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Original article

Affinity and activity profiling of unichiral 8-substituted 1,4-benzodioxane analogues of WB4101 reveals a potent and selective α_{1B}-adrenoceptor antagonist

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ABSTRACT

Unichiral 8-substituted analogues of 2-[(2-(2,6-dimethoxyphenoxy)ethyl)aminomethyl]-1,4-benzodioxane (WB4101) were synthesized and tested for binding affinity at cloned human α_{1a}-, α_{1b}-and α_{1d}-adrenoreceptor (α_{1a}-, α_{1b}-and α_{1d}-AR) and at native rat 5-HT_{1A} receptor and for antagonist affinity at α_{1A}-, α_{1B}-and α_{1D}-AR and at $\alpha_{2A/D}$-AR. Among the selected 8-substituents, namely fluorine, chlorine, methoxyl and hydroxyl, only the last caused significant decrease of α_1 binding affinity in comparison with the lead compound. Functional tests on the *S* isomers confirmed the detrimental effect of OH positioned in proximity to benzodioxane O(1). For the other three substituents (F, Cl, OMe), the α_{1A} and the α_{1D} antagonist affinities were generally lower than the α_{1a} and α_{1d} binding affinities, but not the α_{1B} antagonist affinity, which was similar and sensibly higher compared to α_{1b} binding affinity in the case of F and OMe respectively. This trend confers significant α_{1B}-AR selectivity, in particular, to the 8-methoxy analogue of (*S*)-WB4101, a new potent (pA_2 9.58) α_{1B}-AR antagonist. The *S* enantiomers of all the tested compounds were proved to act as α_1-AR inverse agonists in a vascular model.

1. Introduction

The adrenergic receptors (ARs) have been extensively investigated and their functions have been progressively elucidated. They are classified into α_1, α_2, β_1, β_2 and β_3 [1–3]. The three α_1 subtypes, namely α_{1A}, α_{1B} and α_{1D}, exhibit distinct pharmacology and tissue expression and subtype selective α_1 antagonists have a therapeutic potential in the treatment of several pathologies [4–7]. Indeed, selective α_{1A} and α_{1D} antagonists are used in the therapy of diseases such as hypertension and lower urinary tract symptoms (LUTS) secondary to benign prostatic hyperplasia (BPH) [8–11], but also selective α_{1B} antagonists have been taken into account as therapeutic agents and pharmacological tools [12,13]. In particular, the function of α_{1B}-AR in CNS has been recently reinvestigated [13] and this receptor subtype has been indicated as target for novel drugs useful in the treatment of CNS disorders [14].

The *S* enantiomer of 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane (WB4101) is a potent α_1-adrenoreceptor (α_1-AR) antagonist, which exhibits, in binding experiments, high and similar affinity at α_{1A}-and α_{1D}-AR and one order of magnitude lower affinity for the α_{1B} subtype [15]. In functional studies on animal tissues, *S*-WB4101 shows subnanomolar antagonist affinity at all the three α_1-AR subtypes with a slight selectivity for the α_{1A}-AR [16]. In order to improve potency and subtype selectivity, many α_1-AR ligands structurally related to WB4101 have been designed by making modifications at the benzodioxane nucleus, at the amino function and at the (2,6-dimethoxyphenoxy)ethyl moiety [7]. In particular, our research group has long been involved in characterizing *o*-monosubstituted and *o*-disubstituted phenoxy analogues as well as naphthoxy, naphthodioxane and tetrahydronaphthodioxane analogues [15–18]. The most notable result of such researches was the finding of two potent and selective α_{1D}-AR and α_{1A}-AR antagonists, the former by replacement of the 2,6-dimethoxyphenoxy moiety of *S*-WB4101 with 2-methoxy-1-naphthoxy and the latter by the same modification coupled with substitution of benzodioxane by tetrahydronaphthodioxane [16]. In

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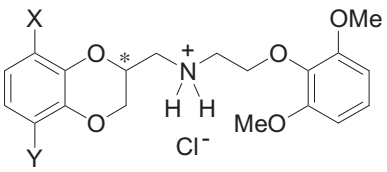
this class of α_1-AR antagonists, however, modifications and substitutions at the aromatic ring of WB4101 benzodioxane have received little attention. In fact, while a large variety of WB4101 analogues modified or substituted at the dioxane have been studied, only the 8-methylbenzodioxane and the three isomeric naphthodioxane analogues have been reported by other research groups and only as racemates [19,20]. Apart from the not ready synthetic accessibility of the derivatives substituted at the aromatic ring of benzodioxane, such a gap is rather surprising since a key role has been attributed to benzodioxane in the interaction with α_1-ARs due to its overall steric and electronic features and its modification has been postulated to be critical, in particular, for α_{1A}-AR subtype selectivity [21,22]. That's why we decided to study the effects of the insertion of a substituent at the aromatic ring of benzodioxane and designed the WB4101 analogues **1**–**5** (Fig. 1). In particular, we focused on the substitution at the 8-position of benzodioxane, namely in proximity to dioxane O(1), which seems to exert a great influence on the conformational properties of the ligand, due to intramolecular interactions with ammonium protons [23], and to play a peculiar and critical role in receptor binding, as suggested by the fact that its replacement, in WB4101 or its analogues, with a methylene, a methine or a hydroxymethine, but not with a carbonyl, is deleterious [24–26]. As a first step, we considered substitution with hydroxyl (**1**), a both HBA and HBD group, able to interfere with the interaction potentiality of O(1) by giving rise to hydrogen bond with it or to contribute itself to binding, if, as reasonable, the counterpart of O(1) is a receptor polar pocket. The 8-methoxy analogue (**2**) was successively chosen as a necessary term for comparison with the 8-hydroxy analogue **1**. Besides the 8-methoxy substitution, for reasons discussed hereinafter, we considered also the 5-methoxy substitution (**5**). The choice of a substituent such as chlorine (**3**) was dictated by its lipophilic character, opposite to OH, and by the steric and electronic effects that it can exert on O(1) when positioned at benzodioxane C(8). Lastly, we considered the introduction of fluorine (**4**), which has a large electronic effect due to high electronegativity and a weak HBA character, but with low additional steric demand in comparison with hydrogen.

We have previously reported the synthesis of **2**, **4** and **5** in both the enantiomeric forms [27]. Herein, we describe the synthesis of the *S* and *R* stereoisomers of the 8-hydroxy and 8-chloro benzodioxane analogues, **1** and **3** respectively, and the biological profile of all the five enantiomeric pairs **1**–**5** in binding tests at α_{1a}-, α_{1b}-and α_{1d}-ARs and the 5-HT_{1A} receptor and, for the *S* enantiomers, in functional experiments at α_{1A}-, α_{1B}-and α_{1D}-and α_2-ARs. The SAFIR and SAR data of **1**–**5** are discussed as well as their nature of inverse agonists, which was studied and semi-quantitatively determined by means of a vascular model.



