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Chiral analogues of (+)-cyclazosin as potent α_{1B} -adrenoceptor selective antagonist

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Abstract

(+)-Cyclazosin [(+)-**1**] is one of most selective antagonists of the α_{1B} -adrenoceptor subtype (selectivity ratios, $\alpha_{1B}/\alpha_{1A} = 13$, $\alpha_{1B}/\alpha_{1D} = 38$ –39). To improve the selectivity, we synthesized and pharmacologically studied the blocking activity against α_1 -adrenoceptors of several homochiral analogues of (+)-cyclazosin featuring different substituents on the carbonyl or amine groups, namely (–)-**2**, (+)-**3**, (–)-**4** – (–)-**8**, (+)-**9**. Moreover, we studied the activity of some their opposite enantiomers, namely (–)-**1**, (–)-**3**, (+)-**6**, and (–)-**9**, to evaluate the influence of stereochemistry on selectivity. The benzyloxycarbonyl and methyl (4a*S*,8a*R*) analogues (+)-**3** and (–)-**6** improved in a significant way the α_{1B} selectivity of the progenitor compound: 4 and 14 time vs. the α_{1D} subtype and 35 and 77 times vs. the α_{1A} subtype, respectively. The study confirmed the importance of the hydrophobic *cis*-octahydroquinoxaline moiety of these molecules for the establishment of interactions with the α_1 -adrenoceptors as well that of their (4a*S*,8a*R*) stereochemistry to grant selectivity for the α_{1B} subtype. Hypotheses on the mode of interaction of these compounds were advanced on the basis of molecular modeling studies performed on compound (+)-**3**.

Graphical Abstract

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Keywords

α_1 -adrenoceptor subtypes; α_1 -adrenoceptor antagonists; α_{1B} -adrenoceptor selective antagonists; (+)-cyclazosin analogues

1. Introduction

α_1 -Adrenoceptors are major mediators of smooth muscle contraction at the level of various animal and human body organs. Three native α_1 -adrenoceptors, α_{1A} , α_{1B} , α_{1D} , have been pharmacologically characterized in animal and human tissues, and have been shown to correspond to the cloned α_{1a} , α_{1b} , and α_{1d} subtypes expressed in various cell lines.¹ Throughout the manuscript, we will refer to α_1 -adrenoreceptors subtypes with uppercase and lowercase letters to indicate tissue functional antagonism and affinity binding data from cloned receptors, respectively. In humans, the α_{1A} -adrenoceptor is mainly found in heart and vascular tissue where it is the principal mediator of blood pressure control.² It is also involved in the contraction of prostate and urinary tissues,³ for which it is the target in the treatment of benign prostatic hyperplasia.^{2,4} The α_{1B} -adrenoceptor is mainly expressed in the heart, vascular tissue, coronary endothelial cells, spleen, and cerebral cortex.^{2,3,5} Beyond vascular contractile activity,¹ which is more important in peripheral vasculature of older individuals,⁶ studies with α_{1B} transgenic mice suggested that the α_{1B} -adrenoceptor is involved in cardiac hypertrophy and neurodegeneration.^{7,8} Also the α_{1D} -adrenoceptor is found in the vascular tissues,² but it is predominantly expressed in bladder detrusor, where its stimulation causes bladder instability and irritability.^{3,9} However, the functional role of all three α_1 -adrenoceptors subtypes is not completely defined because of the lack or shortage of highly selective ligands, useful to elucidate their physiological activities and also to allow the treatment of the diseases in which they are involved. There is particular need for selective α_{1B} -adrenoceptor antagonists which, to date, have been discovered in significantly smaller numbers in comparison to the selective α_{1A} - and α_{1D} -adrenoceptor ligands. Among the ligands that show some selectivity for the α_{1B} -adrenoceptor, (+)-Cyclazosin,¹⁰ (+)-metcyclazosin,¹⁰ and compound L-765,314¹¹ are those endowed with the highest activity. However, their α_{1B} selectivity is still too small for the aforementioned needs.

(+)-Cyclazosin, (+)-2-[(4a*S*,8a*R*)-4-(2-furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(+)-**1**], a prazosin analogue, was shown to display an interesting high binding affinity and selectivity for the α_{1B} -adrenoceptor, both in animal and human cloned α_1 -subtypes (pK_i α_{1b} = 9.16–9.87; selectivity ratios, α_{1b}/α_{1a} = 48–91 and α_{1b}/α_{1d} = 24–39).¹² Subsequent functional experiments confirmed its α_{1B} -adrenoceptor selectivity in rat and rabbit tissues (pA_2/pK_B α_{1B} = 8.85 and 8.86; selectivity ratios, α_{1B}/α_{1A} = 13, α_{1B}/α_{1D} = 38–39).^{10,13} In 2006 (+)-cyclazosin was recognized as the most selective compound for the α_{1b} -adrenoceptor.¹⁴ However, we found that the 5-methylfuryl derivative of (+)-**1**, (+)-metcyclazosin¹⁰ was a competitive antagonist at all three rat α_1 -adrenoceptors with an 11-fold increased α_{1B}/α_{1A} selectivity ratio with respect to (+)-**1** (148 vs 13).

In a previous work¹⁵ we found a number of racemic cyclazosin analogues, having alkyl and aryl groups at the amide function, endowed with a slight preferential binding affinity for human cloned $\alpha_{1d/b}$ -adrenoceptors with respect to α_{1a} . This suggested their candidacy for the development as enantiomer antagonists potentially selective towards the α_{1B} - or α_{1D} -adrenoceptor. In addition, in the ligand-based models developed by MacDougall¹⁴ for the three α_1 -adrenoceptors subtypes, the furyl group of (+)-cyclazosin was indicated as a hydrophobic aromatic (Har) feature important for the α_{1B} -adrenoceptor pharmacophore. Starting from these indications, in the attempt to improve the α_{1B} -adrenoceptor selectivity we synthesized and pharmacologically studied a series of homochiral analogues of (+)-cyclazosin, namely (–)-**2**, (+)-**3**,¹⁰ (–)-**4**, (–)-**5**,¹⁶ (–)-**6** – (–)-**8**, obtained by changing the furyl group with either aliphatic or aromatic substituents. In particular, on the basis of binding affinity experiments published in previous studies, we chose specific arylalkyl-, benzyloxy-, phenoxyalkyl-, or 2,3-dihydro-1,4-benzodioxine-2-yl moieties, as well as methyl, 2,2-dimethylpropyl, and hexyl groups substituents.^{15,16} (Fig. 1).

At the same time, in order to check the influence on the α_1 -adrenoceptors affinity and subtype selectivity of replacement of the 4-amino group of (+)-cyclazosin with a bulky amino function such as the tertiary *N,N*-dipropyl amino moiety, the homochiral analogue (+)-**9** was synthesized (Fig. 1). Similarly, to test the impact of the stereochemistry of these compounds on the affinity and selectivity for specific α_1 -adrenoceptor subtypes, we synthesized and pharmacologically studied three (–)-**1** [(–)-cyclazosin] analogs, namely (–)-**3**,¹⁰ (+)-**6**, and (–)-**9**, that are enantiomers of some above (+)-cyclazosin analogues (Fig. 1). Of note, while most of the compounds are new to this study, compounds (–)-**3**, (+)-**3**, and (–)-**5** had been previously published.^{10,16}

2. Results

2.1. Chemistry

Compounds (+)-**1**, used as reference, and (–)-**1**, (+)-**3**, (–)-**3**, and (–)-**5**, were prepared as reported in the literature,^{10,12,16} whereas the new compounds (–)-**2**, (–)-**4**, (–)-**6** – (–)-**8** were synthesized following a known procedure¹⁰ starting from the common homochiral precursor of (+)-cyclazosin, (–)-6,7-dimethoxy-2-[(4*aS*,8*aR*)-octahydroquinoxalin-1-(2*H*)-yl]quinazolin-4-amine dihydrochloride free base (–)-**10**,¹⁰ which was acylated with the appropriate acyl chloride in dry CH_2Cl_2 and Et_3N . Similarly, reaction of the enantiomer (+)-**10**,¹⁰ homochiral precursor of (–)-cyclazosin, with acetyl chloride afforded the expected compound (+)-**6** (Scheme 1).

Mono alkylation of 2,4-dichloro-6,7-dimethoxyquinazoline with dipropylamine in THF gave the intermediate 2-chloro-6,7-dimethoxy-*N,N*-dipropylquinazolin-4-amine **11** which afforded compounds (+)-**9** and (–)-**9** by reaction with known (4*aR*,8*aS*)-1-(2-furoyl)octahydroquinoxaline (+)-**12**¹ and (4*aS*,8*aR*)-1-(2-furoyl)octahydroquinoxaline (–)-**12**,¹² respectively (Scheme 2).

All chiral compounds were synthesized as hydrochloride salts, purified by crystallization and characterized by elemental analysis, ¹H-NMR, specific rotation and chromatographic parameters.

