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Original article

Antihypertensive profile of 2-thienyl-3,4-methylenedioxybenzoylhydrazone is mediated by activation of the A_{2A} adenosine receptor

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ABSTRACT

Several *N*-acylhydrazone derivatives synthesized from safrole have been found to promote intense vasodilation and antihypertensive activity. The present work describes the synthesis and antihypertensive profile of 2-thienyl-3,4-methylenedioxybenzoylhydrazone (LASSBio-1027), a new analogue of the lead compound 3,4-methylenedioxybenzoyl-2-thienylhydrazone. Thoracic aortas from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were prepared for isometric tension recording. Noninvasive blood pressure measurements were made during 14 days of intraperitoneal (10 mg/kg) or oral (20 mg/kg) administration of LASSBio-1027. LASSBio-1027 exhibited partially endothelium-dependent vasorelaxant activity, which was attenuated in the presence of L-NAME, glibenclamide, or ZM 241385. LASSBio-1027 exhibited an antihypertensive effect in SHR during 14 days of intraperitoneal or oral administration, but did not induce a hypotensive effect in normotensive WKY rats. LASSBio-1027-induced vascular relaxation of aortas from WKY rats was mediated by the activation of A_{2A} adenosine receptors. Docking studies and binding assays suggested that LASSBio-1027 has affinity for A_{2A} and A_3 adenosine receptors. This new *N*-acylhydrazone derivative represents a potential strategy for the treatment of arterial hypertension.

1. Introduction

Arterial hypertension is the most common risk factor for cardiovascular disease, which is the leading cause of death in industrialized societies [1,2]. More than 25% of the adult population worldwide had hypertension in 2000, and almost 30% are projected to have this condition by 2025 [3]. The prevalence of arterial hypertension is increasing worldwide, both in developed and developing countries [4]. Thus, the prevention, detection, and treatment of this disease should be a high priority. Moreover, even with apparently adequate blood pressure (BP) control with conventional antihypertensive drugs, cardiovascular disease risks in hypertensive populations remain increased above those in normotensive populations [5]. This latter finding highlights the importance of the discovery of new drugs for the treatment of arterial hypertension.

Recently, our group studied a series of new *N*-acylhydrazones (NAH) with vasodilator activity synthesized from a Brazilian natural

Abbreviations: DMSO, dimethylsulfoxide; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats; L-NAME, *N*-nitro-L-arginine methyl ester; K_{ATP}, ATP-sensitive potassium channels; NO, nitric oxide; i.p., intraperitoneal; BP, blood pressure; SP, systolic pressure; DP, diastolic pressure; HR, heart rate; IC_{50}, concentration necessary to reduce the phenylephrine-induced contraction by 50%; SEM, standard error of the mean; SR, sarcoplasmic reticulum; VSM, vascular smooth muscle; NAH, *N*-acylhydrazone.

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product obtained from sassafras oil, known as safrole. The lead compound for this series was LASSBio-294 [6], an NAH with potent positive cardiac inotropic action [7]. The activity of this compound was related to its ability to increase Ca^{2+} accumulation in the sarcoplasmic reticulum, which promoted vasodilation in aortic rings mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway [8]. Considering the bioprofile of LASSBio-294, we obtained synthetic analogues by changing the electronic density of the thienyl subunit and modifying the stereoelectronic behavior of the acylhydrazone group through its *N*-alkylation. These analogues were evaluated in vascular smooth muscle (VSM), in an effort to identify new drug candidates with vasodilator properties and fewer side effects. The results showed an important pharmacophoric profile for the thienyl ring [9].

Through knowledge of retroisosterism and inversion of the functional groups present in the lead compound structure (Fig. 1), in this work we describe the discovery of a new analogue of the lead compound, 2-thienyl-3,4-methylenedioxybenzoylhydrazone (LASSBio-1027). We evaluated the *in vitro* effects of LASSBio-1027 on VSM, as well as the possible mechanisms involved in its effects. We also investigated its effects on BP during the treatment of normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Docking experiments and binding assays were performed to confirm the possible molecular mechanisms involved in the vascular effects of the new *N*-acylhydrazone derivative.

2. Chemistry

The synthetic route used to generate the NAH target compound LASSBio-1027 is shown in Scheme 1. The derivative was prepared in ca. 65% overall yield from the thiophene-2-carboxaldehyde **1**. By employing the oxidative Yamada's procedure [10], **1** was 'one-pot' converted, in 89% yield, to the corresponding methyl ester **2** by treatment with 2.6 eq. of KOH and 1.3 eq. of iodine in methanol at 0 °C. Next, the key acylhydrazine intermediate **3** was obtained at 91% yield by treatment of an ethanolic solution of the ester **2** with hydrazine hydrate at 70 °C for 3.5 h [11].

LASSBio-1027 was obtained, in good yield (90%), by condensing compound **3** with piperonal in ethanol, using hydrochloric acid as a catalyst [11] (Scheme 1).

Presence of the (*E*)-diastereomer was detected by analyzing the ^{1}H NMR spectra of LASSBio-1027, on the basis of several previous reports from our group describing the configuration of bioactive

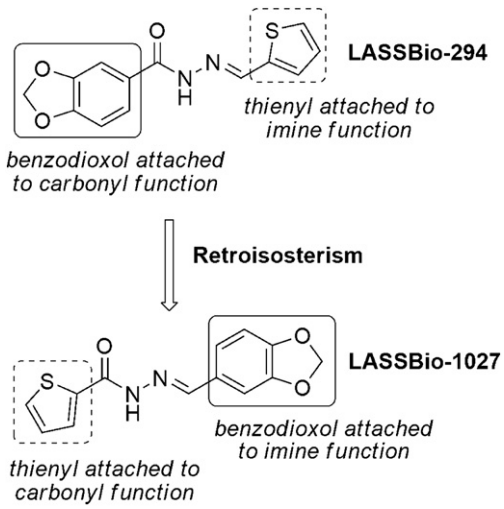


Fig. 1. Retroisosterism applied to the design of LASSBio-1027.

