

## A Novel Adenosine A<sub>2A</sub> Receptor Agonist Attenuates the Progression of Monocrotaline-induced Pulmonary Hypertension in Rats

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### Abstract

**Background and purpose:** Pulmonary hypertension is characterized by enhanced pulmonary vascular resistance, Right Ventricular Hypertrophy (RVH), and increased Right Ventricular Systolic Pressure (RVSP). This study examined the therapeutic effects of (E)-N'-(3,4-dimethoxybenzylidene)-3,4-dimethoxy-N-methylbenzohydrazide (LASSBio-1366) on monocrotaline-induced pulmonary hypertension in rats.

**Experimental approach:** Pulmonary hypertension was induced in male Wistar rats by a single intraperitoneal injection of monocrotaline (MCT) at a dose of 60 mg/kg. Rats were divided into the following groups: control (saline injection only), MCT, MCT+vehicle (dimethyl sulfoxide [DMSO]), and MCT+LASSBio-1366. Starting 14 days after MCT injection, rats were treated daily with orally administered LASSBio-1366 (50 mg/kg/day) or vehicle for 14 days. Experiments were performed at the end of the 2-week treatment period (28 days post-MCT). Hemodynamics, vascular activity, and expression of endothelial nitric oxide synthase (eNOS), adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R), and sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase 2a (SERCA2a) were evaluated in each group.

**Results:** MCT increased the RVSP, RVH, and endothelial dysfunction in the pulmonary artery rings. These changes were attenuated by LASSBio-1366. The RVSP was reduced from 49.59 ± 5.08 mmHg in the MCT group, to 35.50 ± 1.17 mmHg in the LASSBio-1366-treated group (P<0.05). MCT also reduced eNOS, A<sub>2A</sub>R, and SERCA2a levels compared with control, but treatment with LASSBio-1366 rescued their expression.

**Conclusion:** LASSBio-1366 activated A<sub>2A</sub>R and attenuated RVH, endothelial dysfunction, and pulmonary vascular remodeling that occurs in rats with pulmonary hypertension.

**Keywords:** pulmonary hypertension, monocrotaline, ventricular dysfunction, pulmonary vascular remodeling, A<sub>2A</sub> adenosine agonist, N-achylhydrazone derivative

**Abbreviations:** PAH: Pulmonary Arterial Hypertension; RV: Right Ventricle; MCT: Monocrotaline; PASC: Pulmonary Artery Smooth Muscle Cell; PVR: Pulmonary Vascular Resistance; mPAP: Mean Pulmonary Arterial Pressure; PDE: Phosphodiesterase; DMSO: Dimethylsulphoxide; HF: Heart Failure; RVSP: Right Ventricular Systolic Pressure; BW: Body Weight; PhE: Phenylephrine; Ach: Acetylcholine

### Introduction

Pulmonary Arterial Hypertension (PAH) is a rare disorder, with a prevalence of 15-50 people per million [1,2]. The condition can occur idiopathically or develop in the context of other conditions. PAH is characterized by endothelial cell dysfunction, proliferation of endothelial and pulmonary arterial smooth muscle cells, pulmonary vasoconstriction, and *in situ* thrombosis. These changes lead to a sustained increase in pulmonary vascular resistance and pulmonary arterial pressure, culminating in progressive Right Ventricular (RV) dysfunction and death [3]. PAH is diagnosed by invasive hemodynamic measurements after catheterization of the right side of the heart. PAH is defined as a resting mean pulmonary arterial pressure ≥ 25 mmHg [4].

Pulmonary vasoconstriction is considered by many as an early event in the development of PAH. Vasoconstriction has been associated with an abnormal function or expression of K<sup>+</sup> channels, and endothelial dysfunction [5]. Endothelial dysfunction causes decreased production of vasodilators, such as nitric oxide (NO) and prostacyclin, and increased production of vasoconstrictors, such as endothelin-1 [6]. NO is a systemic vasodilator that acts via cyclic guanosine monophosphate

(cGMP) pathways. Therapeutic strategies to increase NO-dependent pulmonary vasodilatation primarily target phosphodiesterase type 5 (PDE5), which degrades cGMP. Sildenafil or tadalafil are PDE5 inhibitors that are effective in patients with PAH [7], confirming NO involvement in the regulation of pulmonary vascular tone.