2.2 Biological evaluation

All compounds were tested in isolated rat tissues to assess their antagonist activity and selectivity at α_1 -adrenoceptor subtypes. The α_{1A} , α_{1B} , and α_{1D} -adrenoceptor blocking activities were evaluated by antagonism of (–)-noradrenaline-induced contractions of rat prostatic vas deferens (α_{1A}) and aorta (α_{1D}), and of phenylephrine-induced contraction of rat spleen (α_{1B}), as previously reported.¹⁰ The antagonist potency at α_1 -adrenoceptor subtypes was expressed as pA_2 values calculated by Schild plots at three different concentrations according to Arunlakshana and Schild.¹⁷ However, when the slope of the Schild plot was significantly different from unity, the potency was expressed as pK_B values, according to van Rossum.¹⁸ In this case the pK_B value was calculated at the lowest antagonist concentration giving a significant rightward shift of the agonist concentration-response curve [$\log(\text{concentration ratio} - 1) = 0.5$].

The affinity profile of all compounds was also evaluated in radioreceptor binding assays on human cloned α_1 -adrenoceptors. Competition experiments were performed using [3H]prazosin to label α_1 -adrenoceptor binding sites on membranes of Chinese Hamster Ovary (CHO) cells expressing human α_{1a} , α_{1b} , and α_{1d} adrenoceptor subtypes.¹⁹ Binding affinities were expressed as pK_i values derived using the Cheng-Prusoff equation.²⁰

Experimental data were subjected to statistical analysis by means of Student's t-test. A p value < 0.05 was taken to indicate a statistically significant difference. The results are reported in Table 1 and Table 2.

In functional experiments, the studied compounds showed either competitive or non-competitive antagonism, as the precursor (+)-**1**. At α_{1D} - and α_{1A} -adrenoceptors, most compounds displayed competitive antagonism, whereas prevalent noncompetitive antagonism was observed at α_{1B} -adrenoceptor (Table 1).

With the exception of compound (–)-**4**, which showed the same low activity at all three α_1 -adrenoceptors, the other homochiral analogues of (+)-**1**, the (4a*S*,8a*R*) stereoisomers (–)-**2**, (+)-**3**, (–)-**5** – (–)-**8**, (+)-**9**, displayed a higher antagonist potency at α_{1B} -adrenoceptor with respect to the α_{1A} and α_{1D} subtypes, retaining the same kind of selectivity of the progenitor compound.

Compounds (+)-**3** and (–)-**6** were shown to be the most interesting of the series, due to their very high α_{1B} selectivity, with an α_{1B}/α_{1A} selectivity ratio of 457 and 1000, respectively, and an α_{1B}/α_{1D} selectivity ratio of 141 and 562, respectively. Their α_{1B}/α_{1A} selectivity was 35 and 77 times higher than (+)-**1**, whereas the α_{1B}/α_{1D} selectivity was 4 and 14 times higher. This pronounced selectivity resulted from a high antagonist potency at the α_{1B} -adrenoceptor, not dissimilar from that shown by (+)-**1** (pA_2 or pK_B , 8.77 and 8.52 vs 8.86 for (+)-**1**), and a low activity at the α_{1A} and α_{1D} subtypes, even lower than that shown (+)-**1** (pA_2/pK_B , 5.52 – 6.11 and 5.77 – 6.62, respectively, vs 7.77 and 7.27).

These high values of selectivity towards the α_{1B} -adrenoceptor, to our knowledge, are unprecedented in the literature, and were never observed in the large group of diaminoquinazoline antagonists or in any other class of compounds. This discovery is

important, because it provides valid and promising tools for pharmacological investigation and receptor characterization, as well for possible use in the treatment of α_{1B} -adrenoceptor related diseases.

The α_{1B}/α_{1A} and α_{1B}/α_{1D} selectivity ratio of other compounds ranged from 9 to 93 and 15 to 44, respectively. Most homochiral analogues of (+)-**1** showed α_{1B} selectivity in the same order of magnitude as the progenitor, whereas compounds (+)-**3** and (-)-**6** displayed 4 (vs α_{1D}) and 38 times (vs α_{1A}) and 14 (vs α_{1D}) and 83 (vs α_{1A}) times higher selectivity than (+)-**1**, respectively.

This observation indicates that the benzyl and, in particular, the methyl group play a crucial role in directing the interaction preferentially towards the α_{1B} -adrenoceptor rather than α_{1A} and α_{1D} subtypes. On the other hand, the only substitution performed on the 4-amino function of (+)-**1** with the tertiary di-propyl amino group, as in (+)-**9**, did not modify the trend of α_{1B} selectivity, which was slightly improved in the α_{1B}/α_{1A} value (93 vs 13). Notably, this occurred despite the fall of antagonism activity on all three subtypes (10–11 times at α_{1B} and α_{1D} -adrenoceptors and 76 times at α_{1A}). These data may indicate that the α_{1B} selectivity is not necessarily related to the primary 4-amino function of these quinazolinyl compounds.

The enantiomers of (+)-**1** and its analogues, namely (-)-**1**, (-)-**3**, (+)-**6**, and (-)-**9**, displayed a high or very high activity at three α_1 -adrenoceptor subtypes, although often not significantly different. Enantiomer (-)-**1** did not discriminate significantly among α_1 -adrenoceptor subtypes, whereas (-)-**3** and (+)-**6** resulted equipotent to each other and more potent at α_{1B} and/or α_{1D} -adrenoceptors than at the α_{1A} subtype, with an α_{1D}/α_{1A} selectivity ratio of 31 and 20 for (-)-**3** and (+)-**6**, respectively, and an α_{1B}/α_{1A} selectivity ratio of 72 for (+)-**6**. Similarly, (-)-**9** displayed low activity in analogy to its (+) enantiomer, but without showing any selectivity. As a result, it can be concluded that the (4a*R*,8a*S*) stereochemistry confers a lower discriminating power to these compounds, in comparison with that conferred by the (4a*S*,8a*R*) stereochemistry, towards α_1 -adrenoceptor subtypes: compounds (-)-**1** and (-)-**9** did not display selectivity, whereas compounds (-)-**3** and (+)-**6** were only slightly selective for α_{1B} and α_{1D} subtypes.

Our results indicate that, with the exception of compound (-)-**4**, also in the presence of a bulky tertiary amino group at position 4 of the quinazoline nucleus, the α_{1B} -adrenoceptor selectivity of cyclazosin and related analogues is mainly due to the (4a*S*,8a*R*) stereochemistry and the nature of the substituent on the carbonyl function. This means that the stereochemistry of the octahydroquinoxaline core controls the selectivity: only the specific spatial orientation of the (4a*S*,8a*R*) stereoisomers satisfies the structural conditions for an optimal and preferential interaction with the α_{1B} -adrenoceptor, whereas the (4a*R*,8a*S*) stereochemistry confers the ability to interact with both α_{1D} and α_{1B} adrenoceptors.

It follows that the comparison of the antagonist potency of the four enantiomer couples at the three α_1 -adrenoceptor subtypes from rat tissues allowed us to identify with certainty the (4a*S*,8a*R*) stereochemistry of the octahydroquinoxaline moiety of the compounds as the absolute configuration necessary to achieve maximum α_{1B} selectivity with cyclazosin based

compounds. In the present work, the pharmacological functional data indicate that within each enantiomer couple the antagonist potency was similarly high at the α_{1B} -adrenoceptor, whereas a significant enantioselectivity was observed at the α_{1A} and even more so at the α_{1D} -adrenoceptor, as revealed by the eudismic ratio. These values were, in fact, very low at the α_{1B} -adrenoceptor (1 to 7), moderately high at the α_{1A} -subtype (14 to 68), and, except for the couple (+)-**9**/(-)-**9**, very high at the α_{1D} -adrenoceptor (457 to 776) (Table 2).

Concerning binding data (Table 3), except for unselective (-)-**4**, (-)-**5**, (-)-**7**, and (-)-**8** compounds, the (4a*S*,8a*R*) stereoisomers displayed higher affinity for the α_{1b} -adrenoceptor than for α_{1a} and α_{1d} subtypes, with a similar or lower selectivity than the progenitor compound. In particular, whereas (-)-**4** showed a significant increase of affinity at the α_{1a} and the α_{1d} -adrenoceptor with respect to (+)-**1**, the methyl analog (-)-**6** displayed an important affinity decrease at all three subtypes (18 to 68 times), especially at the α_{1b} -adrenoceptor. On the contrary, the (+)-cyclazosin 4-N-dipropyl analogue, (+)-**9**, which maintained the preferential affinity of the progenitor for the α_{1b} -adrenoceptor and a similar selectivity profile, seems to suggest that the nature of the 4-amino function on the quinazoline moiety has not a particularly pronounced effect on binding. The most α_{1b} -selective compounds were (+)-**3**, (-)-**6**, (+)-**9**, with an α_{1b}/α_{1a} selectivity ratio of 14, 25 and 74, respectively, and an α_{1b}/α_{1d} selectivity ratio of 11, 12, and 17, respectively. In contrast, all (4a*R*,8a*S*) stereoisomers preferentially showed a slight to moderate affinity and selectivity for the α_{1d} -adrenoceptor. Compounds (+)-**6** and (-)-**9** were the most selective with an α_{1d}/α_{1a} selectivity ratio of 60 and 29, and an α_{1b}/α_{1a} selectivity ratio of 12 and 15.

