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Design, synthesis and in vitro evaluation of (*R*)-4-(2-(*tert*-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)phenyl hydrogen phenylboronate: A novel salbutamol derivative with high intrinsic efficacy on the β_2 adrenoceptor

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ABSTRACT

We tested a set of boron containing arylethanolamine derivatives on the human and guinea pig β_2 adrenoceptor (β_2AR) 3-D structures by docking methodology. The compound with the highest affinity based on docking analysis, (*R*)-4-(2-(*tert*-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)phenyl hydrogen phenylboronate (boronterol) was synthesized, characterized and tested in guinea pig tracheal rings at basal tone and with histamine-induced contractions. Boronterol was at least eightfold more potent than salbutamol as a smooth muscle relaxant drug (judged by the EC_{50} values) and showed a similar maximal relaxant effect as isoproterenol. ICI118,551 showed competitive antagonism on the relaxing effect of boronterol. These results suggest the β_2AR agonist action of boronterol.

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 in the treatment of asthma and chronic obstructive pulmonary disease,[1] as well as in other medical applications.[2]

In order to design β_2AR agonists with high affinity and long half-life, we decided to test boron containing compounds. Several different molecules have shown high potency in biological systems when boron is added.[3,4] Although only a few natural and pharmaceutical boron containing products are known,[3,4] some have an essential role in the binding site through high affinity for serine residues.[3] Indeed, serine, threonine, tyrosine residues in fifth transmembrane domain (TM5), which expose a hydroxyl group in their lateral chains, have been implicated in β_2AR activation, according to some experimental and theoretical studies.[5] Thus boron containing compounds could have high affinity for β_2ARs.

In one of our previous studies, the capacity of compounds containing boron to form hydrogen bonds with hydroxyl groups in lateral chains of residues in TM5 of β_2ARs was suggested. Moreover, since salbutamol is a standard drug for smooth muscle relaxation, we recently designed and tested in vitro a boron containing derivative of this drug. Our test compound showed greater potency than and similar efficacy to salbutamol as a relaxant drug on guinea pig tracheal rings.[6] Also, that compound showed greater half-life than salbutamol in rabbit, which we proposed was due to the boron derivative of salbutamol could avoid the enzymatic biotransformation of the two alcohols (the phenol and the primary alcohol) that takes place in its precursor.[6]

Additionally, we conducted a theoretical study for building a guinea pig β_2AR (gβ_2AR) model based on homology with reported X-ray crystallography human β_2AR (hβ_2AR) models.[7] The 3-D model obtained showed the capacity for predicting affinity values, judging by correlation of such values with those reported in vitro for well-known ligands. We suggest that this model can be a useful tool in drug-design when guinea pig tissues are used in the evaluation of new ligands for β_2ARs.[7]

Based on this information, and by taking the chemical and structural properties of the binding site on the gβ_2AR and hβ_2AR into account, we proposed 28 new compounds (Fig. 1). 3-D representations, which were built for the test compounds as well as for

