antagonists used for the present study suggests the definition of a pharmacophore that explains the observed structure–activity. It consists of five pharmacophoric points that not all the ligands studied in the present study fulfill. The pharmacophore proposed is shown in Fig. 12 and it is defined considering geometrical constraints on ligand moieties. Thus, point 1 is a proton donor center that will interact with Asp^{84} and/or Asp^{266}; point 2 is either a proton accepting/proton donor center that will interact with either Gln^{32} or Gln^{288}; point 3 is a hydrophobic ring that will interact with Trp^{86}; point 4 is a proton accepting center that will interact with Asn^{107} and/or Arg^{169}; point 5 is a hydrophobic/aromatic site that will interact with Trp^{256}, Phe^{259} and Tyr^{295}.

Thus, fasitibant fulfils point 1 by means of the terminal amine; point 3 by means of the dichlorophenoxyl moiety; point 4 by means of one of the sulfonyl oxygens as well as one of the chlorines and point 5 by means of the quinoline moiety. In the case of FR173657 point 2 is fulfilled by means of one of the amide groups; point 3 is fulfilled by means of the dichlorophenoxyl moiety that also fulfills point 4 by means of one of the chlorine atoms; point 5 is fulfilled by means of the quinoline moiety. In the case of anatibant point 2 is fulfilled by means of the pyrrodiline

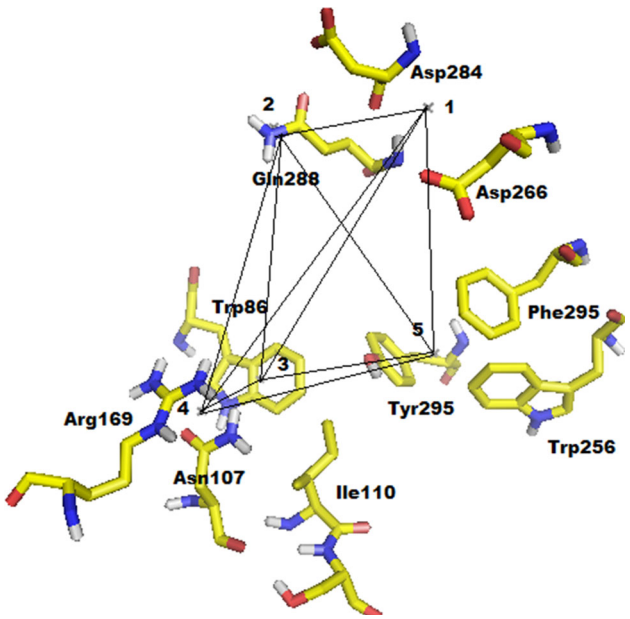
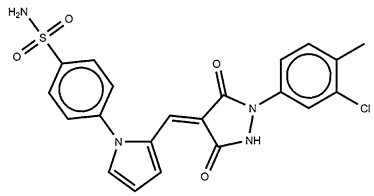
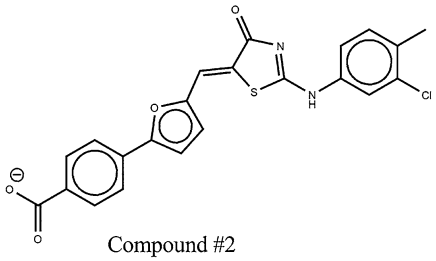
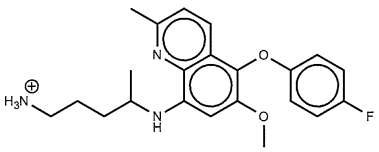
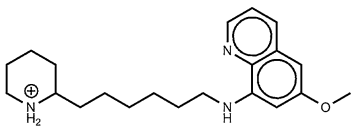
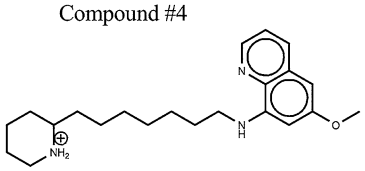