As it can be observed, all tested compounds generally displayed lower values of functional pA_2 or pK_B than binding pK_i affinities. This discrepancy is not new for prazosin analogues and different explanations were offered to rationalize this event as diffusion-related temporal inequilibrium,²¹ inverse agonism and receptor heterodimer formation.^{22–24}

2.3 Molecular modeling

In order to generate hypotheses on the selectivity of our cyclazosin analogs with the α_{1B} -adrenoceptor, we conducted a molecular modeling study coupled with sequence comparison. We focused the structural modeling on a single subtype, namely the α_{1B} -adrenoceptor, and a single compound, namely the antagonist (+)-**3**. We then used sequence comparison in order to identify non-conserved residues that might be at basis of the different selectivity profiles that we observed for our ligands. We deliberately elected to generate coarse information through a sequence comparison-based approach rather than attempting to produce detailed models on multiple subtypes in complex with multiple, as the absence of experimental structures for any of the α -adrenoceptors would have made such a study impractical.

Compound (+)-**3** was chosen for the modeling study because of its high activity and selectivity for the receptor. In particular, compound (+)-**3** was selected instead of (-)-**6**, which shows a similar affinity and selectivity profile, because of the larger nature of the substituent at position Y – a benzyloxy group for (+)-**3** and a methyl group for (-)-**6**. This allowed an exploration of the receptor pocket that putatively accommodates substituents at this position, which would not have been possible with (-)-**6**.

In the absence of experimentally determined structures for the α_{1B} -adrenoceptor, we constructed a homology model of the receptor based on the crystal structure of the D₂ dopamine receptor (PDB ID: 3PBL).²⁵ The latter is a particularly suitable template for our study for two reasons. First, among all the G protein-coupled receptors endowed with crystallographically solved structures, the D₂ receptor is the one that shares the highest percentage of sequence identity with the α_{1B} -adrenoceptor – 48% of sequence identity with respect to the transmembrane domains (TMs). Second, the D₂ structure was solved in complex with an antagonist, and is therefore a viable template for the modeling of receptors in their inactive state. Upon the construction of the α_{1B} -adrenoceptor model, we modeled its interactions with compound (+)-**3** through a molecular docking study followed by energy minimization, obtaining the hypothetical complex shown in Figure 2.

As the figure shows, our results suggest that the compound binds within a cavity lined by TMs 2, 3, 5, 6 and 7, with one of the methoxy groups of its quinazoline ring oriented toward TM5, the other methoxy group oriented toward TM3, and the phenyl moiety of the benzyloxycarbonyl substituent at the opposite end of the molecule sandwiched between TM2 and TM7. Macroscopically, this docking hypothesis is consistent with the orientation of prazosin shown in a previous model of the α_{1B} -adrenoceptor reported in 2013 by Ragnarsson and coworkers.²⁶

A detailed representation of the binding mode hypothesis is shown in Figure 3, which indicates the suggested formation of T-shaped aromatic interactions between the quinazoline ring of the ligand and residues Phe³¹⁰ (TM6), Phe³³⁰ (TM7) and Phe³³⁴ (TM7), as well as a cation aromatic interaction between the quinazoline ring and residue Lys¹⁸⁵ (EL2).

These suggested interactions are consistent with the reported decrease of prazosin affinity when said residues were mutated to alanine.²⁶ Our model also suggests the presence of an interaction between the exocyclic amino group on the quinazoline ring and residue Asp¹²⁵ (TM3), as well as an aromatic-aromatic interaction between the phenyl moiety of the benzyloxycarbonyl substituent and residue Phe³³⁴ (TM7).

A different hypothesis for the binding mode of quinazoline derivatives to the α_1 -adrenoceptors was published in the late 1980s by Campbell and coworkers, who proposed an interaction between the above mentioned aspartate in TM3 and the protonated nitrogen at position 1 of the quinazoline ring.^{27,28} Although we did not detect such a binding pose in this study, such an alternative hypothesis should not be discounted.

A bidimensional (2D) schematic representation of our hypothesized receptor-ligand complex is provided in Figure 4, in which the residues of the α_{1B} -adrenoceptor that are not conserved in the α_{1A} and/or the α_{1D} subtypes are noted.

As the figure shows, the majority of the residues that are predicted to line the binding cavity of the docked compound are shared between the three subtypes, in good agreement with the difficulty in achieving compounds endowed with pronounced selectivity. However, some differences do exist. In particular, three of the residues shown in Figure 4 – namely Leu¹⁰⁵ (TM2), Val¹⁹⁷ (EL2), and Asp³²⁷ (TM7) – are unique to the α_{1B} subtype, being substituted by different residues in the α_{1A} and the α_{1D} subtypes. Moreover, four additional residues

shown in the figure – namely Gly¹⁹⁶ (EL2), Thr¹⁹⁸ (EL2), Ala²⁰⁴ (TM5), and Leu³¹⁴ (TM6) – are only partially conserved, being shared by the α_{1B} and α_{1D} subtypes, but not by the α_{1A} subtype. The possible implications of these non-conserved and partially conserved residues for the selectivity profile of the ligands are discussed in the two paragraphs below.

In our docking hypothesis, two of the three residues unique to the α_{1B} subtype, *i.e.* Leu¹⁰⁵ (TM2) and Asp³²⁷ (TM7), interact with the benzyloxycarbonyl moiety of (+)-**3** (Figure 3 and Figure 4), in good agreement with the noted modulatory effect of substituents at this position on the selectivity of the ligands. Moreover, the third unique residue, *i.e.* Val¹⁹⁷ (EL2), together with one of the four semi-conserved residues, *i.e.* Ala²⁰⁴ (TM5), appears to be located near one of the methoxy groups of the quinazoline ring of (+)-**3** (Figure 2 and Figure 3), suggesting that modification of this group could also modulate the selectivity profile of the compounds.

The remaining three semi-conserved residues, *i.e.* Gly¹⁹⁶ (EL2), Thr¹⁹⁸ (EL2), and Leu³¹⁴ (TM6), contribute to the lining of a hydrophobic pocket that is predicted to accommodate the hydrophobic octahydroquinoxaline ring of (+)-**3**, together with the above mentioned non-conserved residue Val¹⁹⁷ (EL2), which is unique to the α_{1B} subtype, and the conserved residue Phe³³⁰ (TM7) (Figure 3 and Figure 4).

Our hypothesized placement of the octahydroquinoxaline ring within this lowly conserved region of the receptor is supported by our biological data, which indicate that changes to this moiety substantially alter the selectivity profile of the compounds.

3. Discussion

In 2006, starting from the affinity and selectivity data of available antagonists, MacDougall et al.¹⁴ developed ligand-based pharmacophore models for all three α_1 -adrenoceptors. The three generated pharmacophore models, including feature of H-bond acceptor (HBA), hydrophobic aromatic (Har), hydrophobic aliphatic (Hal), and positive ionisable charge (PI), displayed higher statistical significance for the α_{1A} and α_{1D} -adrenoceptors (90% and 95% respectively) than for the α_{1B} subtype (65%). Mapping the α_{1B} pharmacophore on the molecular structure of (+)-cyclazosin, which was considered by the authors the most active and selective α_{1B} -antagonist, the most likely hypothesis that emerged suggested the presence of two HBA features located on the nitrogen and one of the oxygen atoms of the 6,7-dimethoxy quinazoline nucleus, one Hal feature located on the methyl function of second methoxy group, and one Har feature located on the aromatic furan group of molecule.

In agreement with the above models, recent docking experiments²⁶ indicated that prazosin binds to the α_{1B} -adrenoceptor through extensive π – π interactions with amino acid residues in TM6 and TM7. In TM6, the authors suggested the interaction of one methoxy group with a tryptophan residue (W307) and the interaction of the aromatic quinazoline moiety with two phenylalanines (F310 and F311); in TM7, they suggested the interaction of the methyl of the second methoxy group with a phenylalanine residue (F334).

Notably, no pharmacophoric hydrophobic area for the octahydroquinoxaline part of (+)-cyclazosin or the piperazine nucleus of prazosin emerged in either of the above-mentioned studies. In contrast, our previous studies on prazosin analogues, in which the piperazine ring of prazosin was replaced by linear carbon chains²⁹ or a number of dialkylpiperazine moieties,³⁰ demonstrated that the chemical structure linking the quinazoline and furoyl moieties plays an important role for the antagonist potency and in addressing the selectivity towards the α^1 -adrenoceptor with respect to the α^2 type. It was shown that the carbon chain length, the N-methylation, and the stereochemistry of the substituents on the piperazine nucleus are relevant characteristics for both potency and selectivity for α^1 -adrenoceptors. These observations led to the discovery of racemic cyclazosin, which has a *cis*-octahydroquinoxaline moiety in place of the prazosin piperazine group, as the most potent ($pA_2 = 8.97$) and α_1 -selective antagonist of the series (α_1/α_2 selectivity ratio 7800). As a consequence, it was hypothesized^{29,30} that α_1 -adrenoceptors incorporate a lipophilic area, located between the binding sites of the quinazoline and furoyl groups of these structures, able to optimally accommodate either a hexane-1,6-diamine carbon chain or a *cis*-oriented fused cyclohexyl ring of an octahydroquinoxaline moiety.