WB4101	X = H	Y = H
1	OH	H
2	OMe	H
3	Cl	H
4	F	H
5	H	OMe

Fig. 1. 8-and 5-substituted analogues of WB4101.

2. Chemistry

(*S*)-and (*R*)-WB4101, necessary in binding and functional tests as reference compounds, are accessible, as previously reported, from different unichiral synthons, such as (*S*)-isopropylidene glycerol [23], (*R*)-and (*S*)-1,4-benzodioxane-2-carboxylic acid [28–30] and the respective methyl esters [28,31]. The enantiomers of compound **1** were prepared from (*R*)-and (*S*)-2-tosyloxymethyl-8-hydroxy-1,4-benzodioxane, whose synthesis we had previously described [27,32], by conversion into iodomethyl derivatives and amination with 2-(2,6-dimethoxyphenoxy)ethylamine. The enantiomers of compound **3** were synthesized by the same strategy, namely reacting 2-(2,6-dimethoxyphenoxy)ethylamine with enantiopure 2-mesyloxymethyl-8-chloro-1,4-benzodioxane **15**, in turn prepared by using (*S*)- or (*R*)-glycerol acetonide and 2-benzyloxy-3-chlorophenol **10** as building blocks. The synthesis of (*S*)-**3** is illustrated in Scheme 1. The 2,3-disubstituted phenol **10** was obtained from 2-chlorophenol by (a) esterification with acetyl chloride, (b) Fries rearrangement, (c) benzylation of the resulting *o*-acetylphenol **7**, (d) Baeyer-Villiger oxidation to 2-benzyloxy-3-chlorophenyl acetate **9** and (e) methanolysis of the acetate **9**. Displacement of mesylate from the mesyl ester of (*S*)-glycerol acetonide by 2-benzyloxy-3-chlorophenoxide and subsequent hydrolysis of the cyclic ketal provided the phenoxypropanediol (*R*)-**12**, which was dimesylated and then debenzylated. Intramolecular nucleophilic substitution of the mesylate at C(2) of the glycerol skeleton by the *ortho*-phenoxy moiety formed the 1,4-dioxane ring giving (*R*)-2-mesyloxymethyl-8-chloro-1,4-benzodioxane (*R*)-**15**, which was reacted with 2-(2,6-dimethoxyphenoxy)ethylamine to yield (*S*)-**3**. The *R* enantiomer of **3** was synthesized by the same route as illustrated in Scheme 1 but starting from the mesyl ester of (*R*)-glycerol acetonide.

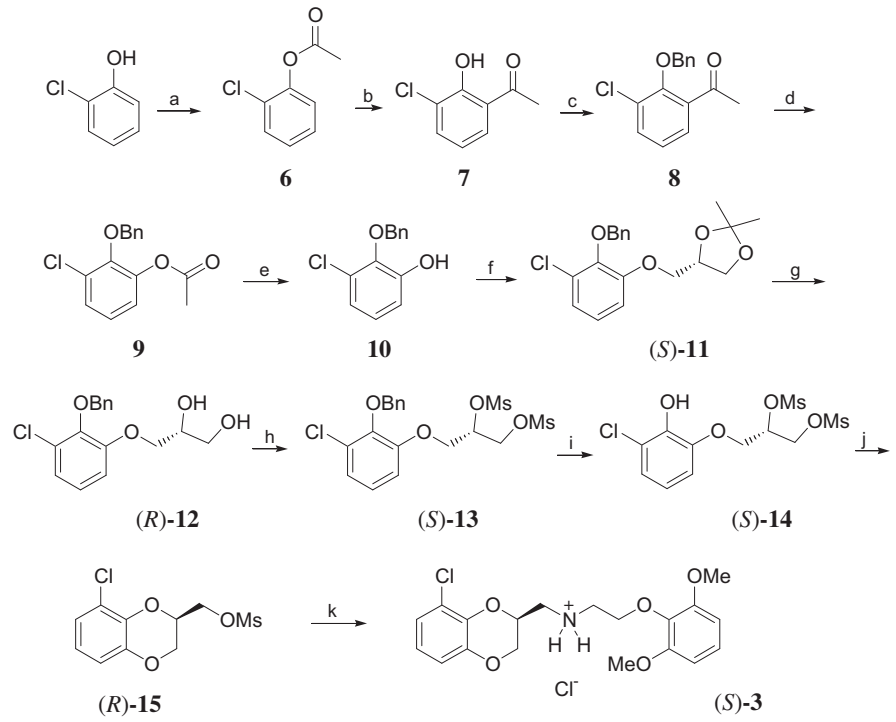
3. Results and discussion

Table 1 reports the affinities, expressed as pK_i values ($-\log K_i$, M), at the three cloned human α_1-AR subtypes and native 5-HT_{1A} serotoninergic receptor from rat hippocampus of the enantiomeric pairs of compounds **1**–**5** and, as comparison terms, of (*S*)-and (*R*)-WB4101.

The affinities of the *S* enantiomers are always higher than those of their antipodes, the eudismic index being generally higher than 1 and, only in the case of some modest affinities of the enantiomers of **5**, significantly lower than 1. Compared to (*S*)-WB4101, the *S* enantiomers of the 8-OMe, 8-Cl and 8-F analogues have quite similar affinity profiles showing α_{1a} and α_{1d} affinities close to those of (*S*)-WB4101 (pK_i 9.39 and 9.29, respectively). For these three compounds as for (*S*)-WB4101, the α_{1a} and α_{1d} affinities are always higher than the α_{1b} affinity, but the difference is less pronounced. Therefore, they are a little less α_{1a}/α_{1b} and α_{1d}/α_{1b} selective than (*S*)-WB4101. A different behaviour is shown by (*S*)-**1**, the 8-hydroxy analogue, for which significant affinity decreases at all the three α_1 subtypes were registered in comparison with (*S*)-WB4101 and with the other 8-substituted derivatives. Concerning the 5-HT_{1A} receptor, the *S* enantiomers of all the four 8-substituted analogues display affinity in the nanomolar range, slightly higher than (*S*)-WB4101. The 5-methoxy analogue, (*S*)-**5**, radically differs from the 8-substituted derivatives, its affinities at the α_1-AR subtypes and 5-HT_{1A} serotoninergic receptor being from one to two orders of magnitude lower.

The functional assays were carried out only for the *S* enantiomers. Their antagonist affinities at α_1-AR subtypes and α_2-AR, expressed as pA_2, are listed in Table 2.