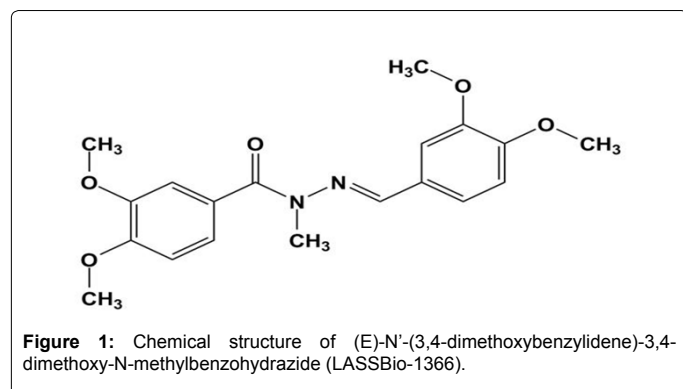
NO can also be released by adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) activation, which is coupled to a G stimulatory protein and activates adenylate cyclase/protein kinase A (PKA) pathways, leading to endothelial NO synthesis and vasodilation [8]. Adenosine is distributed throughout the body and is an important intermediate of purine synthesis and energy metabolism. Experimental evidence suggests that adenosine activation of A<sub>2A</sub>R is critical to the control of vascular tone and remodeling by acting as important regulator of inflammation and a potent vasodilator of pulmonary arteries [9]. A<sub>2A</sub>R-knockout mice develop PAH and undergo increased pulmonary vascular remodeling [9]. Therefore, A<sub>2A</sub>R activation could have beneficial effects on vascular remodeling and vasodilation during PAH.

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Recently, we showed that a new *N*-acylhydrazone derivative (LASSBio-1359), synthesized from a Brazilian natural product in sassafras oil (safrole), reversed monocrotaline (MCT)-induced PAH in rats by activation of A<sub>2A</sub>R [10]. This study investigates the effects of oral administration of (E)-N'-(3,4-dimethoxybenzylidene)-3,4-dimethoxy-N-methylbenzohydrazide (LASSBio-1366) (Figure 1) on PAH. LASSBio-1366 is a novel *N*-acylhydrazone compound that is a structural variant of LASSBio-1359 [11]. We investigated the effects of LASSBio-1366 on pulmonary vascular and RV function in rats with MCT-induced PAH, and explored potential mechanisms of its effects.

## Materials and Methods

### Drugs and reagents

LASSBio-1366 was synthesized by the Laboratório de Avaliação e Síntese de Substâncias Bioativas of the Universidade Federal do Rio de Janeiro. Dimethyl sulfoxide (DMSO, Merck Sharp & Dohme, and Darmstadt, Germany) was used as solvent to the LASSBio-1366, due its low solubility in water. MCT, Phenylephrine (PhE), Acetylcholine (ACh), and ZM 241385 were purchased from Sigma Aldrich (St. Louis, MO). LASSBio-1366 and ZM 241385 were dissolved in DMSO. PhE and ACh were dissolved in distilled water. MCT was dissolved in 1 M HCl, then neutralized with 0.5 M NaOH, and diluted with PBS.

Primary antibody to A<sub>2A</sub>R (catalogue number: AAR-002) was purchased from Alomone Labs Ltd. (Jerusalem, Israel) and the dilution used was 1:1000. The primary antibodies to eNOS (catalogue #9572), SERCA2a (catalogue #4388), and GAPDH (catalogue #5174) were purchased from Cell Signaling Technology (Beverly, MA, USA) and its dilutions were 1:1000. Anti-rabbit IgG-HRP (dilution 1:5000, catalogue number: ab99697) was obtained from Abcam Corporation (Cambridge, MA, USA).

### Animals and experimental protocol

The experimental protocols in this study were approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro. Male Wistar rats (220-300 g) were housed at 20 ± 3°C under a 12-hour light/dark cycle with free access to food and water. Rats were randomly divided into the following groups (*n*=6 per group): (1) control (injected with saline 0.2 mL), (2) MCT, (3) MCT+vehicle 0.2 mL (DMSO), and (4) MCT+LASSBio-1366 (50 mg/kg). Saline or MCT (60 mg/kg) was injected intraperitoneally (i.p.). At 2 weeks after MCT or saline injection, rats were given vehicle (DMSO) or LASSBio-1366 once per day for an additional 2 weeks via oral gavage. Rats were weighed daily, and the amount of LASSBio-1366 administered was adjusted appropriately.