Fig. 12 Proposed pharmacophore for the BK B_2 antagonism. Distance between pharmacophoric points are: d(1,2) = 8.8 A; d(1,3) = 17.4; d(1,4) = 15.5; d(1,5) = 12.2; d(2,3) = 12.1; d(2,4) = 12.2; d(2,5) = 11.6; d(3,4) = 7.2; d(3,5) = 8.8; d(4,5) = 10.3

amide; point 3 by means of the pyrrolidine ring; point 4 by means of the sulfonyl groups and point 5 by means of the quinoline moiety. In the case of WIN64338 point 1 of the pharmacophore is fulfilled by the phosphine group; point 3

Table 1 Structures of the new hits discovered in this work

Structure	Inhibition	Pharmacophore points
 Compound #1	41% @ 10 μM	4
 Compound #2	33% @ 10 μM	3
 Compound #3	45% @ 50 μM	2
 Compound #4	36% @ 50 μM	2
 Compound #5	66% @ 50 μM	2

Their antagonistic potency towards the human B_2 bradykinin receptor and the number of pharmacophore points fulfilled is also shown

is fulfilled by one of the cyclohexanes; point 4 by the amide carbonyl and point 5 by means of the naphtyl moiety. In bradyzide point 1 is fulfilled by the terminal amine; point 2 is fulfilled by one of the sulphonyl oxygens, although in this case the interaction is more likely with Gln^{33} in the vicinity of Gln^{288}; point 3 is fulfilled by means of the nitrobenzene moiety and point 5 by means of the phenyl terminal groups. For CHEMBL442294 point 1 is fulfilled by the guanidinium moiety; point 3 by means of the phenyl substituent of the benzodiazepine scaffold and point 5 by means of the bezyl moiety. In the case of JSM10292 point 1 is fulfilled by means of the pyrazole moiety; point 3 is fulfilled by means of the pyrazone moiety that also fulfils point 4 by means of the carbonyl group. Finally, point 5 is fulfilled by means of the quinoline moiety.

Present pharmacophore includes others previously described in the literature. Specifically, the simple pharmacophore proposed by Salvino et al. [43] consisting in

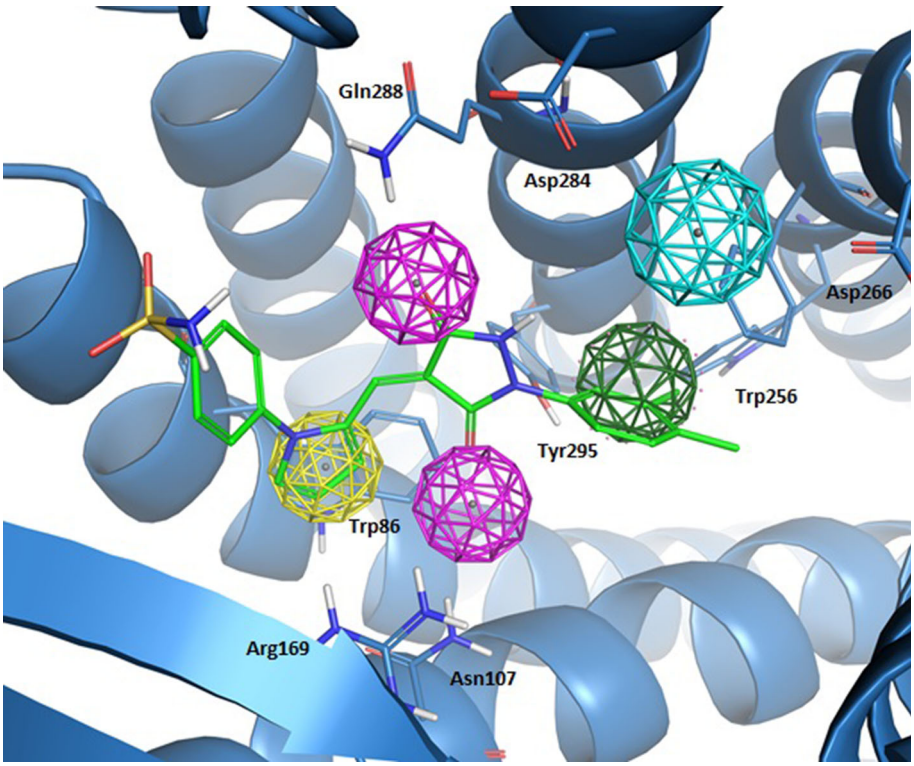


Fig. 13 Pictorial view of the proposed binding mode of compound (**8**) (see Table 1) to the BK B_2 receptor with the pharmacophore points represented as spheres of different colors: *cyan* for a proton donor/positive charge; *magenta* for a proton acceptor/donor; *yellow* for an hydrophobic ring; *dark green* for an aromatic/hydrophobic moiety