α_1-AR subtypes and α_2-AR blocking activity was determined on different rat tissues. In particular, α_1-AR subtypes blocking activity



Scheme 1. Reagents: (a) Acetyl chloride, pyridine, dichloromethane. (b) Aluminium chloride, dichlorobenzene. (c) Benzyl bromide, tetrabutylammonium hydroxide, 10% aqueous NaOH, dichloromethane. (d) 3-Chloroperbenzoic acid, dichloromethane. (e) 2.5 N NaOH, methanol. (f) (*R*)-1-Mesiloxy-2,3-propanediol acetonide, potassium *tert*-butoxide, DMF. (g) 1 N HCl, ethanol. (h) Mesyl chloride, triethylamine, dichloromethane. (i) H_2—Pd/C, MeOH. (j) K_2CO_3, acetone. (k) 2-(2,6-Dimethoxyphenoxy)ethylamine, isobutanol; HCl/EtOH.

was assessed by antagonism of (−)-noradrenaline (NA)-induced contraction of vas deferens prostatic portion (α_{1A}) [33] and of thoracic aorta (α_{1D}) [34] and by antagonism to (−)phenylephrine-induced contraction of spleen (α_{1B}) [34], while α_2-adrenoceptor blocking activity was determined by antagonism to clonidine-inhibited twitch responses of the field-stimulated prostatic portion of vas deferens. Furthermore, considering the demonstrated α_{1A}-AR involvement in maintaining prostate smooth muscle tone and the consequent therapeutic potential of agents reducing the latter for LUTS, the antagonist affinity was also evaluated in rat prostate [35]. Inspection of the results reported in Table 2 reveals trends, which can be summarized as follows: (a) all the compounds exhibit high α_1/α_2 selectivity; (b) the 8-OH and the 5-OMe analogues are significantly less potent α_1 antagonists than the 8-OMe, 8-Cl and 8-F analogues, which display from high to very high antagonist affinities at all the three α_1-AR subtypes; (c) among these three compounds, the 8-F and, even more markedly, the 8-OMe analogue show significant α_{1B} selectivity, opposite to (*S*)-WB4101. It was this last observation that induced us to consider the introduction of methoxy substituent also at the 5 position of benzodioxane and to include the 5-methoxy analogue in the present investigation otherwise devoted only to 8-substituted WB4101 analogues.

The analysis of the functional affinities suggests that the introduction of a substituent at the 8-position of (*S*)-WB4101 benzodioxane, unlike the introduction of a 5-substituent, is well tolerated and relatively different steric and electronic effects, such as those of fluorine, chlorine and methoxyl, are allowed to be exerted by the 8-substituent if not interfering, as in the case of hydroxyl, with the interaction potential of O(1) or with its influence on the ligand conformation. Such an explanation is substantiated by the high antagonist affinities of the 8-OMe analogue (*S*)-**2** compared to the

Table 1
Experimental affinity constants, expressed as pK_i (−logK_i, M) ± SE, of the enantiomers of compounds **1**–**5** and of WB4101 for cloned human α_1-adrenoceptor subtypes and 5-HT_{1A} receptor.

Benzodioxane substituent	Compound	pK_i			
		α_{1a}	α_{1b}	α_{1d}	5-HT_{1A}
–	(*S*)-WB4101[a]	9.39 (±0.06)	8.24 (±0.04)	9.29 (±0.11)	8.61 (±0.04)
	(*R*)-WB4101[a]	7.95 (±0.04)	7.14 (±0.06)	7.98 (±0.08)	7.39 (±0.03)
8-OH	(*S*)-**1**	8.66 (±0.04)	7.51 (±0.07)	8.05 (±0.06)	9.00 (±0.03)
	(*R*)-**1**	7.18 (±0.04)	6.40 (±0.05)	6.49 (±0.03)	7.76 (±0.03)
8-OMe	(*S*)-**2**	9.04 (±0.07)	8.56 (±0.06)	9.43 (±0.09)	9.07 (±0.03)
	(*R*)-**2**	7.78 (±0.04)	7.10 (±0.08)	7.78 (±0.06)	7.89 (±0.04)
8-Cl	(*S*)-**3**	9.05 (±0.11)	8.66 (±0.09)	9.15 (±0.17)	8.77 (±0.07)
	(*R*)-**3**	8.25 (±0.02)	7.47 (±0.05)	8.40 (±0.04)	7.76 (±0.04)
8-F	(*S*)-**4**	9.19 (±0.05)	8.62 (±0.05	9.31 (±0.05)	8.85 (±0.02)
	(*R*)-**4**	7.74 (±0.04)	6.99 (±0.04)	8.13 (±0.05)	7.50 (±0.03)
5-OMe	(*S*)-**5**	7.62 (±0.04)	6.35 (±0.05)	7.10 (±0.09)	7.54 (±0.05)
	(*R*)-**5**	6.09 (±0.07)	6.19 (±0.02)	6.58 (±0.07)	6.91 (±0.06)

[a] Data taken from Ref. [15].

Table 2
S Enantiomers of WB4101 and of compounds **1**–**5**: Antagonist affinities, expressed as pA_2, at α_{1A}-, α_{1B}-, α_{1D}-and α_2-AR on isolated rat tissues and inverse agonism expressed as magnitude of inhibition of calcium-induced increase in the resting tension (IRT) of calcium depleted guinea pig thoracic aorta.

Benzodioxane substituent	Compd	pA_2[a]					Affinity ratios[b]			Inhibition of Ca^{++} induced IRT (%)[c]
		α_{1A}		α_{1B}	α_{1D}	α_2	α_{1A}/α_{1B}	α_{1A}/α_{1D}	α_{1D}/α_{1B}	
		Prostate	Prostatic vas deferens	Spleen	Thoracic aorta	Prostatic vas deferens				
–	(*S*)-WB4101	9.49 (±0.05)	9.98 (±0.01)	9.17 (±0.01)	9.20 (±0.06)	6.92 (±0.02)	6.5	6.0	1.1	84
8-OH	(*S*)-**1**	7.30 (±0.01)	6.78 (±0.07)	7.80 (±0.02)	7.29 (±0.09)	<5	0.10	0.31	0.31	100
8-OMe	(*S*)-**2**	8.55 (±0.02)	8.77 (±0.03)	9.58 (±0.04)	7.93 (±0.03)	6.23 (±0.01)	0.15	6.9	0.02	60
8-Cl	(*S*)-**3**	9.04 (±0.05)	9.56 (±0.01)	8.05 (±0.04)	8.52 (±0.02)	6.02 (±0.01)	32.4	11.0	3.0	80
8-F	(*S*)-**4**	8.92 (±0.03)	7.54 (±0.03)	8.56 (±0.02)	8.03 (±0.03)	6.26 (±0.02)	0.10	0.32	0.30	100
5-OMe	(*S*)-**5**	7.40 (±0.02)	7.27 (±0.02)	7.78 (±0.04)	8.24 (±0.02)	5.67 (±0.01)	0.31	0.11	2.9	79

[a] pA_2 values ± SE were calculated from Schild plots [38], constrained to a slope of −1.0, unless otherwise specified [39]. pA_2 is the positive value of the intercept of line derived by plotting log(DR-1) vs log[antagonist]. The log(DR-1) was calculated at least at three different antagonist concentrations, and each was tested from three to five times. Dose-ratio (DR) values represent the ratio of the potency of the agonist (EC_{50}) in the presence of the antagonist and in its absence. Parallelism of dose-response curves was checked by linear regression, and the slopes were tested for significance ($p < 0.05$).

[b] Antilog of ΔpA_2.