In agreement with the view that α_1 -adrenoceptors bear a specific and spatially oriented hydrophobic pocket where the octahydroquinoxaline moiety of prazosin-related compounds binds, we found¹² that the (+)-cyclazosin enantiomer, endowed with a (4a*S*,8a*R*) stereochemistry,³¹ displayed higher binding affinity and selectivity for the cloned α_{1B} -adrenoceptor than for α_{1A} and α_{1D} -subtypes, as well higher antagonist potency and selectivity for the functional α_{1B} -adrenoceptor¹³ than for α_{1A} and α_{1D} -subtypes.

As we have seen, in the present work we expanded on this concept and found that the two members of each enantiomer couple had similar potency at the α_{1B} -adrenoceptor, whereas a significant enantioselectivity was observed at the α_{1A} and even more so at the α_{1D} -adrenoceptor.

These data may indicate that the α_{1B} -adrenoceptor hydrophobic pocket could be large enough to easily accommodate both the (4a*S*,8a*R*) and the (4a*R*,8a*S*) orientations of the octahydroquinoxaline structure. On the contrary, the (4a*R*,8a*S*) configuration seems preferred for the interaction with the α_{1A} and, even more so, with the α_{1D} -adrenoceptor, suggesting that the lipophilic cavity of these subtypes might have more stringent requirements for binding.

It follows that, in addition to the four pharmacophoric features that emerged from the MacDougall's study for the α_{1B} -pharmacophore (2HBA, 1Har, 1Hal), in the α_{1B} -adrenoceptor structure there could be also a hydrophobic pocket, lined by specific amino acid residues, which is able to accommodate the octahydroquinoxaline moiety of cyclazosin analogues with a (4a*S*,8a*R*) spatial configuration. This might be a further pharmacophoric feature for the α_{1B} -adrenoceptor. As we have seen, in this work we advanced hypotheses on the high selectivity for the α_{1B} -adrenoceptor of (+)-cyclazosin and other homochiral analogues on the basis of the results of a molecular modeling and sequence comparison study. In particular, two specific reasons can be proposed.

One of them can be identified in the interaction of the hydrophobic (4a*S*,8a*R*) octahydroquinoxaline nucleus with the amino acid residue Val¹⁹⁷ (EL2), which is only found in the α_{1B} -adrenoceptor. Indeed, this residue contributes to the lining of the suggested hydrophobic binding pocket of the α_{1B} -adrenoceptor, together with the semi-conserved residues Gly¹⁹⁶, Thr¹⁹⁸, and Leu³¹⁴, which are found in both the α_{1B} - and the α_{1D} -subtypes.

The other specific reason can be identified in the fact that, beyond the conserved residue Phe³³⁴ (TM7), the benzyloxy group of (+)-**3** (or the methyl of (–)-**6**) is suggested to interact with residues Asp³²⁷ (TM7) and Leu¹⁰⁵ (TM2), which are only found in the α_{1B} -adrenoceptor.

4. Conclusion

In conclusion, pharmacological data revealed that two parameters influence the α_1 -adrenoceptor selectivity of investigated cyclazosin analogues: the kind of substituent on the amide function and the stereochemistry of the octahydroquinoxaline group. The (4a*S*,8a*R*) stereoisomers (+)-**3** and (–)-**6**, having a benzyloxy and a methyl substituent, respectively, in place of the furyl group of (+)-cyclazosin, resulted the most α_{1B} -selective compounds, whereas the corresponding (4a*R*,8a*S*) stereoisomers (–)-**3** and (+)-**6**, showed a slight α_{1D} or α_{1B} selectivity. With respect to (+)-**1**, the higher selectivity of (+)-**3** and, in particular, (–)-**6** is due to their similar high potency at α_{1B} -adrenoceptor with a concomitant drop of potency at α_{1D} (5 and 32 times) and, in particular, at α_{1A} subtypes (46 and 178 times). This finding might indicate a reduced ability of benzyloxy and methyl groups to contribute to the interaction with α_{1D} and α_{1A} -adrenoceptors, owing to their physicochemical properties. With support of molecular modelling and sequence comparison, the results of present study also suggest that, for the α_{1B} -adrenoceptor selectivity of cyclazosin analog compounds, a sterically defined configuration of *cis*-octahydroquinoxaline group, (4a*S*,8a*R*), is specifically requested.

5. Experimental section

5.1 Chemistry

5.1.1 General—Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. The IR spectra, taken on a Perkin-Elmer 297 instrument, were consistent with all the assigned structures. The ¹H-NMR spectra were recorded on a Varian Gemini 200 instrument. Except for CDCl₃, used as solvent for compounds (+)-**9** and (–)-**9**, in all other cases the DMSO-*d*₆ solvent was used. The elemental analyses of compounds, performed on a Fisons instrument mod. EA1108CHNS-O, agreed with the calculated values within the range $\pm 0.4\%$. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040–0.063 mm, Merck) by flash chromatography. *R_f* values were determined with silica gel TLC plates (Kieselgel 60 F₂₅₄, layer thickness 0.25 mm, Merck). The composition and volumetric ratio of eluting mixtures were: A, petroleum ether-ethyl acetate-methanol- 28% ammonia (8:6:2:0.2); B, petroleum ether-ethyl acetate-methanol-7% ammonia (7:7:1:0.05); C, petroleum ether-ethyl acetate-methanol-7% ammonia (8:4:1:0.05); D, ethyl acetate-chloroform-methanol (6:1:3); E, cyclohexane-ethyl acetate-methanol

(6:3:1); F, Petroleum ether-cyclohexane (1:1). Petroleum ether refers to the fraction with a boiling point of 40–60 °C. The term "dried" refers to the use of anhydrous sodium sulphate. Compounds were named following IUPAC rules as applied by ACD/Name software, version 7.0 (Advanced Chemistry Development, Inc., Toronto, Canada). Solid compounds were purified by crystallization using the following solvents or mixture of solvents: MeOH, (–)-**2**; *i*-PrOH, (–)-**4**, (–)-**6**, (+)-**6**, (–)-**8**, (+)-**9**, (–)-**9**; MeOH/*i*-PrOH, (–)-**7**; EtOH, **11**. Yields are of purified products and were not optimized. Chemicals and reagents were purchased from Sigma-Aldrich Srl (Milano, Italy) or Lancaster research chemicals (Chiminord, Srl, Cusano Milanino, Milano, Italy).

5.1.2. General procedure for the synthesis of [(–)-2**, (–)-**4**, (–)-**6**, (+)-**6**, (–)-**7**, (–)-**8**]**—A solution of proper acyl chloride (1.05 mmol) in dry CH₂Cl₂ (5 mL) was added dropwise to a stirred and cooled (0 °C) solution of (–)-**10** or (+)-**10** free bases (1.0 mmol) and Et₃N (0.21 mL, 1.5 mmol) dissolved in dry CH₂Cl₂ (10 mL). Then the mixture was stirred at room temperature for 3 h, the solvent evaporated and the residue purified by column chromatography using the proper eluting mixture. The products, eluted as free bases, were transformed into the corresponding hydrochloride salts and crystallized.

5.1.3. 2-[(4a*S*,8a*R*)-4-(2-naphthylacetyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (–)-2****—Obtained from 2-(2-naphthyl)ethanoyl chloride (0.22 g, 1.05 mmol) and (–)-**10** free base (0.34 g, 1.0 mmol); 0.13 g (22%); mp 220–222 °C; *R*_f = 0.47 (eluting mixture A); [α]_D²⁰ = –17.6 (c = 0.5, MeOH); ¹H-NMR (DMSO-*d*₆): δ 1.28–2.32 (m, 8H, H_{5–8} octahydroquinoxaline), 3.70–4.18 (m, 12H; 3.88 (s) OCH₃, 3.92 (s) OCH₃, H_{2–3} octahydroquinoxaline, CH₂CO), 4.32–4.48 (m, 1H, H_{8a} octahydroquinoxaline), 4.60–4.76 (m, 1H, H_{4a} octahydroquinoxaline), 7.34–7.53 (m, 4H, naphthyl), 7.70–7.94 (m, 5H, naphthyl and phenyl), 8.65 (br s, 1H, NH, exchangeable with D₂O), 8.87 (br s, 1H, NH, exchangeable with D₂O), 11.83 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₃₀H₃₃N₅O₃·HCl·1.75H₂O: C %, 62.17; H %, 6.52; N %, 12.08. Found: C %, 62.14; H %, 6.67; N %, 12.14.

