Effects of Rho-kinase inhibition in chronic experimental pulmonary hypertension

Inaugural Dissertation submitted to the
Faculty of Medicine in partial fulfillment of the requirements
for the PhD-Degree of the Faculties of Veterinary Medicine and
Medicine of the Justus Liebig University Giessen

by
Kosanović Đuro
of
Obrenovac, Serbia

Giessen 2011

From the Department of Medicine
Director/Chairman: Prof. Dr. Werner Seeger
Of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member: Prof. Dr. Ralph Schermuly
Second Supervisor and Committee Member: Prof. Dr. Christian M. Kähler
Committee member (Chair): Prof. Dr. Klaus T. Preissner
Committee member: Prof. Dr. Georgios Scheiner-Bobis
Date of Doctoral Defense: 7. September 2011

I dedicate this PhD thesis to my parents and sister for all the love and support.

(Posvećujem ovu doktorsku disertaciju mojim roditeljima i sestri za svu ljubav i podršku.)

"People worry about the large number of diseases. Medical doctors worry about the small number of drugs."

(*3rd Century B.C.*)

Index of figures……1
Index of tables……..……...……...1
Index of photographies……2
Index of schemes……...……...2
1. Introduction……...3
1.1. Pulmonary hypertension……..……4
1.1.1. Definition and classification……..….4
1.1.2. Clinical symptoms and diagnosis of pulmonary arterial hypertension...5
1.1.3. Vasoconstriction and pathomorphological changes of pulmonary arteries……6
1.1.4. *In situ* thrombosis and inflammation in pulmonary hypertension.....7
1.1.5. Current treatment options……..……..……...8
1.2. Animal models of pulmonary hypertension……..……...……...8
1.2.1. The hypoxic animal model of pulmonary hypertension…..…..……..8
1.2.2. Monocrotaline animal model of pulmonary hypertension…..…..……9
1.3. RhoA and Rho-kinase (ROCK) signalling……..…….…….……10
1.4. Role of Rho-kinase in pulmonary hypertension……11
1.5. Inhibition of Rho-kinase as a strategy for the treatment of pulmonary hypertension…….……14
1.6. Azaindole-based Rho-kinase inhibition……15
1.7. Aims of the study……..……16

2. Materials and methods……...…17
2.1. *Materials*……18
2.1.1. Substances and reagents……...…18
2.1.2. Consumables……...…20
2.1.3. Systems and machines……22
2.2. *Methods*……..……24
2.2.1. Experimental animals……...……...….24
2.2.2. Monocrotaline (MCT)-induced pulmonary hypertension model in rats…….…24
2.2.3. Chronic hypoxia-induced pulmonary hypertension model in mice.....24
2.2.4. Isolated murine lungs……..25
2.2.5. Cell culture (proliferation and cytotoxicity assays)…….……...…27
2.2.6. Radio-telemetry study…….……28

2.2.7. Chronic treatment study – experimental design**……..……30**
2.2.8. Hemodynamic and right ventricular hypertrophy measurements**…….…..31**
2.2.9. Measurements of cardiac output and total systemic and pulmonary vascular resistance**……...……....32**
2.2.10. Lung tissue processing**……..33**
2.2.11. Histology and pulmonary vascular morphometry**…….……34**
2.2.12. Immunohistochemistry for phospho-myosin phosphatase target subunit 1 (p-MYPT1) and proliferating cell nuclear antigen (PCNA)**……..……...…36**
2.2.13. Data analysis**…….……....37**

3. Results……..38
3.1. Effects of Rho-kinase inhibitors on acute hypoxic pulmonary vasoconstriction (HPV)**…….……39**
3.2. Effects of Rho-kinase inhibitors on cell cytotoxicity**……40**
3.3. Effects of Rho-kinase inhibitors on proliferation of PASMCs isolated from healthy rats**……..……...……41**
3.4. Effects of Rho-kinase inhibitors on proliferation of pulmonary arterial smooth muscle cells (PASMCs) isolated from MCT-injected rats**…….……...…42**
3.5. Effect of azaindole-1 on MCT-induced progressive elevation of right ventricular systolic pressure (RVSP)**……...…...…43**
3.6. Effects of azaindole-1 on hemodynamics in MCT-induced pulmonary hypertension**……..…44**
3.7. Effect of azaindole-1 on cardiac index and total pulmonary and systemic resistance in MCT-induced pulmonary hypertension **……..45**
3.8. Effects of azaindole-1 on hemodynamics in chronic hypoxia-induced pulmonary hypertension in mice**…….……..……..……46**
3.9. Effects of azaindole-1 on right ventricular hypertrophy in MCT- and chronic hypoxia-induced pulmonary hypertension **……....……..……....47**
3.10. Effects of azaindole-1 on pulmonary vascular remodelling in MCT-induced pulmonary hypertension – Degree of muscularization**…….……...….48**
3.11. Effects of azaindole-1 on pulmonary vascular remodelling in MCT-induced pulmonary hypertension – Medial wall thickness**……..……..49**
3.12. Effects of azaindole-1 on pulmonary vascular remodeling in chronic hypoxia-induced pulmonary hypertension in mice**…….……51**

3.13. Effect of azaindole-1 on pulmonary vascular cell proliferation**……..…..…53**
3.14. Effects of azaindole-1 on Rho-kinase activity**…….55**
3.15. Effect of azaindole-1 on body weight of animals in hypoxia and MCT-induced pulmonary hypertension**……57**
3.16. Effect of azaindole-1 on survival in MCT-induced pulmonary hypertension**.....58**

4. Discussion……..59
4.1. Rho-kinase inhibition in MCT-induced pulmonary arterial hypertension**…….62**
4.2. Rho-kinase inhibition in hypoxia-induced pulmonary hypertension**…….65**
4.3. Future experimental and clinical perspective of azaindole-based Rho-kinase inhibition**…….……..66**

5. Abbreviations and acronyms……..68

6. Summary……...71

7. Zusammenfassung……...…….72

8. Reference list……73

9. Declaration……90

10. Acknowledgments……91

11. Curriculum vitae……..……93

Index of figures

Figure 1. Effects of fasudil, Y-27632 and azaindole-1 on hypoxic pulmonary vasoconstriction (HPV).

Figure 2. Effects of fasudil, Y-27632 and azaindole-1 on pulmonary arterial smooth muscle cell (PASMC) viability.

Figure 3. Effects of fasudil, Y-27632 and azaindole-1 on proliferation of pulmonary arterial smooth muscle cells (PASMCs) isolated from healthy rats.

Figure 4. Effects of fasudil, Y-27632 and azaindole-1 on proliferation of pulmonary arterial smooth muscle cells (PASMCs) isolated from MCT-injected rats.

Figure 5. Effect of azaindole-1 on progressive elevation of right ventricular systolic pressure (RVSP).

Figure 6. Effect of azaindole-1 on hemodynamics in MCT-induced pulmonary hypertension in rats.

Figure 7. Effect of azaindole-1 on cardiac index and total pulmonary and systemic resistance in MCT-induced pulmonary hypertension in rats.

Figure 8. Effect of azaindole-1 on hemodynamics in chronic hypoxia-induced pulmonary hypertension in mice.

Figure 9. Effect of azaindole-1 on right ventricular hypertrophy in MCT- and chronic hypoxia-induced pulmonary hypertension.

Figure 10. Effect of azaindole-1 on degree of muscularization in MCT-induced pulmonary hypertension.

Figure 11. Effect of azaindole-1 on medial wall thickness in MCT-induced pulmonary hypertension.

Figure 12. Effect of azaindole-1 on pulmonary vascular remodelling in hypoxia-induced pulmonary hypertension in mice.

Figure 13. Effect of azaindole-1 on pulmonary vascular cell proliferation.

Figure 14. Effect of azaindole-1 on Rho-kinase activity.

Figure 15. Effect of azaindole-1 on body weight of animals in hypoxia- and MCT-induced pulmonary hypertension.

Figure 16. Effect of azaindole-1 on survival in MCT-induced pulmonary hypertension.

Index of tables

Table 1. Classification of pulmonary hypertension.

Table 2. The composition of Krebs-Henseleit solution.

Table 3. Formulas for calculating the cardiac output (CO), cardiac index (CI), total pulmonary resistance (TPR) and total systemic resistance (TSR).

Table 4. The composition of PBS buffer.

Table 5. The advantages of azaindole-1 in comparison with fasudil and Y-27632.

Index of photographies

Photographies 1. Pulmonary vascular morphological changes in pulmonary hypertension.

Photography 2. Hypoxic chamber.

Index of schemes

Scheme 1. RhoA activated Rho-kinase – molecular structure.

Scheme 2. Roles of Rho-kinase in pulmonary hypertension.

Scheme 2´. Rho-kinase function in vasoconstriction/vasorelaxation processes.

Scheme 3. Rho-kinase signalling.

Scheme 4. Structural chemical formula of azaindole-1.

Scheme 5. Isolated, ventilated and perfused mouse lung system.

Scheme 6. Online telemetry system for monitoring hemodynamics and heart rate.

Scheme 7. Chronic treatment study – experimental design.

Scheme 8. Right heart hypertrophy measurements.

Scheme 9. Lung tissue processing.

Scheme 10. Diagnostic and treatment algorithm for pulmonary hypertension.

Scheme 11. Therapeutic effects of azaindole-1 in experimental pulmonary hypertension.

1. INTRODUCTION

1.1. Pulmonary hypertension

1.1.1. Definition and classification

Pulmonary hypertension (PH) is a chronic severe and fatal disease characterized by a sustained elevation of pulmonary artery pressure and strongly reduced exercise tolerance. Clinically, PH is defined as an augmentation of mean pulmonary arterial pressure of more than 25 mmHg at rest and/or more than 30 mmHg during exercise[1]. As a consequence, the right ventricular afterload increases and ultimately culminates in right ventricular failure and death. The new classification of pulmonary hypertension was created in Dana Point in 2008. (table 1) and PH is classified into 5 heterogeneous groups[2]. Although initial pathological events may be different in various groups, mechanisms of disease progression and pathological manifestations in different groups are often shared[3].

1) Pulmonary arterial hypertension (PAH)
- 1.1. Idiopathic PAH
- 1.2. Heritable (BMPR2; ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia); Unknown
- 1.3. Drug- and toxin-induced
- 1.4. Associated with: Connective tissue disorders; HIV infection; Portal hypertension; Congenital heart disease; Schistosomiasis; Chronic hemolytic anemia
- 1.5. Persistent pulmonary hypertension of the newborn

1´) Pulmonary veno-occlusive (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)

2) Pulmonary hypertension owing to left heart disease
- 2.1. Systolic dysfunction
- 2.2. Diastolic dysfunction
- 2.3. Vascular disease

3) Pulmonary hypertension owing to lung diseases and/or hypoxia
- 3.1. Chronic obstructive pulmonary disease
- 3.2. Interstitial lung disease
- 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4. Sleep-disordered breathing
- 3.5. Alveolar hypoventilation disorders
- 3.6. Chronic exposure to high altitude
- 3.7. Developmental abnormalities

4) Chronic thromboembolic pulmonary hypertension (CTEPH)

5) Pulmonary hypertension with unclear multifactorial mechanisms
- 5.1. Hematologic disorders: myeloproliferative disorders, splenectomy
- 5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis, lymphangioleiomyomatosis, neurofibromatosis, vasculitis
- 5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Table 1. The classification of pulmonary hypertension.

Pulmonary arterial hypertension (PAH) covers the first group in the classification of PH. This subcategory comprises the idiopathic PAH (earlier known as ´primary´), the heritable

(familial) form and PH associated with a wide number of other conditions (table 1). PAH has a multi-complex pathology that includes a combination of pulmonary vascular remodelling, vasoconstriction, *in situ* thrombosis and ultimately development of right ventricular hypertrophy. The progressive pulmonary vascular remodelling is the attribute of PAH pathology and is characterized by abnormalities of vascular cells such as increased proliferation, migration and resistance to apoptosis[4;5]. Idiopathic PAH (IPAH) form mostly affects women in their third and forth decades of life. The disease is rarely diagnosed from the onset of clinical manifestations. As a consequence the disease is rapidly progressive and patients´ survival interval rate is 2-8 years from the moment of diagnosis[1].