### Effects of LASSBio-1366 on isolated pulmonary arteries

Main pulmonary artery was removed from normal male Wistar

rats, cleaned of connective tissue, and prepared for isometric tension recording, as previously described [10,12]. After an equilibration period of 2 h at 1.5 g resting tension, tissues were contracted with phenylephrine (PhE) (10 μmol/L) and then exposed to acetylcholine (ACh) (10 μmol/L) to test endothelial integrity. Vascular endothelium was considered intact when the ACh-induced relaxation was >60% of the PhE-induced contraction. Different concentrations of LASSBio-1366 (5 × 10<sup>-6</sup> to 5 × 10<sup>-4</sup> mol/L) were added at the plateau of PhE-induced contractions. ZM 241385 (10<sup>-7</sup> mol/L), a selective antagonist of A<sub>2A</sub>R [13], was used to evaluate potential mechanisms of LASSBio-1366.

After 2 weeks of treatment with LASSBio-1366 or vehicle, proximal section of the pulmonary artery was removed, cleaned of connective tissue, and prepared for isometric tension recording [10,12]. The rings were placed in vertical chambers filled with saline solution composed of (in mM): NaCl, 120; KCl, 5.9; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 18; CaCl<sub>2</sub>, 1.2; glucose, 11. The saline solution was at pH 7.4, oxygenated with carbogen gas, and maintained at 37.0 ± 0.5°C. After an equilibrium period of 2 h at 1.5 g resting tension, the arteries were exposed to 10 μmol/L PhE. After a contraction plateau, the pulmonary artery rings were exposed to increasing concentrations of ACh (between 1 nmol/L and 10 μmol/L) to determine endothelial dysfunction and the capacity for vasodilation.

### Docking of LASSBio-1366 in A<sub>2A</sub> adenosine receptor crystal structure

The crystal structure of the A<sub>2A</sub> adenosine receptor co-crystallized with the selective antagonist ZM 241385 (PDB ID: 3EML) [14] was chosen to carry out the molecular docking studies, using the genetic algorithm software GOLD 5.2 (CCDC). The set of amino acid residues selected as the binding site to perform docking studies was determined by a distance of 15 Å from the Asn253 residue. A re docking study of ZM 241385 in the A<sub>2A</sub> crystal structure executed to evaluate the quality of the predicted docking pose by comparison with the experimental binding mode showed a good performance for the *GoldScore* fitness function [15], as previously described [16].

The structure of LASSBio-1366 was energy-minimized using the DFT/B3LYP method [17], available in the Spartan'08 software (Wavefunction Inc.). Three consecutive runs were then performed with LASSBio-1366 and the resulting poses were classified according to the chosen fitness function.

### Hemodynamic measurements

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The depth of anesthesia was evaluated by pinching the paws with forceps. The thoracic cavity was opened, and a heparinized 19-G scalp weplast (Embramac) was inserted into the RV. The RV Systolic Pressure (RVSP) was measured with PowerLab (ADInstruments, Sydney, Australia) monitoring equipment. Rats were euthanized at the end of the experiment while still anaesthetized. Hemodynamic values were automatically calculated with the physiological data acquisition system LabChart 7.0 (ADInstruments, Sydney, Australia).

### RV hypertrophy (RVH) measurements

After euthanasia, hearts were isolated, flushed with saline, and dissected to separate the RV from the left ventricle (LV) and septum (S). RVH was detected from the ratio of the weight RV/LV+S.

### Western blotting

Tissues were harvested, submerged in lysis buffer with protease inhibitors, and immediately frozen in liquid N<sub>2</sub> for homogenization.

Lung and cardiac tissues were homogenized in a potter glass homogenizer using a lysis buffer (12.5% sucrose, 20 mM Tris-HCl [pH 7.4], and 1 mM EDTA) in the presence of 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM benzamidine, 1 mM dithiothreitol (DTT), and 1 µg/mL of a protease inhibitor cocktail (pepstatin, chymostatin, aprotinin, leupeptin, and antipain). The homogenate was centrifuged for 5 min at 1000 × g. The supernatant was collected and frozen [18] for further analyses. Total protein concentrations in each sample were determined spectrophotometrically using the Bradford method [19].

Identical amounts of protein for each blot (50-100 µg) were separated [20] by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gels and transferred to nitrocellulose membranes in a semi-dry system (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Phosphate Buffered Saline (PBS) containing 0.1% Tween 20 and incubated with antibodies against A<sub>2A</sub>R, SERCA2a, eNOS, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Membranes were treated with Super Signal West Pico Chemiluminescence reagents (Pierce, Rockford, IL, USA) and exposed to radiographic films. Films were scanned and densitometry was measured using Scion Image (Scion Corporation, Frederick, MD). Expression of each protein was normalized to GAPDH expression.

