European Journal of Medicinal Chemistry 44 (2009) 2840–2846

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, pharmacological and in silico evaluation of 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol, a compound designed to act as a β_2 adrenoceptor agonist

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ARTICLE INFO

Article history:
Received 16 May 2008
Received in revised form
13 November 2008
Accepted 15 December 2008
Available online 25 December 2008

Keywords:
BR-AEA
Boron
Docking
β_2 Adrenoceptor agonist

ABSTRACT

In this study, 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol, (BR-AEA), was designed, synthesized, characterized and tested in docking studies and in vitro. Previous to its synthesis, a set of compounds, including well-known ligands and boron containing compounds, were studied under docking simulations. BR-AEA showed greater affinity than these well-known agonists and was found to be slightly closer than salbutamol to the residues in the TM5 and TM3 of the β_2 adrenoceptor (β_2AR), making a greater number of interactions with them, including some that are apparently key to greater affinity and β_2AR activation. This study suggests that affinity is closely related to the interactions of the boron atom, as well as the capacity of boronic acid moieties to make a network of hydrogen bonds with the β_2AR. In vitro, the relaxing effects of BR-AEA on isolated guinea pig tracheal rings were compared with salbutamol. The EC_{50} values for BR-AEA were at least five-fold lower than for salbutamol, showing the greater potency of the former. Additionally, propranolol and ICI 118,551 showed competitive antagonism in relation to the relaxing effect of the test compound (pA_2 6.204 ± 0.367 and 9.089 ± 0.470, respectively).

1. Introduction

Development of β_2 adrenoceptor (β_2AR) agonists is an attractive area of research, since their biological effects have many applications in the medical field. These drugs are used alone or in combination with corticosteroids in the treatment of asthma and Chronic Obstructive Pulmonary Disease (COPD) [1]. In addition, β_2AR agonists have been studied in relation to Alzheimer disease [2], diabetes mellitus [3] and some vascular diseases [4,5].

Currently, only a few natural and pharmaceutical boron containing products are known, since nature lacks biosynthetic pathways to form a boron–carbon bond and probably lacks metabolic enzymes able to break such a bond down. This biological condition could represent an advantage if these molecules were employed as therapeutic agents [6–9]. Among the different organic boron containing compounds, boronic acids $RB(OH)_2$ are particularly suited for drug design owing to their low toxicity, adequate chemical stability under physiological conditions, and good lipophilicity [10]. Their strong Lewis acid character allows boronic acids to readily convert from the trigonal, planar sp^2 form to anionic, tetrahedral sp^3 complexes, by coordination with various nucleophilic centers. Some synthetic boron containing compounds have been shown to form very tight complexes with several serine proteases, such as chymotrypsin, β-lactamases, trypsin and thrombin [6]. In addition, X-ray crystal structures show that boronic acids inhibit these enzymes by the formation of tetrahedral adducts, analogous to the deacylation tetrahedral intermediate formed by the normal catalytic activity of these enzymes [6].

On the other hand, recent experimental and theoretical studies have suggested that some serine, threonine and tyrosine, which are amino acids that expose hydroxyl groups in their lateral chains, are implicated in β_2AR activation [11,12].

Abbreviations: BR-AEA, 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol; β_2AR, β_2 adrenoceptor; TM, transmembranal domain.

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doi:10.1016/j.ejmech.2008.12.016

Accordingly, by taking the structural characteristics of the binding site on the β_2AR into account, we designed of a set of boron containing compounds that can act as ligands for this receptor.

The β_2AR is a prototype for the G-protein-coupled receptor (GPCR) family, which makes it an excellent model system for studying the mechanism of activation and signalling of such receptors [12,13]. The catecholamine binding site of β_2AR is well known and has affinity for a wealth of structurally related ligands with a diverse functionality. Recent reports have demonstrated the existence of different binding sites on the β_2AR for various ligands, and have provided evidence that activation of a GPCR is a multistep process [14].

Other studies have demonstrated that the β_2AR can activate not only G-proteins of the Gs type, but also the Gi- and Gq-family, and that it interacts with other proteins such as β-arrestin [12–15]. This evidence is not divergent from what Kobilka and co-workers reported recently when using models developed by Rasmussen et al. [16] and Cherezov et al. [17] with X-ray crystallography, and when observing the differences in signalling from β_1 and β_2 adrenoceptors [18]. All these information help in the development of new ligands for the β_2AR.