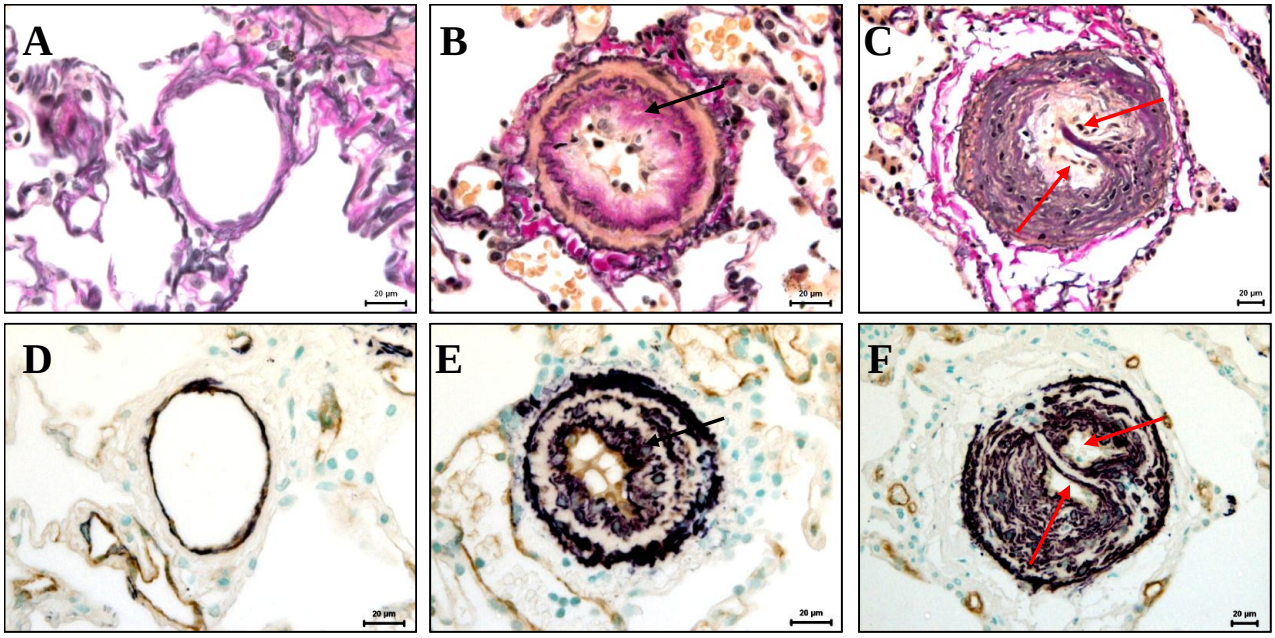
Pulmonary hypertension owing to lung diseases and/or hypoxia covers the third group in the classification of PH (table 1). This group comprises many diseases or pathological conditions that are associated with persistent or intermittent hypoxia, either globally or regionally, within confined areas of the lung[6]. Chronic hypoxia may induce pathological changes in the structure of pulmonary arteries and finally leads to the development of remodelling and persistent vasoconstriction of the pulmonary circulation[6;7].

1.1.2. Clinical symptoms and diagnosis of pulmonary arterial hypertension

Initial symptoms, like dyspnea and fatigue are usually nonspecific. These symptoms, together with chest pain, syncope and peripheral edema characterize advanced stage of disease when heart is already unable to adapt to the progressive increase in pulmonary vascular resistance[1;8]. Non-invasive echocardiography is a very useful diagnostic approach for diagnosis of PAH in patients and can provide important information about the parameters related with right heart hypertrophy (such as right ventricular dimensions and right ventricular wall thickness) and can help to estimate the hemodynamic parameters (such as pulmonary arterial pressure)[1]. Additionally to that, many other non-invasive tests can be included: 6 minutes walking test, chest radiography, perfusion lung scanning or computer tomography scanning. Although these approaches provide important information about the disease, for the final diagnosis of PAH the right heart catheterization should be performed. The right heart catheterization, although invasive approach, still remains the gold standard for the diagnosis of pulmonary hypertension[1;9].

1.1.3. Vasoconstriction and pathomorphological changes of pulmonary arteries in PAH

PAH is a complex pulmonary vascular disease with a plethora of different pathophysiological, molecular and histopathological events. An imbalance between vasodilators and vasoconstrictors strongly characterize this disease[10]. The patients suffering from PAH have reduced levels of vasodilator and anti-proliferative agents, such as nitric oxide (NO) and prostacyclin, while the production of vasoconstrictors, such as endothelin-1, angiotensin II and thromboxane, is a noticeably augmented[10-13]. Additionally to vasoconstriction, the disease is associated and characterized with complex and progressive morphological changes of all structural layers of the pulmonary arteries. The progressive pulmonary vascular remodelling is the attribute and hallmark of PAH pathology and is a consequence of abnormalities in vascular cells, such as increased proliferation and resistance to apoptosis[4;5;14]. The remodelling process mostly affects the small pulmonary arteries and arterioles where the neomuscularization of peripheral normally non-muscularized vessels occurs[10;15]. The development of complex histopathological features, such as neointima and plexiform lesions (photographies 1), adventitial proliferation and *in situ* thrombosis, also takes a place in pathology of PAH[10;15;16].



Photographies 1. Morphological changes of pulmonary arteries in PAH. Lung tissues from healthy donors (**A, D**) and patients with IPAH (**B, C, E, F**) were stained with Elastica van Gieson (**A**, **B**, **C**) and immunostained for detection of media layer (α-smooth muscle actin (violet)) and von Willebrand factor as a marker for endothelium (brown) (**D**, **E**, **F**). Black arrow indicates the neointima formation and red arrow indicates the plexiform lesions formation. Scale = 20µm. Photomicrographs are done by author and Ewa Bieniek.

Neomuscularization of normally non-muscularized distal pulmonary arteries is associated with increased proliferation of vascular smooth muscle cells and consequently the thickening of media layer[10;15;16]. The proliferation and hypertrophy of medial smooth muscle cells in the proximal muscular arteries also take a place in pathogenesis of PAH. Additionally to the changes in the media layer, there is proliferation of fibroblasts in *adventitia* along with collagen deposition[15;17].

Neointima is a typical pathomorphological formation in patients with PAH and represents a novel structural layer localized between the vascular endothelium and *lamina elastica interna*[10;15]. Neointima layer is composed of vascular cells and extracellular matrix. The accumulating body of evidence suggests that proliferating vascular cells from the *media* and *adventitia* of arteries migrate to the sub-endothelial space. As the neointima formation shows positive staining for α-smooth muscle actin, it is supposed that the present cells in this novel layer may represent a specialized subpopulation of smooth muscle cells[18]. However, the exact origin of the cells that create the neointima is not known. The fibroblasts can also migrate into the media and transform into smooth muscle cells[19]. On the other side, the endothelial cells can differentiate into smooth muscle cells when stimulated with platelet derived growth factor (PDGF)[20].

Plexiform lesions are another important structural feature of the pulmonary hypertension pathology and are found in 80% of PAH cases[15]. The disorganized proliferation of endothelial cells leads to the development of plexiform lesions[15]. These formations are characterized by a small neovessels, arising from the arteries. The cells that are involved in creation of plexiform lesions are endothelial cells, supported by stroma containing matrix proteins and myofibroblasts that express α-smooth muscle actin[15;21].

1.1.4. *In situ* thrombosis and inflammation in pulmonary hypertension

In situ thrombosis is another important finding in pulmonary vessels from patients with PAH. It can contribute significantly to the progression and prognosis of the disease[22]. The different coagulation and fibrinolytic abnormalities are found in plasma samples from patients with pulmonary hypertension, such as increased levels of fibrinolytic inhibitor plasminogen activator 1 and decreased levels of a soluble thrombomodulin[23].

An accumulating body of evidence implicates the role of inflammation in pathogenesis of pulmonary hypertension[24;25]. The augmented levels of inflammatory cell infiltrates (macrophages, mast cells, T and B lymphocytes and dendritic cells) are found in remodelled pulmonary vessels[25;26]. Upon pathological stimulation these cells release a plethora of different mediators that may contribute to the pulmonary vascular remodelling process.

1.1.5. Current treatment options

The pulmonary hypertension pathogenesis and pathology are the subjects of intensive research, but the precise mechanisms of the disease are not fully understood and successful therapeutic strategy to cure the disease is still needed[27]. During the years many different therapeutic options were investigating for the treatment of pulmonary hypertension, such as phosphodiesterase (PDE)-5 inhibitors (sildenafil), prostacyclin analogs (treprostinil, iloprost and beraprost) and endothelin-receptor antagonists (bosentan, sitaxentan)[27-31]. Although these therapeutic approaches importantly improved the quality of life and prolonged survival of the patients with pulmonary hypertension, a novel clinical options to achieve the ultimate goal of reversing the progressive pulmonary vascular remodelling and right ventricular hypertrophy are more than needed.

1.2. Animal models of pulmonary hypertension

1.2.1. The hypoxic animal model of pulmonary hypertension

The pathological changes associated with high altitude disease in cows (*Brisket* disease) were described almost one hundred years ago by the work of George Glover and Issac Newsom. In 1946. Ulf von Euler and Goran Liljestrand found that the reduction of the alveolar oxygen pressure provokes strong pulmonary arterial vasoconstriction. A short exposure to hypoxia leads to acute pulmonary vasoconstriction, while prolonged (chronic) exposure induces many different pathological events additionally to vasoconstriction (production of vascular endothelial growth factors, decrease of apoptosis, activation of hypoxia-inducible factor (HIF)-1α) that finally result in development of pulmonary vascular remodelling[7]. Hypoxic exposure of mice and rats are well accepted models of pulmonary hypertension. The chronic hypoxia is induced either by normal air at hypobaric pressure or oxygen-poor air at normal pressure. Hypoxia-induced pulmonary hypertension in rats and mice is characterized by *de*

novo muscularization of small, normally non-muscular arteries, elevation of pulmonary arterial pressure and development of right ventricular hypertrophy[32;33]. Although in both species the alterations caused by hypoxia are similar, the remodelling process and hemodynamic changes in rats are more severe than in mice[6;34]. As every other animal model of human disease, the hypoxic model also has a several limitations. In contrast to human situation where the patients do not respond to oxygen therapy, the hypoxia-induced pulmonary hypertension in mice and rats is reversible after return the animals to normoxic conditions. Additionally to that, this model is not associated with the development of neointima or plexiform lesion formation that strongly characterize the human disease. However, it was shown recently that hypoxia in combination with vascular endothelial growth factor receptor (VEGFR)-2 inhibitor SU5416 injection in rats leads to development of pulmonary vascular changes like neointima and this model may be more relevant with human situation [35;36].

1.2.2. Monocrotaline (MCT) animal model of pulmonary hypertension

A pyrrolizidine alkaloid monocrotaline (MCT), the main toxic substance extracted from the plant *Crotalaria spectabilis*, is often used to induce experimental pulmonary hypertension and this model was introduced in 1961[37]. Upon a single injection of MCT, usually at the dose of 60mg/kg, the severe pulmonary vascular disease is developed in experimental animals. Although this model was very frequently in use for many years and decades, the basic mechanism that underlies the pulmonary hypertension induction by MCT is still not fully understood. It is well accepted that MCT is not intrinsically toxic, but it must be activated by hepatic cytochrome P450 3A. The cytochrome P450 3A activates the MCT pyrrolizidine alkaloid to the reactive MCT pyrrole, which is the initial dehydrogenation product of MCT[38;39]. The initial and early target of MCT intoxication is pulmonary vascular endothelium that also plays a central role in human pulmonary hypertension[40]. The injury of vascular endothelium is followed by the extravascular leakage of proteases that react on the components of extracellular matrix. These pathological events are followed by the triggering of early inflammatory response[41]. After that the phase of increased reactivity to vasoconstrictors occurs and subsequently leads to the progressive thickening of the media layer of pulmonary arteries. Finally, these underlying mechanisms result in progressive elevation of pulmonary arterial pressure and right ventricular systolic pressure, and ultimate development of right ventricular hypertrophy.

Different animal species react differently to MCT injection. Some animals, for example mice, are resistant to MCT intoxication and do not develop pulmonary hypertension[42]. The most accepted and widely used animals for MCT-induced pulmonary hypertension are rats.

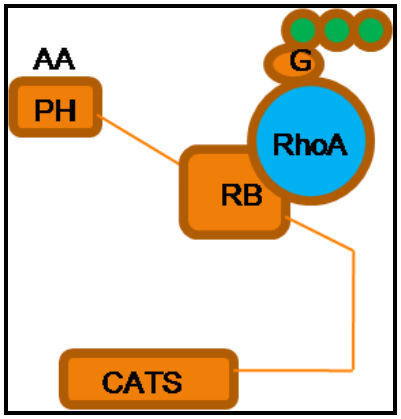
Although some features of human pathology (neointima and plexiform lesions) are not covered by this model, some other important pathological characteristics, such as initial endothelial injury, increased perivascular inflammation and *de novo* muscularization of small pulmonary arteries are shared features between human disease and MCT animal model. These facts clearly suggest this model as a useful tool not only for the investigation of the mechanisms of the disease, but also for the discovery and evaluation of a novel therapeutic strategies to cure this severe and life-threatening disease. Recently it was found that combination of MCT injection and one-sided pneumonectomy causes the development of neointima and vascular obliteration of small pulmonary arterioles, suggesting this improved MCT model for future use as it reproduces these important morphological features of human disease[16].