[c] Data are the percent decreases of the Ca^{2+} (1.8 mM)-induced IRT in the presence of the antagonist at 10 nM concentration, calculated assuming as a reference IRT the Ca^{2+} (1.8 mM)-induced IRT in the absence of any agent. Data represent the mean ± SE from 4 to 7 experiments.

modest ones of (*S*)-**1**, whose 8-OH can give rise to hydrogen bond with O(1).

Matching of binding with functional tests indicates that only the overall lower potencies of (*S*)-**1** and (*S*)-**5**, compared to (*S*)-**2**-(*S*)-**4**, were correctly predicted by the binding assays. Indeed, (*S*)-**1** and (*S*)-**5** are both less affinitive and less potent than (*S*)-**2**, (*S*)-**3** and (*S*)-**4**. Apart from such a parallelism, the α_{1A}-, α_{1B}-and α_{1D}-antagonist affinities of compounds (*S*)-**1**–(*S*)-**5** significantly diverge, for the most part, from the binding affinities at the corresponding cloned AR subtypes being almost always lower at α_{1A}-and α_{1D}-AR and higher at α_{1B}-AR. Such a trend is particularly evident for the 8-OMe analogue (*S*)-**2**, whose subnanomolar α_{1B} antagonist affinity is one order of magnitude greater than the α_{1b} binding affinity, while its α_{1A} and α_{1D} antagonist affinities are significantly lower than the α_{1a} and α_{1d} binding affinities.

As previously for WB4101 and some of its derivatives [16], the discrepancy between binding and functional data prompted us to verify if these antagonists behave as inverse agonists thus showing affinity values not system-independent, but different according to the relative proportion of receptor in the resting state and receptor in the active state typical of the system employed for the determination. Inhibition of calcium induced increase in the resting tension (IRT) of calcium depleted guinea pig thoracic aorta was assumed to indicate inverse agonist behaviour [36], in particular at the α_{1A}-AR since the α_1-AR subtype involved in the contraction of guinea pig thoracic aorta is pharmacologically similar to α_{1A}-AR of rat vas deferens. In Table 2, the inhibition of the IRT produced by 1.8 mM Ca^{++} administered after incubation with 10 nM antagonist is expressed in comparison to the IRT produced by the same concentration of Ca^{++} in the absence of the antagonist. For instance, (*S*)-**3** shows a 80% inhibition, which means that 1.8 mM Ca^{++} induced IRT is, after incubation with 10 nM (*S*)-**3**, the 20% of the 1.8 mM Ca^{++} induced IRT in the absence of the antagonist. In the case of (*S*)-**1** and (*S*)-**4** (100% inhibition), 1.8 mM Ca^{++} doesn't induce any IRT after incubation with the antagonist at 10 nM. This semi-quantitative test indicates that all the five compounds would be α_{1A}-AR inverse agonists. Ranking their potencies is difficult, because, at the tested concentration (10 nM), four compounds, namely (*S*)-WB4101, (*S*)-**2**, (*S*)-**3** and (*S*)-**5**, exert percentual IRT inhibitions, which are similar and lower than 100%, while (*S*)-**1** and (*S*)-**4** give the maximum response (100%). This notwithstanding, it is worthy of note that these two maximum 100% inhibitions are associated with the two largest negative divergences of α_{1A} pA_2 (vas deferens) from α_{1a} pK_i (−1.88 and −1.65 for (*S*)-**1** and (*S*)-**4** respectively), while the α_{1A} pA_2–α_{1a} pK_i differences are more modest and either negative or positive for the other compounds not producing the maximum inhibition under the adopted experimental conditions.

4. Conclusion

The present investigation focused on substitution of the hydrogen at the 8-position of 1,4-benzodioxane of WB4101, a quite neglected modification of this lead compound of α_1-AR selective antagonists. Fluorine, chlorine, hydroxyl and methoxyl were selected as 8-substituents; methoxyl was introduced also at the 5 position. Binding assays at the three α_1-AR subtypes indicated that F, Cl and OMe at the 8 position don't substantially modify the affinity profile of (*S*)-WB4101, whereas OH at the same position and, more markedly, OMe at the 5 position are invariably deleterious. Interference with the role played by benzodioxane O(1) and steric hindrance might be respectively invoked to justify the negative effects of these two substitutions. The antagonist affinities at the three α_1-AR subtypes, which confirm the 8-hydroxy and the 5-methoxy analogues as the least potent derivatives in this set of compounds, diverge from the corresponding binding affinities and such a difference might be explained by inverse agonist nature, demonstrated and quantified at the α_{1A}-AR for the *S* enantiomers of all the five analogues. For the most part, the α_{1A} and α_{1D} antagonist affinities are lower than the α_{1a} and α_{1d} binding affinities, while the opposite behaviour was observed for the α_{1B} subtype. This trend results in a new potent and significantly α_{1B}-AR selective antagonist, the (*S*)-WB4101 analogue 8-methoxy substituted at the benzodioxane nucleus.

5. Experimental protocols

5.1. Chemistry

Melting points were measured on Buchi melting point apparatus and are uncorrected. ^1H NMR spectra were recorded operating at 300 MHz and ^{13}C NMR at 75 MHz. Chemical shifts are reported in ppm relative to residual solvent ($CHCl_3$ or DMSO) as internal standard. Signal multiplicity is designed according to the following abbreviations: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, br s = broad singlet, br t = broad triplet. Coupling constants are reported in Hertz (Hz). Optical rotations were determined by a Perkin–Elmer 241 Polarimeter at 25 °C. Elemental analyses (C, H, N, S, Cl, I) of the new substances are within 0.40% of theoretical values. Purifications were performed by flash chromatography using silica gel (particle size 40–63 μm, Merck).

5.1.1. (R)-2-Iodomethyl-8-hydroxy-1,4-benzodioxane

A mixture of (*R*)-2-tosyloxymethyl-8-hydroxybenzodioxane (4.0 g, 11.9 mmol) and NaI (5.35 g, 35.7 mmol) in acetone (100 mL) was refluxed for 3 h. The solvent was evaporated and the residue treated with 10% aqueous HCl (100 mL) and ethyl acetate. The organic phase was separated, washed with a saturated aqueous solution of $Na_2S_2O_5$, dried and concentrated. The residue was purified by chromatography on silica gel (eluent: cyclohexane/ethyl acetate 80:20) yielding 3 g (86%) of (*R*)-2-iodomethyl-8-hydroxy-1,4-benzodioxane as a white solid: m.p. 82 °C; $[\alpha]_D^{25} = -13.3$ (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.71–6.80 (t, 1H), 6.59 (d, 1H), 6.46 (d, 1H), 5.44 (s, 1H, exchangeable with D_2O), 4.29–4.41 (m, 2H), 4.11–4.19 (m, 1H), 3.36 (d, 2H). Anal. Calcd for $C_9H_9IO_3$ (292.07).