5.1.4. 2-[(4a*S*,8a*R*)-4-[(2-isopropyl-6-methoxyphenoxy)acetyl]octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (–)-4****—Obtained from 2-(2-*i*-propyl-6-methoxyphenoxy)ethanoyl chloride (0.26 g, 1.05 mmol) and (–)-**10** free base (0.34 g, 1.0 mmol); 0.19 g (30%); mp 227–229 °C; *R*_f = 0.45 (eluting mixture C); [α]_D²⁰ = –11.8 (c 1, MeOH); ¹H-NMR (DMSO-*d*₆): δ 1.13 (d, *J* = 7.00 Hz, 6H, (CH₃)₂CH), 1.30–1.72 (m, 4H, H_{5–8} octahydroquinoxaline), 1.80–2.38 (m, 4H, H_{5–8} octahydroquinoxaline), 3.30 (sept, *J* = 7.00 Hz, 1H, (CH₃)₂CH), 3.60–3.90 (m, 11H; 3.78 (s) OCH₃, 3.85 (s) OCH₃, 3.90 (s) OCH₃, H_{2–3} octahydroquinoxaline), 4.00–4.28 (m, 2H, H_{2–3} octahydroquinoxaline), 4.30–4.43 (m, 1H, H_{8a} octahydroquinoxaline), 4.45–4.80 (m, 3H, CH₂CO, H_{4a} octahydroquinoxaline), 6.80–6.92 (m, 2H, H₃ and H₅ phenoxy), 7.06 (t, *J* = 8.89 Hz, 1H, H₄ phenoxy), 7.56 (s, 1H, aromatic), 7.73 (s, 1H, aromatic), 8.62 (br s, 1H, NH, exchangeable with D₂O), 8.90 (br s, 1H, NH, exchangeable with D₂O), 12.00 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₃₀H₃₉N₅O₅·HCl·2H₂O: C %, 57.92; H %, 7.13; N %, 11.26. Found: C %, 58.26; H %, 7.44; N %, 11.09.

5.1.5. 2-[(4a*S*,8a*R*)-4-acetyloctahydroquinoxalin-1(2*H*)-yl]-6,7-

dimethoxyquinazolin-4-amine hydrochloride (–)-6—Obtained from acetyl chloride (0.08 g, 1.05 mmol) and (–)-**10** free base (0.34 g, 1.0 mmol); 0.17 g (36%); mp 218–220 °C; $R_f = 0.47$ (eluting mixture D); $[\alpha]_D^{20} = -57.1$ (c 0.5, MeOH); $^1\text{H-NMR}$ (DMSO- d_6): δ 1.32–2.30 (m, 11H; H_{5–8} octahydroquinoxaline and 2.10 (s) CH₃CO), 3.66–4.17 (m, 10 H; 3.87 (s) CH₃O, 3.92 (s) CH₃O and H_{2–3} octahydroquinoxaline), 4.20–4.42 (m, 1H, H_{8a} octahydroquinoxaline), 4.58–4.72 (m, 1H, H_{4a} octahydroquinoxaline), 7.52 (s, 1H, aromatic), 7.76 (s, 1H, aromatic), 8.68 (br s, 1H, NH, exchangeable with D₂O), 8.90 (br s, 1H, NH, exchangeable with D₂O), 11.96 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₀H₂₇N₅O₃·HCl·0.25 *i*-PrOH·2.25H₂O: C %, 52.20; H %, 7.28; N %, 14.67. Found: C %, 52.22; H %, 7.31; N %, 14.58.

5.1.6. 2-[(4a*S*,8a*R*)-4-(2,2-dimethylpropanoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (–)-7

—Obtained from 2,2-dimethylpropanoyl chloride (0.13 g, 1.05 mmol) and (–)-**10** free base (0.34 g, 1.0 mmol); 0.13 g (25%); mp 291–293 °C; $R_f = 0.30$ (eluting mixture B); $[\alpha]_D^{20} = -32.5$ (c 0.5, MeOH); $^1\text{H-NMR}$ (DMSO- d_6): δ 1.15–2.30 (m, 17H; 1.24 (s) C(CH₃)₃, H_{5–8} octahydroquinoxaline), 3.70–4.38 (m, 11H; 3.82 (s) OCH₃, 3.88 (s) OCH₃, H_{2–3} and H_{8a} octahydroquinoxaline), 4.62–4.81 (m, 1H, H_{4a} octahydroquinoxaline), 7.70 (s, 1H, aromatic), 7.80 (s, 1H, aromatic), 8.62 (br s, 1H, NH, exchangeable with D₂O), 8.97 (br s, 1H, NH, exchangeable with D₂O), 12.22 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₃H₃₃N₅O₃·HCl·0.25 *i*-PrOH·1.5H₂O: C %, 56.37; H %, 7.77; N %, 13.84. Found: C %, 56.39; H %, 8.10; N %, 13.61.

5.1.7. 2-[(4a*S*,8a*R*)-4-hexanoyloctahydroquinoxalin-1(2*H*)-yl]-6,7-

dimethoxyquinazolin-4-amine hydrochloride (–)-8—Obtained from hexanoyl chloride (0.14 g, 1.05 mmol) and (–)-**10** free base (0.34 g, 1.0 mmol); 0.11 g (20%); mp 228–230 °C; $R_f = 0.31$ (eluting mixture A); $[\alpha]_D^{20} = -35.4$ (c 0.5, MeOH); $^1\text{H-NMR}$ (DMSO- d_6): δ 0.86 (t, $J = 6.58$ Hz, 3H, CH₃CH₂), 1.18–2.20 (m, 14H, H_{5–8} octahydroquinoxaline, (CH₂)₃CH₃), 2.22–2.45 (m, 2H, COCH₂), 3.65–4.20 (m, 10H; 3.83 (s) OCH₃, 3.88 (s) OCH₃ and H_{2–3} octahydroquinoxaline), 4.22–4.38 (m, 1H, H_{8a} octahydroquinoxaline), 4.61–4.78 (m, 1H, H_{4a} octahydroquinoxaline), 7.65 (s, 1H, aromatic), 7.80 (s, 1H, aromatic), 8.64 (br s, 1H, NH, exchangeable with D₂O), 8.96 (br s, 1H, NH, exchangeable with D₂O), 12.18 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₄H₃₅N₅O₃·HCl·0.25 *i*-PrOH·2.0H₂O: C %, 56.19; H %, 8.00; N %, 13.24. Found: C %, 55.87; H %, 7.84; N %, 13.28.

5.1.8. 2-[(4a*R*,8a*S*)-4-acetyloctahydroquinoxalin-1(2*H*)-yl]-6,7-

dimethoxyquinazolin-4-amine hydrochloride (+)-6—Obtained from acetyl chloride (0.08 g, 1.05 mmol) and (+)-**10** free base (0.34 g, 1.0 mmol); 0.09 g (18%); mp 235–237 °C; $R_f = 0.47$ (eluting mixture D); $[\alpha]_D^{20} = -57.0$ (c 0.5, MeOH); $^1\text{H-NMR}$ (DMSO- d_6): δ 1.28–2.22 (m, 11H; H_{5–8} octahydroquinoxaline and 2.08 (s), CH₃CO), 3.63–4.32 (m, 11H; 3.84 (s), OCH₃, 3.91 (s) OCH₃, H_{2–3} and H_{8a} octahydroquinoxaline), 4.60–4.78 (m, 1H, H_{4a} octahydroquinoxaline), 7.65 (s, 1H, aromatic), 7.80 (s, 1H, aromatic), 8.65 (br s, 1H, NH, exchangeable with D₂O), 8.98 (br s, 1H, NH, exchangeable with D₂O), 12.22 (br s, 1H, NH,

exchangeable with D₂O). Anal. Calcd for C₂₀H₂₇N₅O₃·HCl·0.5*i*-PrOH·3H₂O: C %, 51.03; H %, 7.57; N %, 13.84. Found: C %, 51.27; H %, 7.25; N %, 13.97.

5.1.9. 2-[(4a*S*,8a*R*)-4-(2-furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxy-*N,N*-dipropylquinazolin-4-amine hydrochloride (+)-9—A mixture of **11** (0.32 g, 1.0 mmol), (+)-**12** (0.35 g, 1.5 mmol) and *i*-AmOH (15 ml) was refluxed for 48 h then the solvent distilled and the residue purified by column chromatography eluting with mixture C. Obtained 0.16 g (29%); mp 211–213 °C; *R*_f = 0.55 (eluting mixture C); [α]_D²⁰ = + 67.4 (c 1, MeOH); ¹H-NMR (CDCl₃): δ 1.0 (t, *J* = 7.09 Hz, 6H, CH₂CH₃), 1.32–2.20 (m, 11H, CH₂CH₃, H_{5–8} octahydroquinoxaline), 2.41–2.64 (m, 1H, H_{5–8} octahydroquinoxaline), 3.45–3.72 (m, 4H, NCH₂CH₂), 3.88 (s, 3H, OCH₃), 4.00–4.55 (m, 8H; 4.03 (s) OCH₃, H_{2–3} and H_{8a} octahydroquinoxaline), 4.62–4.80 (m, 1H, H_{4a} octahydroquinoxaline), 6.46 (m, 1H, H₄ furan), 7.18 (m, 2H, H₃ furan, aromatic), 7.50 (s, 1H, aromatic), 8.58 (m, 1H, H₅ furan), 13.20 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₉H₃₉N₅O₄·HCl·0.25H₂O: C %, 61.92; H %, 7.26; N %, 12.45. Found: C %, 62.25; H %, 7.59; N %, 12.58.