### Noninvasive and invasive blood pressure (BP) measurements

Noninvasive BP measurements were performed on Wistar rats through a tail-cuff plethysmograph (Letica model LE 5001, Cornell, Barcelona, Spain). Animals were treated daily, for 14 days, with either vehicle (DMSO) or LASSBio-1366 (50 mg/kg) via oral gavage. The BP of rats was measured before treatment began, and at treatment days 1, 3, 5, 7, 11, and 14 [10].

Wistar rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and a catheter was placed into the right carotid artery for arterial BP measurement using a pressure transducer (MLT884, ADInstruments). A pair of external electrodes was placed on the chest for electrocardiogram recording. A catheter was placed in the jugular vein for intravenous (i.v.) injection of LASSBio-1366. Thirty minutes after the surgical procedure, rats received a bolus injection of LASSBio-1366 (10 mg/kg). Both BP and electrocardiogram were recorded continuously before and during the administration of LASSBio-1366.

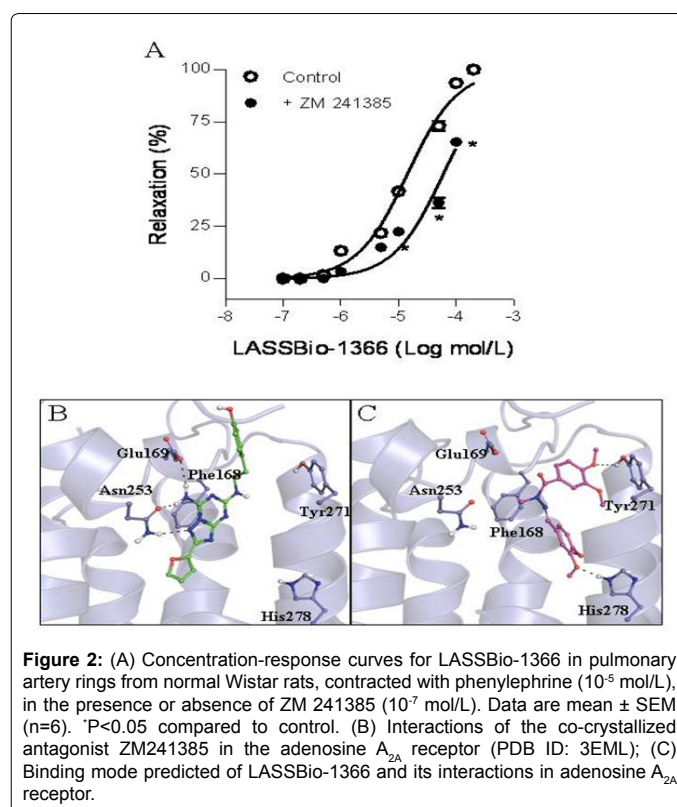
### Data analyses

All data are presented as the mean ± Standard Error of the Mean (SEM). To compare multiple groups, one-way analysis of variance (ANOVA) followed by Newman-Keuls' tests was used. *P* values <0.05 were considered statistically significant.

## Results

### LASSBio-1366 activates the adenosine A<sub>2A</sub>R to induce pulmonary arterial relaxation

The vasodilatory activity of LASSBio-1366 was investigated in pulmonary artery rings from normal Wistar rats. LASSBio-1366 induced relaxation of PhE-contracted vessels (10<sup>-5</sup> mol/L) in a concentration-dependent manner. The concentration of LASSBio-1366 that reduced the PhE-induced contraction by 50% (IC<sub>50</sub>) was 25.1 ± 12.9 µmol/L (Figure 2A). The effect of LASSBio-1366 on vasodilation in the presence of ZM 241385 (10<sup>-7</sup> mol/L), a selective antagonist of the adenosine A<sub>2A</sub>R, was investigated. Pretreatment of pulmonary arteries with ZM 241385 induced a rightward shift of the concentration-response curve and reduced the maximal relaxation by 34.7% (*P*<0.05, Figure 2A).



**Figure 2:** (A) Concentration-response curves for LASSBio-1366 in pulmonary artery rings from normal Wistar rats, contracted with phenylephrine (10<sup>-5</sup> mol/L), in the presence or absence of ZM 241385 (10<sup>-7</sup> mol/L). Data are mean ± SEM (n=6). \**P*<0.05 compared to control. (B) Interactions of the co-crystallized antagonist ZM241385 in the adenosine A<sub>2A</sub> receptor (PDB ID: 3EML); (C) Binding mode predicted of LASSBio-1366 and its interactions in adenosine A<sub>2A</sub> receptor.