1.3. RhoA and Rho-kinase (ROCK) signalling

The small GTPase RhoA is one of the members of the Rho (**R**as **ho**mologous) protein family that regulate cellular functions such as contraction, motility, proliferation and apoptosis, and Rho-kinases (ROCKs) are the best characterized downstream targets for RhoA[43]. In general, RhoA act can be considered as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound molecular conformation that interacts with downstream effectors to induce a certain cellular response[44].

GTP-bound active form of RhoA translocates to the plasma membrane and activates its targets, including the two isoforms of the serine/threonine Rho-kinase (ROCK): ROCK-1 and ROCK-2[45-48]. ROCK protein structure comprises a kinase (catalytic) domain (CATS) located at the amino (N)-terminus of protein, followed by a coiled-coil region that contains Rho-binding domain (RB) and pleckstrin-homology domain (PH)[44]. The C-terminal region of ROCKs has a function as a negative regulatory region responsible for autoinhibition of the ROCKs activity in resting cells, probably via interaction with the catalytic part of the molecule[49]. The activation of ROCKs can be done by binding of the active GTP-bound RhoA

to RB domain (scheme 1)[44]. Additionally, ROCKs can be activated also by a lipid messengers such as arachidonic acid.



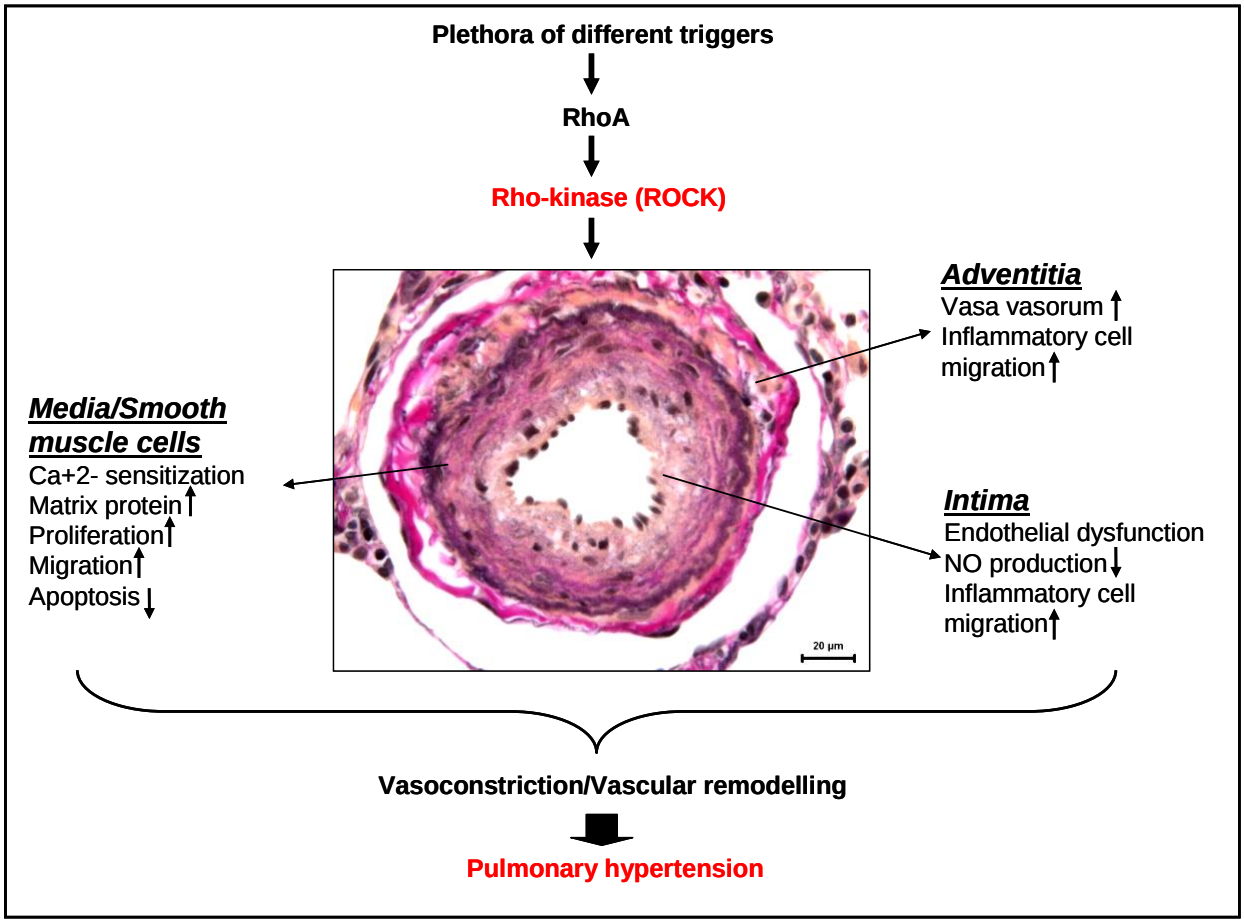
Scheme 1. RhoA activated Rho-kinase – molecular structure. Legend: **PH** – pleckstrin-homology domain; **RB** – RhoA binding domain; **G** – GTP; **CATS** – catalytic domain; **AA** – arachidonic acid.

Both ROCK isoforms are ubiquitously expressed in tissues including vasculature and heart[50]. Owing to its role in key cell functions, hyperactive ROCK signalling results in cardiovascular disorders associated with sustained abnormal vasoconstriction and promotion of vascular remodelling[44;48].

1.4. Role of Rho-kinase in pulmonary hypertension

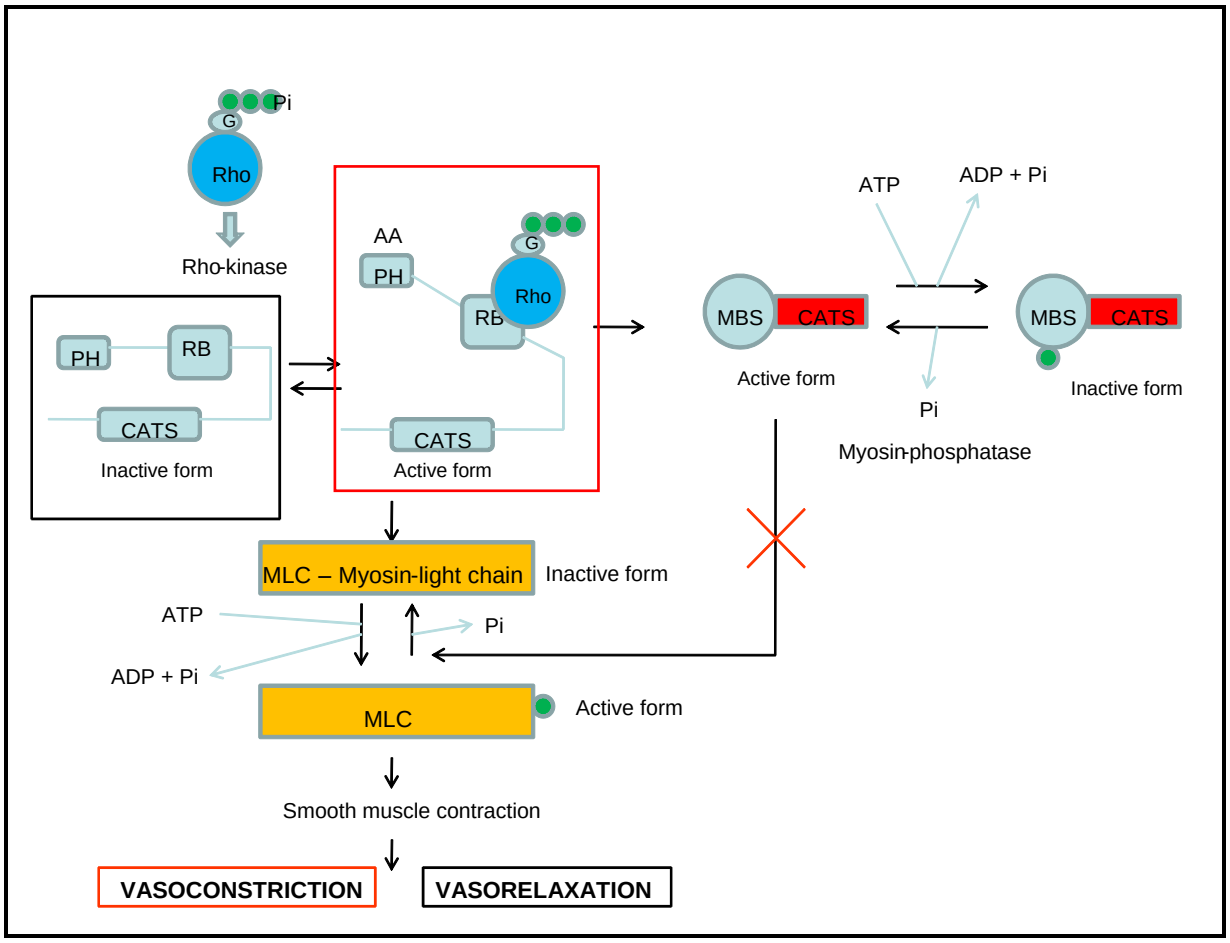
Abnormally activated Rho-kinase (ROCK) signalling may be involved in the pathomorphological changes in all three layers of pulmonary arteries (scheme 2)[51]. Hyperactive ROCKs may affect the *intima* layer of pulmonary vessels and cause the endothelial dysfunction, decreased nitric oxide (NO) production and increased inflammatory cell migration. Also, ROCK signalling may be involved in pathological events related to the *media* layer, such as increased proliferation, migration and resistance to apoptosis of smooth muscle cells and induction of vasoconstriction. Finally, augmented Rho-kinase activity may contribute to the increase of inflammatory cell migration and of *vasa vasorum* in *adventitia* of pulmonary arteries[48;51].

Rho-kinase is significantly involved in contraction, differentiation, proliferation and migration of pulmonary vascular smooth muscle cells[44;51].



Scheme 2. Roles of Rho-kinase in pulmonary hypertension. The possible involvement of Rho-kinase in different pathological events related to the development of pulmonary hypertension. (↑) – increase or (↓) – decrease of a specific event. Modified from Fukumoto *et al*, 2007.

Rho-kinase plays an important role in smooth muscle cell (SMC) contraction and it is suggested from the literature that this signalling is one of the most important regulators of vascular tone[52]. The major regulatory mechanism of SMC contraction is phosphorylation/dephosphorylation of myosin-light chain (MLC)[44;53]. Active Rho-kinase phosphorylates MLC and translates it from inactive to active form that further induces the smooth muscle contraction[44;54]. Additionally to that, active Rho-kinase phosphorylates myosin-phosphatase (MYPT) and inactivates it. Inactivation of MYPT prevents the active MYPT-mediated dephosphorylation of MLC and its subsequent deactivation[54]. Thus, Rho-kinase and MYPT co-ordinately regulate the phosphorylation state of MLC (scheme 2´).