The aim of this study was to employ docking studies to design a compound with a better fit on the β_2AR than the well-known ligands. One of the boron containing compounds with the best fit was 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol, a derivative of salbutamol whose existence had only been suggested [19] which in this paper is denominated as BR-AEA (acronym of borate salt of R-Arylethylamine), was synthesized and chemically characterized in this study. Once characterized, an attempt was made to establish its pharmacodynamic effects on β_2AR, first by utilizing docking studies to examine the binding site on the receptor for all the ligands proposed. The K_d values of BR-AEA and those of other well-known ligands and boron containing compounds were compared based on these theoretical studies. After that, in vitro assays were carried out and the K_d value obtained for BR-AEA was compared with reported in vitro data for salbutamol and other well-known ligands.

2. Results

2.1. Chemistry

By placing R-salbutamol in an alkaline medium with NaOH, the electrophilic attack of the boron atom of boric acid on the electron rich oxygen atoms was increased, allowing them to bind to the hydroxyl groups located in the aromatic ring, yielding BR-AEA. This derivative was synthesized as depicted in Scheme 1.

The spectroscopic data obtained (see below) show signals which correspond with the proposed structure, and these signals are in agreement with those reported for other analogous structures [20].

2.2. Effects of BR-AEA on guinea pig tracheal rings

By testing BR-AEA and salbutamol on guinea pig tracheal rings (precontracted with carbachol, 1 μM, or histamine, 10 μM), it was found that both induced concentration-dependent relaxation at concentrations greater than 0.1 nM. The maximum relaxant effect was observed at 1 μM for BR-AEA and 100 μM for salbutamol. EC_{50} values of BR-AEA and salbutamol were 0.072 nM and 0.454 nM, respectively, when histamine was used as a precontractile agent (Fig. 1), and were 2.097 nM and 268 nM, respectively ($n = 6$), when carbachol was used (Data not shown). The maximum relaxation values in relation to both drugs correspond to more than 100% of the histamine or carbachol-induced contraction.

Propranolol at concentrations greater than 50 nM, and ICI 118,551 at concentrations greater than 1 nM shifted the dose-response curve of BR-AEA to the right, thus showing competitive antagonist effects when used with isolated guinea pig tracheal rings precontracted with histamine. The pA_2 value for the antagonist activity of propranolol in the presence of salbutamol was 7.740 ± 0.470 and in the presence of BR-AEA was 6.204 ± 0.367. On the other hand, this value for the antagonist activity of ICI 118,551 in the presence of BR-AEA was 9.089 ± 0.470 (Fig. 2).

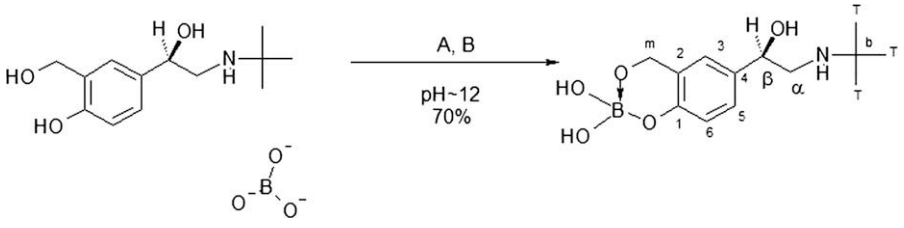
2.3. Molecular modeling

A set of ligands, including the proposed boron containing compounds (Scheme 3), salbutamol (a partial agonist with known affinity on the β_2AR) and other well-known agonists, the inverse agonist ICI 118,551, and the antagonist propranolol were docked with their lowest conformational energy on the β_2 adrenoceptor binding site. Several well-known residues (Asp113, Thr118, Tyr199, Ser203, and Ser207) close to the ligands are involved in the recognition process. The K_d value of BR-AEA obtained with computational methods was smaller than that for salbutamol and other known ligands. Additionally, from the set of proposed compounds (Scheme 3) with similar structure, the role of boron and the number of interactions between any given ligand and TM5 amino acids were identified (Scheme 2).

The interactions and binding site for each ligand on β_2AR were identified among one hundred low energy conformations. BR-AEA and salbutamol, which were tested in vitro and compared, were at the lowest energy conformation (Fig. 3). According the docking studies, the binding sites for both on the β_2AR were found to be close to each other. Hydrogen bond interaction between the carboxyl group of Asp113 and the N atom of BR-AEA, was observed to have a distance of 2.63 Å. This amino acid, together with the side chains of Tyr316 and Asn312, forms an assembly surface of polar groups for the alkylamine and alcohol groups of BR-AEA, while Val114, Val117, Phe193, Phe289 and Phe290 form a hydrophobic cluster that involves hydrophobic and π–π interactions. The proximity of the boron atom of BR-AEA and the hydroxyl groups of Tyr199, Ser203, Ser204, and Ser207 residues of the transmembranal domain 5 (TM5) is illustrated in Fig. 3. The resulting hydrogen bond is <4.1 Å in each case.