### The proposed mode of interaction of LASSBio-1366 in A<sub>2A</sub>R

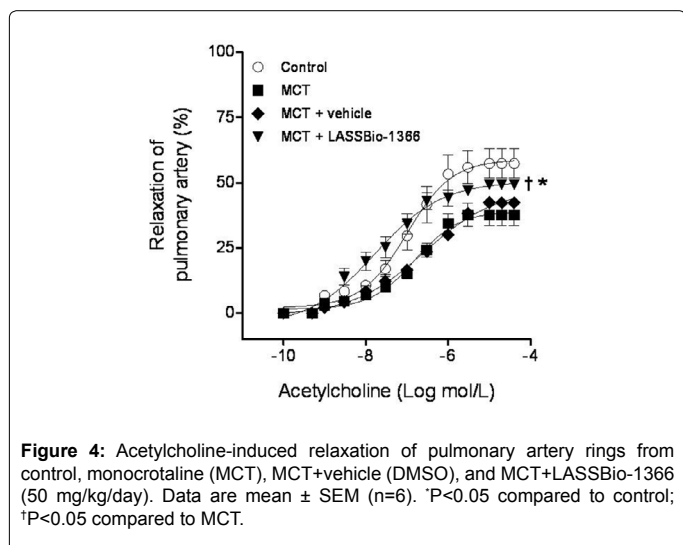
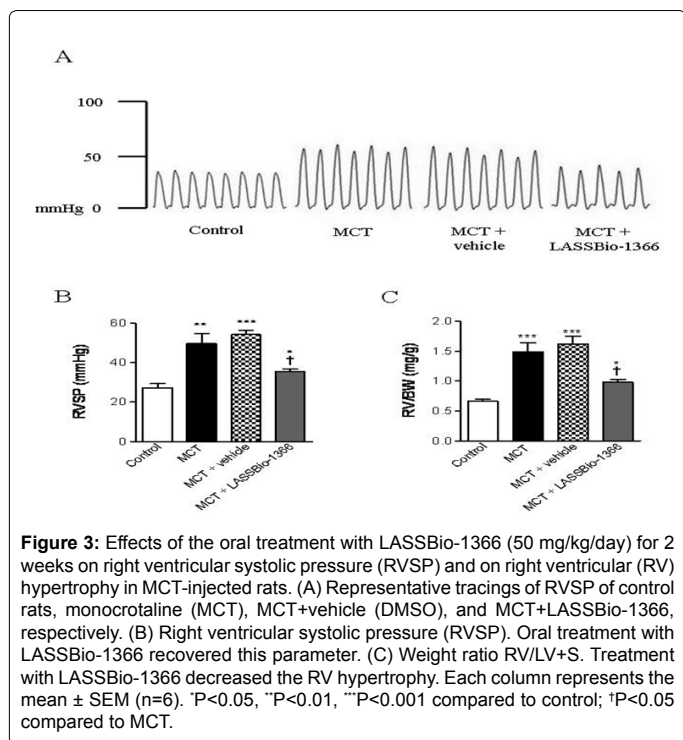
After the three consecutive runs, the highest score pose of LASSBio-1366 in A<sub>2A</sub> adenosine receptor was analyzed and compared with the interactions of ZM 241385 (Figures 2B and 2C). The scale of the score gives a guide as to how good the pose is; the higher the score, the better the docking result is likely to be. It can be observed that LASSBio-1366 has a different binding mode when compared with ZM241385. The triazolo-triazine subunit of ZM241385 interacts with Phe168 by  $\pi$ -stacking interactions and with Glu169 and Asn253 by hydrogen bonds (Figure 2B). LASSBio-1366 is involved in two hydrogen bonds with the side chains of Tyr271 and His278 and its planar *N*-acylhydrazone subunit is positioned near the hydrophobic side chain of Phe168 (Figure 2C).

### Effects of orally administered LASSBio-1366 on pulmonary hemodynamics and RVH

We demonstrated that RVH in MCT-treated rats led to the enhancement of RVSP (Figure 3B). RVSP was significantly increased in rats after MCT treatment (49.59 ± 5.08 mmHg) compared to normal rats (27.28 ± 2.09 mmHg) (*P*<0.05; Figure 3B). Similarly, MCT increased the RV/BW ratio from 0.67 ± 0.03 in normal control rats to 1.49 ± 0.15 (*P*<0.05; Figure 3C). LASSBio-1366 treatment significantly decreased RVSP to 35.50 ± 1.18 mmHg compared to 54.17 ± 2.21 mmHg in the MCT-vehicle group (*P*<0.05). Additionally, LASSBio-1366 significantly decreased RV/BW+S from 1.62 ± 0.12 in the MCT-treated group to 0.98 ± 0.04 (*P*<0.05).

