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Short communication

Neutral/negative α_1-AR antagonists and calcium channel blockers at comparison in functional tests on guinea-pig smooth muscle and myocardium

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ABSTRACT

Background: Constitutive (agonist-independent) activity is a prerogative of many G protein-coupled receptors (GPCRs) including α_1-adrenoceptors (α_1-ARs). Inhibition of such an activity at α_1-AR subtypes by antagonists with negative efficacy is difficult to be adequately tested.

Methods: In the present experimental approach, we compared the activity of three calcium channel blockers (nifedipine, diltiazem and verapamil) and of three potent benzodioxane-based α_1-AR antagonists, differing for subtype selectivity and inverse agonist properties, in producing smooth muscle relaxation and negative inotropy under the same test conditions. We selected, as benzodioxane derivatives, (*S*)-WB4101, inverse agonist with slight α_{1A}/α_{1B}-α_{1D} AR selectivity, and two previously developed analogues. Both of these are potent antagonists at α_{1D}-AR, that is the α_1- AR subtype suspected of the highest susceptibility to inverse agonists for its high degree of basal activity, but only one is inverse agonist.

Results: We found that all the three benzodioxane-related α_1-AR antagonists have significant intrinsic relaxant activity on non-vascular smooth muscle and moderate negative inotropic effect, while they do not relax aorta. Their potency is always lower than that of three calcium channel blockers.

Conclusions: Intrinsic myorelaxant and negative inotropic activity of the three benzodioxane-based α_1-AR antagonist is related neither to a particular profile of α_1-AR subtype selectivity nor to whether or not being an inverse agonist, but it parallels the calcium antagonists effects indicating a direct interaction of the three α_1-AR antagonists with L-type Ca^{2+} channels.

Introduction

The α_1-adrenoceptors subtypes (α_{1A}-, α_{1B}- and α_{1D}-AR) play a key role in modulating many physiological functions. Great advances in understanding their functions have come from the availability of subtype-selective antagonists. In particular, many efforts have been made to modulate the subtype-selectivity of (*S*)-WB4101 [1], the prototype of α_1-AR antagonists based on the widely employed benzodioxane scaffold [2–6] and one of the most potent and α_1-selective antagonists [7] (Fig. 1), by modifications at its 1,4-benzodioxane and/or 2,6-dimethoxyphenyl moiety. The 8-methoxy substitution at 1,4-benzodioxane results in a potent and selective α_{1B}-AR antagonist [8], while replacement of 2,6-dimethoxyphenyl by 2-methoxy-1-naphthyl impressively exalts α_{1D} antagonism (Fig. 1: compound **1**) and it is optimized, in terms of α_{1D} selectivity, when 2-methoxy-1-naphthyl is in turn replaced by 2,3-dihydro-6-methoxy-7-benzofuranyl (Fig. 1: compound A175) [9].

(*S*)-WB4101 and a series of its structural analogues, including **1**, but not A175, behave as inverse agonists when tested on guinea pig thoracic aorta, where the α_{1A}-AR subtype predominates. In fact, they inhibit calcium induced increase in the resting tension (IRT) of this tissue depleted of calcium until irresponsive to noradrenaline [7–9]. Such behaviour, indicative of inverse agonism [10], is not apparently related to their subtype selectivity profiles and it incongruously coincides with both positive and negative differences between pK_i binding affinities and corresponding pA_2 antagonist affinities at α_1-AR subtypes.

Therefore, we were interested in more deeply studying the intrinsic activity of some of these benzodioxane-based, subtype-selective α_1-AR antagonists and in better understanding the role of calcium in their activity. We thus decided to accomplish further

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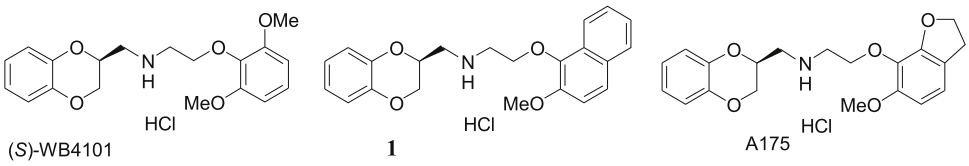