3. Discussion

The aim of this study was the design, synthesis and characterization (chemical and pharmacodynamic) of a compound with



Scheme 1. Synthesis of 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol (BR-AEA). Reagents and conditions: (A) acetonitrile:methanol (v:v 1:2) and NaOH, reflux 2 h; (B) boric acid and water, reflux 2 h.

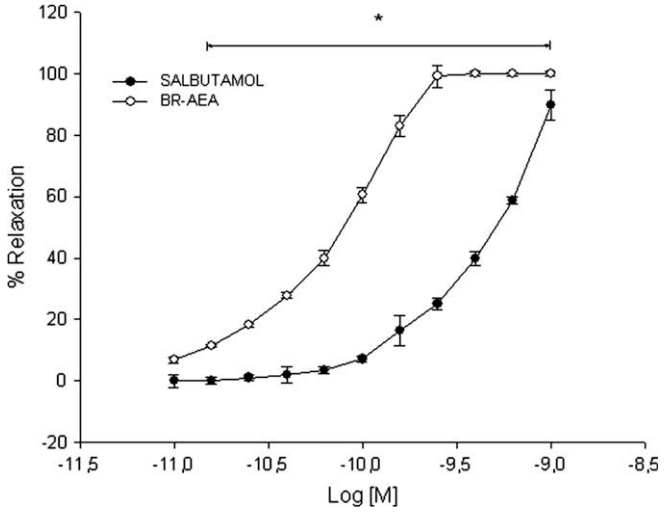


Fig. 1. Effect of BR-AEA and salbutamol on isolated guinea pig tracheal rings precontracted by histamine 10 μM. Each point represents the mean effect of 6 experiments; vertical bars represent the S.E.M. * indicates a statistically significant difference $p < 0.05$.

capacity for interacting with proposed key amino acids involved in β_2AR activation. Each of these residues has a hydroxyl group exposed in its lateral chains (Scheme 2). Accordingly, we considered boron containing compounds that have greater affinity for hydroxyl groups on the β_2AR, and compared them to salbutamol, regarded as a selective agonist agent on this receptor [21]. Among the boron containing compounds, the one that has the structure most like salbutamol is BR-AEA. A careful consultation of the literature in relation to this compound showed that its synthesis has not been described, nor has it been characterized spectroscopically or tested biologically on the β_2AR [19]. In the current study, it was considered useful to do such characterization and testing due to the biological advantages of boron containing compounds.

As there are several pharmacological tools to test compounds which have an analogous structure [22], we compared the pharmacodynamic parameters of BR-AEA and salbutamol in vitro on isolated tracheal rings from guinea pigs. Previously, docking studies

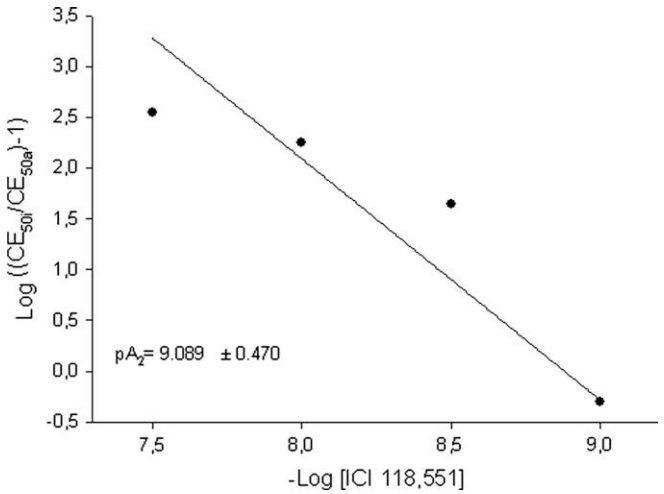
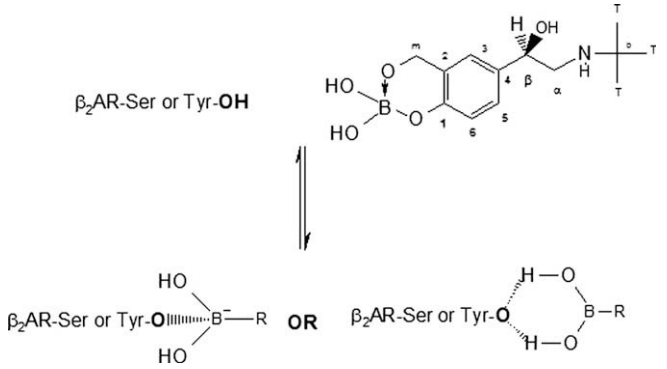