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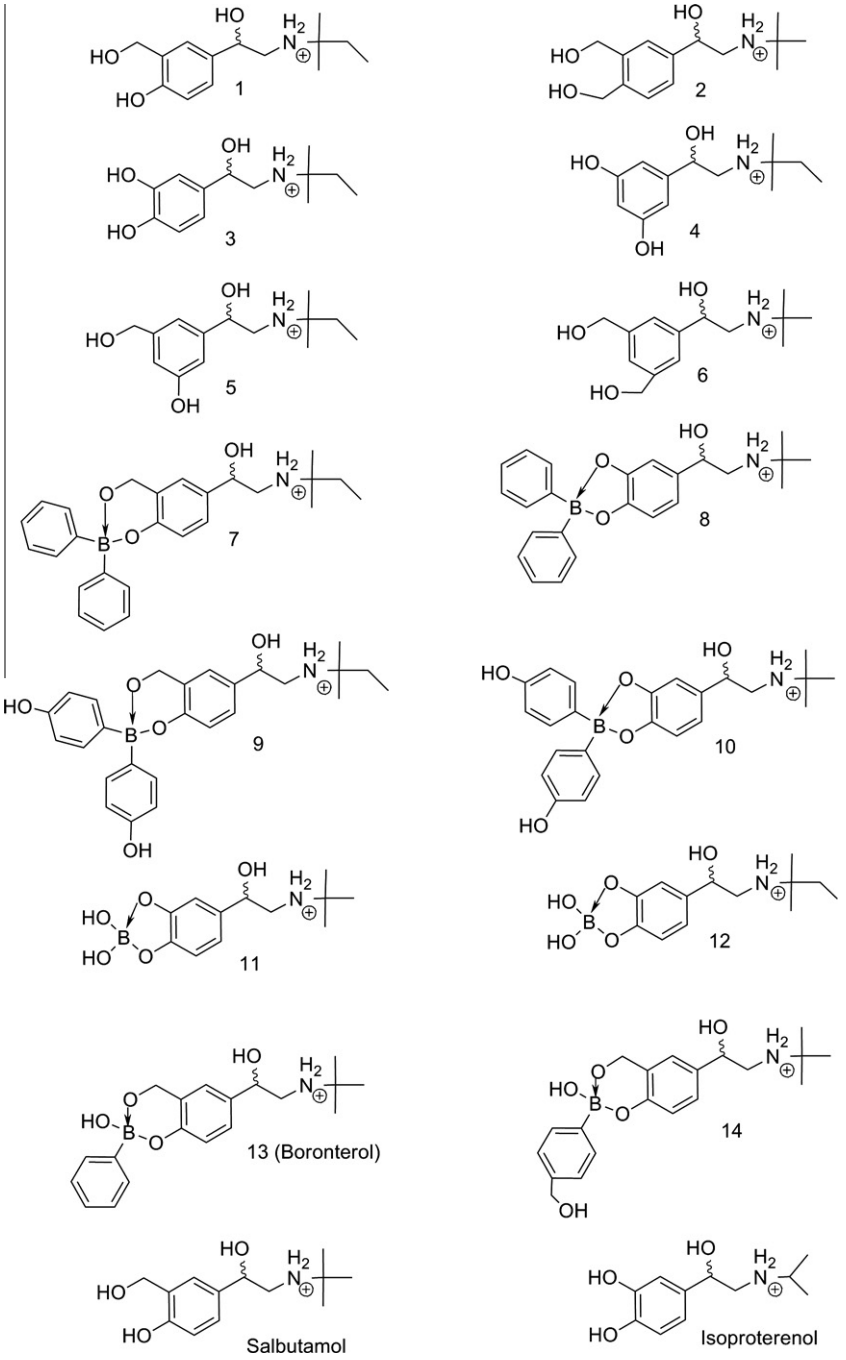

Figure 1. Ligands included in drug screening on human and guinea pig β_2AR 3-D models. Wavy bonds were considered for building *R*/*S* ligand 3-D models.

salbutamol and isoproterenol, were geometrically optimized and docked on human and guinea pig β_2AR 3-D models, as was done in previous studies in order to study atomic-molecular details about the fit on the β_2AR.[8]

Docking simulations were carried out with these 28 compounds on the β_2AR models by using AutoDock software for identifying the recognition site and to determine β_2AR-ligand affinities.[9] A box with an 80 × 80 × 80 Å point grid and a 0.375 Å spacing was constructed over the Cα of Asp113 for each β_2AR model in order to limit the binding site explored on the β_2AR.[10] The lowest energy cluster returned for each compound was used for further analysis. The interactions of the ligands on β_2AR were visualized and figures were created using AutoDock Tools v1.4.5 and VMD 1.8.6 Software.[9,11]

Thus, the enantiomeric R form of compound **13** ((*R*)-4-(2-(*tert*-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)phenyl hydrogen phenylboronate, named boronterol) was selected based on the highest affinity values (obtained by docking simulations) compared to the other compounds, and the capacity to interact with the reported key residues in β_2AR models.[12] The K_d value of boronterol from docking simulations was smaller than that for other known and proposed ligands on both β_2AR models (Fig. 2), indicating that among these ligands it has the greatest affinity. The difference in estimated affinities between these models could be due to the fact that affinity values are underestimated in the gβ_2AR model, as previously reported.[7] However, the affinity of the ligands for both models of β_2AR showed the same tendency.