N-acylhydrazone compounds [11–13]. The analytical results for C, H, and N for LASSBio-1027 were within ±0.4% of the theoretical values.

3. Pharmacology

3.1. Effects of LASSBio-1027 on VSM

The vasodilator activity of LASSBio-1027 was investigated in aortic rings from WKY rats. The maximal contractile response induced by phenylephrine (10 μM) in aortic rings with and without endothelium was recorded after exposure to increasing concentrations of LASSBio-1027, which induced relaxation of the precontracted aorta in a concentration-dependent manner (Fig. 2). The concentration necessary to induce 50% relaxation (IC_{50}) of aortic rings with endothelium was 6.9 ± 1.4 μM ($n = 6$). Removal of the endothelium reduced the vasodilator response induced by the compound, which indicates that its vasorelaxant effect was partially dependent on the integrity of vascular endothelium.

Considering the endothelial involvement in the relaxation of aortic rings induced by LASSBio-1027, we investigated which signaling pathways were involved in its mechanism of vasodilation. Endothelium-intact aortic rings were pretreated with L-NAME, a nitric oxide synthase inhibitor, which significantly reduced the maximal relaxation to 9.1 ± 1.8% (Fig. 3A). Pretreatment of the aorta with intact endothelium with ZM 241385, a selective antagonist of the A_{2A} adenosine receptor, induced a rightward shift of the concentration–response curve and reduced the maximal relaxation to 3.6 ± 2.0% (Fig. 3B). This finding indicates that LASSBio-1027 promotes vasorelaxation through NO production mediated by activation of the A_{2A} adenosine receptors.

We also investigated the mechanism of LASSBio-1027-induced vasodilation when the endothelium-denuded aorta was pretreated with glibenclamide, a K_{ATP} channel blocker. Glibenclamide completely abolished vascular relaxation of the aorta (Fig. 4). This result suggests that these channels have an important role in the vascular effect of LASSBio-1027, because the activation of the A_{2A} adenosine receptors in VSM promotes the opening of K_{ATP} channels, inducing hyperpolarization and vasodilation.

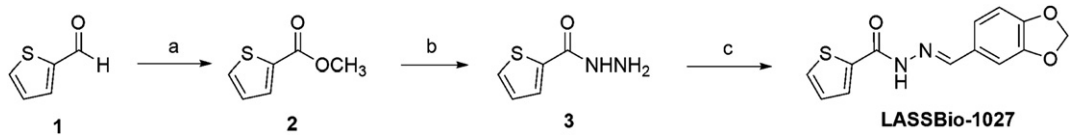
Maximal relaxation values for LASSBio-1027 in phenylephrine-contracted aortic rings from WKY rats are summarized in Table 1.

3.2. Effects of LASSBio-1027 during prolonged treatment of WKY rats and SHR

LASSBio-1027 was administrated intraperitoneally (i.p.) (10 mg/kg/day) to WKY rats and SHR for 14 days. LASSBio-1027 had no significant effect on BP in WKY rats (Fig. 5, Table 2). However, i.p. treatment of SHR for 14 days significantly reduced both systolic and diastolic BP (SP and DP) (Fig. 5, Table 2). Prolonged treatments with LASSBio-1027 did not alter significantly the heart rate (HR) of WKY rats or SHR. Oral administration of LASSBio-1027 (20 mg/kg/day) to SHR for 14 days resulted in significant reduction of SP and DP, but did not change the HR of the rats (Fig. 6, Table 3).

3.3. Docking of LASSBio-1027 in A_{2A} and A_3 adenosine receptors

To confirm the interaction of LASSBio-1027 with the adenosine receptors, we performed a docking study using GOLD 5.1 (CCDC). The crystal structure of A_{2A} receptor was obtained from the RCSB Protein Data Bank (PDB code: 3EML) and until now no crystal structure of A_3 receptor is available on this Data Bank. Thus, an A_3 receptor model was construct by homology modeling using the Swiss Model Server [14,15] using the A_{2A} crystal structure as template (PDB code: 3EML). To validate the *GoldScore* fitness function, a redocking of the cocrystallized antagonist ZM 241385



Scheme 1. General pathway for the synthesis of LASSBio-1027: (a) I_2, KOH, 0 °C, 2 h; (b) $NH_2NH_2 \cdot H_2O$, EtOH, 70 °C, 3.5 h; (c) piperonal, HCl cat, EtOH, 1 h.

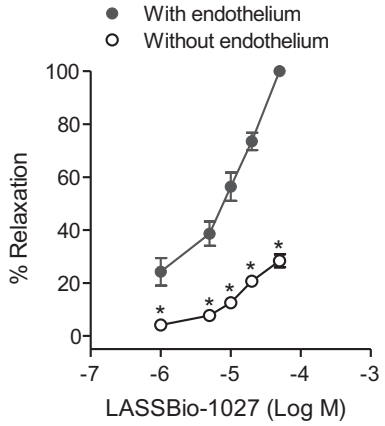


Fig. 2. Concentration-response curves for LASSBio-1027 in rat aorta with and without endothelium, precontracted with phenylephrine. Data are mean ± SEM ($n = 6$). $^*P < 0.05$ vs. with endothelium.

was done. Redocking results show the ability of the function to predict an experimental binding mode. Redocked ZM 241385 and crystal ZM 241385 of the complex were located very close to each other in the active site, with an RMSD of 0.52 Å for all atoms (Fig. 7).