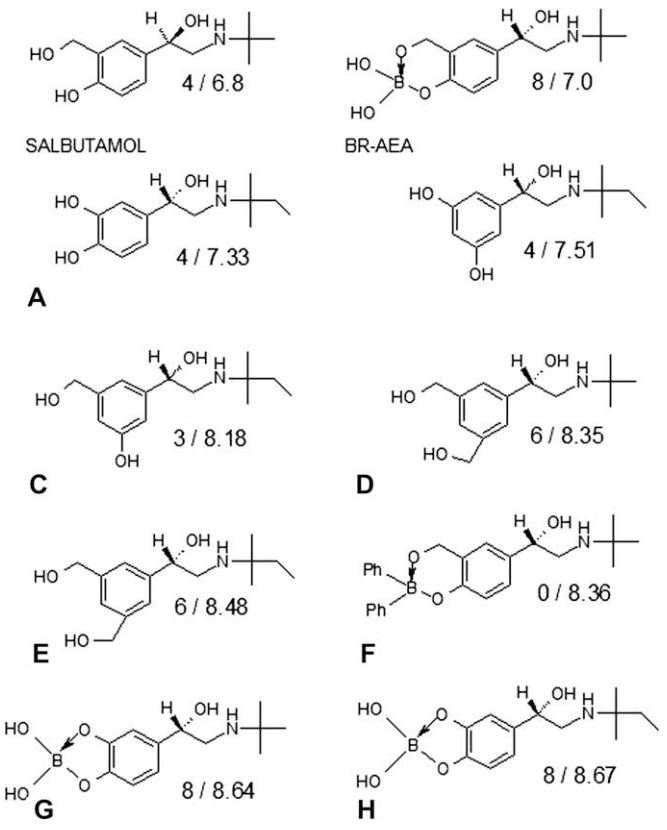
Fig. 2. Schild regression applied to the antagonist action of ICI 118,551 on the relaxant effect of BR-AEA on guinea pig tracheal rings precontracted with histamine ($n = 6$).



Scheme 2. Possible interactions between a boronic acid compound (BR-AEA) and some key amino acid residues of the β_2 adrenergic receptor (β_2AR).

were done to identify and explain the BR-AEA interaction mechanism on the β_2AR.

The pharmacological effect of both compounds was evaluated after the tracheal rings were precontracted with histamine or carbachol, in the presence of propranolol (a non-selective β adrenoceptor antagonist) or ICI 118,551 (a well-known β_2 adrenoceptor selective inverse agonist that acts as an antagonist, blocking the effect of agonists) [23]. These contraction-triggering agents produce broncoconstriction by the activation of G-protein-coupled receptors, the functioning of which seems to be altered in patients with asthma and COPD [24].



Scheme 3. Salbutamol, BR-AEA and compounds with similar structure were tested in docking simulations. Number of hydrogen bonds (HB) observed between ligands and residues on transmembranal domain 5 of the β_2 adrenergic receptor and pK_d values are added for each structure (HB/pK_d).