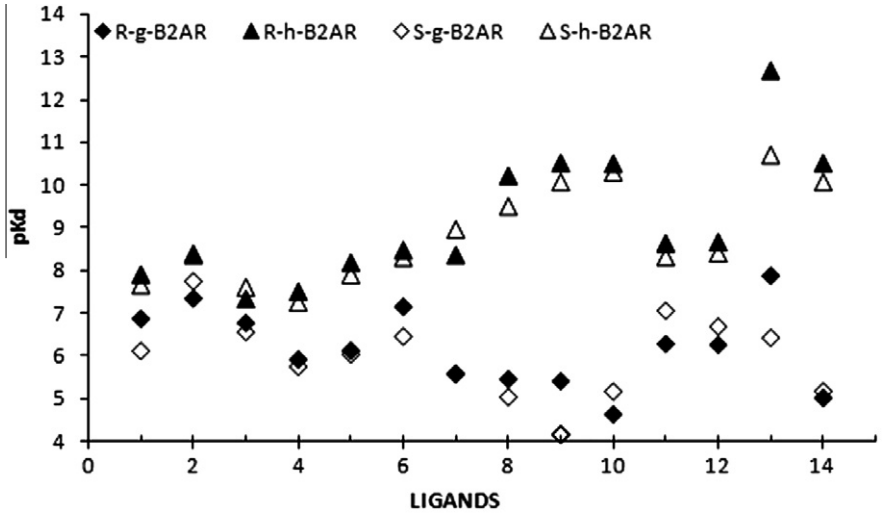

Figure 2. Estimated affinity of proposed *R*(filled)/*S*(blank) ligands on gβ_2AR (rhombi) and hβ_2AR (triangles) models. Numbers on the '*X*' axis correspond to R/S ligands in Figure 1.

In the current study, a similar binding mode in docking simulations was found for almost all ligands tested. The binding sites for boronterol on the hβ_2AR involved a electrostatic interaction between the N^+ atom of boronterol and the oxygen in the carboxyl group of Asp113, as well as hydrophobic and $\pi-\pi$ interactions between a hydrophobic cluster of hβ_2AR and the aromatic ring linked to the alkylaminoethyl moiety. The side chain of the carboxyl group of Asp113, together with those of Trp109, Thr110 and Asn312, form an assembly surface of polar groups for the alkylamine and alcohol groups of boronterol, while the side chains of Phe193, Phe286, Phe289, and Phe290 form the aforementioned hydrophobic cluster. Furthermore, in boronterol the proximity of both the boron atom and the hydroxyl gem to boron with the Tyr199 and Ser203 residues of the TM5 domain make hydrogen bond interactions.

Other ligands under docking study on the β_2AR models also showed interactions with Thr110, Thr118, Val114, Val117, Ser204, and Ser207. Although similar interactions were found between gβ_2AR and hβ_2AR (Fig. 3), critical differences were observed. For example, in hβ_2AR (a) Phe166 is at the top of the binding site, (b) Asn293 is not included in the binding site, as it forms part of the second extracellular loop, and (c) a lower number of contacts were found between the amino acids in TM5 and the ligands.

There was a greater affinity of some boron containing ligands, compounds **7–14** in Figure 1, to the hβ_2AR. The essential role of boron and the interactions between any given ligand and the TM5 amino acids of hβ_2AR were in accordance with previous reports.[6,13] However, this was not the case for simulations of all ligands under study on the gβ_2AR model. Whereas some ligands showed greater affinity for the TM5 domain of gβ_2AR, others (*R*/*S* 1,3,11 and 12) showed greater affinity for the amino acids in TM3 and ECL2 (Supplementary Fig. 1). The latter site is in a slightly extracellular situation, unlike the well-known binding site described in crystal structures of hβ_2AR.[13]

Because of the results of the docking studies, boronterol was synthesized by mixing equimolar amounts of *R*-salbutamol and NaOH (pH ~12). This mixture was vigorously stirred at 115 ± 5 °C for 30 min in a reflux system, using 50 ml toluene for each gram