5.1.10. 2-[(4a*R*,8a*S*)-4-(2-furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxy-*N,N*-dipropylquinazolin-4-amine hydrochloride (–)-9—A mixture of **11** (0.33 g, 1.0 mmol), (–)-**12** (0.36 g, 1.5 mmol) and *i*-AmOH (15 ml) was refluxed for 48 h then the solvent distilled and the residue purified by column chromatography eluting with mixture C. Obtained 0.2 g (35%); mp 210–212 °C; *R*_f = 0.55 (eluting mixture C); [α]_D²⁰ = – 67.1 (c 1, MeOH); ¹H-NMR (CDCl₃): δ 1.02 (t, *J* = 7.11 Hz, 6H, CH₂CH₃), 1.32–2.18 (m, 11 H, CH₂CH₃, H_{5–8} octahydroquinoxaline), 2.45–2.68 (m, 1H, H_{5–8} octahydroquinoxaline), 3.46–3.75 (m, 4H, NCH₂CH₂), 3.90 (s, 3H, OCH₃), 4.00–4.58 (m, 8H; 4.08 (s) OCH₃, H_{2–3} and H_{8a} octahydroquinoxaline), 4.65–4.82 (m, 1H, H_{4a} octahydroquinoxaline), 6.50 (m, 1H, H₄ furan), 7.18 (m, 2H, H₃ furan, aromatic), 7.52 (s, 1H, aromatic), 8.60 (m, 1H, H₅ furan), 13.22 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₉H₃₉N₅O₄·HCl·0.5H₂O: C %, 61.42; H %, 7.29; N %, 12.35. Found: C %, 61.64; H %, 7.65; N %, 12.54.

5.1.11. 2-chloro-6,7-dimethoxy-*N,N*-dipropylquinazolin-4-amine (11)—Dipropylamine 0.5 g (4.97 mmol) in THF (10 ml) was added to a solution of 2,4-dichloro-6,7-dimethoxyquinazoline (0.6 g, 2.3 mmol) in THF (40 ml) then the mixture stirred at room temperature for 12 h. The precipitate was filtered off and the solution evaporated to dryness. The residue purified by crystallization with EtOH gave **11** as solid product, 0.51 g (68%), mp 123–124 °C; *R*_f = 0.5 (eluting mixture F). ¹H-NMR (DMSO-*d*₆): δ 0.94 (t, *J* = 7.02 Hz, 6H, CH₂CH₃), 1.78 (sext, *J* = 7.02 Hz, 4H, CH₂CH₂CH₃), 3.59–3.69 (m, 4H, NCH₂CH₂), 3.89 (s, 3H, OCH₃), 3.94 (s, 3H, OCH₃), 7.14 (s, 1H, aromatic), 7.18 (s, 1H, aromatic). Anal. Calcd for C₁₆H₂₂ClN₃O₂: C %, 59.35; H %, 6.85; N %, 12.98. Found: C %, 59.06; H %, 7.21; N %, 13.00.

5.2. Biological evaluation

5.2.1. Functional experiments—Male Wistar rats (275–300 g; Charles River, Como, Italy) were killed by cervical dislocation and the required organs were isolated. Vas deferens prostatic portion, spleen and aorta were freed from adhering connective tissue and set up rapidly, under a suitable tension, in 20-mL organ baths. The bath medium, containing

physiological salt solution (pH 7.4), was kept at 37 °C and aerated with 5% CO₂: 95% O₂. Concentration-response curves were constructed by cumulative addition of agonist. The agonist concentration in the bath was increased approximately 3-fold at each step, with each addition being made only after the response of the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a force displacement transducer connected to the MacLab System PowerLab/800.

In all experiments a control agonist concentration-response curve (vehicle) was constructed in the presence of the maximum DMSO concentration (0.5%) contained in the bathing solutions being the solvent used for dissolution of tested antagonists on preparing the initial stock solution. These curves were not different from the previous one indicating no interference of solvent in the agonist effect. The agonist-elicited concentration-response curves obtained in the presence of the tested concentrations of antagonist were related to the vehicle control curve, of which the maximal response was taken as 100%. Parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. The experimental conditions used for the investigation at α_1 -adrenoceptor subtypes are procedures taken from quoted literatures.

All pharmacological graphics were drawn by a Prism 3.0 computer program (GraphPad Software, Inc., San Diego, CA, USA). Chemicals, (–)-noradrenaline bitartrate, (–)-phenylephrine hydrochloride, cocaine hydrochloride, normetanephrine hydrochloride and (±)-propranolol hydrochloride were purchased from Sigma-Aldrich Srl (Milano, Italy).

5.2.1.1. Prostatic rat vas deferens: Affinity at α_{1A} adrenoceptor was evaluated on prostatic rat vas deferens according to a reported procedure.¹⁰ Prostatic portions of 2 cm length were mounted under 0.35 g tension at 37 °C in Tyrode solution of the following composition (mM): NaCl, 130; KCl, 2; CaCl₂, 1.8; MgCl₂, 0.89; NaH₂PO₄, 0.42; NaHCO₃, 25; glucose, 5.6. To prevent the neuronal uptake of noradrenaline, used as agonist, cocaine hydrochloride (10 μ M) was added to the Tyrode solution 20 min before the agonist cumulative concentration-response curve. Vas deferens were equilibrated for 45 min, with washing every 15 min. After the equilibration period, tissues were primed twice by addition of 10 μ M noradrenaline in order to obtain a constant response. After another washing and equilibration period of 45 min, a cumulative isotonic noradrenaline concentration-response curve was constructed to determine the relationship between agonist concentrations and contractile response. When measuring the effect of the antagonist, it was allowed to equilibrate with the tissue for 30 min before constructing a new concentration-response curve to the agonist. The noradrenaline solution contained 0.05% Na₂S₂O₅ to prevent oxidation.

5.2.1.2. Aorta: Affinity at rat aorta α_{1D} adrenoceptor was evaluated using a procedure already reported.¹⁰ Two strips (15 mm $\times$ 3 mm) were cut helically from rat thoracic aorta beginning from the end most proximal to the heart. The endothelium was removed by rubbing with filter paper: the absence of 100 μ M acetylcholine-induced relaxation to preparations contracted with 1 μ M noradrenaline was taken as an indicator that the vessel was denuded successfully. The strips were then tied with surgical thread and suspended in an organ bath containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; CaCl₂, 1.9; MgSO₄, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11.7. Cocaine

hydrochloride (10 μ M), normetanephrine hydrochloride (1 μ M), and propranolol hydrochloride (1 μ M) were added to prevent the neuronal and extraneuronal uptake of the agonist noradrenaline and to block the β -adrenoceptors, respectively. In the absence of these inhibitors the noradrenaline concentration-response curve was significantly displaced to the right (data not shown).

After an equilibration period of at least two hours under an optimal tension of 1 g, cumulative noradrenaline concentration-response curves were recorded isometrically at 1 h intervals, the first being discarded and the second one taken as control. After inspection of vehicle activity, the antagonist was allowed to equilibrate with the tissue for 30 min before generation of the third cumulative concentration-response curve to the agonist. Noradrenaline solutions contained 0.05% K₂EDTA in 0.9% NaCl to prevent oxidation.

5.2.1.3. Spleen: The spleen was removed and bisected longitudinally in two strips, which were suspended in tissue baths containing Krebs solution of the following composition (mM): NaCl, 120; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.5; KH₂PO₄, 1.2; NaHCO₃, 20; glucose, 11; EDTA, 0.01. Propranolol hydrochloride (4 μ M) was added to block β -adrenoceptors. Following a reported procedure¹⁰ the spleen strips were placed under 1 g resting tension and equilibrated for 2 h. A first cumulative concentration-response curve to the agonist phenylephrine was quickly taken isometrically, followed by 30 min washing. Subsequently, a second cumulative curve was constructed followed by 30 min washing. Each tissue was then incubated for 30 min either with vehicle or different antagonist concentrations and a new agonist concentration-response curve constructed (third curve).

5.2.2. Binding assays—Competition binding assays to cloned human α_{1a} , α_{1b} , and α_{1d} -adrenoceptor subtypes were performed in membrane preparations from CHO (Chinese Hamster Ovary) cell line transfected by electroporation with DNA expressing the gene encoding each α_1 -adrenoceptor. Cloning and stable expression of the human α_1 -adrenoceptor gene was performed as previously described.¹⁹ Briefly, CHO cells membranes (30 μ g proteins) were incubated in 50 mM Tris-HCl buffer, pH 7.4, with 0.1–0.4 nM [³H]prazosin, in a final volume of 1.02 mL for 30 min at 25 °C, in the absence or presence of competing drugs (1 pM–10 μ M). Non-specific binding was determined in the presence of 10 μ M phentolamine. The incubation was stopped by addition of ice-cold Tris-HCl buffer and rapid filtration through 0.2% poly(ethylenimine)-pretreated Whatman GF/B or Schleicher & Schuell GF52 filters.