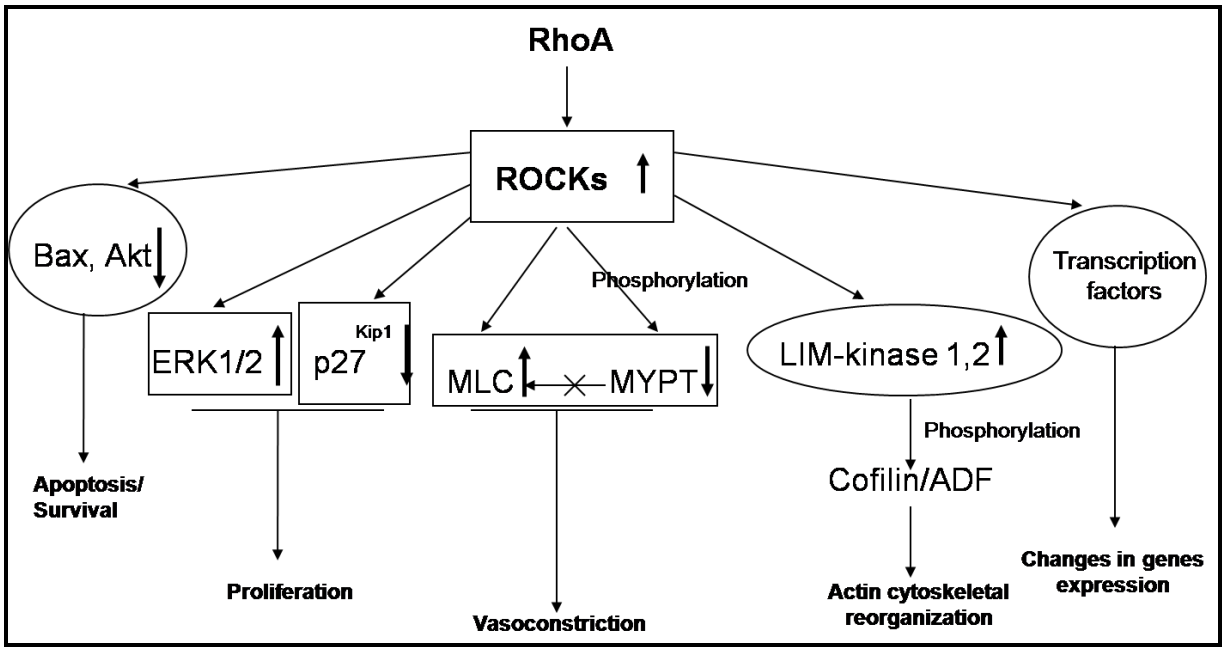


Scheme 2´. Rho-kinase function in vasoconstriction/vasorelaxation processes. Legend: **CATS** – catalytic domain; **RB** – RhoA binding domain; **PH** - pleckstrin-homology domain; **G** – GTP; **AA** – arachidonic acid; **MBS** – myosin-binding subunit.

Rho-kinase also plays a role in vascular smooth muscle cell (VSMC) proliferation (scheme 3). Rho-kinase participate in platelet-derived growth factor (PDGF)-BB-induced activation of extracellular-regulated kinase ½ (ERK ½) and proliferation of VSMCs[55]. Additionally, it was found that VSMC proliferation induced by thrombin and urotensin-II is inhibited by Rho-kinase blockers, suggesting an involvement of Rho-kinase in G-protein-coupled receptor-stimulated cell proliferation[56;57]. Also, active Rho-kinase downregulates cyclin-dependent kinase inhibitor $p27^{Kip1}$ expression and subsequently accelerates the cell cycle progression[58;59].

The literature suggests the role of Rho-kinase in VSMC migration process. It was found that blockade of Rho-kinase leads to inhibition of VSMCs migration induced by PDGF and

lysophosphatidic acid[60]. Additionally, Rho-kinase inhibition blocks the UTP- and thrombin-induced SMCs migration[61;62].



Scheme 3. Rho-kinase signalling. Rho-kinase involvement in different cellular processes.

Accumulating body of evidence incriminates the role of Rho-kinase in endothelial cell functions. ROCK is involved in the regulation of endothelial permeability via Rho-kinase-dependent regulation of actin cytoskeleton organization and cellular contractility. ROCK also activates LIM-kinase 1, which is responsible for thrombin-induced endothelial barrier disrupction involved microtubule disassembly[44;63;64]. Additionally and importantly, it was found that Rho-kinase negatively regulates NO production by endothelial cells, suggesting the contribution of ROCK in disturbing the balance of NO/endothelial NO synthase (eNOS) system[65-67].

1.5. Inhibition of Rho-kinase as a strategy for the treatment of pulmonary hypertension

A plethora of different studies on animal models of pulmonary hypertension, such as chronic hypoxia-, MCT-, vascular endothelial growth factor receptor inhibition and chronic hypoxia-, pneumonectomy and MCT- and bleomycin-induced pulmonary hypertension suggest that increased ROCK expression and activity is involved in the pathogenesis of this disease[68-75]. Moreover, accumulating data clearly implicate ROCK signalling in clinical pulmonary

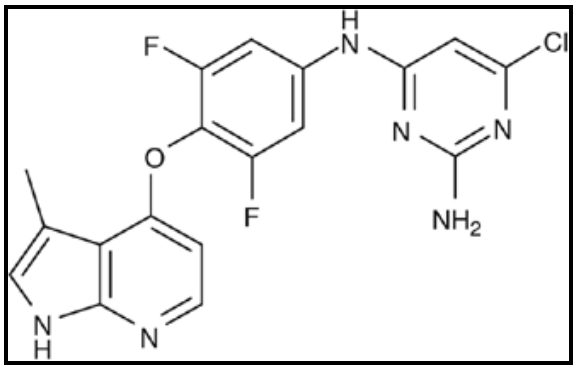
hypertension and suggest that ROCK inhibition provides beneficial acute effects in patients suffering from this severe and life-threatening disease[76-82].

Fasudil and Y-27632 are two most commonly investigated ROCK inhibitors[83;84]. These inhibitors have been used to evaluate and establish the role of Rho-kinase in the pulmonary hypertension pathology[75;85]. It was shown *in vivo* that Fasudil significantly reduced the MCT-induced pulmonary hypertension in rats and inhibited the development of pulmonary hypertension in chronic hypoxia mice, suggesting Rho-kinase as a promising target for the treatment[68;69].

Although Fasudil exerts the beneficial effects, the data obtained with this compound should be interpreted with caution because it is also a relatively potent inhibitor of other kinases, for example protein kinase C[86]. Additionally to that, ROCK inhibitors show the discrepancy in their efficacy depending on the dose, route of administration and animal model, suggesting the importance and need to find more potent and selective Rho-kinase inhibitor[48].

1.6. Azaindole-based Rho-kinase inhibition

Recently, a highly selective and orally active azaindole-based ROCK inhibitor, **azaindole-1** (6-chloro-N^4-{3,5-difluoro-4-[(3-methyl-1H-pyrrolo[2,3-b]pyridin-4-yl)oxy]phenyl}pyrimidine-2,4-diamine) has been reported (scheme 4)[87;88]. This novel ROCK inhibitor acts in an ATP-competitive manner with activity in the lower nanomolar range (half maximum inhibitory concentration (IC_{50}) of 0.6nM and 1.1nM for human ROCK-1 and ROCK-2, respectively), suggesting that this inhibitor is a very potent compound[87].



Scheme 4. Structural chemical formula of azaindole-1.

Importantly, although ATP-competitive, azaindole-1 was inactive against 89 of different kinases and exhibited only a weak activity against 21 kinases, suggesting that this compound is a highly selective ROCK inhibitor[87].

Moreover, oral administration of azaindole-1 induces a dose-dependent decrease in blood pressure without inducing a significant reflex increase in heart rate of normotensive and spontaneously hypertensive rats[87]. However, the therapeutic potential of azaindole-1 in animal models of pulmonary hypertension has not yet been investigated.

1.7. Aims of the study

The aims of this study were to investigate the effects of a novel Rho-kinase inhibitor azaindole-1 on:

1) acute hypoxic pulmonary vasoconstriction (HPV) in isolated, ventilated and buffer-perfused mice lungs
2) proliferation of rat pulmonary arterial smooth muscle cells (PASMCs) *in vitro*
3) hemodynamics, right ventricular hypertrophy and pulmonary vascular remodelling in experimental pulmonary hypertension induced by MCT-injection in rats
4) hemodynamics, right ventricular hypertrophy and pulmonary vascular remodelling in experimental pulmonary hypertension induced by chronic hypoxic exposure in mice
5) pulmonary vascular cell proliferation *in situ* by immunohistochemistry of the lungs of MCT-injected rats for proliferating cell nuclear antigen (PCNA)
6) Rho-kinase activity by immunohistochemistry of the lungs of MCT-injected rats for phospho-myosin phosphatase target subunit 1 (p-MYPT1).

2. MATHERIALS AND METHODS

2.1. MATHERIALS

2.1.1. Substances and reagents

Animal experiments

Monocrotaline (Crotaline®)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Sodium-hydroxide 1N (1mol/l)	*Merck, Darmstadt, Germany*
Chlorhidric acid 1N (1mol/l)	*Merck, Darmstadt, Germany*
Enrofloxacine (Baytril 2.5%®)	*Bayer Vital GmbH, Leverkusen, Germany*
Isoflurane	*Baxter Deutschland GmbH, Unterschleissheim, Germany*
Transcutol (32230) (Diethylene glycol monoethyl ether)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Cremophor (Cremophor®, 95921)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Distilled water	*B.Braun Melsungen AG, Melsungen, Germany*
Fasudil (H139)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Y-27632 (Y0503)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Azaindole-1	*BayerHealth Care AG, Wuppertal, Germany*
Ketamin-hydrochloride (100 mg/ml) (Ketavet®)	*Pharmacia GmbH, Berlin, Germany*
Medetomidin-hydrochloride (1mg/ml) (Domitor®)	*Pfizer GmbH, Berlin, Germany*
Povidone-iodine solution (Braunoderm®)	*B.Braun Melsungen AG, Melsungen, Germany*
Physiological saline solution (0.9% NaCl)	*B.Braun Melsungen AG, Melsungen, Germany*
Heparin (Heparin-Natrium-25.000-ratiopharm®)	*Ratiopharm GmbH, Ulm, Germany*
Formaline (Formaldehyd-Lösung 3.5-3.7%)	*Otto Fischar GmbH&Co.KG, Saarbrücken, Germany*

- Specific reagents for transmitter implantation

Bepanthen crème	*Bayer, Leverkusen, Germany*
Antisedan	*Pfizer GmbH, Berlin, Germany*
Anticoagulant gel	*Dataquest A.R.T.2.1. Data Sciences Inc., MN, USA*

- Specific reagents for catheterization of rats and mice

Ventilation gas (50%O_2, 50% N_2)	*Air Liquid, Siegen, Germany*
Rompun 2% (Xylazin-hydrochloride)	*Bayer, Leverkusen, Germany*

- Specific reagents for isolated murine lungs

Dimethyl-sulfoxide (DMSO)	*Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Krebs-Henseleit electrolyte solution	*Serag-Wiessner KG, Naila, Germany*
Pentobarbital sodium (100 mg/kg)	*Merial GmbH, Hallbergmoos, Germany*

Normoxic ventilation gas *Air Liquide, Ludwigshafen, Germany*
(21% O_2, 5.3% CO_2, balanced with N_2)
Hypoxic ventilation gas *Air Liquide, Ludwigshafen, Germany*
(1% O_2, 5.3% CO_2, balanced with N_2)

Cell culture

DMEM/F12 medium *Invitrogen, Darmstadt, Germany*
Fetal calf serum (FCS, 0.1% and 10%) *PAA Laboratories GmbH, Pasching, Austria*
DMSO *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
HBSS *Gibco BRL, Eggenstein, Germany*
3-4,5-dimethylthiatol-2,5 diphenyl tetrabromide (MTT) *Promega, Mannheim, Germany*
(CellTiter 96AQ kit)
Methyl-^{3}H-Thymidine *Amersham Biosciences, Munich, Germany*
Trichloroacetic acid (TCA) *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
0.1M NaOH *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*

Histology

Paraffin embedding medium (Paraplast Plus®) *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Sodium-chloride *Carl Roth GmbH&Co, Karlsruhe, Germany*
Potassium-chloride *Carl Roth GmbH&Co, Karlsruhe, Germany*
Disodiumhydrogenphosphat dihydrat ($Na_2HPO_4 \cdot 2H_20$) *Merck, Darmstadt, Germany*
Potassiumdihydrogenphosphat (KH_2PO_4) *Merck, Darmstadt, Germany*
Roti-Histol (Xylol) (Roti®-Histol) *Carl Roth GmbH&Co, Karlsruhe, Germany*
Ethanol 70%, 96%, 99.6% *Otto Fischar GmbH&Co.KG, Saarbrücken, Germany*
Isopropyl-alcohol (99.8%) *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Mounting medium (Pertex®) *Medite GmbH, Burgdorf, Germany*

- Specific reagents for medial wall thickness determination

Resorcin-Fuchsin *Waldeck GmbH&co.KG, Münster, Germany*
Nuclear Fast Red (Kernechtrot Aluminiumsulfat) *Waldeck GmbH&co.KG, Münster, Germany*

- Specific reagents for degree of muscularization assessment

Methanol *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*
Hydrogen-peroxide (50%) *Merck, Darmstadt, Germany*
Trypsin (Digest All2®) *Invitrogen Corporation, Camarillo, CA, USA*

Normal Horse Serum *Alexis Biochemicals, Grünberg, Germany*

Streptavidin-Biotin-Blocking Kit *Lunaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*

Vectastain ABC Kit *Lunaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*

Horseradish peroxidase streptavidin *Vector Laboratories, Burlingame, CA, USA*

Vector VIP (Vector®VIP substrate Kit for peroxidise SK-4600) *Vector Laboratories, Burlingame, CA, USA*