two charges separated 10 Å that gave rise to the discovery of WIN64338, includes points 1 and 4 of the present pharmacophore. On the other hand, in order to mimic the C-terminus of BK after a thorough exploration of the conformational space of the five bradykinin analogues, it was suggested a few years ago a partial pharmacophore for BK antagonism [44, 45]. This includes an ionizable positive charge, a hydrophobic group and an aromatic/hydrophobic group in a specific spatial arrangement of 4.5–7.5, 5.5–8–5 and 8.5 Å. This pharmacophore is included in the more general pharmacophore described in the present work. Distances are not directly comparable since in the present pharmacophore distances are defined on the side chains of receptor residues and not on the chemical moieties. Thus, the ionizable positive charge is the moiety facing point 2 of the present pharmacophore; the hydrophobic group is the one facing point 5 of the present pharnacophore and the aromatic ring is the moiety interacting with point 3 in the present pharmacophore.

Proof of concept

We used the pharmacophore described above for the discovery of new structures with antagonistic activity for the B_2 bradykinin receptor by virtual screening. For this purpose we searched for compounds fulfilling at least three pharmacophore points in different data bases of 3D structures of compounds including, the Available Chemical Directory (ACD), the Derwent World Drug Index, the National Cancer Institute (NCI) and Maybridge for a total of approximately 500,000 compounds. The search yielded a set of compounds that were subsequently classified into a few clusters. Specifically, molecules were first encoded into vectors using the fulfillment of three-point pharmacophores as criterion; second, a distance between vectors was computed using the Tanimoto index and, third a hierarchical clustering algorithm was used to classify the molecules [46]. A representative member of each of the clusters if available was purchased and tested for their B_2 antagonistic activity.

Biological assays permitted to identify new hits with structures that do not resemble those used for pharmacophore development. The success rate was approximately one-third of the molecules tested as previously found by other authors in similar studies [47]. Table 1 shows the structures as well as the antagonistic activity to the human bradykinin B_2 receptor of a selected group of hits, disclosed to give support to the pharmacophore hypothesis developed in this work. These molecules were docked onto the receptor model and inspected for fulfillment of the pharmacophore. Information regarding the number of pharmacophore points fulfilled by each of the hits is also included in Fig. 11. As an example, Fig. 13 shows the proposed binding mode of compound (**8**) to the B_2 receptor, showing the fulfillment of the pharmacophoric points. Interestingly, the antagonistic activity observed

experimentally correlates well with the number of pharmacophoric points fulfilled by these molecules.

Conclusions

We have constructed models of the bound conformation of diverse non-peptide B_2 bradykinin antagonist and analyzed the stereochemical features of the complexes with the aim to find common trends. For this purpose we first constructed an atomistic model of the receptor by homology modeling, using the CXC4 chemokine receptor as template. Antagonists selected for the present study include fasitibant, FR173657, anatibant, WIN64338, bradyzide, CHEMBL442294 and JSM10292, encompassing the maximum possible diversity. Complexes with the bound conformation of each of the antagonists were constructed by docking the molecules into the receptor. Due to the flexibility of the ligands and the size of the orthosteric site of the receptor, several docking attempts were carried out for each of the molecules. The final conformation was selected by the scoring function and the results on site directed mutagenesis studies available.

Our results suggest that there are certain anchoring points that are found in more than one compound permitting the definition of a common pharmacophore. This consist of five points that defined on the features of the ligand include a proton donor/positive charge (point 1), a proton acceptor/proton donor (point 2), an aromatic/planar hydrophobic moiety (point 3), a proton acceptor/proton donor (point 4) and a hydrophobic/aromatic moiety (point 5).

The pharmacophore was used in a subsequent study to guide a virtual screening process. The results permitted to identify a set of compounds some of which were purchased and in vitro tested for their capability to antagonize the bradykinin B_2 receptor. In the present work we disclose a subset of these compounds that give support to the validity of the pharmacophoric hypothesis described in this work.

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