To perform the docking of LASSBio-1027, three consecutive runs were conducted and the highest-scoring conformations from each run were analyzed. Water molecules were removed from the A_{2A} pdb file and the A_3 model was constructed without water molecules. The docking of ZM 241385 was performed with the same protocol.

All of the docking runs showed the same pattern, except for the orientation of the thiophene ring at the acyl unit of LASSBio-1027. According to the results, the 1,3-benzodioxole ring interacts by a hydrogen bond with ASN253 (distance of 2.5 Å) and through π–π stacking contacts with PHE168. Moreover, the *N*-acylhydrazone hydrogen interacts with GLU169 through a hydrogen bond (distance of 1.9 Å), as shown in Fig. 8A. In the A_3 receptor the GLU169 is substituted by a hydrophobic amino acid residue VAL169 which might be responsible for the change in the orientation of LASSBio-1027 in the active site showed in Fig. 8B.

The average of the three docking scores was calculated for LASSBio-1027 in A_{2A} and A_3 receptors and for ZM 241385 in A_{2A} receptor. The obtained values were 51.6, 49.9 and 62.7, respectively. This result indicates that ZM 241385 has more favorable interactions than LASSBio-1027, which will be the subject of further studies.

To compare the binding modes of ZM 241385 and LASSBio-1027, the crystal structure was superimposed with one of the conformations (Fig. 9). Based on the superpositions, LASSBio-1027 seems to bind in the same active site as ZM 241385, which corroborates the *in vitro* pharmacological findings in the VSM of rat aorta.

Score values of the *GoldScore* fitness function did not identify differences in the affinity of LASSBio-1027 for A_{2A} and A_3 receptors. One possible explanation is that the hydrogen-bond energy terms of *GoldScore fitness function* could have a higher representative value than the van der Waals energy terms. Indeed the A_3 receptor has more hydrophobic amino acid residues in the active site, which may explain why the score value of LASSBio-1027 was lower than in A_{2A} receptor (Fig. 10).

LASSBio-1027 had greater affinity for A_3 adenosine receptor and it could be explained by the hydrophobic nature of the A_3 receptor active site in comparison to the A_{2A} receptor active site, which sum of hydrophobic interactions may be more energetic favorable than isolated hydrogen-bond interactions.

3.4. Binding assay

Binding of the agonist [^{3}H]CCPA (1 nM) to the A_1 receptor ($n = 2$) was inhibited by an average of 29.1% by LASSBio-1027 (10 μM). Binding of agonist [^{3}H]CGS21680 (6 nM) to the A_{2A} receptor ($n = 2$) and binding of agonist [^{125}I]AB-MECA (0.15 nM) to the A_3 receptor ($n = 2$) were inhibited by 47.5% and 67.5% by LASSBio-1027 (10 μM).

4. Discussion

Adenosine modulates various physiological processes in mammals. Many of the responses mediated by adenosine are caused by its interaction with specific membrane-bound receptors. From pharmacological and molecular biology studies, four adenosine receptor subtypes have been characterized, named A_1, A_{2A}, A_{2B},

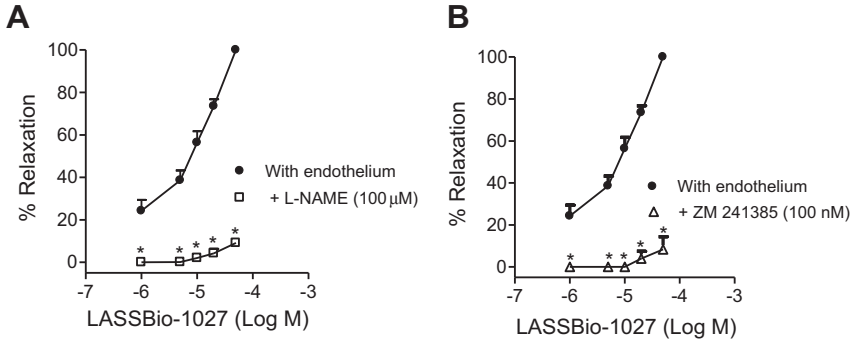


Fig. 3. Concentration-response curves for LASSBio-1027 in aorta with endothelium precontracted with phenylephrine in the presence of (A) L-NAME and (B) ZM 241385. Data are mean ± SEM ($n = 6$). $^*P < 0.05$ vs. with endothelium.

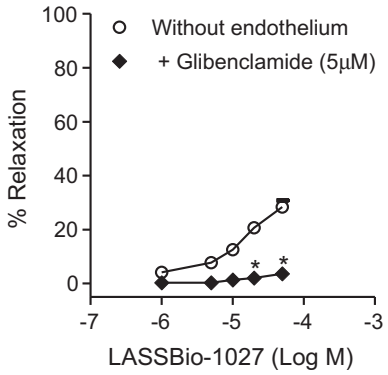


Fig. 4. Concentration-response curves for LASSBio-1027 in endothelium-denuded aorta precontracted with phenylephrine in the presence of glibenclamide. Data are mean ± SEM ($n = 6$). *$P < 0.05$ vs. without endothelium.