DAB (substrate Kit for peroxidase SK-4100) *Vector Laboratories, Burlingame, CA, USA*

Methyl-green (H-3402) *Vector Laboratories, Burlingame, CA, USA*

- Specific reagents for in situ proliferation and Rho-kinase activity assessments

Citrate buffer *Zymed Laboratories Inc., Invitrogen, Carlsbad, USA*

Methanol *Sigma-Aldrich Chemie GmbH, Steinheim, Germany*

Hydrogen-peroxide (50%) *Merck, Darmstadt, Germany*

Proteinase K (Dako Real™, Proteinase K (40x) *Dako Denmark A/S, Glostrup, Denmark*

Proteinase K diluent (Dako Real ™, Proteinase K diluent) *Dako Denmark A/S, Glostrup, Denmark*

Peroxidase (ImmPress™ Reagent Kit) *Lunaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*

Nova RED Substrate (Vector® Nova RED™ Substrate Kit for Peroxidase) *Lunaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*

Hematoxylin (Hematoxylin QS H-3404) *Vector Laboratories, Burlingame, CA, USA*

- Antibodies used for immunohistochemistry

Anti-α-smooth muscle actin antibody (Monoclonal, mouse anti-human) *Sigma-Aldrich, Steinheim, Germany*

Anti-von Willebrand factor antibody (Polyclonal, rabbit anti-human) *Dako Cytomation, Hamburg, Germany*

Anti-PCNA antibody (Polyclonal, rabbit) *Santa Cruz Biotechnology, Santa Cruz, CA, USA*

Anti-p-MYPT1 antibody (Polyclonal, goat) *Santa Cruz Biotechnology, Santa Cruz, CA, USA*

2.1.2. Consumables

Animal experiments

Gloves (Nitra-Tex®)	*Ansell Ltd., Tamworth, Staffordshire, UK*
Napkins	*Tork, Mannheim, Germany*
Needles (BD Microlance 3®) (18G /1.2mm x 40mm), 20G (0.9mm x 40mm), 26G (0.45mm x 13mm)	*Becton Dickinson GmbH, Heidelberg, Germany*
Syringes (Injekt®-F) (1ml, 2ml, 5ml, 20ml)	*B.Braun Melsungen AG, Melsungen, Germany*
Black thread no.16	*Coats GmbH, Kenzingen, Germany*
Medical adhesive bands 2.5cm/9.2m (3M™ Durapore™ Surgical Tape)	*3M Health Care, St.Paul, MN, USA*
Gauze 4x5 cm (Purzellin®)	*Lohmann und Rauscher, Rengsdorf, Germany*
Gauze balls size 6, unsteril	*Fuhrmann Verrbandstoffe GmbH, Munich, Germany*
Surgical instruments	*Fine Science Tools GmbH, Heidelberg, Germany* *Martin Medizintechnik, Tuttlingen, Germany*
Cannula for vein catheter support 22G (Vasocan Braunüle®)	*B.Braun Melsungen AG, Melsungen, Germany*
Instrument for venous catheterization (Intradyn ™ Venous Hemostasis Introducer)	*B.Braun Melsungen AG, Melsungen, Germany*
Silicone catheter for right heart catheterization	*Custom-made*
Polyethylene cannula for insertion into the carotid artery (Vasofix® Safety®, 22G)	*B.Braun Melsungen AG, Melsungen, Germany*
Tracheal cannula	*Custom-made from BD Microlance 3® 15 or 20G needles (Becton Dickinson, Germany) shortened to 1.5cm*
Stopcock for infusion therapy and pressure monitoring (Discofix® C-3)	*B.Braun Melsungen AG, Melsungen, Germany*
Rat restrainer (model 81)	*IITC Life Science Inc., Woodland Hills, CA, USA*
Heating underlay (ThermoLux®)	*Witte + Sutor GmbH, Murrhardt, Germany*
Eppendorf tubes (Microtubes 1.5ml)	*Sarstedt, Nürnbrecht, Germany*
Scalpels (Feather Disposable Scalpel)	*Feather Safety Razor Co, LTD, Osaka, Japan*

Cell culture

Gloves (Nitra-Tex®)	*Ansell Ltd., Tamworth, Staffordshire, UK*
Tissue Culture Dish	*Greiner Bio-One GmbH, Frickenhausen, Germany*
Tissue Culture Flask	*Greiner Bio-One GmbH, Frickenhausen, Germany*
Tissue Culture 6-well Plate	*Greiner Bio-One GmbH, Frickenhausen, Germany*

Histology

Gloves (Nitra-Tex®)	*Ansell Ltd., Tamworth, Staffordshire, UK*

Histological glass slides 25x75x1mm (SuperFrost UltraPlus®)	*R. Langenbrinck, Emmendingen, Germany*
Embedding cassettes	*Leica Microsystems, Nussloch, Germany*
Coverslips 24x36mm	*Menzel GmbH&Co.KG, Braunschweig, Germany*
Microtom blades (Microtome blade type A35)	*Feather Safety Razor Co Ltd, Osaka, Japan*
Tips for automatic pipettes 200µl, 1000µl, 10µl	*Sarstedt, Nürnbrecht, Germany*
Automatic pipettes 10-100µl, 1-10µl (Eppendorf PhysioCare concept)	*Eppendorf AG, Hamburg, Germany*

2.1.3. Systems and machines

Animal experiments

Weighing machine for animals (Sauter RP 3000)	*August Sauter GmbH, Albstadt- Ebingen, Bayer Leverkusen, Germany*
Balance for substances (Mettler Toledo PB303 Delta Range®)	*Mettler Toledo, Switzerland*
Transducers (Combitrans Monitoring Set Mod.II for Arterial Blood Pressure Management)	*B.Braun Melsungen AG, Melsungen, Germany*
Ventilation system for rats (SAR- 830/P Ventilator)	*IITH Life Science Inc., Woodland Hills, CA, USA*
Computer and screen	
Blood analyzer (Rapidlab™ 348)	*Bayer Healthcare, Fernwald, Germany*
Centrifuge (Hettich Mikro 200R)	*Hettich-Zentrifugen GmbH & Co. KG, Tuttlingen, Germany*
O_2 controller (Model 4010)	*Labotect, Göttingen, Germany*
Peristaltic pump (REGLO Digital MS-4/12)	*Ismatech SA, Glattbrugg, Switzerland*
Piston pump Minivent Type 845	*Hugo Sachs Elektronik Harvard Apparatus GmbH, March-Hugstetten, Germany*
Analog-to-digital transformer PCLD	*Advantech, Feldkirchen, Germany*
Force Transducer	*Hottinger Baldwin Messtechnik, Fuchstal, Germany*
DSI Receiver	*Dataquest A.R.T.2.1. Data Sciences Inc., MN, USA*
DSI Matrix	*Dataquest A.R.T.2.1. Data Sciences Inc., MN, USA*
DSI software	*Dataquest A.R.T.2.1. Data Sciences Inc., MN, USA*
Transmitter (TA 11PA-C40)	*Dataquest A.R.T.2.1. Data Sciences Inc., MN, USA*

Cell culture

β-counter	*Canberra Packard, Central Europe GmbH*

Spectrophotometer	*Eppendorf, Hamburg, Germany*
Cell culture incubator	*Heraeus GmbH, Hanau, Germany*
Cell culture microscope	*Helmut Hund GmbH, Wetzlar, Germany*
Counter plate reader	*TECAN, Mainz-Kastel, Germany*
Culture Hood	*Heraeus GmbH, Hanau, Germany*

Histology

Tissue dehydrating machine (Leica TP 1050)	*Leica Microsystems, Nussloch GmbH, Germany*
Tissue embedding machine (Leica EG 1140 H)	*Leica Microsystems, Nussloch GmbH, Germany*
Cooling table (Leica EG 1150 C)	*Leica Microsystems, Nussloch GmbH, Germany*
Automated microtome (Leica RM 2165)	*Leica Microsystems, Nussloch GmbH, Germany*
Flattening table (Leica HI 1220)	*Leica Microsystems, Nussloch GmbH, Germany*
Flattening bath for paraffin sections (Leica HI 1210)	*Leica Microsystems, Nussloch GmbH, Germany*
Computer (Q 550 IW)	*Leica Microsystems, Nussloch GmbH, Germany*
Software (Q Win V3)	*Leica Microsystems, Nussloch GmbH, Germany*
Light microscope (DMLA)	*Leica Microsystems, Nussloch GmbH, Germany*
Ice flake machine (Icematic F100 Compact)	*Castelmac SPA, Castelfranco, Italy*

2.2. METHODS

2.2.1. Experimental animals

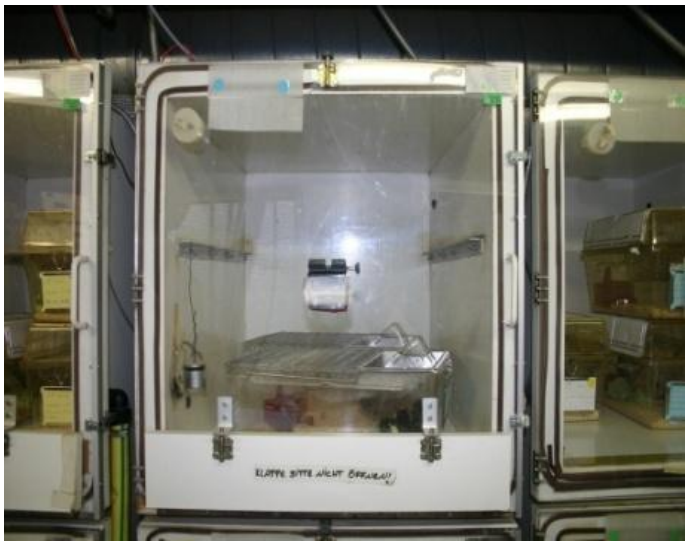
Adult male Sprague-Dawley rats (Species: *Rattus norvegicus*) (300-350g of body weight (BW)) and C57BL/6 mice (Species: *Mus musculus*) (20-22g BW) were obtained from Charles River Laboratories, Sulzfeld, Germany. Rats and mice were kept under controlled temperature conditions (~22ºC), with food and water provided *ad libitum*. All studies were performed according to the guidelines of the University of Giessen and were approved by the local authorities.

2.2.2. Monocrotaline (MCT)-induced pulmonary hypertension model in rats

A pyrrolizidine alkaloid monocrotaline (MCT), the main toxic substance extracted from the plant *Crotalaria spectabilis* is often used to induce experimental pulmonary hypertension[70;89;90]. Pulmonary hypertension in this study was induced in adult male Sprague-Dawley rats of 300 to 350g of BW via single subcutaneous administration of monocrotaline (dose: 60mg/kg) in the area of animal neck. Monocrotaline solution was freshly prepared by dissolving the alkaloid in 1N HCl and 1N NaOH. In details, 250mg of monocrotaline was dissolved in 3ml of 1N HCl and 2ml of 1N NaOH and pH was adjusted at 7.4. An injection was administered at day 0, after light inhalation anesthesia with isoflurane. Healthy control rats received only 500µl of saline solution subcutaneously under the same conditions. To avoid unwanted infections, rats were receiving an antibiotic solution (2.5% baytril) from day 1 to 15 upon MCT injection. Baytril was dissolved in the drinking water, at a concentration of 2ml of baytril in 500ml of water.

2.2.3. Chronic hypoxia-induced pulmonary hypertension model in mice

Pulmonary hypertension in C57BL/6 mice was induced by chronic hypoxic (Hox) exposure (10% O_2)[70;89-91]. The animals were kept in ventilated hypoxic chambers under the temperature range from 22-24°C (photography 2). The constant level of hypoxia was held by an autoregulatory control unit (model 4010). The humidity in the system was prevented by condensation in a cooling system and CO_2 was removing by soda lime. Mice exposed to normoxia (Nox) conditions (21% O_2) in a ventilated chamber served as a healthy control.



Photography 2. Hypoxic chamber.