Fig. 1. Chemical structures of (*S*)-WB4101 and of its analogues **1** and A175.

functional tests on two tissues, smooth muscle and myocardium, which differ for α_1-ARs population and function [11,12], including, by comparison, calcium antagonists such as verapamil, diltiazem and nifedipine. In particular, we selected (*S*)-WB4101 and its analogues **1** and A175 because their different profiles of inverse agonism and α_{1A} and α_{1D} antagonism, qualitatively ranked for simplicity in Table 1, could result in differentiated and thus informative responses in intrinsic activity tests. Here, we report and discuss the results of our comparative investigation.

Materials and methods

Animals

All animals employed in this study were housed and treated according to the directives on the protection of animals used for scientific purposes (Directive 2010/63/EU of the European Parliament and of the Council) and the WMA Statement on Animal Use in Biomedical Research.

Chemicals

Nifedipine, verapamil and diltiazem were purchased from Sigma Aldrich. (*S*)-WB4101, A175 and compound **1** were prepared as previously reported [9,13].

Guinea-pig atrial preparations and treatments

Guinea-pigs (males and females, 200–400 g) obtained from Charles River (Calco, Como, Italy) were housed in a controlled environment with a 12:12-h light-dark cycle at 22 °C and provided with chow diet and water *ad libitum*.

Guinea-pigs were sacrificed by cervical dislocation. After thoracotomy the heart was immediately removed and washed by perfusion through the aorta with oxygenated Tyrode solution containing (mM): NaCl 136.9; KCl 5.4; $CaCl_2$ 2.5; $MgCl_2$ 1.0; $NaH_2PO_4xH_2O$ 0.4; $NaHCO_3$ 11.9; and glucose 5.5. Spontaneously beating left atria driven at 1 Hz were used. The entire left and right atria were dissected from the ventricles, cleaned of excess tissue, hung vertically in a 15 mL organ bath containing the physiological salt solution (PSS) continuously bubbled with 95% O_2 – 5% CO_2 at 35 °C, pH 7.4. The contractile activity was recorded isometrically by means of force transducer (FT 0.3, Grass Instruments Corporation, Quincy, MA, USA) using Power Lab® software (AD-Instruments Pty Ltd, Castle Hill, Australia). The left atria were stimulated by rectangular pulses of 0.6–0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 Stimulator). After several min, a length-tension curve was determined, and the muscle length was maintained at the value which elicited 90% of maximum contractile force observed at the optimal length. After a stabilization period of 45–60 min the atria were challenged by various agents. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to check (*S*)-WB4101, **1** and A175 at increasing doses on the inotropic and chronotropic activity as well as nifedipine, verapamil and diltiazem.

Table 1
Qualitative ranking of the three selected benzodioxane derivatives for α_{1A}- and α_{1D}-AR antagonist activity and for inverse agonism (IRT inhibition): +++: very potent; ++: potent; +: moderately potent; –: poorly or not active.

Compound	pA_2		Ca^{2+}-induced IRT inhibition
	α_{1A}	α_{1D}	
(*S*)-WB4101	++	+	+
1	+	+++	+
A175	–	++	–

Guinea-pig aortic and ileum strips preparation

The thoracic aorta (vascular smooth muscle) and ileum (non-vascular smooth muscle) were removed and placed in Tyrode solution containing (mM): NaCl, 118; KCl 4.75; $CaCl_2$ 2.54; $MgSO_4$ 1.20; KH_2PO_4 1.19; $NaHCO_3$ 25; and glucose 11; bubbled with 95% O_2-5% CO_2, pH 7.4. The smooth muscle strips were cleaned of extraneous connective tissue. Two helicoidal strips (10 mm x 1 mm) were cut from aorta beginning from the end proximal to the heart and ileum near the ileocecal valve. Smooth muscle strips were then tied with surgical thread (6-0) and suspended in 15 mL of aerated PSS at 35 °C in a jacketed tissue bath. Strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction and washed every 20 min with fresh PSS for 1 h. Strips were subjected to a resting force of 1 g. After the equilibration period, guinea-pig vascular and non-vascular strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K^+ for Na^+) When the contraction reached a plateau (about 45 min or 15 min respectively) (*S*)-WB4101, **1** and A175 were added to the bath in cumulative manner allowing for any relaxation to obtain an equilibrated level of force together with nifedipine, verapamil and diltiazem.