Table 1
Maximal relaxation induced by LASSBio-1027 in phenylephrine-contracted aortic rings from WKY rats.

	With endothelium	Without endothelium
Control	100.0 ± 0.0	28.4 ± 2.5#
+L-NAME (100 μM)	9.1 ± 1.8*	ND
+ZM 241385 (100 nM)	8.3 ± 6.0*	ND
+Glibenclamide (5 μM)	ND	3.6 ± 2.0*

*$P < 0.05$ vs. control; #$P < 0.05$ vs. with endothelium.
All values are mean ± SEM, $n = 6$.
ND: not determined.

and A_3 [16]. These receptors belong to the large family of G protein coupled receptors. Activation of A_1 and A_3 receptors leads to inhibition of adenylate cyclase by an inhibitory G protein, whereas A_{2A} and A_{2B} receptors stimulate the enzyme through a stimulatory G protein [17]. In the cardiovascular system, activation of the A_1 receptor subtype produces an inhibitory action on the heart, promoting bradycardia, negative dromotropy, and inotropy, and consequently reduces the cardiac output [18–20]. Stimulation of the A_{2A} receptor subtype elicits various effects, including vasodilation, inhibition of both platelet aggregation and neutrophil adhesion, and reduction in the generation of oxygen free radicals [20–22]. A_3 adenosine receptors are expressed on the surface of most immune cell types, including neutrophils, macrophages, dendritic cells, lymphocytes and mast cells [23]. A_3 activation on immune cells governs a broad array of immune cell functions, which include cytokine production, degranulation, chemotaxis, cytotoxicity, apoptosis and proliferation [23].

LASSBio-1027-induced concentration-dependent relaxation of the aorta mainly by activating A_{2A} adenosine receptors and releasing NO. These actions were observed in pharmacological experiments and confirmed in the molecular docking study, which demonstrated an interaction between LASSBio-1027 and the A_{2A} receptor. The compound induced hypotension and an antihypertensive effect in SHR during prolonged treatment, but it had no effect on the BP of normotensive WKY rats. Moreover, LASSBio-1027 had no effect on the HR of either WKY rats or SHR, which suggests the selectivity of the compound for the A_{2A} receptor subtype relative to the A_1 subtype, which was shown by the low affinity of LASSBio-1027 for the A_1 receptor in the binding assay. Both docking studies and binding assays suggest that LASSBio-1027 could be an agonist of A_{2A} and A_3 subtypes but the vasodilator activity of LASSBio-1027 is probably mediated by the activation of the A_{2A} receptor.

The activation of A_3 receptor improves myocardial function after ischemia-reperfusion protocol in guinea-pig, reduces myocardial injury in rat and also helps protecting isolated cardiomyocytes from cell death [24,25]. Additionally, A_3 agonist exerts anti-apoptotic and

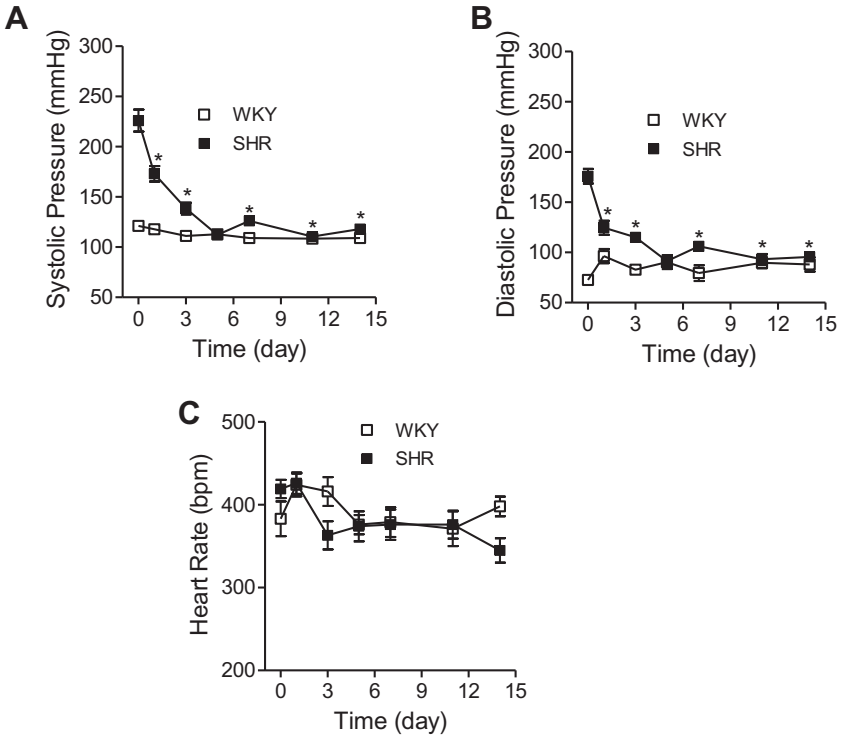


Fig. 5. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were treated with i.p. LASSBio-1027 (10 mg/kg/day) for 14 days. Effects on (A) systolic blood pressure, (B) diastolic blood pressure, and (C) heart rate are shown. Data are mean ± SEM ($n = 6$). *$P < 0.01$ vs. day zero.

Table 2
Intraperitoneal treatment of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) with LASSBio-1027 (10 mg/kg/day) for 14 days.