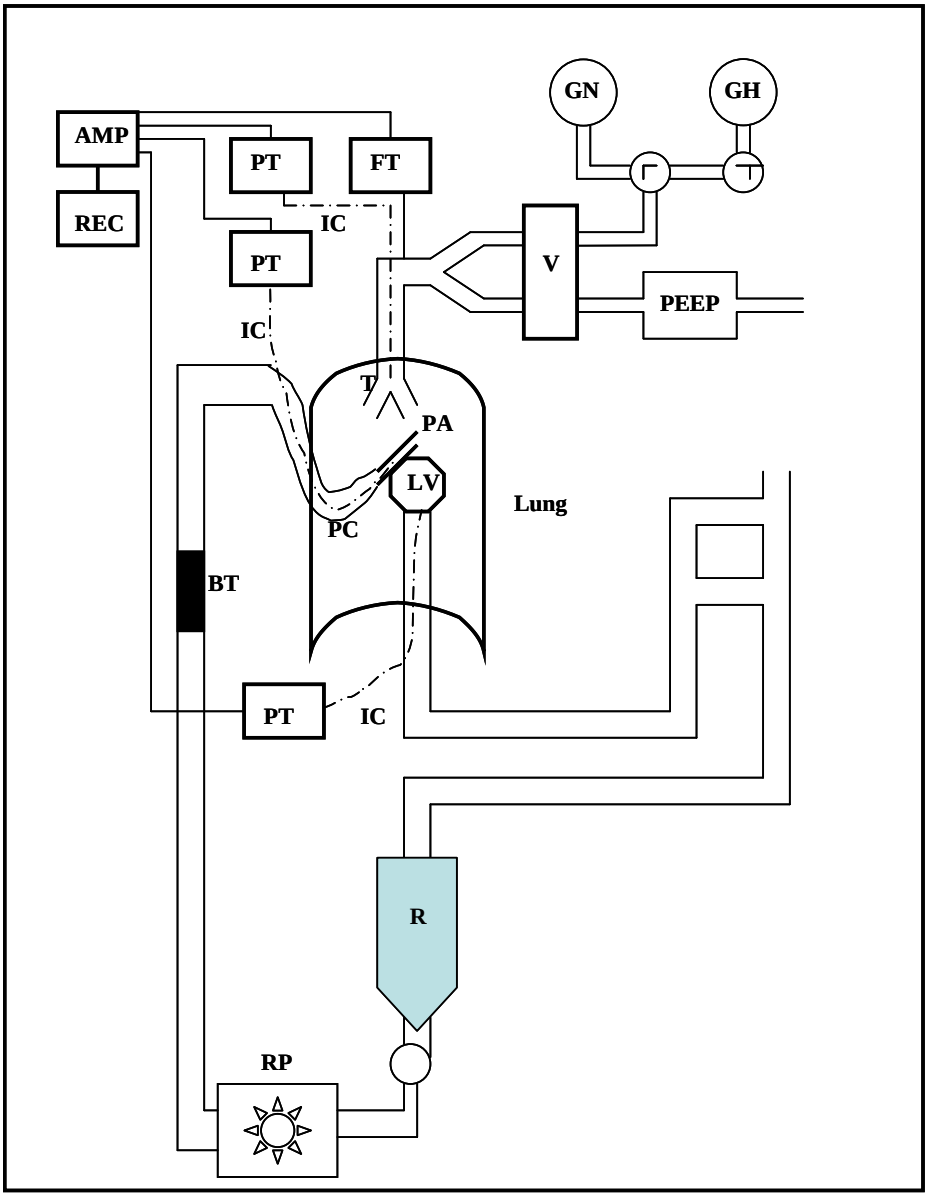
2.2.4. Isolated murine lungs

The technique of successive hypoxic maneuvers was employed in isolated, ventilated and buffer-perfused mice lungs to investigate the effect of different Rho-kinase (ROCK) inhibitors (azaindole-1, fasudil and Y-27632) on acute hypoxic pulmonary vasoconstriction (HPV)[70;92]. The technique of mouse lung isolation was performed as previously described (scheme 5)[93-95]. Mice were initially anesthetized intraperitoneally with pentobarbital sodium at the dose of 100mg/kg of BW and injected intravenously with heparin to prevent the coagulation. A median cut was made in the center of mice neck and the trachea was exposed by blunt dissection and was partially transected. After that mice were intubated and ventilated by room air with a specific piston pump (Minivert Type 845). Then the midsternal thoracotomy was performed, the ribs were spread followed by the incision of the apical part of the heart. The right ventricle was cut and a fluid-filled perfusion catheter was forwarded into pulmonary artery. After catheter insertion, the perfusion (REGLO Digital MS-4/12) with sterile and ice-cold Krebs-Henseleit solution was started at 4°C and a flow of 0.2ml/min. The composition of Krebs-Henseleit solution is depicted below (table 2).

Sodium chloride	**120mM**
Potassium chloride	**4.3mM**
Potassium dihydrogen phosphate	**1.1mM**
Calcium chloride	**2.4mM**
Magnesium chloride	**1.3mM**
Glucose	**13.32mM**
Hydroxyethylamylopectin	**5%**

Table 2. The composition of Krebs-Henseleit solution.

In parallel, the ventilation was changed from room air to a normoxic gas mixture of 21% O_2, 5.3% CO_2 balanced with N_2. Without interruption of ventilation and perfusion, the lungs, trachea and heart were excised from the thorax and were freely suspended from a force transducer to monitor lung weight gain. The second perfusion catheter was inserted via the left ventricle into the left atrium and the flow was increased from 0.2 to 2ml/min. The lungs were then washed with buffer to remove the blood and perfusion system was closed for recirculation. The left atrial pressure was set at 2mmHg and the system of isolated, ventilated and perfused lungs was placed in a temperature-equilibrated chamber and was heated to 37.5°C. The pulmonary arterial pressure was registered by pressure transducers connected to the perfusion catheters and were digitised with an analog-to-digital converter[95]. The ROCK inhibitors were prepared freshly in dimethyl-sulfoxide (DMSO). Sequential hypoxic maneuvers of 10 min duration interrupted by 15 min periods of normoxia were performed. The effect of the Rho-kinase inhibitors on pressure responses provoked by alveolar hypoxia (1% O_2, 5.3% CO_2 and balanced N_2 gas mixture) was determined within such a sequence of repetitive hypoxic maneuvers. The Rho-kinase inhibitors were added to the buffer fluid 5 min after a hypoxic challenge, with the addition starting after the second hypoxic maneuver was accomplished. Cumulative dose effect curves were established by addition of the inhibitors (dose range: 0.1-30.0μM). Controls received the vehicle (DMSO) only[70].



Scheme 5. Isolated, ventilated and perfused mouse lung system. AMP-amplifier, **BT**-bubble trap, **FT**-force transducer, **GN**-normoxic gas, **GH**-hypoxic gas, **IC**-intraluminal catheter, **LV**-left ventricle, **PA**-pulmonary artery, **PC**-perfusion catheter, **PEEP**-positive end-expiratory pressure, **PT**-pressure transducer, **R**-reservoir, **RP**-roller pump, **REC**-recording device, **T**-trachea, **V**-ventilator. Modified from Seeger W. *et al* (1994).

2.2.5. Cell culture (proliferation and cytotoxicity assays)

The isolation, culture and proliferation assay of primary pulmonary artery smooth muscle cells (PASMCs) were done as described in the literature[70;89]. The cells were isolated from

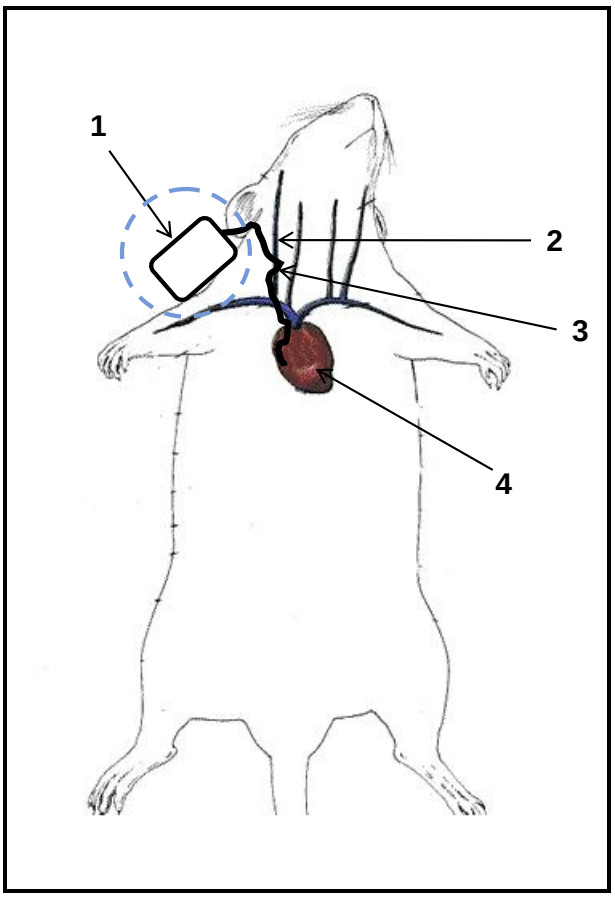
healthy and monocrotaline-injected rats (day 21, n = 3) and cultures were maintained at 37°C in a humidified 5% CO_2 - 95% O_2 atmosphere. Equal number of PASMCs (~ $4x10^4$ cells/well) was seeded and the following day the medium was substituted with DMEM/F12 containing 0.1% fetal calf serum (FCS) to render the cells quiescent. After 24h serum starvation, cells were induced to cell cycle re-entry by FCS (10 %) together with different concentrations of ROCK inhibitors (fasudil, Y-27632 and azaindole-1 (500, 1000 and 5000nM in DMSO)) for 24h, including in the last 12h the incorporation of [^{3}H]-thymidine (1.5μCi/ml). Cells were then washed twice with 500μl chilled HBSS, fixed with 250μl ice-cold methanol and precipitated by 250μl 10% trichloroacetic acid (TCA). Finally samples were lysed in 0.1M NaOH and transferred to 4ml scintillation solution and counted by a β-counter. The values are expressed as disintegration per minute (dpm). All the experiments were done in triplicate. Cell viability/cytotoxicity was assessed by the MTT assay using a CellTiter 96AQ kit (Promega) according to the manufacturer´s instructions. The cells were plated in 96-well plates and allowed to attach for 6h, and then cultured under serum-free conditions with various concentrations of fasudil, Y-27632 and azaindole-1 for 48h. The number of surviving cells was determined by measuring the absorbance at 560nm (*A*560 nm) of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt for 1 hour. All of the experiments were carried out in triplicate[70].

2.2.6. Radio-telemetry study

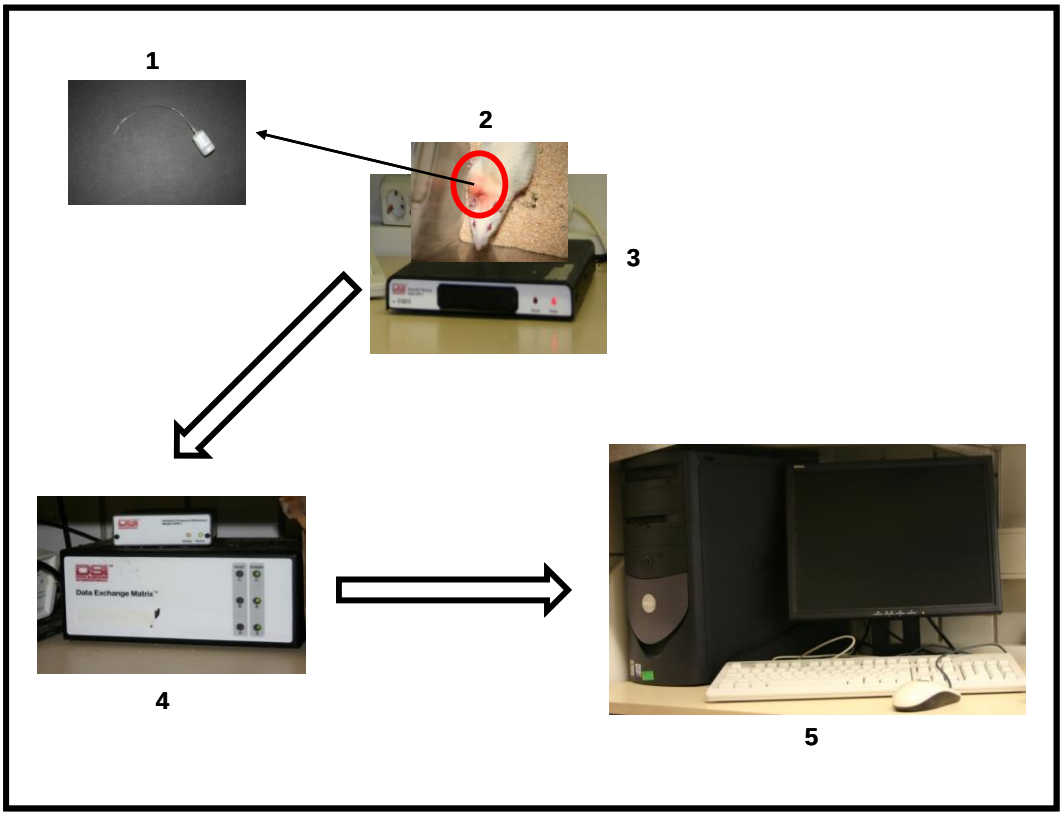
To investigate the effects of azaindole-1 on progressive elevation of right ventricular systolic pressure (RVSP) and on heart rate (HR), the online radio-telemetry measurement (Dataquest A.R.T. 2.1; Data Sciences Inc.) was performed as described previously[90;96;97]. A catheter connected to a fluid filled sensor (transmitter) was inserted into the jugular vein and forwarded to the right ventricle (RV) of rats under anesthesia. To anesthetize the animals the combination of ketamine and domitor was used in the ratio of 10 : 1 (v/v). The transmitter was placed under the skin in the area of animal back. The signal from the transmitter (model TA11PA-C40) was transferred to a remote receiver and a data-exchange matrix connected to a computer (scheme 6b). The top of the telemetry catheter was filled up with anticoagulant gel to avoid the blood coagulation. The waveform was displayed on the computer and used to ensure correct positioning of the catheter. After the surgery the animals received a light dose of anti-anesthetic (antisedan) to wake up easier and for the next 2 weeks were receiving antibiotic baytril to reduce the unwanted infections due to surgery. Animals were allowed to