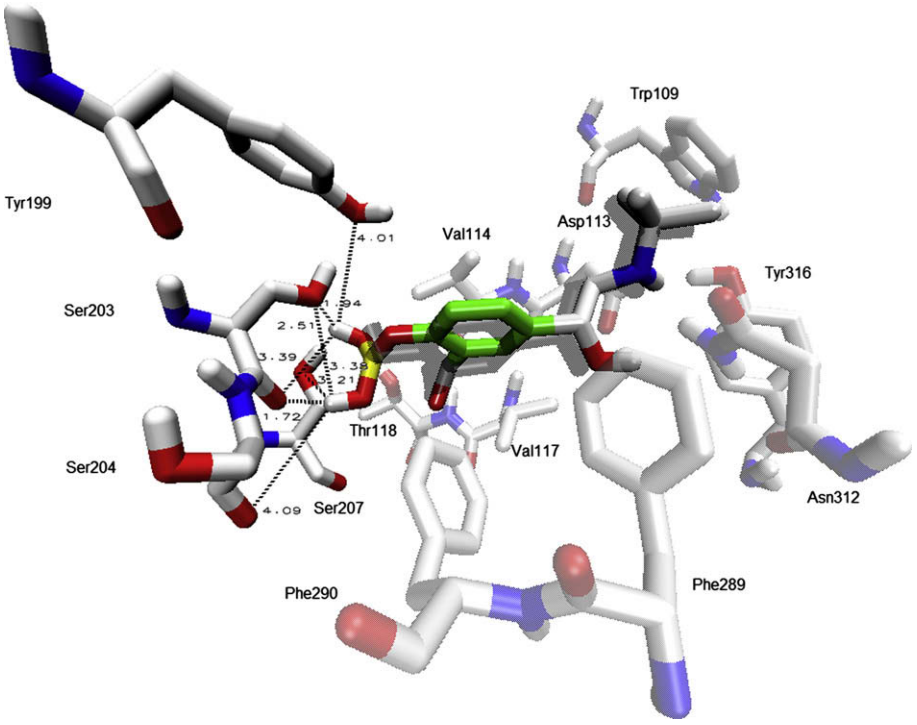


Fig. 3. A close-up extracellular view of the predicted binding site for 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol; its hydroxyl groups are closer to the Tyr199, Ser203, Ser204 and Ser207 residues in TM5 than that of salbutamol (black). Atoms are coloured by type. The amino acids which form the binding pocket, as well as the possible network of hydrogen bonds formed in the interaction between the test compound and the mentioned residues of the β_2 adrenoceptor and the distance (in Angström units) of the bonds are all shown.

A relaxing effect on guinea pig tracheal rings was observed with concentrations of BR-AEA lower than 0.1 nM, whereas its EC_{50} was at least five-fold lower than for salbutamol under all experimental conditions. This, together with the fact that the relaxation induced by BR-AEA and salbutamol was blocked competitively by propranolol, suggests that the BR-AEA effect is mediated by a β adrenoceptor. The competitive antagonist activity of ICI 118,551 on the relaxing effect of BR-AEA (as seen by using the Schild regression according to the Kenakin analysis [25]) supports the idea that this effect was mediated by selective β_2AR activation (Fig. 2). This information leads us to conclude that BR-AEA and salbutamol have similar mechanisms for producing tracheal smooth muscle relaxation. According to the proposed mechanism, which is in agreement with previous studies of other related compounds [26,27], the activation of this receptor is possible when a ligand is able to interact with TM 3, 4, 5 and 6, and generate a conformational change that allows for the triggering of the signalling pathway, which in turn results in an interaction with TM3 and the formation of at least two hydrogen bonds with TM5 or TM6.

The binding sites for the test compound and other ligands at their lowest docking energy on the β_2AR were identified by docking studies. All of these sites seem to be in the same domain, but the groups of residues that each of these compounds reacts are not identical.

The few differences that were identified in relation to the interactions of salbutamol or BR-AEA on the β_2AR appear to be crucial for making insights into the activation mechanism of this receptor, as the residues unique to BR-AEA are probably optimum for creating affinity. The binding site for BR-AEA is slightly closer to TM5 than that for salbutamol, and therefore the lengths of the hydrogen bonds formed between the hydroxyl groups located at the boron atom of BR-AEA and the hydroxyl groups of the Tyr199, Ser203 and Ser207 residues in TM5 are shorter than those for salbutamol. The greatest bond length for BR-AEA is 4.09 Å, and the smallest is 1.72 Å, while for salbutamol these values are 4.2 and 1.97, respectively (Fig. 3). In each case, these bonds correspond with the active site described for other ligands [28,29]. There is also a hydrogen bond formed between the hydroxyl groups linked to the boron atom of BR-AEA and the hydroxyl group of the Thr118 residue in TM3, an interaction which does not exist with salbutamol. Since the latter residue has been reported as a key point in the interaction for full agonists on the β_2AR [11], the bond formed between Thr118 and BR-AEA could be the main reason for the greater pharmacological effects of this compound. Another difference between BR-AEA and salbutamol is that the former has hydrogen interaction with the oxygen backbone of the Ser204 residue in TM5, while the latter does not. The amino acids found in TM5 could be particularly important, as they seem to be involved in controlling the equilibrium between the active and inactive forms of the β_2AR [11,26]. Some amino acids found in the TM3 and TM6 domains seem to form switch and toggle mechanisms that regulate receptor activation [11,30].

Some residues included in TM 3, 4, 5, and 6, constitute the binding pocket reported recently [12,28]. With BR-AEA there are important interaction sites that allow it to fit in the binding pocket, as has been reported for other ligands [28,31] Thus, the higher affinity of BR-AEA than salbutamol for the β_2AR, evidenced by the lower experimental K_d values for the former compound (Table 1), could be due to its greater number of interactions in this binding pocket. If true, this would mean that the greater surface area of interaction of BR-AEA activates the receptor more efficiently than salbutamol. On the other hand, there are many similarities in interactions between the β_2AR and BR-AEA or salbutamol.