	WKY			SHR		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
SP (mmHg)	121.2 ± 4.5	109.0 ± 3.3	109.0 ± 2.8	226.0 ± 11.0	126.2 ± 3.2*	118.0 ± 1.2*
DP (mmHg)	72.6 ± 4.7	79.4 ± 7.8	88.0 ± 7.1	175.9 ± 7.3	106.2 ± 4.1*	95.5 ± 4.3*
HR (bpm)	383.0 ± 21.0	379.0 ± 17.9	398.0 ± 11.8	419.0 ± 11.0	376.0 ± 18.3	344.8 ± 14.7

*$P < 0.01$ vs. day 0.
All values are mean ± SEM, $n = 6$.
SP, systolic pressure; DP, diastolic pressure; HR, heart rate.

anti-necrotic effects after ischemia/reoxygenation protocol in isolated adult rat myocytes [24]. Thus, A_3 agonists, such as LASSBio-1027, are of great interest in cardioprotection. On the other hand, activation of A_3 receptors could promote hyper responsiveness of mast cells [26] which would be contraindicated in patients with asthma or chronic obstructive pulmonary disease.

According to the results, LASSBio-1027 caused pronounced vascular relaxation of the endothelium-intact aortic rings, which was significantly reduced in endothelium-denuded aorta. The relaxation was also reduced when the aortas were incubated with a NO synthase inhibitor (L-NAME) or with a selective antagonist of the A_{2A} adenosine receptor (ZM 241385). LASSBio-1027 induced a minor vasodilator effect on endothelium-denuded aorta, and this effect was completely abolished in the presence of glibenclamide, a K_{ATP} channel blocker. These findings indicate that LASSBio-1027 activates the A_{2A} receptor located in the vascular endothelium and VSM of the rat aorta, thereby promoting vasorelaxant activity.

The A_{2A} adenosine receptor is located not only in the vascular endothelium but also in the VSM of the rat aorta [27], and its activation is involved in vasodilation [21,22]. Activation of endothelial A_{2A} receptors, which are coupled with a G stimulatory protein, induces NO release by activating the adenylate cyclase-PKA pathway [28,29]. In addition, activation of A_{2A} receptors in the VSM increases activation of cAMP and PKA, which leads to phosphorylation and opening of K_{ATP} channels. This effect, in turn, causes hyperpolarization and vasodilation [30]. Therefore, blocking of these channels by glibenclamide prevented the vasodilator action of LASSBio-1027.

Dubey and collaborators [31] reported lower levels of extracellular adenosine in the smooth muscle cells of conduit arteries (aorta) and resistance microvessels (renal arterioles) from SHR versus normotensive WKY rats, due to the increased activity of adenosine deaminase, which is responsible for the degradation of adenosine. Dysregulation of extracellular adenosine in the VSM cells of SHR contributes to the enhanced proliferative response that is involved in the pathophysiology of vascular remodeling induced by arterial hypertension [32], because vascular-derived adenosine inhibits cell growth [33]. Therefore, the development of new adenosine receptor agonists, especially A_2 agonists, is of great importance, given their vasodilator and antiproliferative effects.

Several reports have described the synthesis and antihypertensive effects of adenosine A_1 and A_{2A} agonists in conscious SHR [34–39]. In these studies, the adenosine analogues were synthesized from structural modifications of adenosine. Hypotension with

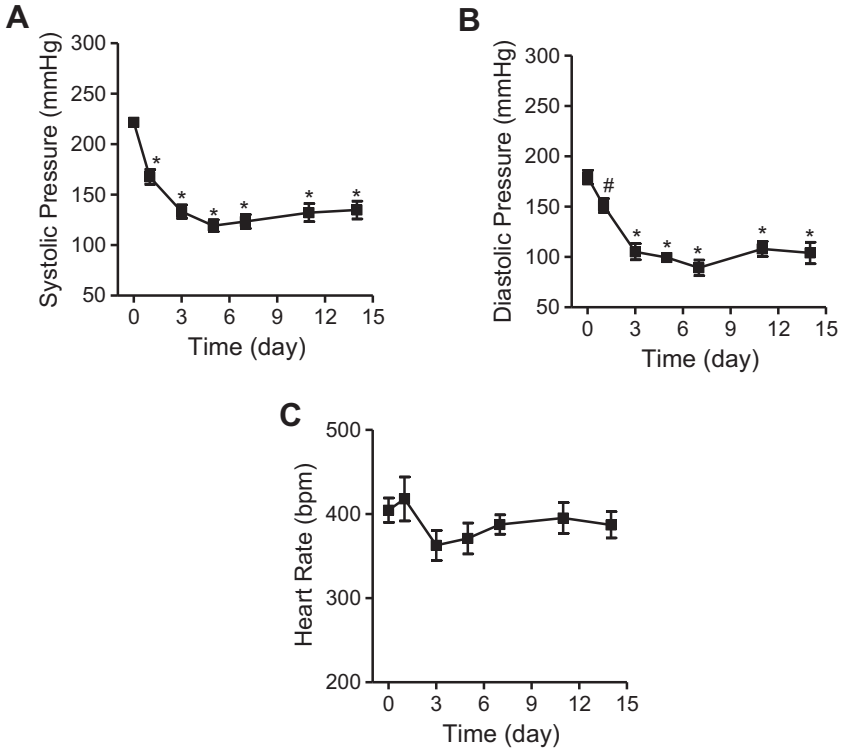


Fig. 6. Spontaneously hypertensive rats (SHR) were treated with oral LASSBio-1027 (20 mg/kg/day) for 14 days. Effects on (A) SP, (B) DP, and (C) HR are shown. Data are mean ± SEM ($n = 6$). #$P < 0.05$ vs. day zero; *$P < 0.01$ vs. day zero.