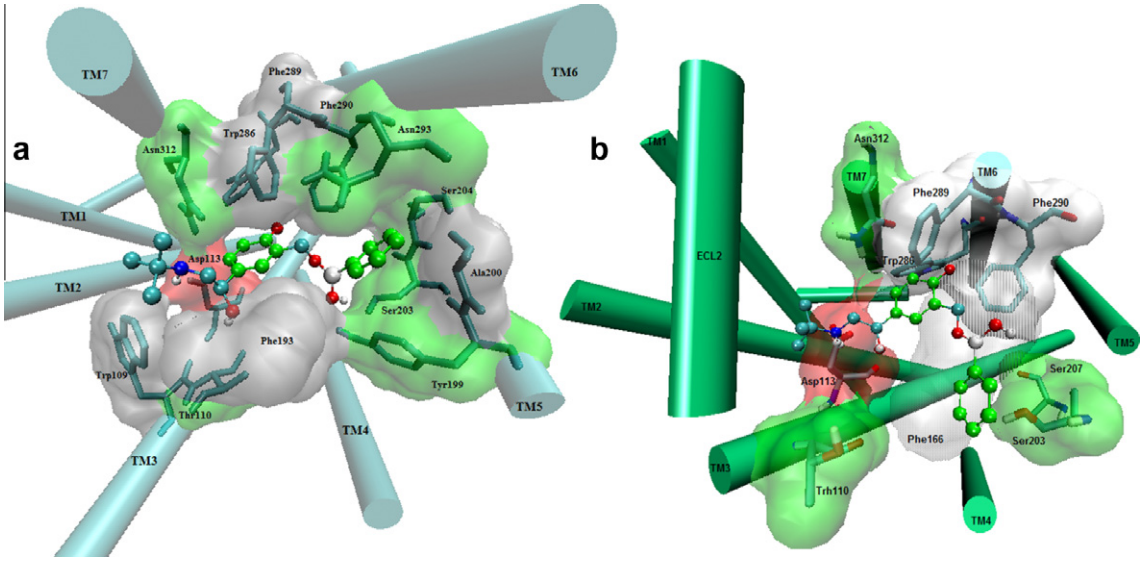

Figure 3. The binding pocket for boronterol on (a) hβ_2AR and (b) gβ_2AR. In gray is the non-polar surface, in green the surface formed by polar-neutral amino acids, and in red the charged amino acids.

of salbutamol. After, phenylboronic acid was added and the mixture reaction was vigorously stirred for another 4 h in a reflux system maintained at 115 ± 5 °C. Hence, the electrophilic attack of the boron atom of phenylboronic acid on the electron rich oxygen atoms was increased, allowing them to bind to the hydroxyl groups located in the aromatic ring, yielding boronterol (Fig. 4). The solution was filtered and the product obtained was washed with distilled H_2O (3 × 30 ml). The resulting product was dried at 40 °C for 4 h. The spectroscopic data obtained[14] show signals which correspond with the proposed structure, and these signals are in accordance with those reported for other analogous structures.[6,15]

The presence of tetravalent boron in the boronterol structure implies a great capacity of interactions with TM5 of the β_2AR, as indicated in several reports about the interactions between the moieties of other ligands with tetravalent boron and the lateral chains of TM5 residues in the β_2AR.[3,6] For example, the boronterol boron atom is very close to the Tyr199 and Ser203 residues to which it binds in TM5 of the hydroxyl group (Fig. 3), and these residues probably play an essential role in β_2AR, as suggested by in vitro and in silico experiments.[5] Moreover, the interaction of tetravalent boron and the lateral chains of TM5 residues has been clearly advantageous in the formation of other related complexes with biomedical applications.[3,6,16] Thus, the presence of a boron atom in boronterol and the great number of contacts of this ligand with the β_2AR probably account for its greater efficacy. These same factors could be key in the affinity of other β_2AR agonists with nucleophilic moieties in their aromatic rings, such as clenbuterol, SPFF compound, and carmoterol.[17]

There are several notable characteristics of boronterol that could explain its great number of contacts with the β_2AR. Like other known ligands, boronterol is capable of interacting with the Asp113 and Asn312 residues of the β_2AR by means its *N*-terbutyl moiety. Also, like other ligands with high affinity for the β_2AR, the aromatic rings of boronterol fit in the hydrophobic region of the binding site constituted by amino acids in TM3, TM4, and TM6 and the hydrophobic top. This top is formed by Phe193 in the hβ_2AR and by this residue and Phe166 in the gβ_2AR. Hence, the high affinity of boronterol is based on hydrogen bonds, electrostatic interactions, the great number of hydrophobic interactions, and probably on the partial negative charge of tetravalent boron, as suggested by reports of such interactions in similar complexes.[3,16]

Although docking simulations on β_2AR models have proven to be reliable for identifying potential ligands on this receptor,[18] and we reported the correlation of affinity values for known ligands on hβ_2AR and gβ_2AR models between experimental and theoretical results,[6–8] we are aware of the possible limitations of this model in the prediction of affinity values. It is difficult to identify a ligand-specific conformational state by computational tools,[5,18,19] especially in relation to unavailable molecules (for which the intrinsic activity is unknown) by considering the probable activity of screened ligands on the β_2AR. In spite of these limitations, we consider that docking simulations provide valuable information on β_2AR models that is useful in drug-design.