*5.1.2. (S)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-hydroxy-1,4-benzodioxane hydrochloride [(S)-**1**]*

A solution of (*R*)-2-iodomethyl-8-hydroxy-1,4-benzodioxane (1.3 g, 4.45 mmol) and 2-(2,6-dimethoxyphenoxy)ethylamine (1.71 g, 8.9 mmol) in 2-propanol (5 mL) was refluxed for 20 h. After cooling, dichloromethane (10 mL) and 1 M aqueous NaOH (10 mL) were added. The organic phase was separated, washed with water, dried and concentrated. The residue was purified by chromatography on silica gel (eluent $CH_2Cl_2/CH_3OH/Et_3N$ 95:5:1) yielding 700 mg (44%) of (*S*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino) methyl]-8-hydroxy-1,4-benzodioxane as a solid: m.p. 122–124 °C; $[\alpha]_D^{25} = -43.3$ (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.96–7.05 (t, 1H), 6.67–6.75 (t, 1H), 6.41–6.61 (m, 4H), 3.89–4.40 (m, 5H), 3.84 (s, 6H), 2.94–2.97 (m, 4H). The free amine (500 mg, 1.39 mmol) was dissolved in ethanol (6 mL) and added with 2 M ethanolic HCl (1.5 mL). Recovery of the precipitated solid by filtration yielded 300 mg of (*S*)-**1** as a white solid: m.p. 211–212 °C; $[\alpha]_D^{25} = -53.9$ (*c* 1, CH_3OH); ^{1}H NMR ($CDCl_3$) δ 9.40 (br s, 2H, exchangeable with D_2O), 9.06 (s, 1H, exchangeable with D_2O), 7.06–7.14 (t, 1H), 6.39–6.77 (m, 5H), 4.65 (m, 1H), 4.45 (dd, 1H), 4.22 (m, 2H), 4.05 (dd, 4H), 3.82 (s, 6H), 3.39 (m, 4H).^{13}C NMR (d_6-DMSO) δ 47.3, 47.4, 56.6, 65.6, 68.4, 69.6, 106.1, 108.1, 109.4, 121.5, 125.2, 131.3, 136.1, 144.1, 147.2, 153.7. Anal. Calcd for $C_{19}H_{24}ClNO_6$ (397.86).

5.1.3. (S)-2-Iodomethyl-8-hydroxy-1,4-benzodioxane

Prepared in 87.3% yield from (*S*)-2-tosyloxymethyl-8-hydroxybenzodioxane as described for the *R* enantiomer: $[\alpha]_D^{25} = +2.7$ (*c* 1, $CHCl_3$); m.p. and ^{1}H NMR identical to (*R*)-2-iodomethyl-8-hydroxy-1,4-benzodioxane. Anal. Calcd for $C_9H_9IO_3$ (292.07).

*5.1.4. (R)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-hydroxy-1,4-benzodioxane hydrochloride [(R)-**1**]*

Prepared from (*S*)-2-iodomethyl-8-hydroxy-1,4-benzodioxane as described for the *S* enantiomer. (*R*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-hydroxy-1,4-benzodioxane: $[\alpha]_D^{25} = +39.6$ (*c* 1, $CHCl_3$); m.p. and ^{1}H NMR identical to (*S*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-hydroxy-1,4-benzodioxane. (*R*)-**1**: m.p. 210–211 °C; $[\alpha]_D^{25} = +52.9$ (*c* 1, CH_3OH); ^{1}H NMR and ^{13}C NMR identical to (*S*)-**1**. Anal. Calcd for $C_{19}H_{24}ClNO_6$ (397.86).

*5.1.5. 2-Chlorophenyl acetate (**6**)*

Acetyl chloride (66.6 mL, 933 mmol) was added drop-wise to a solution of 2-chlorophenol (120 g, 933 mmol) and pyridine (85.3 mL) in dichloromethane (600 mL) at 25 °C. The mixture was stirred for 4 h at room temperature and then washed with water (480 mL). The organic phase was washed with 10% HCl (180 mL) and then with water (120 mL), dried and concentrated to give 159 g (quantitative yield) of the desired product as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.44 (dd, 1H), 7.29 (dd, 1H), 7.19 (dt, 1H), 7.13 (dt, 1H), 2.36 (s, 3H).

*5.1.6. 2-Hydroxy-3-chloroacetophenone (**7**)*

At room temperature, **6** (152.5 g, 893.7 mmol) was added dropwise to a solution of aluminium chloride (119.0 g, 893.7 mmol) in dichlorobenzene (120 mL). The reaction mixture was stirred at 100 °C for 5 h. After cooling to room temperature, dichloromethane was added and the resulting mixture was poured into H_2SO_4 2 N at 0 °C. Precipitated 3-chloro-4-hydroxyacetophenone was removed by filtering the mixture. The aqueous layer was separated and extracted with dichloromethane. The organic phases were combined, rinsed with water, dried and concentrated. The residue was crystallized from cyclohexane, yielding additional undesired 3-chloro-4-hydroxyacetophenone (23.6 g) as a yellowish solid and, by concentration of the crystallization mother liquor, 47.3 g (31.0%) of the desired product **7** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 12.84 (br s, 1H, exchangeable with D_2O), 7.67 (dd, 1H), 7.57 (dd, 1H), 6.87 (dd, 1H), 2.66 (s, 3H). 3-Chloro-4-hydroxyacetophenone: m.p. 109 °C; ^{1}H NMR ($CDCl_3$) δ 7.98 (d, 1H), 7.81 (m, 1H), 7.08 (m, 1H), 6.30 (br s, 1H, exchangeable with D_2O), 2.56 (s, 3H).

*5.1.7. 2-Benzyloxy-3-chloroacetophenone (**8**)*

Tetrabutylammonium bromide (8.94 g, 27.7 mmol) and 10% aqueous NaOH (222 mL) were added to a solution of **7** (47.3 g, 277.3 mmol) in dichloromethane (500 mL). Benzyl bromide (36.5 mL, 305.1 mmol) was added dropwise to the mixture while vigorously stirring. After 5 h at room temperature, the organic phase was separated, treated with 10% HCl (450 mL), separated again, washed with water, dried and concentrated. Column chromatography on silica gel (eluent: cyclohexane/ethyl acetate 95:5) of the resulting residue allowed 48.5 g (67.1%) of **8** to be isolated as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.56 (dd, 1H), 7.48 (m, 3H), 7.38 (m, 3H), 7.15 (t, 1H), 5.02 (s, 2H), 2.55 (s, 3H).

*5.1.8. 2-Benzyloxy-3-chlorophenyl acetate (**9**)*

3-Chloroperbenzoic acid (55%) (64.24 g, 372.3 mmol) was added in small portions to a solution of **8** (48.53 g, 186.1 mmol) in dichloromethane at 0 °C. The reaction mixture was stirred for 7 days at room temperature and then poured into a saturated $NaHCO_3$ solution. The aqueous layer was separated and extracted with dichloromethane. The organic phases were combined, washed with water, dried and concentrated to give 51.50 g (100%) of **9** as a dark oil: ^{1}H NMR ($CDCl_3$) δ 7.40 (m, 5H), 7.29 (dd, 1H), 7.07 (t, 1H), 7.00 (dd, 1H), 5.03 (s, 2H), 2.17 (s, 3H).