Table 3
Oral treatment of spontaneously hypertensive rats (SHR) with LASSBio-1027 (20 mg/kg/day) for 14 days.

	SHR		
	Day 0	Day 7	Day 14
SP (mmHg)	121.2 ± 4.5	109.0 ± 3.3*	109.0 ± 2.8*
DP (mmHg)	72.6 ± 4.7	79.4 ± 7.8*	88.0 ± 7.1*
HR (bpm)	383.0 ± 21.0	379.0 ± 17.9	398.0 ± 11.8

*$P < 0.01$ vs. day 0.
All values are mean ± SEM, $n = 6$.
SP, systolic pressure; DP, diastolic pressure; HR, heart rate.

bradycardia or tachycardia was induced after administration of A_1 or A_{2A} agonists, respectively. In addition, Webb and collaborators [40] reported the development of tolerance to the antihypertensive effect of adenosine A_{2A} agonists after two weeks of administration.

The present study describes an innovative compound, LASSBio-1027, which is classified as an NAH derivative and does not have structural similarity to adenosine. The activation of the A_{2A} receptor is responsible for the vasodilator and antihypertensive actions of the new compound. Chronic administration of the substance in SHR did not induce tolerance to the antihypertensive effect, suggesting a new candidate for the prolonged oral treatment of hypertensive subjects.

5. Conclusions

We describe the successful synthesis of a new NAH derivative, LASSBio-1027, which exhibited vasodilator and antihypertensive actions that were mediated by activation of the A_{2A} adenosine receptor. Molecular docking studies and binding assays showed interaction of the compound with both A_{2A} and A_3 receptors. This work highlights the importance of this new class of compounds, which could be explored as an original structural pattern for the activation of adenosine receptors.

6. Experimental section

6.1. Chemistry

Melting points were determined with a Quimis 340 apparatus. Uncorrected melting points are reported. ^{1}H NMR spectra were determined in deuterated chloroform and dimethylsulfoxide containing ca. 1% tetramethylsilane as an internal standard, with a Bruker Avance-500 at 500 MHz ^{13}C NMR spectra were obtained in the same spectrometer at 125 MHz.

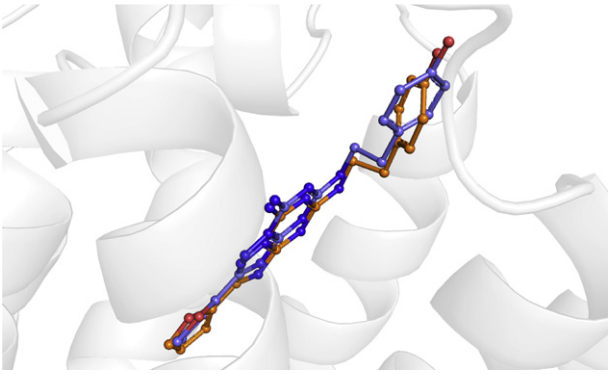


Fig. 7. Superposition of ZM 241385 in the crystal structure of the A_{2A} receptor (orange) and superposition obtained after redocking (blue) with the program GOLD. RMSD = 0.52 Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Microanalyses were performed with a Perkin Elmer 240 analyzer and Perkin Elmer AD-4 balance. The progress of all reactions was monitored by thin-layer chromatography (TLC), which was performed on 2.0 cm × 6.0 cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–365 nm) and treated with iodine vapor. Reagents and solvents were purchased from commercial suppliers and used as received.

6.1.1. Synthesis of methyl thiophene-2-carboxylate (**2**)

Methanolic solutions (each 20 mL) of iodine (1.00 g, 3.9 mmol) and KOH (0.51 g, 9.0 mmol) at 0 °C were added successively to a solution of thiophene-2-carboxaldehyde **1** (0.34 g, 3.0 mmol) in absolute methanol (15 mL) that was cooled to 0 °C. After stirring for 2 h at 0 °C, small amounts of saturated $NaHSO_3$ solution were added until the brown color disappeared [10]. Next, the methanol was almost totally evaporated under reduced pressure. Water (30 mL) was added to the residue, followed by a partition with CH_2Cl_2 (3 × 30 mL). The desired methyl thiophene-2-carboxylate **2** was obtained after the organic phase was dried with Na_2SO_4 and evaporated under vacuum, to 89% yield, as brown oil. ^{1}H NMR (300 MHz $CDCl_3$-d_6) δ 3.87 (s, 3H), 7.08 (dd, 1H, *Jab* = 4.5 Hz,

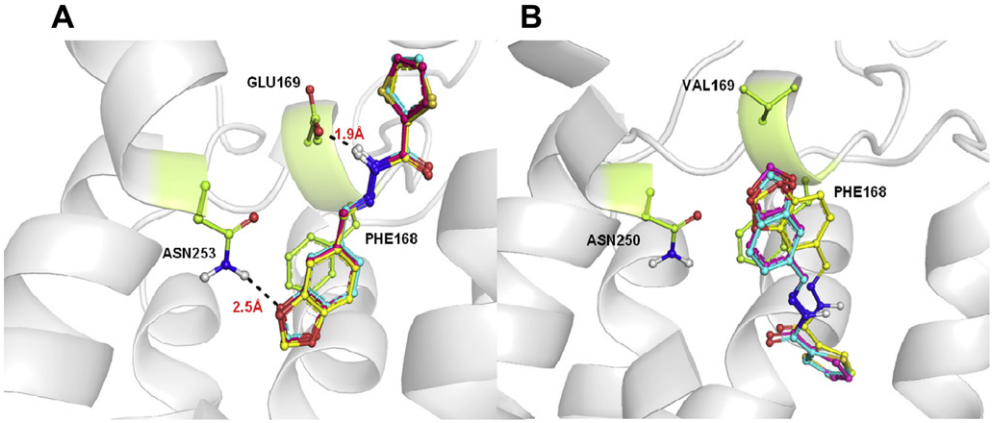


Fig. 8. Possible conformations of the compound LASSBio-1027 (yellow, blue, pink) in (A) A_{2A} adenosine receptor (PDB ID: 3EML) and (B) A_3 adenosine receptor model. Amino acid residues in green are part of the active site of these receptors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