After synthesizing and chemically characterizing boronterol, in vitro assays were carried out in order to establish its pharmacodynamic effects. For studying the in vitro boronterol-β_2AR agonist activity, the effects on guinea pig tracheal rings were assessed with a protocol 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.[20,21] Boronterol produced a relaxant effect on guinea pig tracheal rings at basal tone and on those precontracted with histamine at 10 μM. On tracheal rings at basal tone, there was a concentration-dependent relaxation at concentrations greater than 0.031 nM for boronterol and 0.1 nM for salbutamol. On precontracted rings, there was a concentration-dependent relaxation effect at concentrations greater than 0.31 nM for boronterol and 10 nM for salbutamol. The maximum relaxant effect was observed at 10 μM for boronterol and salbutamol on rings at basal tone, and at 3.1 μM for boronterol and 10 μM for salbutamol on precontracted rings (Fig. 5). At basal tone EC_{50} (related to pK_d) values for boronterol and salbutamol were 4.677 nM (8.33) and 112.202 nM (6.95), respectively. When histamine was used as a precontractile agent, these values were 30.903 nM (7.51) and 245.471 nM (6.61), respectively.

Although the maximum relaxation values in relation to both drugs correspond to more than 100% of the histamine-induced contraction, a notably greater relaxation was observed by boronterol than salbutamol. Taking into account that salbutamol is regarded as a partial β_2AR agonist, experiments were carried out using isoproterenol, a potent non-selective but β adrenoceptor full-agonist.[22] A similar maximum effect was found for boronterol and isoproterenol, but not for salbutamol (Fig. 6).

The greater affinity of boronterol that was suggested by in silico experiments probably accounts for the greater potency found experimentally for this compound. Whereas the estimated EC_{50} value for boronterol was ~24-fold lower than that for salbutamol on tracheal rings at basal tone, it was only ~eightfold lower than that for salbutamol on precontracted rings. The difference in the slopes of the dose–response curves between basal tone and precontracted ring experiments, which has been reported in experiments for other β_2AR agonists, probably is responsible for this variation in comparative EC_{50} values.[17,22] The specific mechanisms related to this difference are still unknown.

With isolated guinea pig tracheal rings precontracted with histamine, the dose–response curve of boronterol was shifted to the right when using the well-known β_2AR selective inverse agonist, ICI118,551,[22] at concentrations greater than 1 nM, thus showing competitive antagonist effects. The pA_2 value for the antagonist activity of ICI118,551 in the presence of boronterol was 9.43 ± 0.27 (Supplementary Fig. 2).

The similarity between data obtained in silico and in vitro for salbutamol and isoproterenol on the hβ_2AR is remarkable, as is

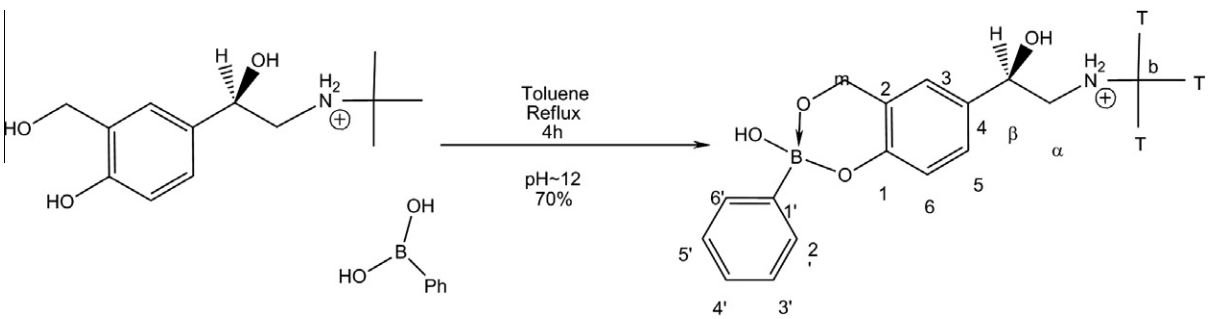

Figure 4. Synthesis of (*R*)-4-(2-(*tert*-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl) phenyl hydrogen phenylboronate (boronterol).