Statistical analysis

Data on atria, vascular and non-vascular strips were analyzed by the Student's *t*-test and presented as means ± SEM [14] in the appropriate pharmacological preparations; *p* value less than 0.05 has been considered significant.

Results

Myorelaxant activity on guinea pig aorta and ileum

Table 2 summarizes the previously reported antagonist affinities, expressed as pA_2, of (*S*)-WB4101, **1** and A175 at α_{1A}-, α_{1B}- and α_{1D}-AR on isolated rat tissues. In the same Table, the inverse agonism of the three benzodioxane derivatives is expressed as per cent inhibition of calcium-induced IRT of calcium-depleted guinea pig thoracic aorta.

Table 2
α_1-AR Subtypes antagonist affinity on isolated rat tissues and percent inhibition of calcium-induced increase in the resting tension of calcium-depleted guinea pig thoracic aorta [a].

Compound	pA_2			Ca^{2+}-induced IRT inhibition (%)
	α_{1A} prostatic vas deferens	α_{1B} spleen	α_{1D} thoracic aorta	
(*S*)-WB4101	9.98	9.17	9.20	84
1	8.96	9.69	10.68	90
A175	7.47	8.49	9.58	0

[a] pA_2 and percent inhibition values taken from references [7,9].

Table 3
Nifedipine, verapamil, diltiazem, (*S*)-WB4101, **1** and A175: relaxant activity on K^+-depolarized Guinea pig smooth muscle (aorta and ileum) and negative inotropy on Guinea pig left atrium.

Compound	Aorta			Ileum			Left atrium		
	Activity[a] (M ± SEM)	IC_{50}[c] (μM)	95% conf. lim. ($x10^{-6}$)	Activity[a] (M ± SEM)	IC_{50}[c] (μM)	95% conf. lim. ($x10^{-6}$)	Activity[b] (M ± SEM)	EC_{50}[c] (μM)	95% conf. lim. ($x10^{-6}$)
Nifedipine	82 ± 1.3[d]	0.009	0.003-0.02	70 ± 0.4[f]	0.0015	0.0011-0.0022	97 ± 2.0	0.26	0.19 – 0.36
Verapamil	95 ± 1.7[d]	0.38	0.20-0.70	98 ± 0.1[d]	0.014	0.010-0.019	84 ± 2.1	0.61	0.40-0.80
Diltiazem	88 ± 2.3[e]	2.6	2.2-3.1	98 ± 1.5[d]	0.11	0.085-0.13	78 ± 3.5	0.79	0.70-0.85
(*S*)-WB4101	3 ± 0.1			54 ± 1.5	8.29	6.91 – 9.22	45 ± 1.3		
1	13 ± 1.2			79 ± 1.4	2.81	2.20 – 3.57	48 ± 2.3		
A175	15 ± 1.1			61 ± 2.4	3.55	2.05 – 6.16	42 ± 2.6		

[a] Percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) guinea pig aortic strips and ileum longitudinal smooth muscle at 10^{-5} M.
[b] Decrease in developed tension in isolated guinea-pig left atrium at 10^{-5} M, expressed as percent changes from the control (n = 4–6). The left atria were driven at 1 Hz. The 10^{-5} M concentration gave the maximum effect for all compounds.
[c] Calculated from log concentration-response curves (Probit analysis by Litchfield and Wilcoxon [14] with n = 6–7 (aorta and ileum) or 4–7 (left atrium)). When the maximum effect was <50%, the IC_{50} and EC_{50} values were not calculated.
[d] At the 10^{-6} M.
[e] At the 10^{-4} M.
[f] At the 5×10^{-9} M.