Both the binding site as well as the steps in the activation of the β_2 adrenoceptor by related compounds that were previously reported [28,29], are congruent with the observations in relation to

Table 1
Free energy (kcal/mol), K_d and interactions between ligands and β_2 adrenoceptor.

Ligand	DOP	ORC	NOR	TER	SAL	ADR	BRA	ISO	PRO	ICI
K_d [17,32–34] (experimental, μM)	64.56	4.81	3.98	2.51	1.16[a]	0.63	0.11[a]	0.05	7.9×10^{-4}	6.3×10^{-4}
K_d (calculated, μM)	2.45	0.70	1.62	1.48	0.16	1.62	0.09	0.01	0.60	0.10
HB with TM5 residues of β_2AR	3	3	3	4	4	4	8	4	0	0

DOP = dopamine, NOR = noradrenaline, ADR = adrenaline, ISO = Isoprenaline, TER = terbutaline, ORC = Orciprenaline, SAL = salbutamol, BRA = BR-AEA, PRO = propranolol and ICI = ICI 118,551. All calculated K_d values were estimated from docking data. Hydrogen bonds (HB) observed in docking studies between ligands and transmembranal domain 5 (TM5) residues.

[a] Experimental K_d value was estimated from effect on guinea pig tracheal rings, other K_d values are from the literature [17,32–34].

BR-AEA. First, a ligand interacts with the Asp113 residue, followed by conformational change interactions with hydroxyl lateral groups of the Ser203, Ser204, and Ser207 residues. This conformational change is generated by particular movements in TM 3, 4, 5, and 6 of the β_2AR [11,26].

In docking studies, BR-AEA interacted with the Trp109, Asp113, Val114 and Val117 residues in TM3 of the β_2AR. Similar interactions were found for salbutamol. In other words, there is a hydrophobic interaction between the tert-butyl group of the ligand and the side chain of Trp109, which constitutes an exposed aliphatic and aromatic noncharged region. A hydrogen bond is formed between the hydroxyl group located at the β carbon of both BR-AEA and salbutamol, and oxygen atoms from the side chain of Asn312, as well as between the same hydroxyl group of the ligand and the oxygen of the carboxyl group of the Asp113 residue. The latter residue interacted with the amino group in the ligand, making the well-known salt bridge group bond.

Similarities and differences observed in interaction and affinity of well-known ligands in relation to β_2AR, shown both by in vitro and in silico data from recent studies [11,17,32–35], lead one to consider that the presence of a boron atom as well as the number of possible hydrogen bonds among ligands and residues involved in the receptor activation is of utmost importance. A set of ligands (Scheme 3) with both a boron atom and the possibility of these hydrogen bonds was developed and tested using computational tools. Compounds with a similar structure to salbutamol were included in this set, taking into account the essential requirements for interaction with the β_2AR [36], including the capacity for interacting with residues in TM5. The major differences between the test compounds and well-known ligands were the presence of a boron atom in some compounds, and an additional methyl moiety bonded to one of a free methyl fragment of the tert-butyl group. The latter change was included because it is known that bulky moieties are bonded to the amino group in the β_2AR agonist with long action (LABAS) [37], but

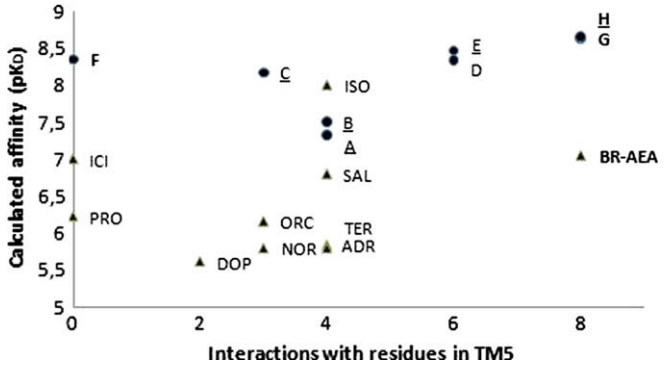


Fig. 4. The number of interactions and affinity values calculated by docking simulations for well-known ligands on the β_2AR (▲) and compounds included in Scheme 3 (●). Labels for compounds with boron are in bold and for compounds with a bulkier group than tert-butyl bonded to the amino moiety are underlined; see Table 1 for code key of abbreviations.

the effect of small additional groups has not been reported. The greater the possibility for making hydrogen bond interactions with side chain residues in TM5, the greater affinity of compounds for the receptor (Fig. 4). In this sense, boron containing compounds showed greater affinity than those without boron atoms. Although compound F does not contain hydroxyl groups (see Scheme 3), this boron containing compound showed additional interactions with amino acids residues as well as great affinity for the receptor. In this case, the partial negative charge probably explains the high affinity, since boron is near the hydrogen of Ser203 and Ser207 in TM5.