*5.1.9. 2-Benzyloxy-3-chlorophenol (**10**)*

A solution of **9** (51.5 g, 186 mmol) in methanol (520 mL) was added to 2.5 N aqueous NaOH 2.5 N (83 mL). After stirring for 2 h, the reaction mixture was concentrated under vacuum. The residue was dissolved in dichloromethane and water. The aqueous layer was separated, acidified with HCl (pH 1) and extracted with dichloromethane. The organic phases were combined, dried and concentrated to give 35.7 g (81.7%) of **10** as a dark oil: ^{1}H NMR ($CDCl_3$) δ 7.41 (m, 5H), 6.97 (m, 2H), 6.85 (m, 1H), 5.62 (br s, 1H, exchangeable with D_2O), 5.10 (s, 2H).

*5.1.10. (S)-3-(2-Benzyloxy-3-chlorophenoxy)-1,2-propanediol acetonide [(S)-**11**]*

(*R*)-1-Mesiloxy-2,3-propanediol acetonide (5.91 g, 28.1 mmol) in DMF was added dropwise to a solution of **10** (6.6 g, 28.1 mmol) and potassium *tert*-butoxide (3.31 g, 29.5 mmol) in DMF (100 mL). After 4 h the solvent was removed under vacuum; the residue was dissolved in dichloromethane and treated with 2.5 N aqueous NaOH. The aqueous layer was separated and extracted with dichloromethane.

The organic phases were combined, washed with HCl 10% and then with water, dried and concentrated to give 7.64 g (77.8%) of (*S*)-**11** as a dark oil: $[\alpha]_D^{25} = +9.1$ (*c* 0.5, EtOH); ^{1}H NMR ($CDCl_3$) δ 7.58 (m, 2H), 7.37 (m, 3H), 7.00 (m, 2H), 6.85 (m, 1H), 5.05 (s, 2H), 4.50 (m, 1H), 4.15 (m, 2H), 4.10 (m, 1H), 3.93 (m, 1H), 1.43 (s, 3H),1.38 (s, 3H).

5.1.11. (*R*)-3-(2-Benzyloxy-3-chlorophenoxy)-1,2-propanediol [(*R*)-**12**]

1 N HCl (40 mL) was added to a solution of (*S*)-**11** (7.64 g, 21.9 mmol) in ethanol (20 mL) and the mixture was heated at 75 °C for 4 h. After cooling, ethanol was removed under vacuum and the residual aqueous phase was extracted with dichloromethane. The organic extract was washed with water, dried and concentrated to give an oily residue which was chromatographed on silica gel. Elution with dichloromethane/methanol (95:5) yielded 2.66 g (39.2%) of (*R*)-**12** as a light brown solid: m.p. 86–87 °C; $[\alpha]_D^{25} = -5.2$ (*c* 0.5, EtOH); ^{1}H NMR (DMSO-d_6) δ 7.50 (m, 2H), 7.38 (m, 3H), 7.01 (m, 3H), 5.01 (m, 2H + 1H exchangeable with D_2O), 4.71 (t, 1H, exchangeable with D_2O), 4.07 (m, 1H), 3.96 (m, 1H), 3.86 (m, 1H), 3.48 (m, 2H). Anal. Calcd for $C_{16}H_{17}ClO_4$ (308.76).

5.1.12. (*S*)-3-(2-Benzyloxy-3-chlorophenoxy)-1,2-dimesyloxypropane [(*S*)-**13**]

Mesyl chloride (1.33 mL, 17.2 mmol) was added dropwise to a solution of (*R*)-**12** (2.66 g, 8.6 mmol) and triethylamine (2.4 mL, 17.2 mmol) in dichloromethane (50 mL) at 0–5 °C. The mixture was stirred for 2 h and then diluted with dichloromethane (50 mL) and treated with a saturated aqueous solution of $NaHCO_3$ (30 mL). The organic phase was separated, washed with water, dried and concentrated under vacuum to give 4.6 g of crude product, which was triturated in diisopropyl ether (25 mL) yielding 2.23 g (55.6%) of (*S*)-**13** as a light brown solid: m.p. 85 °C; $[\alpha]_D^{25} = -6.2$ (*c* 1, $CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 7.50 (m, 2H), 7.35 (m, 3H), 7.08 (m, 3H), 5.27 (m, 1H), 5.00 (dd, 2H), 4.60 (m, 1H), 4.50 (m, 1H), 4.37 (m, 2H), 3.28 (s, 6H). Anal. Calcd for $C_{18}H_{21}ClO_8S_2$ (464.93).

5.1.13. (*S*)-3-(2-Hydroxy-3-chlorophenoxy)-1,2-dimesyloxypropane [(*S*)-**14**]

A solution of (*S*)-**13** (2.23 g, 4.8 mmol) in methanol (56 mL) was added with 10% Pd/C (0.2 g) and vigorously shaken under hydrogen at room temperature until hydrogen uptake ceased. The catalyst was removed by filtration and the filtrate concentrated to give 1.77 g of crude product. Purification by chromatography on silica gel (eluent: cyclohexane/ethyl acetate 1:1) yielded 1.16 g (64.4%) of (*S*)-**14** as an orange oil: $[\alpha]_D^{25} = -12.2$ (*c* 1, $CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 9.25 (br s, 1H, exchangeable with D_2O), 6.98 (m, 2H), 6.78 (m, 1H), 5.20 (m, 1H), 4.75 (m, 1H), 4.60 (m, 1H), 4.25 (m, 2H), 3.30 (s, 3H), 2.25 (s, 3H).

5.1.14. (*R*)-2-Mesyloxymethyl-8-chloro-1,4-benzodioxane [(*R*)-**15**]

Potassium carbonate (0.43 g) was added to a solution of (*S*)-**14** (1.16 g, 3.1 mmol) in acetone (12 mL) and the mixture was refluxed for 24 h. After evaporating the solvent, the resultant residue was treated with ethyl acetate (80 mL) and aqueous 10% HCl (70 mL). The organic phase was separated, washed with water, dried and concentrated to give 0.99 g of crude product, which was crystallized from diisopropyl ether yielding 0.44 g (50.9%) of (*R*)-**15** as a white solid: m.p. 76–77 °C; $[\alpha]_D^{25} = +7.5$ (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.90 (m, 1H), 6.75 (m, 3H), 4.50 (m, 1H), 4.48 (m, 3H), 4.26 (dd, 1H), 4.07 (dd, 1H), 3.08 (s, 3H). Anal. Calcd for $C_{10}H_{11}ClO_5S$ (278.71).