5.2.3. Data analysis—In functional studies, responses were expressed as percentage of the maximal contraction observed in the agonist concentration-response curve taken as control. Each response was plotted graphically as a mean from at least four separate experiments. Curves were fitted to all the data by a non-linear regression using the Prism 3.0 program to calculate pEC₅₀ values. In all cases, 50% of the maximum for each concentration-response curve was used to evaluate the EC₅₀. This value, calculated in presence and in absence of antagonist in a single tissue, was used to determine the concentration ratio.

Schild plots were constructed to estimate the pA_2 values and the slope of the regression line using experimental series obtained from at least three different concentrations.¹⁷ The Schild diagrams were constructed by plotting the log (concentration ratio – 1) against the log [antagonist] and deriving it from a linear regression using the Prism 3.0 program. When the Schild plot slope was not significantly different from unity ($P > 0.05$), the regression was recalculated with a constrained slope of 1 and the result given as a pA_2 value. In a number of cases, Schild analysis could not be performed due to the nonparallel slopes of concentration-response curves and variable depression. As consequence, pK_B values were calculated at only one concentration, according to van Rossum.¹⁸ Thus, whereas for a high percentage of compounds a pA_2 value was determined at α_{1A} - and α_{1D} -adrenoceptors, in all other cases the antagonist potency was expressed by pK_B values calculated with the equation $pK_B = \log (\text{concentration ratio} - 1) / [\text{antagonist}]$ at the lowest antagonist concentration giving a significant rightward shift (> 0.5) of the agonist concentration-response curve. Conversely, at α_{1B} -adrenoceptor the antagonist potency of majority of compounds was calculated as pK_B values instead that pA_2 .

Data were compared by Student's *t*-test and presented as means $\pm$ s.e. mean of 4 to 6 experiments. A *p* value < 0.05 was taken to indicate a statistically significant difference.

Data from binding assays were analysed using a non-linear curve-fitting program Allfit.³² Scatchard plots were linear in all preparations and the pseudo-Hill coefficients non significantly different from the unity ($p > 0.05$). The inhibition of the radioligand specific binding by tested compounds allowed the estimation of IC_{50} values that were converted to affinity constants (K_i) by the Cheng-Prusoff equation [10]: $K_i = IC_{50} / (1 + L / K_d)$, where *L* and K_d are the concentration and the equilibrium dissociation constant of the radioligand. Data are expressed as mean of pK_i values of 2 to 3 separate experiments performed in triplicate.

5.3 Molecular modeling methodology

Template receptor: The template for the construction of the homology modeling of the α_{1B} -adrenoceptor, namely the crystal structure of dopamine D_2 receptor solved in complex with the antagonist eticlopride (PDB ID: 3PBL),²⁵ was downloaded from the Protein Databank (www.rcrb.org).

Sequence alignment: The sequence alignment of the template and the target receptors was performed with the Swiss-PDB Viewer (Deep View, <http://spdbv.vital-it.ch>),³³ following a manual procedure based on the identification of the seven TMs that we described in recent articles.^{34,35} An initial automatic sequence alignment was first obtained with the Blosom 62 matrix, with penalties for the opening and the extension of gaps set to 4 and 3, respectively. This initial alignment was then inspected and manually adjusted, if needed, to ensure the matching of the motifs that identify each of the seven TMs and the absence of internal gaps within the boundaries of the membrane-spanning domains. Moreover, in the alignment of the second extracellular loop domain (EL2), we ensured the matching of the Cys residues found in the α_{1B} -adrenoceptor and the D_2 receptor, namely Cys¹⁹⁵ and Cys¹⁸¹, respectively – of note, in the crystal structure of the D_2 receptor, Cys¹⁸¹ shows a disulfide with Cys¹⁰³ in

the third transmembrane domain (TM3) that is known to exist in the great majority of G protein-coupled receptors.³⁴ Differences in sequence length between the two aligned receptors were accounted for by placing gaps in the loop domains, following our recently described procedures for the alignment of domains that have fewer residues in the modeled receptor than in the template (*deletions in the modeled receptor*) and for the alignment of domains that have more residues in the modeled receptor than in the template (*insertions in the modeled receptor*).³⁵ Finally, as a number of residues in the N-terminus, C-terminus and third intracellular loop (IL3) of the D₂ receptor are not solved in the crystal structure, we expunged from the sequence alignment the residues of the α_{1B} -adrenoceptor that extend beyond those crystallographically solved for the template. The resulting alignment is shown in Figure 5.

Construction of the homology models: The homology modeling procedure was performed with the program Modeller.³⁶ Following the sequence alignment, 10 alternative models were built with the “automodel” class. The parameters for the variable target function method (VTFM) optimization were set as follows: library_schedule = autosched.slow; deviation = 4; max_var_iterations = 300. The parameters for the molecular dynamics optimization were set as follows: md_level = refine.slow. The formation of a disulfide bridge between Cys¹¹⁸ (TM3) and Cys¹⁹⁵ (EL2) in the model of the α_{1B} -adrenoceptor was enforced in the modeling procedure. These two Cys residues correspond to the abovementioned Cys¹⁰³ (TM3) and Cys¹⁸¹ (EL2) residues of the D₂ receptor, which are involved in the formation of a conserved disulfide bridge between TM3 and EL2. After the construction of the models, for each of them the structural viability of each residue was then assessed through Modeller’s probability density function (PDF). Models were required to have PDF values lower than 0.06 for each of their residues in order to be accepted. The accepted models were evaluated on the basis of the Modeller’s Discrete Optimized Protein Energy (DOPE) method to identify the top-scoring model, which was then used for the molecular docking studies.

Preparation of the α_{1B} model for molecular modeling: The top-scoring molecular model of the α_{1B} -adrenoceptor was optimized with the Protein Preparation Wizard of the Schrodinger suite, version 2016-2.^{37,38} Through the workflow, hydrogen atoms were added and the protonation state at pH 7 for all of the ionizable groups was calculated with the PROPKA method. Moreover, the orientation of hydroxyl groups, as well as Asn, Gln and His residues was optimized. Finally, the structure was minimized with the Impact molecular mechanics engine and the OPLS3 force field, allowing a maximum root mean square deviation (RMSD) of 0.30 Å from the original structure.

Preparation of the ligand for molecular modeling: Compound (+)-**3** was sketched through the Maestro interface and subsequently optimized with the LigPrep tool of the Schrodinger suite, version 2016-2,^{37,38} calculating the most favorable protonation and tautomeric states available at pH 7.0 through the Epik engine.^{39,40} In the LigPrep procedure, the configuration of the chiral center of the compound was set to be maintained as in the input structure.

Identification of the ligand-binding cavity: The cavities present in the model the α_{1B} - adrenoceptor were identified with the SiteMap module of the Schrodinger suite, version

2016-2.³⁷ The parameters were set as follows: require at least 15 site points per reported site; report up to 5 sites; use more restrictive definition of hydrophobicity; use standard grid; crop site maps at 4 Å from nearest site point. The cavity located within the helical bundle of the receptor, near its opening toward the extracellular space, was selected as the ligand-binding cavity.

Molecular docking: Molecular docking experiments were conducted with the Glide module of the Schrödinger suite, version 2016-2.³⁷ The docking grid was generated without scaling down the *van der Waals* radii of the receptor atoms, was centered on the identified ligand-binding cavity, and was given a size capable of accommodating compounds as large as the cavity. An inner box, within which the midpoint of the diameter of the docked compound was required to fall, was also centered on the cavity and was given a diameter of 10 Å. Molecular docking was conducted with the HTVS algorithm and the OPLS3 force field. The scaling factor for the *van der Waals* radii of the docked compounds was set to 0.70 for all of the atoms with a partial charge lower than 0.15 *e*. The sampling method for ring conformations was set to include that of the input structure. After docking, a post-docking minimization was set to be performed on the 25 lowest energy complexes, with a threshold of 0.50 kcal/mol for rejecting minimized poses. The procedure resulted in the final production of four docking complexes.

Minimization: The four docking complexes produced by the above-mentioned docking procedure were then subjected to a further energy minimization with the MacroModel molecular mechanics engine of the Schrodinger suite, version 2016-2.³⁷ In particular, the minimization was conducted with the OPLS3 force field, using water as the implicit solvent, and protracted until the potential energy gradient reached the cutoff value of 0.05 kJ/(mol Å). Flexibility was granted to the entire ligand as well as all of the residues located within 5 Å from it. An additional shell of residues located within 3 Å from the flexible atoms were considered for the calculation of the potential energy but were held rigid. The selection of water as the implicit solvent is justified by the fact that the energy minimization was confined to the ligand-binding cavity. The complex endowed with the lowest energy after the MacroModel minimization was taken as the final solution.

Bi-dimensional representation of the receptor ligand interactions: The bi-dimensional representation of the receptor-ligand complex shown in Figure 4 was plotted with the Ligand Interaction Diagram tool of the Schrodinger suite, version 2016-2,³⁷ manually repositioning the residues around the ligand to reflect the three-dimensional arrangement of the binding site.

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Highlights

1. An improved α_{1B} -adrenoceptor selectivity of (+)-cyclazosin analog antagonists was obtained
2. Compounds (+)-**3** and (–)-**6** have to date the highest α_{1B} -selectivity in functional antagonism
3. The (4a*S*,8a*R*) stereochemistry of compounds resulted crucial for the α_{1B} -selectivity

Figure 1.