recover for 3 to 4 days before the induction of pulmonary hypertension by monocrotaline and were housed individually in standard rat cages. Monocrotaline (MCT)-injected rats were randomized into two groups and they received either azaindole-1 or placebo from day 21 for two weeks. Azaindole-1 was prepared daily in transcutol-based vehicle. Briefly, the compound was dissolved in a special vehicle (the mixture of transcutol, cremophor and distilled water in the ratio of 10 : 20 : 70 (v/v/v)) to reach the wanted dose. Rats were treated by oral gavage at the dose of 10 mg/kg BW/day. The placebo group received only vehicle. RVSP and HR were recorded once per day in duration of 10 minute over the next 35 days from the time of MCT injection. The dose of azaindole-1 was selected based on literature and our own pilot experiments[87].

a)



b)

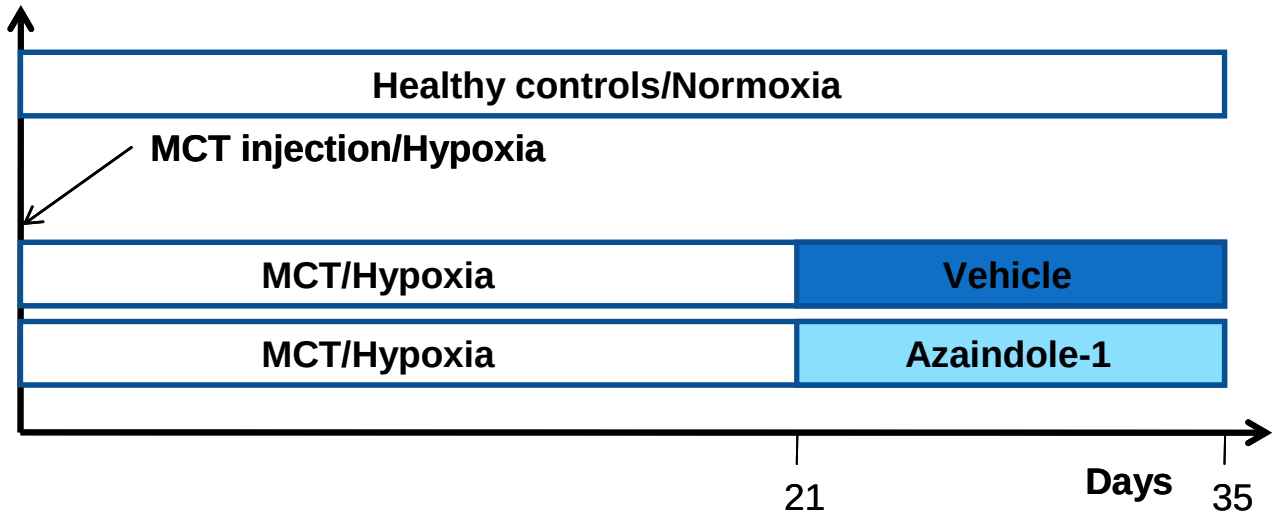


Scheme 6. Online telemetry system for monitoring hemodynamics and heart rate. (**a**) Transmitter surgical implantation. **1** – transmitter, **2** – right jugular vein, **3** – fluid-filled catheter, **4** – heart. (**b**) Radio-telemetry system. **1** – transmitter; **2** – the animal with inserted transmitter/catheter complex placed on **3** – receiver; **4** – matrix; **5** – software for data analysis (Data Sciences Inc., MN, USA).

2.2.7. Chronic treatment study – experimental design

To investigate the therapeutic efficacy of azaindole-1 in animal models of pulmonary hypertension (PH), chronic treatment studies were performed. The MCT-rats (n=30) were randomized into two groups and treated orally by gavage from 21 to 35 days either with azaindole-1 (dose: 10mg/kg BW/day) or placebo (a transcutol-based vehicle). Saline injected rats served as healthy control (n=10). Mice exposed to chronic hypoxia (n=16) were treated daily with azaindole-1 (dose: 30mg/kg BW/day) or placebo from 21 to 35 days. As

mentioned, the dose of azaindole-1 was selected based on literature and our own pilot experiments and prepared daily for oral application[87]. Control mice (n=6) remained under normoxia (21% O_2). At the end of experiment (day 35 of the MCT-injection in rats or chronic hypoxic exposure of mice) the animals were sacrificed for hemodynamic and right ventricular hypertrophy measurements. The experimental protocol is depicted below (scheme 7).

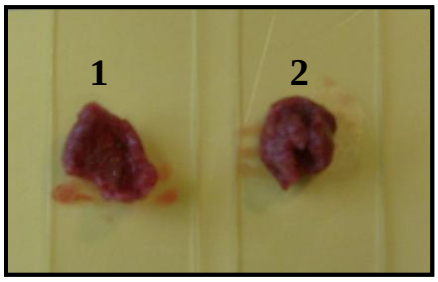


Scheme 7. Chronic treatment study – experimental design.

2.2.8. Hemodynamic and right ventricular hypertrophy (RVH) measurements

Right ventricular systolic pressure (RVSP) was measured by a catheter inserted into the right ventricle (RV) via the right jugular vein and for systemic arterial pressure (SAP) the left carotid artery cannulation was performed as described[70;89;90]. Rats were initially anesthetized by intraperitoneal injection with combination of ketamine and domitor solutions in the volume ratio of 10:1. After that the tracheotomy was performed and animals were artificially ventilated with a mixture of oxygen and nitrogen (1:1), at a constant frequence of 60 breaths/min, with an inspiratory flow rate of 500 – 600cc/min. The inspiratory time was 0.5 seconds and the positive end expiratory pressure (PEEP) was set to 1cmH_2O. The left carotid artery was isolated and cannulated for the measurement of SAP. A cannula was connected to a fluid-filled force transducer. The right heart catheterization was performed for measurement of RVSP. The right jugular vein was isolated and the home-made silicone catheter was connected to a fluid-filled force transducer. The catheter was inserted into the jugular vein and

forwarded into the right ventricle under the guidance of pressure tracing. To prevent the blood coagulation a volume of 1ml of heparin solution was administered through the jugular vein. The fluid force transducers were calibrated at 0 to the hillum level before the beginning of experiment. The Labtech Notebook Runtime Version 9.02 computer software was recording the ventilation pressure, SAP and RVSP for 5 – 10 minutes. Hemodynamic changes in mice were measured similarly as for the rats with small modifications. Mice were anesthetized with combination of ketamine, rompun and saline in the volume ratio of 1:1:2. Before receiving the anesthesia the mice were injected with heparin to reduce the effect of blood coagulation. Mice were tracheotomised and artificially ventilated. The rest of the procedure was the same as for the rats. At the end of experiments, the hearts of both animal models were extracted and dissected to separate right ventricle (RV) from left ventricle plus septum (LV+S), and the weight ratio RV/(LV+S) was calculated as a measurement of right ventricular hypertrophy (RVH) (scheme 8).



Scheme 8. Right heart hypertrophy measurements. The heart ratio was determined as the weight ratio of right ventricle (**1**) and left ventricle plus septum (**2**).

2.2.9. Measurements of cardiac output and total systemic and pulmonary vascular resistance

After hemodynamic measurements the rat blood was collected from jugular vein and carotid artery. Hemoglobin (Hb), arterial and venous saturation were measured by blood gas analyzer (Rapid lab^{TM} 348). Cardiac output was calculated using the Fick principle, by employing the mixed venous oxygen and the arterial oxygen content as previously described[98]. Cardiac index

(CI) was assessed as a cardiac output normalized to body weight (BW) of rats. Total pulmonary resistance (TPR) was determined by dividing RVSP with CI, while total systemic resistance was assessed by dividing SAP with CI. The detailed formulas with measure units are presented in the table 3[99].

CO (ml/min) = 5.46/ ((Hb·arterial saturation·0.0134) – (Hb·venous saturation·0.0134))
CI (ml/min·100 g BW) = CO·100/BW
TPR (mmHg·100 g BW ·min / ml) = RVSP / CI
TSR (mmHg·100 g BW·min / ml) = SAP / CI

Table 3. Formulas for calculating the cardiac output (CO), cardiac index (CI), total pulmonary resistance (TPR) and total systemic resistance (TSR). Legends: **Hb** – hemoglobin; **BW** – body weight; **RVSP** – right ventricular systolic pressure; **SAP** – systemic arterial pressure.

2.2.10. Lung tissue processing

After the hemodynamic measurement and collection of the blood samples, the abdomen and thoracic cavity were opened and the both heart ventricles were incised in order to allow the removal of blood[89]. The right ventricle was incised at approximately 5mm below the base of pulmonary artery. The lungs were flushed out of blood at a pressure of 30cmH_2O above the pulmonary hillum by a cannula inserted into the pulmonary artery through the right ventricle and connected to a reservoir filled with saline solution. The left lungs were prepared for histology using a cannula inserted into the pulmonary artery and connected to a reservoir filled with 3.5-3.7% formalin solution. The left lung was perfused at a pressure of 30cmH_2O above the pulmonary hillum for 5 to 10 minutes, further isolated and stored in formalin

solution at 4ºC over night and then in phosphate buffer (PBS, pH=7.4). After that the lungs were placed in histological cassettes, dehydrated in an automatic dehydration machine and then embedded in paraffin (scheme 9). Sections of 3µm in diameter were cut from the paraffin blocks using a microtome.

Scheme 9. Lung tissue processing. 1 – embedding machine, **2** – microtome.

2.2.11. Histology and pulmonary vascular morphometry

In general, the assessment of pulmonary vascular remodelling was done by determination of the degree of muscularization of the rats and mice small peripheral pulmonary arteries. Intra-acinar arteries in rats and mice were analyzed by categorizing them as fully muscular, partially muscular and non-muscular. In addition, the medial wall thickness of the vessels was analyzed as another well-known parameter for determination of pulmonary vascular remodelling[70;89;90]. All analyses were done in a blinded fashion.

Medial wall thickness. After dehydration and paraffin embedding, a 3 µm sections were stained for Elastin - Nuclear Fast Red to assess the medial wall thickness, as described previously[70;89]. Medial wall thickness was defined as the distance between the *lamina elastica interna* and *lamina elastica externa*. Percentage of medial wall thickness (% MWT) was examined by light microscopy using a computerized morphometric system (Qwin, Leica, Wetzlar, Germany) and was calculated by a formula:

$$\textbf{\% MWT} = (2 \cdot \text{wall thickness}/\text{external diameter}) \cdot 100$$

Degree of muscularization. A 3 µm sections of formalin-fixed and paraffin embedded lung tissues were obtained and double immunostaining was performed with an anti-α-smooth

muscle actin antibody (αSMA) (dilution 1:900, clone 1A4, Sigma, Saint Louis, Missouri) and anti-human von Willebrand factor antibody (vWF, dilution 1:900, Dako, Hamburg, Germany), as described previously[89;90;100]. The sections were initially maintained for 60 minutes at 58-60˚C in the heating chamber. After that the sections were deparaffinized in xylol and progressively rehydrated in a graded ethanol series. The endogenous peroxidase activity was blocked by using a freshly prepared solution of hydrogen peroxide (H_2O_2) in methanol in the volume ratio of 1:1. Similarly, the endogenous biotin and streptavidine were eliminated by using a specific biotin/streptavidine blocking solutions. Antigen retrieval was performed by treatment with trypsin for 10 minutes at 37°C. For staining of pulmonary vascular smooth muscle layer and endothelium, the slides were first incubated with normal horse serum for 30 minutes to avoid the non-specific bindings caused by immunoglobulin cross-reactivity and after that incubated with primary antibodies. For vascular smooth muscle staining the slides were incubated with anti-α-smooth muscle actin primary antibody at room temperature. To detect the vascular endothelium the slides were incubated with anti-von Willebrand factor primary antibody at 37°C. Both antibodies were diluted 1:900 in 10% BSA and maintained for 30 minutes. After that the slides were washed a couple of times with phosphate-buffered saline (PBS, pH 7.4) and further incubated with the corresponding biotinylated secondary antibodies. The composition of PBS buffer is depicted below (table 4).