Table 3 reports the relaxant activity of the three calcium antagonists, nifedipine, verapamil and diltiazem, and of (*S*)-WB4101, **1** and A175 on guinea pig aorta and distal ileum, expressed as percent inhibition of calcium-induced contraction on the two K^+-depolarized (80 mM) tissues. On guinea pig aorta, all the three calcium antagonists exert >80% inhibition at concentrations ranging between 1 and 100 μM, even if with significantly different potencies. Nifedipine is the most potent one (8.05 pIC_{50}), followed by verapamil (6.42 pIC_{50}) and diltiazem (5.59 pIC_{50}). At 10 μM concentration and under the same experimental conditions, (*S*)-WB4101, **1** and A175 produce only negligible or very weak relaxation with maximum effects much lower than 50% and their intrinsic activity become actually null at 10 nM concentration. It is worth pointing out that, at the same concentration and on the same tissue preparation, (*S*)-WB4101 and **1** show high inverse agonism activity (84% and 90% inhibition of calcium-induced IRT respectively; Table 2).

On a non-vasal smooth muscle, namely distal ileum, where calcium channel blockers have proved to be more effective, nifedipine, verapamil and diltiazem show similar efficacy as on aorta but with from 6-fold to 26-fold higher potency. Analogously, (*S*)-WB4101, **1** and A175 also produce a notable relaxation unlike what was observed in aorta. At 10 μM concentration, all the three responses are >50% and the pIC_{50} values range between 5.55 and 5.08.

Cardiac activity

Table 3 reports also the negative inotropic activity of the six compounds on guinea pig left atrium. At 10 μM concentration, nifedipine, verapamil and diltiazem all exert negative inotropic effects with very high activity, decreasing from nifedipine through verapamil to diltiazem, and submicromolar potency. At the same concentration, (*S*)-WB4101, **1** and A175 show lower intrinsic activity, with effects, in any case, ranging between 40 and 50%.

Discussion

Intrinsic myorelaxant activity of α_1-AR antagonists, not counteracting norepinephrine stimulus but attenuating smooth muscle contraction consequent to membrane depolarization and intracellular calcium increase, might be an indicator of inverse agonism. In order to evidence a nexus between inverse agonism and calcium antagonism, we compared three calcium antagonists (nifedipine, verapamil and diltiazem) with (*S*)-WB4101and its analogues **1** and A175 in inhibiting the calcium-induced contraction of K^+-depolarized vascular and non-vascular smooth muscle, namely guinea pig aortic strips and ileum longitudinal smooth muscle. In guinea pig aorta, adrenoceptors are predominantly α_{1A}-ARs [15], while guinea pig ileum has been not characterized for its α_1-AR sub-types population.

The results reported in Table 3 show that nifedipine, verapamil and diltiazem have all high myorelaxant activity, especially on ileum, but with markedly different potencies. Conversely, **1**, A175 and (*S*)-WB4101 display a high intrinsic relaxant activity on ileum smooth muscle with modest 5–5.5 pIC_{50}, but they do not appreciably inhibit calcium-induced contraction of aortic strips even at supramicromolar concentration. Notably, all the three benzodioxane derivatives do not relax aorta although two of them, (*S*)-WB4101 and **1**, act on guinea pig aorta as inverse agonists [7]. Furthermore, (*S*)-WB4101, which is the most potent α_{1A}-AR antagonist of the three [7], is the most inactive, despite predominance of α_{1A}-AR assimilable receptors in this tissue. Rather, in ileum relaxation, their potency and efficacy increase from (*S*)-WB4101 through A175 to **1**, paralleling their α_{1D}-AR

antagonist affinity ranking (Table 1: (*S*)-WB4101 < A175<**1**). A175, which has the highest α_{1D}-AR pA_2 (10.68), produces the maximum inhibition of calcium-induced ileum contraction (79%) and it has the lowest IC_{50} (2.8 μM). This might be the effect of α_{1D}-AR inverse agonism attenuating the calcium-induced contraction and the phenomenon would be consistent with the observation that the α_{1D}-AR is generally coupled to increases in intracellular calcium and that it is constitutively active [16]. Another suggestion comes from the observation that our three benzodioxane derivatives display higher relaxant activity on ileum than on aorta exactly as the three calcium antagonists. This might indicate that their action is connected to a direct interaction with L-type Ca^{2+} channels, a hypothesis supported by the affinity of WB4101 for Ca^{2+} channels documented in literature [17].