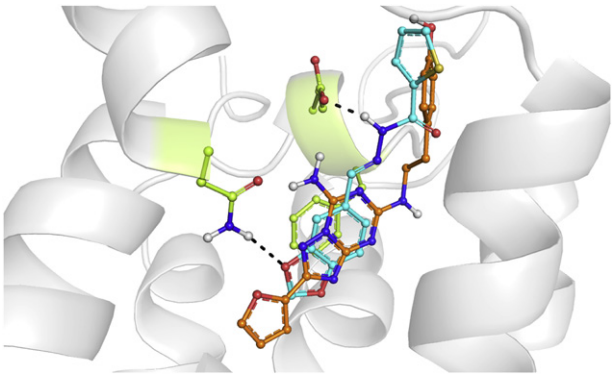


Fig. 9. Superposition of the A_{2A} adenosine receptor antagonist ZM 241385 (orange) with the agonist LASSBio-1027 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Jac = 3.9 Hz), 7.53 (d, 1H, *Jab* = 3.9 Hz), 7.79 (d, 1H, *J* = 4.5 Hz); ^{13}C NMR (75 MHz $CDCl_3$-d_6) δ 52.1, 127.7, 132.3, 133.5, 133.6, 162.7.

*6.1.2. Synthesis of thiophene-2-carbohydrazide (**3**)*

A 0.6-mL aliquot of 100% hydrazine monohydrate was added to a solution of 0.30 g (1.24 mmol) of **2** in 6 mL of ethanol [11]. The reaction mixture was maintained at 70 °C for 3.5 h, when TLC indicated the end of the reaction. The media was poured on ice. The resulting precipitate was partitioned with AcOEt (5 × 30 mL) to generate the title compound with 81% yield, as a brown solid, m.p. 136–138 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.46 (br, 2H), 7.11 (dd, 1H, *Jab* = 4.8 Hz, *Jac* = 3.9 Hz), 7.69–7.72 (m, 2H), 9.74 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 127.2, 127.6, 130.0, 138.0, 160.9.

6.1.3. Synthesis of (E)-N′-(benzo[d][1,3]dioxol-5-ylmethylene) thiophene-2-carbohydrazide (LASSBio-1027)

Piperonal (0.174 g, 1.16 mmol) was added to a solution of thiophene-2-carbohydrazide **3** (0.150 g, 1.01 mmol) in absolute EtOH (7 mL) containing 2 drops of 37% hydrochloric acid [11]. The mixture was stirred at room temperature for 1 h, until extensive precipitation was visualized. Next, the solvent was partially concentrated at reduced pressure, and the resulting mixture was poured into cold water. After neutralization with 10% aqueous sodium bicarbonate solution, the precipitate that formed was filtered out and dried under vacuum to give the desired acylhydrazone derivative, LASSBio-1027 (90%) as a cream-colored solid; m.p. 228–231 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.08 (s, 2H, OCH_2O), 6.98 (d, $H_{3,4\text{-metilenodioxy}}$, *J* = 7.9 Hz), 7.15–7.27 (m, $1H_{3,4\text{-metilenodioxy}}$, $1H_{tiophene}$), 7.51 (d, 1H, H–Ar, *J* = 8.1 Hz), 7.27 (s, $H_{3,4\text{-metilenodioxy}}$), 7.37 (s, $H_{3,4\text{-metilenodioxy}}$), 7.84–8.00 (m, $2H_{tiophene}$), 8.02 (s, 1H, NCH), 8.32 (s, 1H, NCH), 11.70 (s, 1H, CONH), 11.75 (s, 1H, CONH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 102.1, 105.7, 105.8, 109.0, 123.8, 124.0, 127.1, 128.6, 129.0, 129.1, 129.3, 132.2, 133.4, 135.2, 135.4, 138.9, 144.3, 147.9, 148.5, 149.5, 149.6, 158.1, 161.6; MS (ESI) *m/z* 275 ($[M + H]^+$). Anal. Calcd for: $C_{13}H_{10}N_2O_3S$: C 56.92; H 3.67; N 10.21; Found: C 56.87; H 3.69; N 10.15.

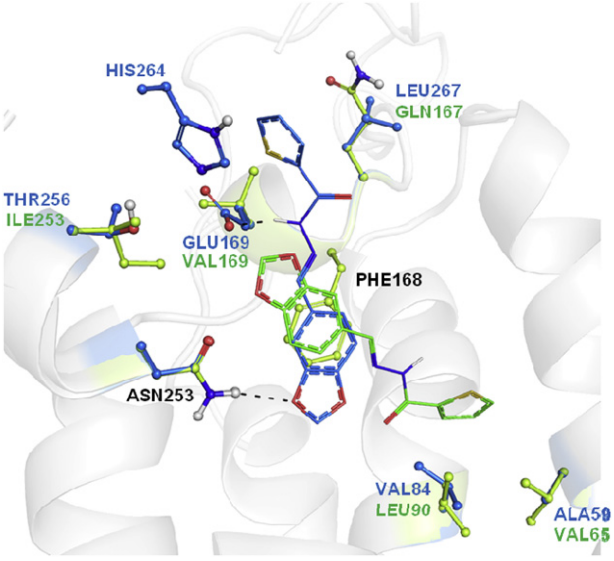


Fig. 10. Superposition of LASSBio-1027 and some amino acid residues in A_{2A} adenosine receptor active site (blue); LASSBio-1027 and some amino acid residues in A_3 adenosine receptor model (green). The amino acid in black is conserved in both receptors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6.2. Pharmacology

The protocols used in the present study were approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro.