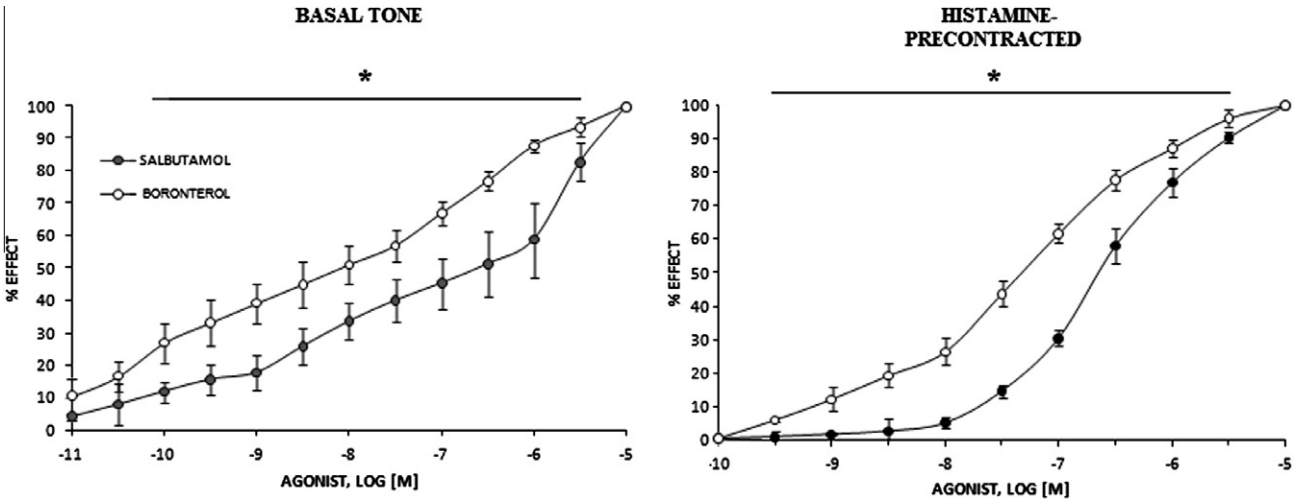

Figure 5. Effect of bronterol and salbutamol on isolated guinea pig tracheal rings with basal tone (left panel), and precontracted with histamine 10 μM (right panel). The maximum relaxant effect of each β_2AR agonist was considered 100%. Each point represents the mean effect and vertical bars represent the S.E.M. ($n = 6$). * Statistically significant difference $p < 0.05$.

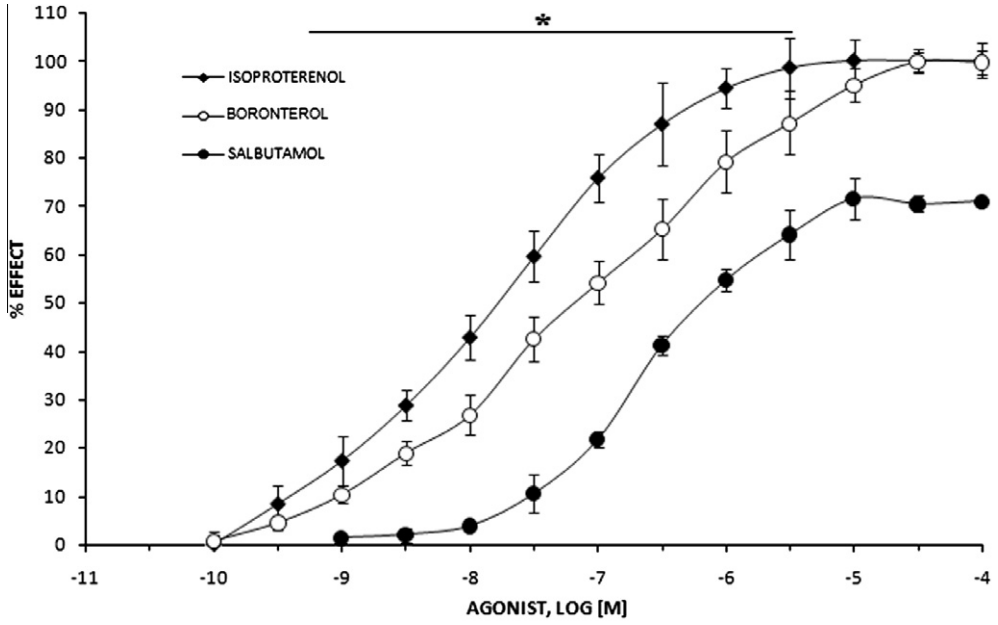

Figure 6. Effect of bronterol, salbutamol and isoproterenol on isolated guinea pig tracheal rings precontracted by histamine at 10 mM. The maximum relaxant effect by isoproterenol was considered 100%. Each point represents the mean effect and vertical bars represent the S.E.M. ($n = 5$). * Statistically significant difference compared with isoproterenol, $p < 0.05$.

the lower calculated affinity values on gβ_2AR for both of these (and other included) compounds. Regarding bronterol, the in silico and in vitro data are not completely in agreement. Whereas bronterol proved to be a more potent β_2AR agonist than salbutamol and isoproterenol in silico, the affinity of bronterol was not greater than that for isoproterenol in vitro (Table 1). Although the relationship between the in silico affinity values of bronterol and salbutamol on the gβ_2AR model was similar to that observed in vitro, such a relationship was not found with the affinity values of these two compounds on the hβ_2AR model.