### Oral treatment with LASSBio-1366 attenuates endothelial dysfunction of pulmonary artery rings in MCT-induced PAH

At the end of the treatment course, pulmonary artery rings were removed from rats and prepared for isometric tension recording to



evaluate the endothelial dysfunction. The maximum relaxation induced by ACh was reduced from  $57.3 \pm 5.5\%$  in control rats to  $37.5 \pm 4.0\%$  in MCT-treated rats and  $42.36 \pm 1.3\%$  in rats treated with MCT and vehicle ( $P<0.05$  vs. control; Figure 4). This finding indicates that MCT causes endothelial dysfunction in the pulmonary artery rings. Oral administration of LASSBio-1366 (50 mg/kg) to MCT-treated rats increased the maximum relaxation induced by ACh to  $49.31 \pm 1.4\%$  ( $P<0.05$  vs. MCT; Figure 4). The concentration of LASSBio-1366 to induce 50% of the maximal relaxation ( $IC_{50}$ ) in the phenylephrine-induced contraction of pulmonary artery from vehicle-treated and LASSBio-1366-treated rats were  $21.9 \pm 2.0$  and  $10.2 \pm 1.7$ , respectively ( $P<0.001$ ).

### Effects of LASSBio-1366 on A<sub>2A</sub>R and eNOS expression in the lungs of MCT-treated rats

Western blotting showed that MCT significantly decreased expression

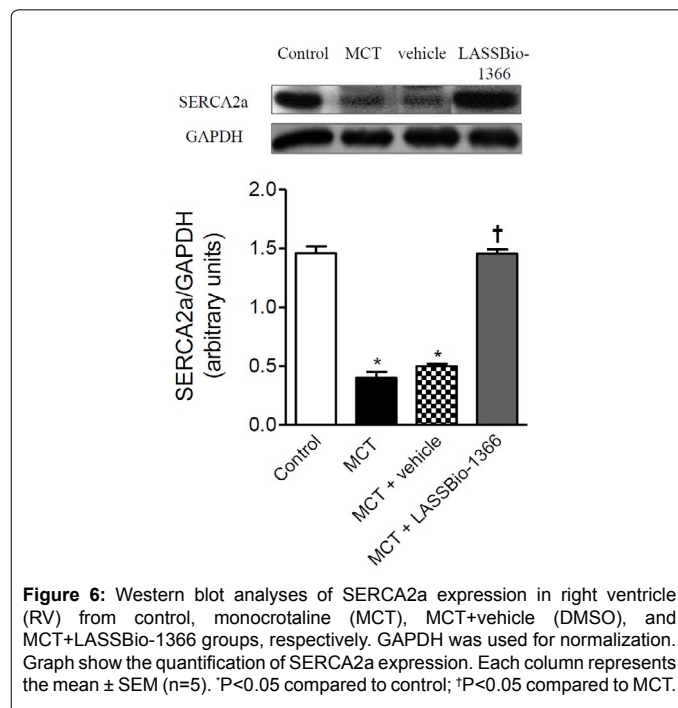
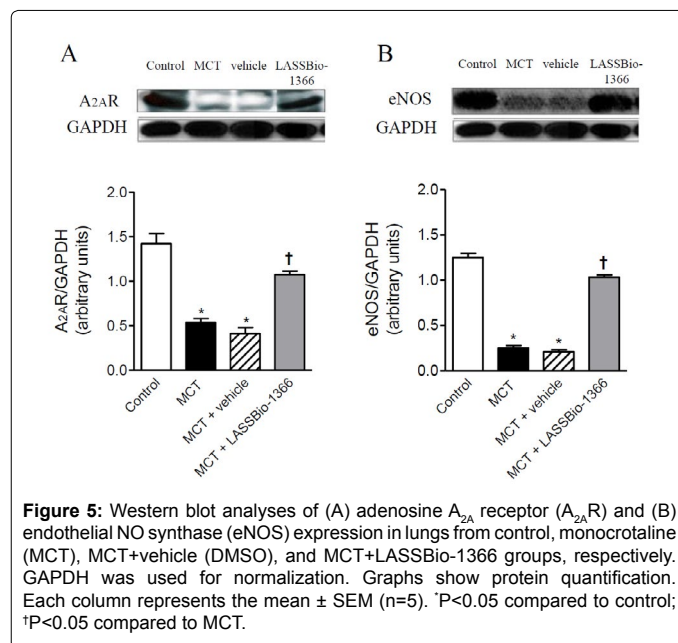
of A<sub>2A</sub>R and eNOS in the lungs. Notably, LASSBio-1366 (50 mg/kg/day) increased the expression of A<sub>2A</sub>R (Figure 5A) and eNOS (Figure 5B) in MCT-injured lungs compared to treatment with vehicle alone.