6.2.1. In vitro experiments

Thoracic aortas were removed from 13-week-old male WKY rats. The aortas were cleaned of connective tissue and prepared for isometric tension recording, as previously described [41]. After a 2-h equilibrium period of 1 g resting tension, the aortic rings were contracted with phenylephrine (10 μM), followed by exposure to acetylcholine (10 μM) to test the integrity of the endothelium. Acetylcholine-induced relaxation of >80% denoted the presence of intact endothelium. In some experiments, LASSBio-1027 was tested in aortas in which the endothelium had been mechanically removed [41].

To investigate the ability of LASSBio-1027 to induce relaxation of the aortic rings, intact or endothelial-denuded rings were precontracted with a single concentration of phenylephrine (10 μM) and exposed to increasing concentrations of LASSBio-1027 (1–300 μM). Control experiments were performed in the presence of dimethylsulfoxide (DMSO) alone.

To evaluate the possible mechanisms involved in the effects of the derivative, aortic rings were pretreated with: *N*-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor (100 μM) [42,43]; glibenclamide, a K_{ATP} channel blocker (5 μM) [44]; and ZM 241385, a selective antagonist of the A_{2A} adenosine receptor (100 nM) [29,42]. The antagonists were added to the solution 20 min before the contraction with phenylephrine.

6.2.2. In vivo experiments

Male WKY rats and SHR (13-week old) were treated i.p. daily for 14 days with a single dose of LASSBio-1027 (10 mg/kg). In addition, SHR were treated orally daily for 14 days with LASSBio-1027 (20 mg/kg). Noninvasive blood pressure measurements were performed through tail-cuff plethysmography (Letica model LE 5001) to acquire SP, DP, and HR. The BP of rats was measured before and at 1, 3, 5, 7, 11, and 14 days of treatment. The BP measurements were performed at 4 h after administration.

6.2.3. Drugs

Phenylephrine, acetylcholine, glibenclamide, and L-NAME were purchased from Sigma Chemical (St Louis, MO, USA). ZM 241385 was purchased from Tocris Bioscience (Ellisville, MO, USA). LASSBio-1027, glibenclamide, and L-NAME were dissolved in dimetylsulphoxide (DMSO) (Merck Darmstadt, Germany). Phenylephrine and acetylcholine were dissolved in distilled water.

6.2.4. Statistics

All data are expressed as the mean ± standard error of the mean (SEM). The LASSBio-1027 concentration necessary to reduce the phenylephrine-induced contraction by 50% (IC_{50}) was determined for each experiment. The concentration–response curve was fitted to the following equation: $y_{max} = y_{min} + a/(1 + e^{-x-x/b}_{0})$, where y is the percentage of isometric tension; $a = y_{max} - y_{min}$; b = slope; and $x_0 = IC_{50}$. Differences between 2 groups were determined with the unpaired Student's t-test and were considered significant when P was <0.05.

6.3. In silico experiments

The docking experiment was performed with the crystal structure of the A_{2A} adenosine receptor (PDB ID: 3EML) [45] and the A_3 adenosine receptor homology model made by our group using the same crystal structure as the template. The docking program used was GOLD 5.1 (CCDC) and its fitness function *GoldScore* [46]. The *GoldScore* fitness function is calculated from the sum of the following energy terms: hydrogen bond of the protein–ligand complex, van der Waals energy of the protein–ligand complex, van der Waals energy of the ligand, and ligand internal torsional energy. Empirical parameters were used for the calculation. The resulting nondimensional score represents the relative affinity when comparing ≥2 ligands. A higher score represents more favorable interactions in the ligand–receptor complex [46]. Before the experiment, energy minimization was performed on LASSBio-1027 with the semi-empirical AM1 method [47].

The set of amino acid residues selected as the binding site to perform docking studies was determined by a distance of 10 Å from the conserved amino acid residue ASN253 located in the A_{2A} and A_3 receptors. Three consecutive runs were conducted, each of which generated 10 conformations. The highest-scoring conformation of each run was chosen and analyzed.

6.4. Binding assay

A binding assay was performed between LASSBio-1027 (10 μM) and the A_1, A_{2A} or A_3 receptors of human recombinant CHO cells (A_1) or human recombinant HEK-293 cells (A_{2A} and A_3). [^{3}H]CCPA (1 nM), [^{3}H]CGS21680 (6 nM) and [^{125}I]AB-MECA (0.15 nM) were used as the A_1, A_{2A} and A_3 agonists radioligands, respectively. The data were expressed as a percent inhibition of control specific binding obtained in the presence of LASSBio-1027 using the equation: % inhibition = 100 − [(measured specific binding/control specific binding) × 100].

Disclosure statement

The authors declared no conflict of interest. All co-authors have agreed with the submission of the final manuscript and all authors participated in the research and article preparation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, online version, at http://dx.doi.org/10.1016/j.ejmech.2012.06.056.

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