The corrected theoretical values[23] in relation to the hβ_2AR model, on the other hand, are closer to the in vitro values obtained in the current study. Thus, the experimental and calculated affinity values for salbutamol were 6.61–6.95 and 7.37, respectively, and for bronterol 7.51–8.33 and 10.05, respectively. However, due to this correction the bronterol/salbutamol affinity value relationship is increased from 8.11 (unmodified data) to 479.24 (corrected data;[23] Table 1), which is inconsistent with experimental values.

In order to explore a probable explanation for the higher theoretical than experimental affinity values for bronterol on the hβ_2AR model, differences that were not found on the gβ_2AR model, it is useful to analyze the few differences that exist between the interactions of bronterol with each of these receptor models. Accordingly, the different aromatic residue on the top of the hβ_2AR (Phe193) and gβ_2AR (Phe166), the availability of aromatic amino acids in each model, and the greater number of possible contacts of ligands with amino acids in TM5 on the hβ_2AR model could account for the higher calculated affinity on this receptor. This explanation is in agreement with the 'fit-disruption' by a single amino acid conformation (Phe290) in ligand-β_2AR recognition described by Costanzi.[19]

Table 1
Affinity values for salbutamol, boronterol and isoproterenol estimated from in vitro and in silico systems

Compounds	pK_d			
	In vitro		In silico[a]	
	Basal tone	Precontracted	$h\beta_2$AR model	$g\beta_2$AR model[b]
Salbutamol	6.95	6.61	6.96	4.97 (7.37)
Boronterol	8.33	7.51	12.68	5.89 (10.05)
Isoproterenol	—	7.82	7.89	6.12 (10.73)
K_d salbutamol/K_d boronterol ratio	23.87	7.81	528,846.16	8.11 (479.24)

[a] Data shown were for *R*-forms of ligands.
[b] In parenthesis are the corrected values.[23]

Another probable cause for the higher theoretical affinity value on $h\beta_2$AR for boronterol is a difference between the actual conformational state of $h\beta_2$AR induced by boronterol and that evaluated in silico. Moreover, docking reliability seems to differ for ligands with high and low affinity values, which indicates the need for improvement in ligand affinity estimation. Vilar et al., upon probing several methods for this task, found that most yielded low correlation with experimental data.[24]

The mechanism for the boronterol behavior as a β_2AR full agonist should be studied further. It is possible that the higher potency of boronterol than salbutamol is due to the greater hindrance effects of the former ligand as well as its capability to induce a conformationally active state (which has also been reported for other ligands with great hindrance effects, such as indacaterol, carmoterol, and SPFF).[17] The important changes induced by these moieties in the amino acids of TM4–TM6 are implicated in the activation process.[5,19]

Based on the distinct signaling cascades described in $h\beta_2$AR activation,[19] the different types of β_2AR signaling involved after ligand recognition could also account for the higher potency of boronterol than salbutamol on this receptor. Further molecular biological studies are required to clarify this question. However, the reliability of the data indicating a high β_2AR-affinity of boronterol is confirmed by the similar response-curves obtained in the present study, as well as by the competitive antagonism shown by ICI118,551, the latter evidenced by the Schild regression with a slope near the unit value.

In conclusion, we found the docking of ligands on refined structures of the β_2AR to be an effective tool for evaluating the potency of the test compounds. The in silico approach showed greater affinity of boronterol than salbutamol for the β_2AR. The in vitro experimental data based on relaxation of guinea pig tracheal rings confirm this greater proposed affinity of boronterol than salbutamol. Contrarily, although the theoretical results indicated that boronterol has higher affinity for this receptor than isoproterenol (Fig. 2), the experimental data show the opposite. However, boronterol produced a maximal relaxant effect similar to isoproterenol, which shows that boronterol behaves as a full β_2AR agonist. The blocking activity of ICI118,551 on the relaxant effect of boronterol strongly suggests that this effect is mediated by selective β_2AR activation.