4. Conclusion

Both the experimental data in relation to tracheal rings from guinea pigs and docking studies evidence that BR-AEA has greater affinity than classical agonists such as salbutamol for the β_2AR. The key factor that probably provides BR-AEA with its experimentally observed higher affinity for the receptor than salbutamol is the larger number of interactions in the active site of the β_2AR. Some of these interactions, such as that with Thr118 in TM3 and those with some residues in TM5, in which BR-AEA is closer to the residues than salbutamol, apparently play a crucial role in the activation of the adrenergic receptors. In TM5 it is the boron atom of BR-AEA that probably causes the closer interaction of the ligand with the residues. Accordingly, docking simulations of the test compounds support the idea that the presence of a boron atom increases the affinity for the β_2AR. The reason that the boron atom in the ligand probably causes greater affinity is the bond between this atom and the hydroxyl groups of the β_2AR. This bond may play a similar role with this receptor as the corresponding bond of boronic acid derivatives with serin proteases (see Scheme 2) that are inhibitors and have nanomolar concentration affinity [6]. Currently, the role of boron in molecules with biological activity is being intensively researched. The presence of a boron atom in 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino) ethanol makes it the first of boronic acids derivatives tested with β_2AR agonist action.

5. Experimental protocols

The protocol was revised and approved by the bioethical committee of our Institution and is in accordance with the Mexican Health Law in relation with the use of experimental animals.

5.1. General procedure for synthesis of 1-(4-di-hydroxy-3, 5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol

BR-AEA was obtained by mixing equimolar amounts of salbutamol and NaOH, using 20 ml of a solution of acetonitrile:methanol (v:v 1:2) for each gram of salbutamol; the mixture was vigorously stirred at 75 °C for 2 h, in a reflux system. A yellow solution was obtained, to which boric acid, previously dissolved in 1 ml of

bidistilled water, was added. The mixture was vigorously stirred at 75 °C for another 2 h in the reflux system. Then, the solution was submitted to vaporization and the product obtained was precipitated and washed with distilled H_2O (3×30 ml). The resulting suspension was filtered and the products dried at 40 °C for 24 h.

5.2. Chemical characterization

The reaction was monitored by thin-layer chromatography (*TLC*, silica gel 60 F_{254}, 0.25 mm), and the product visualization was done using a 254 nm UV lamp. The uncorrected melting point was obtained in open-ended capillary tubes with an Electrothermal 9300 digital apparatus. The molecule obtained was identified by IR spectrum (recorded on a MIDAC M2000 FT-IR instrument, KBr), and by ^{1}H, ^{13}C and ^{11}B NMR spectra (recorded at 270 MHz, 67.5 MHz, and 86.5 MHz, respectively) on a Jeol GSX-270 spectrometer, using DMSO-d_6 as solvent and TMS as internal reference. EIMS (70 eV) experiments were determined on a Hewlett Packard 5989A mass spectrometer under the electron-impact ionization mode and by direct insertion.

1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol. Yellow powder; yield: 70%; m.p.: 161 °C; IR (KBr) ν_{max}/cm^{-1}, 3658, 2968, 1617, 1365; ^{1}H NMR (DMSO, 270 MHz) δ/ppm 1.1 (9H, s, H-T); 2.50 (1H, t, $J = 1.7$ Hz, H-β); 2.66 (2H, d, J 6.2 Hz, H-α); 4.43 (2H, t, $J = 5.9$ Hz, H-β), 4.59 (1H, s, H-m), 6.40 (1H, d, $J = 8.1$ Hz, H-6), 6.78 (1H, d, H-3), 6.89 (1H, dd, $^3J = 8.1$ Hz, $^4J = 2.0$ Hz, H-5), ^{13}C NMR (DMSO, 67.5 MHz) δ/ppm 28.0 (C-T), 50.4 (C-b), 52.5 (C-α), 61.9 (C-m), 71.8 (C-β), 116.5 (C-6), 122.5 (C-3) 124.9 (C-5), 126.41 (C-2), 131.0 (C-4), 157.3 (C-1), ^{11}B NMR (DMSO, 86.5 MHz) δ/ppm 1.1 (B); m/z (EI) 284 [M$^+$], 81 (100%).