The comparison was extended to cardiac myocytes, where α_{1A}- and α_{1B}-AR subtypes are predominant, both in rodents and in humans, and the α_{1D}-AR subtype seems to be not expressed. The selective α_{1A}-AR stimulation has been proved to potentiate L-type Ca^{2+} current through a specific intracellular signalling pathway in cardiomyocytes [18]. The α_{1A}-AR subtype mediates positive inotropic responses, while the α_{1B}-AR subtype seems to be associated with depressed contractile function. Overall, α_1-AR-mediated inotropic responses are not required for basal contractile function, but might prevent contractile decline in response to pathologic stress [12] and mediate important protective and adaptive functions in hearth.

In our experiments, all the three benzodioxane-related α_1-AR antagonists diminish the baseline contractile function and with similar potencies and efficacies. Their negative inotropic effect is irrespective of both α_{1A}-AR antagonist potencies (A175 and (*S*)-WB4101 do not differentiate from each other) and of inverse agonist behaviour (both A175 and **1** exert intrinsic negative inotropy). Moreover, the absence of α_{1D}-ARs excludes an α_{1D}-AR mediated effect, in particular by inverse agonism. These results and the above literature evidences further support the suggestion resulting from the ileum experiments that the observed intrinsic negative inotropic activity is connected to a direct interaction with L-type Ca^{2+} channels rather than with α_1-ARs. Consistently with such an explanation, the three benzodioxane derivatives, in these experiments based on K^+ activation in the absence of α_1-AR agonist, prove to be more similar to diltiazem than to the other two calcium channel blockers in the order of potency. Indeed, well-established literature reports that: (a) diltiazem does not interact with α_1-ARs unlike verapamil, to which competitive α_1-AR antagonism is attributed, or interacts but at concentrations higher than the effective one for Ca-entry blocking activity [19], and (b) it can control the phenylephrine α_1-AR stimulation with modest potency [20] and indirectly by inhibiting basal Ca^{2+} influx *via* L-type Ca^{2+} channels [21].

In conclusion, our comparative functional studies on the myorelaxant effect and negative cardiac inotropy of (*S*)-WB4101, its analogues **1** and A175 and three well-known calcium antagonists show that both inverse agonists, namely (*S*)-WB4101 and **1**, and a non-inverse agonists, such as A175, have intrinsic activity, albeit lower than that of calcium channel blockers. The α_{1A}-AR seems not to be involved, as we observed very weak intrinsic myorelaxation of guinea pig aorta, where α_{1A}-AR predominates, and negative inotropy by both potent ((*S*)-WB4101 and **1**) and weak (A175) α_{1A}-AR antagonists. On the other hand, intrinsic reverse activity at α_{1D}-AR might explain ileum relaxation and, indirectly, also the lack of aorta relaxation, but not the non-negligible negative cardiac inotropy. Indeed, the observed constitutive activities of the three α_1-AR antagonists cannot be unitedly and univocally interpreted on the basis of their known inverse agonism properties and/or subtype selectivity profiles. Conversely, the parallelism with the higher calcium antagonist activity of nifedipine, verapamil and diltiazem on ileum contraction than on aorta and the indistinct negative inotropy supports direct interaction of the three benzodioxane-related antagonists with L-type Ca^{2+} channels.

Authorship contribution

R. Budriesi: Study design, funds collection; M. Micucci: Data collection, statistical analysis, data interpretation; A. Chiarini: Acceptance of final manuscript version, literature search.

Declarations of interest

None.

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