In order to obtain a better correlation between experimental and theoretical data, further molecular modeling and dynamic simulation studies are required on the β_2AR. Docking simulations suggest that the greater affinity of boronterol than salbutamol on the β_2AR can be explained by the boron atom in the structure of the former ligand, as well as by its capability of interacting with residues in TM5 of the receptor.[25] The capacity of boronterol to act as a full agonist could also be related to these interactions. Additional studies are required in order to analyze the behavior of chemical and pharmacological kinetics as well as the pathways involved in the maximal relaxant effect of boronterol.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.040.

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14. The reaction was monitored by thin-layer chromatography (*TLC*, Silica Gel 60 F_{254}, 0.25 mm), and the product visualized 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 Joel 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. Additionally, the optical activity of our product was measured with an ERMA-1704 Optical Works polarimeter. The physical-spectral data of (*R*)-4-(2-(*tert*-butylamino)-1-hydroxyethyl)-2-(hydroxymethyl)phenyl hydrogen phenyl-boronate. White-beige powder; yield: 70%; mp: 201 ± 1 °C; $[\alpha]_D^{25} = -0.41$ ($c = 0.0075$, water); IR (KBr) ν_{max}/cm^{-1}, 3595, 3214, 2972, 1677, 1272; ^{1}H NMR (DMSO, 270 MHz) δ/ppm 1.20 (9H, s, H-T); 2.91 (2H, d, *J* 6.2 Hz, H-α), 4.57 (1H, s, H-m), 4.70 (2H, t, *J* = 6.0 Hz, H-β), 6.44 (1H, d, *J* = 8.1 Hz, H-6), 6.84 (1H, s, H-3), 6.94 (2H, d, *J* = 8.4 Hz, H-2′6′), 7.32 (2H, m, *J* = 8.4 Hz, H-3′5′), 7.38 (1H, m, H-4′), 8.24 (1H, s, *J* = 1.8 Hz, *J* = 8.0 Hz, H-5); ^{13}C NMR (DMSO) δ/ppm 25.2 (C-T), 48.4 (C-α), 55.8 (C-q), 61.4 (C-m), 69.1 (C-β), 116.2 (C-6), 122.3 (C-3) 124.6 (C-2′6′), 126.0 (C-4), 127.9 (C-3′5′), 129.6 (C-1′), 130.6 (C-4′), 134.9 (C-5), 157.2 (C-1); ^{11}B NMR (DMSO) δ/ppm 1.6 (B); *m*/*z* (EI) 332 [M$^+$], 224 (100%).

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20. For evaluating the relaxation effects on isolated guinea pig tracheal ring; male Hartley guinea pigs (weighing 325 ± 25 g) were sacrificed by an intraperitoneal overdose of sodium pentobarbital. Immediately, 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. In all experiments, indomethacin 2.8 μM was added to inhibit endogenous prostanoid synthesis. The relaxant effect induced by salbutamol or boronterol was determined on basal tone or with precontracted (with histamine, 10 μM) isolated guinea pig tracheal rings. The maximum relaxant effect for each compound was taken as 100% and the cumulative concentration–response curves were established for salbutamol and boronterol (from 0.01 nM to 0.1 mM). Additional experiments were carried out by comparing the maximal relaxant effect induced for isoproterenol, salbutamol or boronterol on histamine (10 μM) precontracted isolated guinea pig tracheal rings.
    The effects of ICI-118,551 on the boronterol response were evaluated in guinea pig tracheal rings precontracted with histamine (10 μM), then incubated with ICI 118,551 (1 nM–1 μM). Five minutes later, boronterol was added to the organ bath at concentrations from 0.01 nM to 0.1 mM, and the maximum relaxation produced by boronterol was taken as 100%. The concentration–response curves of boronterol in the absence and presence of ICI 118,551 were established. EC_{50} and pA_2 values were obtained by WinNonlin™ Ver 2.1 (Pharsight Corporation, CA, U.S.A.), and antagonist potency was expressed with pA_2 values obtained from Schild plotting. Histamine phosphate, isoproterenol, ICI 118,551, and (*R*)-salbutamol were purchased from Sigma–Aldrich© (St. Louis MO, U.S.A.).
21. The biological test results are expressed as mean values ± standard error of the mean (SEM) of at least six experiments. Statistical significance of differences between groups was verified with two-way analysis of variance (ANOVA), with a confidence limit of 95%.
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23. Data corrected by equation: Experimental affinity = 2.92 × (calculated affinity) − 7.14; which was obtained from reported experimental and calculated data in Ref. 7
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