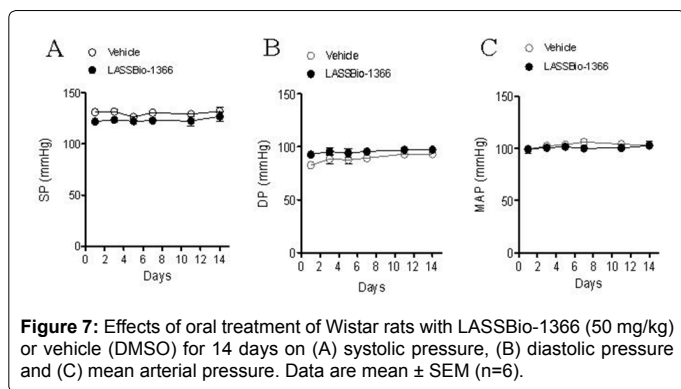
### Effects of LASSBio-1366 on SERCA2a expression in RV tissue from MCT-treated rats

Western blotting of RV tissue revealed that MCT reduced SERCA2a expression compared to controls ( $P<0.05$ ; Figure 6). LASSBio-1366 restored SERCA2a expression in MCT-treated rats.

### Effects of LASSBio-1366 on systemic BP during prolonged or acute treatment in normal Wistar rats

LASSBio-1366 was administrated orally (50 mg/kg/day) to





**Figure 7:** Effects of oral treatment of Wistar rats with LASSBio-1366 (50 mg/kg) or vehicle (DMSO) for 14 days on (A) systolic pressure, (B) diastolic pressure and (C) mean arterial pressure. Data are mean  $\pm$  SEM (n=6).

| Wistar Rat   | SP (mmHg)       | DP (mmHg)       | MAP (mmHg)      |
|--------------|-----------------|-----------------|-----------------|
| Before       | 144.5 $\pm$ 0.4 | 101.4 $\pm$ 0.3 | 131.1 $\pm$ 0.5 |
| LASSBio-1366 | 139.8 $\pm$ 0.6 | 102.8 $\pm$ 0.7 | 129.9 $\pm$ 0.7 |
| 5 min after  | 141.0 $\pm$ 0.2 | 99.2 $\pm$ 0.4  | 128.0 $\pm$ 1.2 |
| 10 min after | 143.0 $\pm$ 0.4 | 104.2 $\pm$ 0.3 | 125.0 $\pm$ 1.0 |
| 20 min after | 141.0 $\pm$ 0.4 | 98.9 $\pm$ 0.5  | 130.0 $\pm$ 0.9 |

\*All values are mean  $\pm$  SEM, n=6 per group

<sup>b</sup>SP: Systolic Pressure; DP: Diastolic Pressure; MAP: Mean Arterial Pressure

**Table 1:** Blood systemic pressure data of Wistar rats receiving i.v. LASSBio-1386 (10 mg/kg).

normal Wistar rats for 14 days. Neither vehicle nor LASSBio-1366 had significant effects on systemic BP (Figure 7), indicating a lack of systemic hypotension. After i.v. administration of LASSBio-1366 in normal rats, we did not observe changes in BP at 5, 10, or 20 minutes post-injection (Table 1).

## Discussion

The present study shows that LASSBio-1366 attenuates pulmonary vasoconstriction, RV systolic pressure, and RV hypertrophy in rats with MCT-induced PAH. PAH is a disease of the small pulmonary arteries, characterized by vascular narrowing leading to a progressive increase in pulmonary vascular resistance. The consequence of this increased right ventricle afterload is the heart failure [6]. LASSBio-1366 induced relaxation of PhE-contracted pulmonary vessels indicating an interesting pharmacological action for intervention in the dysfunctional arterioles during PAH. Pretreatment of pulmonary artery rings with the  $A_{2A}$ R antagonist ZM 241385 significantly decreased the vasodilator effect of LASSBio-1366 and this finding suggested the involvement of  $A_{2A}$ R in this process.

It is interesting to notice that ZM241385 is an  $A_{2A}$  antagonist while LASSBio-1366 has an agonist activity potential and this could be related to the different patterns of interactions observed in the docked structures. The amino acid residue His278, which interacts only with LASSBio-1366, has been described as one of the residues that interact with the endogenous agonist adenosine and with its analogues in crystallographic experiments [14]. This information is supported by mutagenesis data which have shown that mutation of the His278 to alanine abolishes the affinity of  $A_{2A}$  agonists [21,22].