Chemical structures of (+)-1 [(+)-cyclazosin], the homochiral analogues (-)-2 – (+)-9, and some enantiomers (-)-1, (-)-3, (+)-6, (-)-9

Figure 2. Molecular model of the α_{1B} -adrenoceptor in complex with the antagonist (+)-**3**. The backbone of the receptor is schematically represented as a ribbon, with a gradient of colors ranging from red to purple going from the N-terminus to the C-terminus. The ligand is shown as balls and sticks, with pink carbons. The seven transmembrane domains are labeled (TM1 to TM7).

Figure 3.

Detailed view of the ligand-binding site in the molecular model of the α_{1B} -adrenoceptor in complex with the antagonist (+)-**3**, showing the ligand and the amino acid residues located within 4 Å from it – Lys¹⁸⁵ was included in the figure although outside of 4 Å radius because it establishes a cation-aromatic interaction with the ligand. The ligand is shown as balls and sticks, with pink carbons. The amino acid residues are shown with grey carbons and are represented as thin tubes if conserved in both the α_{1A} and α_{1D} subtypes, or as balls and sticks with green carbons if non-conserved in at least one of the two subtypes. Hydrogen bond are shown as yellow dotted lines; aromatic-aromatic interactions are shown as blue dotted lines, cation-aromatic interactions are shown as green dotted lines.

Figure 4.

Bi-dimensional representation of the antagonist (+)-**3** and the amino acid residues of the α_{1B} -adrenoceptor located within 4 Å from it. For the non-conserved residues, the corresponding residues in the α_{1A} or the α_{1D} subtypes are indicated. The residues are colored as follows: green = hydrophobic; cyan = polar; red = negatively charged; light blue: positively charged; dark blue = unspecified.

Scheme 1.

Reagents and conditions: (a) acyl chloride [2-(2-naphthyl)ethanoyl chloride for **(-)-2**; 2-(2-*i*-propyl-6-methoxyphenoxy)ethanoyl chloride for **(-)-4**; acetyl chloride for **(-)-6** and **(+)-6**; 2,2-dimethylpropanoyl chloride for **(-)-7**; hexanoyl chloride for **(-)-8**], CH_2Cl_2 , Et_3N

Scheme 2.

Reagents and conditions: (a) Dipropylamine, THF; (b) *i*-AmOH, reflux

Table 1

Functional antagonist affinities, expressed as pA_2 or pK_B , of compounds (–)-**2** – (+)-**9**, (–)-**3**, (+)-**6**, (–)-**9** and (+)-**1** and (–)-**1** as references, at α_1 -adrenoceptor subtypes of isolated rat prostatic vas deferens (α_{1A}), spleen (α_{1B}) and thoracic aorta (α_{1D}).

Compound	pA_2^a or pK_B^b		Selectivity ratio c			
	α_{1A}	α_{1B}	α_{1D}	α_{1B}/α_{1A}	α_{1D}/α_{1A}	α_{1D}/α_{1B}
(+)- 1	7.77 ± 0.03^a	8.86 ± 0.03^b	7.27 ± 0.07^a	13	39	0.3
(–)- 2	6.56 ± 0.13^b	8.05 ± 0.06^b	6.87 ± 0.06^a	30	15	2
(+)- 3	6.11 ± 0.03^a	8.77 ± 0.03^b	6.62 ± 0.03^a	457	141	3
(–)- 4	6.82 ± 0.03^a	6.72 ± 0.02^a	7.23 ± 0.06^a	0.8	0.3	3
(–)- 5	6.70 ± 0.03^a	8.61 ± 0.15^b	7.10 ± 0.10^b	81	32	3
(–)- 6	5.52 ± 0.03^a	8.52 ± 0.11^a	5.77 ± 0.05^a	1000	562	2
(–)- 7	7.17 ± 0.04^b	8.26 ± 0.14^b	6.85 ± 0.05^a	12	26	0.5
(–)- 8	7.06 ± 0.08^b	7.99 ± 0.18^b	6.81 ± 0.06^a	9	15	0.6
(+)- 9	5.89 ± 0.06^b	7.86 ± 0.19^b	6.22 ± 0.06^b	93	44	2
(–)- 1	9.23 ± 0.08^a	9.67 ± 0.04^b	10.12 ± 0.06^a	3	0.4	8
(–)- 3	7.79 ± 0.04^a	8.74 ± 0.12^a	9.28 ± 0.07^a	9	0.3	31
(+)- 6	7.35 ± 0.03^a	9.21 ± 0.05^a	8.66 ± 0.04^a	72	4	20
(–)- 9	7.02 ± 0.02^b	7.17 ± 0.13^b	7.23 ± 0.06^a	1	1	1

^a pA_2 values, expressed as means $\pm$ SEM of three different concentrations, each tested at least four times.

^b pK_B values ($\pm$ SEM) calculated according to van Rossum.

^c Calculated by the antilog of the difference between pA_2 or pK_B values at different α_1 -adrenoceptor subtypes.

Table 2

Antagonist potency, pA₂ or pK_B, and Eudismic Ratio (ER) for enantiomer couples of **1**, **3**, **6**, **9** at α₁-adrenoceptor subtypes

Compound (stereochemistry)	α _{1A}	ER ^c	pA ₂ ^a (pK _B) ^b	α _{1B}	ER ^c	pA ₂ ^a (pK _B) ^b	α _{1D}	ER ^c
(+)- 1 (4a <i>S</i> ,8a <i>R</i>)	7.77 ± 0.03 ^a	29	8.86 ± 0.03 ^b	7	7.27 ± 0.07 ^a	708		
(-)- 1 (4a <i>R</i> ,8a <i>S</i>)	9.23 ± 0.08 ^a		9.67 ± 0.04 ^b		10.12 ± 0.06 ^a			
(+)- 3 (4a <i>S</i> ,8a <i>R</i>)	6.11 ± 0.03 ^a	48	8.77 ± 0.03 ^b	1	6.62 ± 0.03 ^a	457		
(-)- 3 (4a <i>R</i> ,8a <i>S</i>)	7.79 ± 0.04 ^a		8.74 ± 0.12 ^a		9.28 ± 0.07 ^a			
(-)- 6 (4a <i>S</i> ,8a <i>R</i>)	5.52 ± 0.03 ^a	68	8.52 ± 0.11 ^a	5	5.77 ± 0.05 ^a	776		
(+)- 6 (4a <i>R</i> ,8a <i>S</i>)	7.35 ± 0.03 ^a		9.21 ± 0.05 ^a		8.66 ± 0.04 ^a			
(+)- 9 (4a <i>S</i> ,8a <i>R</i>)	5.89 ± 0.06 ^b	14	7.86 ± 0.19 ^b	5	6.22 ± 0.06 ^b	10		
(-)- 9 (4a <i>R</i> ,8a <i>S</i>)	7.02 ± 0.02 ^b		7.17 ± 0.13 ^b		7.23 ± 0.06 ^a			

^a pA₂ values, expressed as means ± SEM of three different concentrations, each tested at least four times.

^b pK_B values (± SEM) calculated according to van Rossum.

^c Eudismic Ratio, calculated by the antilog of difference between pA₂ or pK_B values of distomer and automer at different α₁-adrenoceptor subtypes.

Table 3

Binding affinity constants, expressed as pK_i , of compounds **(-)-2** – **(+)-9**, **(-)-3**, **(+)-6**, **(-)-9** and **(+)-1** as references, at cloned human α_1 -adrenoceptor subtypes expressed in CHO cells.

Compound	pK_i^a		Selectivity ratio ^b			
	α_{1a}	α_{1b}	α_{1b}/α_{1a}	α_{1b}/α_{1d}	α_{1d}/α_{1a}	
(+)-1	7.91	9.87	8.49	91	24	4
(-)-2	7.77	8.93	8.65	14	2	8
(+)-3	8.26	9.42	8.40	14	11	1
(-)-4	9.06	9.82	9.41	6	3	2
(-)-5	8.47 ^c	8.85 ^c	8.41 ^c	2	3	1
(-)-6	6.65	8.04	6.98	25	12	2
(-)-7	8.18	9.11	8.21	9	8	1
(-)-8	8.24	9.04	8.43	6	4	2
(+)-9	7.65	9.52	8.29	74	17	4
(-)-1	9.23	10.15	10.22	8	1	10
(-)-3	9.30	9.72	10.40	3	0.2	13
(+)-6	8.04	9.13	9.82	12	0.2	60
(-)-9	8.73	9.89	10.20	15	0.5	29

^aEquilibrium dissociation constants (K_i) were calculated from IC_{50} values using the Cheng-Prusoff equation. The affinity estimates, derived from displacement of [3H]prazosin binding from α_1 -adrenoceptors and expressed as mean values of $pK_i \pm$ SEM, were from two to three experiments performed in triplicate, which agreed within $\pm 20\%$.

^bCalculated by the antilog of the difference between pK_i values at different α_1 -adrenoceptor subtypes.

^cData are from Ref [6].