5.3. Biological experiments

5.3.1. Chemicals

Histamine phosphate, carbachol, propranolol, ICI 118,551, and R-Salbutamol were purchased from Sigma–Aldrich© (St. Louis MO, U.S.A.).

5.3.2. Bronchorelaxation effects on precontracted isolated guinea pig tracheal rings

Male Hartley guinea pigs (weighing 325 ± 25 g) were sacrificed by cervical dislocation under light ether anesthesia. After the trachea was dissected and withdrawn, tracheal rings (length = 5 mm) were prepared and mounted under a resting tension of 2 g in an organ bath containing 10 ml of Krebs–Henseleit solution: (in mM, pH = 7.4): NaCl 189.6, KCl 5.98, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 0.16, and glucose 11.1, which was continuously perfused with a gas mixture (O_2 95%, CO_2 5%) and maintained at 37 °C. The isolated guinea pig tracheal rings were precontracted with carbachol (1 μM) or histamine (10 μM). The maximum contraction induced by histamine or carbachol was taken as 100% and the cumulative concentration-response curves (determined by relaxing tracheal rings) were established for salbutamol as well as BR-AEA (from 0.01 nM to 0.1 mM).

5.3.3. Effects of propranolol and ICI 118,551 on the BR-AEA response

The isolated guinea pig tracheal rings were precontracted with carbachol (1 μM) or histamine (10 μM), then incubated with propranolol (10 nM) or ICI 118,551 (1 nM). Five minutes later, BR-AEA was added to the organ bath under cumulatively concentrations from 0.01 nM to 0.1 mM, and the maximum relaxation produced by BR-AEA was taken as 100%; the concentration-response curves of BR-AEA in the absence and presence of propranolol or ICI 118,551 were established. EC_{50} and pA_2 values were obtained by WinNonlin™ Ver 2.1 (Pharsight Corporation, CA, U.S.A.), antagonist potency was expressed with pA_2 values obtained from Schild plotting according to the method described by Kenakin [25].

5.4. Molecular modeling

In this study, the target compounds were docked on β_2AR, which was previously characterized in 3D by X-ray crystallography studies [17]. The β_2AR was obtained from Protein Data Bank (PDB code: 2rh1).

The compounds were geometrically optimized by Gaussian 98 software [38] at the B3LYP/6-31G* level. Parameters for the boron atom were based on those described by Tafi et al. [6]. To identify the recognition site and to determine β_2 adrenoceptor-ligands affinities, docking simulations were done on the human 3D protein structure, which is the main target for drug design as was previously mentioned [39]. Before docking analysis, the T4-lysozyme and water molecules were removed from the β_2 adrenoceptor, then the protein hydrogens were added, and finally the whole protein was minimized in 2000 steps by using the CHARMM27 parameters implemented in the Nanoscale Molecular Dynamics (NAMD) program [40]. Then, all possible flexible bonds of the ligands, the partial atomic charges (Gasteiger–Marsili formalism), and the Kollman charges for all atoms in the β_2 adrenoceptor were assigned using AutoDock Tools 1.4.5 [41]. Finally, by using the sam2e program, hydrogen placed at the polar atoms was maintained on the amino acids, considering a ~7.4 pH value. All other parameters were kept at their default values. Binding was modeled using AutoDock 3.05, as its algorithm allows full flexibility for small ligands. The input preparations of the protein, ligand structures and binding sites definitions were carried out under a GRID-based procedure [42]. Then, a box with $60 \times 60 \times 60$ Å point grid with 0.375 Å spacing was constructed over the Cα of Asp113. All simulations used the hybrid Lamarckian Genetic Algorithm, with an initial population of 100 randomly placed individuals and 10 million maximum number of energy evaluations. Docked orientations within a root-mean square deviation of 0.5 Å were clustered together. The lowest energy cluster returned for each compound was used for further analysis. The interactions of the ligands on β_2AR were visualized and the figures were created using AutoDock Tools v1.4 and VMD 1.8.5 Software.

5.5. Statistics

The biological test results are expressed as mean values ± standard error of the mean (SEM) of six experiments. Antagonistic potency was expressed as pA_2 values, obtained from Schild plotting. Statistical significance of differences between groups was verified with two-way analysis of variance (ANOVA), with a confidence limit of 95%.

Acknowledgments

The present work and the scholarships for the authors were supported partially by COFAA, SIP-IPN(20080026), and CONACYT(62488). We thank Bruce Allan Larsen for his review of the use of English in the manuscript.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.12.016.

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