5.1.15. (*S*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-chloro-1,4-benzodioxane hydrochloride [(*S*)-**3**]

A solution of (*R*)-**15** (850 mg, 3.05 mmol) and 2-(2,6-dimethoxyphenoxy)ethylamine (1.2 g, 6.1 mmol) in isobutanol (5 mL) was refluxed for 24 h. The solvent was evaporated and the residue treated with 10% aqueous NaOH (30 mL) and dichloromethane. The organic phase was separated, washed with water, dried and concentrated. Purification of the residue by chromatography on silica gel (eluent: cyclohexane/ethyl acetate 1:1) gave 690 mg of (*S*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino) methyl]-8-chloro-1,4-benzodioxane as an oil: $[\alpha]_D^{25} = -21.0$ (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.92 (t, 1H), 6.85 (m, 1H), 6.70 (m, 2H), 6.49 (d, 2H), 4.27 (m, 2H), 4.04 (m, 3H), 3.77 (s, 6H), 3.01 (dd, 1H), 2.90 (m, 3H), 2.01 (br s, 1H). The free amine was dissolved in ethanol (4 mL) and 2.6 m ethanolic HCl (1.1 mL) was added while stirring. After 10 min, ethanol was evaporated and ethyl acetate (10 mL) was added to the residue. Ethanol (2 mL) was added to the mixture while refluxing. After cooling to 0 °C, the suspension was filtered to give 293 mg (24.4%) of (*S*)-**3** as a white solid: m.p. 161–162 °C; $[\alpha]_D^{25} = -41.7$ (*c* 1, CH_3OH); ^{1}H NMR (DMSO-d_6) δ 9.63 (br s, 1H), 9.36 (br s, 1 H), 7.05 (m, 2H), 6.88 (m, 2H), 6.68 (m, 2H), 4.85 (m, 1H), 4.45 (br d, 1H), 4.16 (m, 3H), 3.76 (s, 6H), 3.28–3.58 (m, 4H). ^{13}C NMR (DMSO-d_6) δ 46.3, 47.4, 56.1, 56.2, 65.2, 67.9, 70.7, 105.6, 116.4, 121.1, 121.9, 122.5, 124.8, 136.6, 138.7, 144.2, 153.2. Anal. Calcd for $C_{19}H_{23}Cl_2NO_5$ (416.30).

5.1.16. (*R*)-3-(2-Benzyloxy-3-chlorophenoxy)-1,2-propanediol acetonide [(*R*)-**11**]

Prepared in quantitative yield from (*S*)-1-mesyloxy-2,3-propanediol acetonide as described for the *S* enantiomer: $[\alpha]_D^{25} = -9.0$ (*c* 0.5, EtOH); ^{1}H NMR identical to (*S*)-**11**.

5.1.17. (*S*)-3-(2-benzyloxy-3-chlorophenoxy)-1,2-propanediol [(*S*)-**12**]

Prepared in 28.0% yield from (*R*)-**11** as described for (*R*)-**12**: $[\alpha]_D^{25} = +5.7$ (*c* 0.5, EtOH); m.p. and ^{1}H NMR identical to (*R*)-**12**. Anal. Calcd for $C_{16}H_{17}ClO_4$ (308.76).

5.1.18. (*R*)-3-(2-Benzyloxy-3-chlorophenoxy)-1,2-dimesyloxypropane [(*R*)-**13**]

Prepared in 51.4% yield from (*S*)-**12** as described for (*S*)-**13**: $[\alpha]_D^{25} = +7.1$ (*c* 1, $CHCl_3$); m.p. and ^{1}H NMR identical to (*S*)-**13**. Anal. Calcd for $C_{18}H_{21}ClO_8S_2$ (464.93).

5.1.19. (*R*)-3-(2-Hydroxy-3-chlorophenoxy)-1,2-dimesyloxypropane [(*R*)-**14**]

Prepared in 45.3% yield from (*R*)-**13** as described for (*S*)-**14**: $[\alpha]_D^{25} = +11.8$ (*c* 1, $CHCl_3$); ^{1}H NMR identical to (*S*)-**14**.

5.1.20. (*S*)-2-Mesyloxymethyl-8-chloro-1,4-benzodioxane [(*S*)-**15**]

Prepared in 53.3% yield from (*R*)-**14** as described for (*R*)-**15**: $[\alpha]_D^{25} = -6.4$ (*c* 1, $CHCl_3$); m.p. and ^{1}H NMR identical to (*R*)-**15**. Anal. Calcd for $C_{10}H_{11}ClO_5S$ (278.71).

5.1.21. (*R*)-2-[((2-(2,6-Dimethoxyphenoxy)ethyl)amino)methyl]-8-chloro-1,4-benzodioxane hydrochloride [(*R*)-**3**]

Prepared from (*S*)-**15** as described for (*S*)-**3**: $[\alpha]_D^{25} = +43.9$ (*c* 1, CH_3OH); m.p. and ^{1}H NMR and ^{13}C NMR identical to (*S*)-**3**.Anal. Calcd for $C_{19}H_{23}Cl_2NO_5$ (416.30).

5.2. Biology

5.2.1. Binding assays

The pharmacological profile of both the *S* and *R* enantiomers of compounds **1**–**5** was assessed by measuring their affinities for α_{1a}, α_{1b}, α_{1d} AR-subtypes and 5-HT_{1A} serotoninergic receptor with in vitro binding studies.

Briefly, membranes derived from Chinese Hamster Ovary (CHO) cells expressing α_1-AR subtypes [37] were resuspended in Tris HCl,

50 mM, pH = 7.7 containing 10 μM pargyline and 0.1% ascorbic acid, and incubated for 30 min at 25 °C with 0.5 nM [^{3}H]-Prazosin (NEN, 80.5 Ci/mmol) in the absence or presence of different concentrations of the tested compounds (from 0.3 to 1000 nM depending on the affinity). Prazosin 1 μM was used to determine non-specific binding.

Binding studies at 5-HT$_{1A}$ receptor were carried out using crude membrane preparations from rat hippocampus, which were resuspended in Tris HCl 50 mM (pH = 7.7, 10 μM pargyline and 4 mM $CaCl_2$) and incubated for 30 min at 25 °C with 1 nM [^{3}H]-8-OH-DPAT, in the absence or presence of different concentrations of the tested compounds. 5-HT 1 μM was used to determine non-specific binding.

Incubations were stopped by rapid filtration, through GF/B fibre filters, which were then washed, dried and counted in an LK1214 rack β Liquid scintillation Spectrometer.

At least three different experiments, in triplicate, were carried out for each compound and usually each compound was tested simultaneously on the different α_1-AR subtypes. Prazosin or 5-HT was always tested in parallel, as reference drugs. The % inhibitory effects obtained in the different experiments were pooled together and the inhibition curves were analyzed using the "one-site competition" equation built into GraphPad Prism 4.0 (GraphPAD Software, San Diego, CA). This analysis gives the IC50 (i.e. the drug concentration inhibiting specific binding by 50%), calculated with the relative standard error. K_i values were then calculated by IC50 using the Cheng and Prusoff equation in which the K_d of [^{3}H]-Prazosin for α_{1a}, α_{1b}, α_{1d} AR-subtypes were 0.4, 0.4 and 0.7 nM, respectively, whereas the K_d of [^{3}H]-8-OH-DPAT for 5-HT$_{1A}$ receptors was 1.2 nM.