$A_{2A}$ R-knockout mice show pulmonary vascular features that resemble idiopathic PAH, suggesting an important role for  $A_{2A}$ R in PAH development [9].  $A_{2A}$ R agonists may emerge as new compounds to treat PAH. Furthermore,  $A_{2A}$ R agonists may be important regulators of inflammation [21]. Adenosine is a potent pulmonary vasodilator that has been used to treat clinical and experimental pulmonary

hypertension [21]. Thus,  $A_{2A}$ R activation by LASSBio-1366 may be an important therapeutic strategy for the treatment of PAH.

In addition, LASSBio-1366 effectively attenuates endothelial dysfunction of pulmonary artery rings, as measured by the increased ACh-induced relaxation and it probably occurs because  $A_{2A}$ R is an important regulator in the development of PAH and pulmonary vascular remodeling [9]. The  $A_{2A}$ R is located in the vascular endothelium and is coupled to a G stimulatory protein. It induces NO release to promote vasodilatation by activating adenylate cyclase/PKA pathways [8]. Adenosine also produces endothelium-independent relaxation, which is partly induced by the activation of  $A_{2A}$ R in smooth muscle, which regulates a mixed population of  $K^+$  channels [23]. LASSBio-1366 could, therefore, activate  $A_{2A}$ R in the pulmonary arteries and restore normal levels of endothelial NO, leading to improvement of endothelial dysfunction and reduction of pulmonary vascular resistance.

Xu et al. [9] provided the first evidence that  $A_{2A}$ R contributes to the development of PAH in mice. At 14 to 16 weeks of age,  $A_{2A}$ R-knockout mice exhibited hemodynamic, histological, and ultrastructural changes associated with PAH [9]. In our experiments, MCT-treated rats showed a reduction of  $A_{2A}$ R expression and LASSBio-1366 upregulated  $A_{2A}$ R level in lung tissue, further implicating  $A_{2A}$ R in PAH progression.

To investigate the impact of endothelial injury on microvascular function, we measured eNOS expression in lung tissue from MCT-treated rats and found that eNOS was down-regulated. Previous reports have demonstrated that eNOS is decreased in rat lungs after MCT-induced PAH [24,25]. Considering that eNOS is mainly expressed in normal endothelial cells, we hypothesize that the inflammatory response to MCT over 28 days disrupts endothelial cell membranes resulting in loss of eNOS molecules [26]. In the present study, we found that LASSBio-1366 restored eNOS expression in the lungs of rats with PAH.

Considerable evidence has been collected concerning the role of intracellular  $Ca^{2+}$  signaling in the development of heart failure. SERCA2a regulates intracellular  $Ca^{2+}$  handling and plays a crucial role in cardiac contraction and relaxation. In human patients with and animal models of heart failure, SERCA2a expression and activity are reduced [27]. This study provides the first evidence that in a rat model of MCT-induced PAH, there is a reduction of RV SERCA2a at 28 days after MCT injection. Impaired SERCA2a expression may be implicated in the RV dysfunction induced by PAH. The rescue of RV SERCA2a expression by LASSBio-1366 was likely the result of reduced load in right side of the heart.

Oral treatment of spontaneously hypertensive rats with LASSBio-1366 promoted a pronounced hypotensive effect (data not shown). Although treatment of normotensive Wistar rats with the compound had no effect on blood pressure. This feature is important because it indicates a low potential of LASSBio-1366 to induce side effects on the blood pressure of patients with PAH.

Previously, we demonstrated that the activation of  $A_{2A}$ R by a new agonist named LASSBio-1359 attenuated the development of MCT-induced PAH in a rat model [10]. LASSBio-1366 was designed and synthesized to improve the molecular recognition by the receptor. The rationale to its synthesis was to facilitate its interaction to the  $A_{2A}$ R. The molecular similarity between the two aromatic rings of compound LASSBio-1359 was improved by the introduction of the dimethoxyl group in the left ring which resulted in LASSBio-1366. Our hypothesis was that because of the molecular symmetry of LASSBio-1366, it should interact easily to the  $A_{2A}$ R and should contribute to its superior therapeutic effects to reduce the vascular remodeling in HAP.

It was confirmed that oral treatment with LASSBio-1366 reduced cardiac and vascular dysfunction partially mediated by the activation of the adenosine A<sub>2A</sub>R which was similar to those effects observed with LASSBio1359. Future studies are required to determine whether LASSBio-1366 could be used in clinical settings.

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