5.2.2. Functional antagonism in isolated rat tissues

Male Sprague-Dawley rats (Charles River, Italy) were killed by cervical dislocation under ketamine anaesthesia and the organ required were isolated, freed from adhering connective tissue, and set up rapidly under resting tension in organ bath (15 mL) containing physiological salt solution kept at appropriate concentration (see below) and gassed with 95%O_2 and 5%CO_2 at pH 7.4. Concentration-response curves were constructed by cumulative addition of agonist. The concentration of agonist in the organ bath was increase approximately 5-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady.

All experimental data were recorded by means of isometrically or isotonically FT.03 Grass force transducers using Power Lab® software (AD-Instruments Pty Ltd, Castle Hill, Australia). In addition parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

5.2.2.1. Prostate. This tissue (from rats of 200–250 g) was used to assess α_{1A}-adrenoceptor antagonist activity [35]. Prostatic strips measuring 8–10 mm in length and 1–2 mm in width were placed under a resting tension of 2 g in modified Krebs solution of the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$ 1.18; $NaHCO_3$, 25.0; KH_2PO_4, 1.18; glucose, 5.5. The preparations were equilibrated for 60 min; during this time the bathing solution was changed every 20 min. Before the concentration-curves were started, tissues were exposed to (−)-noradrenaline at a concentration of 1.0 μM. A minimum response of 0.5 g of tension was required for the tissue to be used for concentration-response curves. After a 90 min time period, a cumulative response-curve to (−)-noradrenaline was constructed. After completion of the concentration-response curve, the tissue was washed for 90 min, and the antagonist was added and incubated for 30 min before a second cumulative concentration-response curve was obtained.

5.2.2.2. Vas deferens prostatic portion. This tissue (from rats of 200–250 g) was used to assess α_{1A}-adrenoceptor antagonist activity [33]. Prostatic portions of 2 cm length were set up in Tyrode solution of the following composition (mM): NaCl, 130.0; KCl, 2.0; $CaCl_2 \cdot 2H_2O$, 1.8; $MgCl_2$ 0.89; $NaHCO_3$, 25.0; $NaH_2PO_4 \cdot 2H_2O$, 0.42; glucose, 5.6; desipramine hydrochloride (0.01 μM) was added to prevent the neuronal uptake of (−)-noradrenaline. The medium was maintained at 37 °C The preparations were equilibrated for 1 h under a resting tension of 0.35 g. The preparations were equilibrated for 45–60 min and during this time the bathing solution was changed every 10 min. Contraction-response curves for isotonic contractions in response to (−)-noradrenaline were recorded at 30 min intervals; the first one being discarded and the second one taken as control. After the incubation with antagonist concentration for 30 min, a third dose-response curve was obtained.

α_2-Adrenoreceptor antagonist activity was determined also on prostatic portions of 1.5–2 cm length which were set up in organ bath containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.52; $MgSO_4$, 0.6; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.1. Propranolol hydrochloride (1 μM) and desipramine hydrochloride (0.01 μM) were present in the above described Krebs solutions throughout the experiments to block β-adrenoreceptors and to prevent the neuronal uptake of (−)-noradrenaline respectively. The physiological salt solution was kept at 37 °C. Field stimulation of the tissues was carried out by means of two platinum electrodes, connected to a Grass S88 stimulator, placed near the top and bottom of the vas deferens at 0.1 Hz, using square pulses of 3 ms duration at voltage of 20–40 V. A 1 h equilibration period under a resting tension of 0.35 g was allowed. A first clonidine concentration-response curve, taken as control, was obtained cumulatively. The antagonist concentration was allowed to equilibrate with the tissue for 30 min before obtaining a second dose-response curve.

5.2.2.3. Spleen. This tissue (from rats of 250–300 g) was used to assess α_{1B}-adrenoceptor antagonist activity [34]. The spleens were bisected transversally into two strips and were suspended in organ baths maintained at 37 °C and containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 1.9; $MgSO_4$ 1.2; $NaHCO_3$, 25.0; $NaH_2PO_4 \cdot 2H_2O$, 1.2; glucose, 11.7; desipramine hydrochloride (0.01 μM) and (±)-propranolol hydrochloride (1 μM) were added to prevent the neuronal uptake of (−)-phenylephrine and to block β-adrenoreceptors, respectively. The spleen strips were placed under 1 g of resting tension and equilibrated for 1 h. The cumulative concentration-response curves to phenylephrine were measured isometrically and obtained at 30 min intervals, the first one being discarded and the second one taken as control. The antagonist was allowed to equilibrate with the tissue for 30 min, and then a new concentration-response curve to the agonist was constructed.

5.2.2.4. Thoracic aorta. This tissue (from rats of 250–300 g) was used to assess α_{1D}-adrenoceptor antagonist activity [34]. The thoracic portion of aorta was cleaned from extraneous connective tissue and placed in organ bath containing Krebs solution maintained at 37 °C of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 1.9; $MgSO_4$ 1.2; $NaHCO_3$, 25.0; NaH_2PO_4, 1.2; glucose, 11.7; desipramine hydrochloride (0.01 μM) and (±)-propranolol hydrochloride (1 μM) were added to prevent the neuronal uptake of (−)-noradrenaline and to block β-adrenoreceptors, respectively. Two helicoids strips were cut in strips from each aorta of about 1.5 cm length. The endothelium was removed by rubbing with filter paper: the functional loss of endothelial cells was confirmed by the absence of the relaxing response to acetylcholine. After at least 1 h equilibration period under an optimal tension of 1 g, cumulative

(−)-noradrenaline dose-response curves were recorded, the first two being discarded and the third one taken as a control. The antagonist was allowed to equilibrate with the tissue for 30 min before the generation of a fourth cumulative dose-response curve with (−)-noradrenaline.

5.2.3. Inverse agonism

The guinea pig thoracic aorta was used to assess the activity of α_1-antagonist as inverse agonist [16,36]. Aortic strips were isolated and cleaned as previously described and placed in organ bath containing the Krebs solution maintained at 37 °C of the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 1.8; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; glucose, 11. Tissue were equilibrated for 1 h under an optimal tension of 1 g, and the effect of a single dose of (−)-noradrenaline (1 μM) was recorded. During 1 h of wash in Ca^{2+}-free Krebs solution containing EDTA (0.1 mM) the agonist was applied and washed with Ca^{2+}-free solution until no contraction was elicited, indicating depletion of internal Ca^{2+} stores sensitive to NA. After incubation with the antagonist for 30 min, addition of Ca^{2+} (1.8 mM) induced increase in the resting tension (IRT). The magnitude of the inhibition was expressed as a percent decrease of the reference IRT, namely of the IRT induced by calcium (1.8 mM) in the absence of any agent.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.09.049.

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