NaCl	80g
KCL	2g
$Na_2HPO_4x2H_2O$	11.5g
KH_2PO_4	2g
Distilled water	900ml

Table 4. The composition of PBS buffer.

Two different substrates were used to develop a colour by reaction with horseradish peroxidise/streptavidine complex coupled to the secondary antibodies: VIP substrate

determined the purple/violet colour of the smooth muscle layer and DAB determined the brown colour of the vascular endothelium. Finally, the sections were counterstained with methyl-green, then progressively dehydrated, coverslipped using mounting medium and examined by light microscopy using a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). For monocrotaline-induced pulmonary hypertension rat model the vessels of 20-50µm in size were used for analysis. For hypoxia-induced pulmonary hypertension mice model the vessels of 20-70µm in size were used for analysis. Each vessel was categorized as non-muscularized (N), partially muscularized (P) or fully muscularized (F). The percentage of pulmonary vessels in each category was determined by dividing the number of vessels in that category with the total number counted in the same experimental group.

2.2.12. Immunohistochemistry for phospho-myosin phosphatase target subunit 1 (p-MYPT1) and proliferating cell nuclear antigen (PCNA)

Paraffin-embedded lung tissue sections with thickness of 3µm were deparaffinized in xylol and rehydrated in a graded ethanol series to PBS. Antigen retrieval was performed by pressure cooking in citrate buffer (pH 6.0). The sections were pretreated with hydrogen peroxide (15%) to quench endogenous peroxidase activity. Following blocking with BSA (10%) for one hour and then with blocking serum (Impress reagent kit, Vector Laboratories, CA) for 20 minutes, the sections were incubated overnight at 4°C with primary antibodies. Rabbit polyclonal anti-PCNA and goat polyclonal anti-p-MYPT1 (Thr 696) antibodies (1: 100 and 1:20 dilutions, respectively; Santa Cruz Biotechnology Inc.) were used as primary antibodies. Development of the dye was carried out with peroxidase and substrate (NovaRed substrate kit, Vector Laboratories, CA) according to manufacturer´s instruction (Vector laboratories, CA). Finally, sections were counterstained with hematoxylin (Zymed laboratory, UK) and coverslipped using mounting medium[70]. Additionally, the PCNA positive pulmonary vascular cells were counted using a light microscope throughout the entire section and the index of proliferation (IOP) was determined as the number of PCNA positive cells per pulmonary vessel.

IOP = The number of PCNA-positive cells in the vessels/The number of the vessels

The IOP (in %) for placebo and azaindole-1 treated groups was calculated by assuming the average IOP of healthy control lungs as 100 %.

2.2.13. Data analysis

All data are expressed as mean ± SEM. The different experimental groups were analyzed by one-way ANOVA and Newman-Keuls post-hoc test for multiple comparisons. Values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered as statistically significant. Two-way ANOVA analysis with Bonferroni multiple comparison post-hoc test was performed to compare the RVSP values derived by telemetric measurement.

3. RESULTS

3.1. Effects of Rho-kinase inhibitors on acute hypoxic pulmonary vasoconstriction (HPV)

First we investigated the pulmonary vasorelaxant potency of azaindole-1 and the other commonly used ROCK inhibitors fasudil and Y-27632. The experiment was performed with isolated, ventilated and buffer-perfused murine lungs as described in methods. All the ROCK inhibitors significantly reduced the HPV in a dose dependent manner as compared with vehicle control. The maximum inhibitory effects on HPV were ~75 % for fasudil and Y-27632 and ~90 % for azaindole-1 at the highest concentration, 30μM (figure 1). The effects of fasudil and Y-27632 were comparable; however, a clear leftward shift of the dose response curve was observed with azaindole-1 as compared with fasudil and Y-27632. At the higher doses (10 and 30μM) there were significant reductions of HPV by azaindole-1 versus fasudil and Y-27632. The findings suggest that azaindole-1 is a potent pulmonary vasorelaxant and shows a stronger effects on HPV in comparison with other Rho-kinase inhibitors.

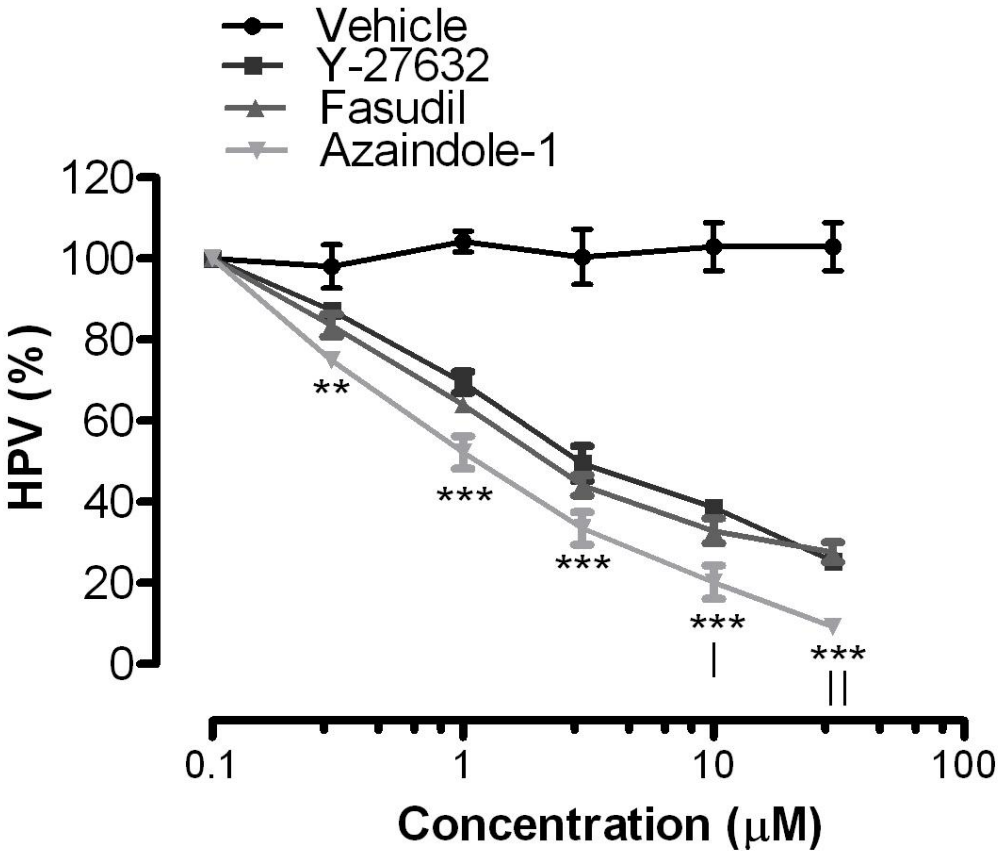


Figure 1. Effects of fasudil, Y-27632 and azaindole-1 on hypoxic pulmonary vasoconstriction (HPV). The effects of Rho-kinase inhibitors on acute HPV in isolated, ventilated and buffer-perfused murine lungs (n=5) were investigated as described in methods section. Dose-response curves are shown. Data are presented as mean ± SEM. **p<0.01 and ***p<0.001 versus vehicle; |p<0.05 and ||p<0.01 versus fasudil/Y-27632.

3.2. Effects of Rho-kinase inhibitors on cell cytotoxicity

Then we investigated if azaindole-1 and other two Rho-kinase inhibitors show any toxic effects on the pulmonary arterial smooth muscle cells (PASMCs). PASMCs viability/cytotoxicity was assessed by the MTT assay (figure 2). We did not observe any cytotoxic effects of all Rho-kinase inhibitors (fasudil, Y-27632 and azaindole-1) at the concentrations tested in our study.

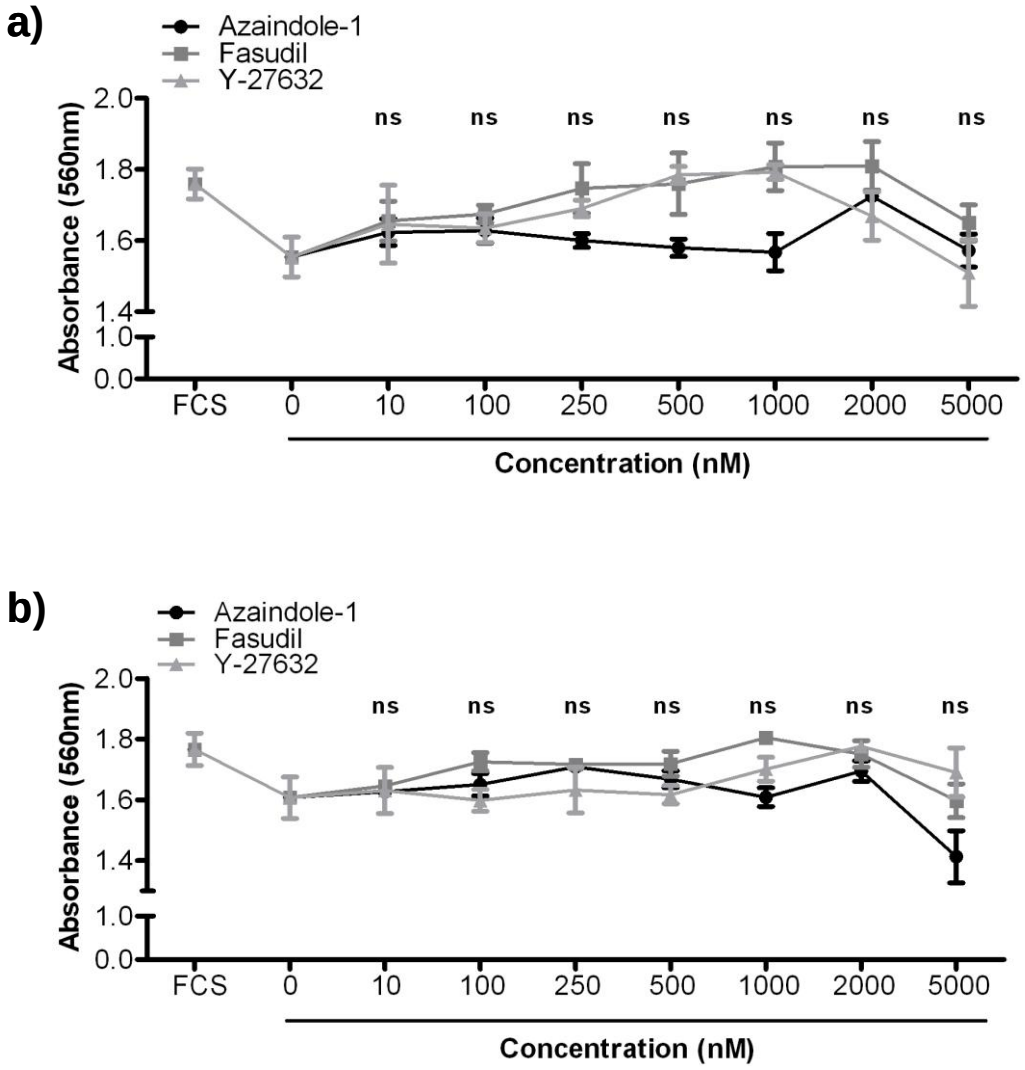


Figure 2. Effects of fasudil, Y-27632 and azaindole-1 on pulmonary arterial smooth muscle cell (PASMC) viability. The effects of Rho-kinase inhibitors on viability of PASMCs isolated from healthy and MCT-injected rats were investigated using MTT assay as described in methods. Absorbance measured at 560nm as an estimation of the number of surviving PASMCs from (**a**) healthy and (**b**) MCT-injected rats is shown (n=3). Each dot represents mean ± SEM. **ns** – not statistically significant.

3.3. Effects of Rho-kinase inhibitors on proliferation of PASMCs isolated from healthy rats

The effects of fasudil, Y-27632 and azaindole-1 on PASMCs proliferation were investigated by thymidine incorporation assay (figure 3). There were no significant effects of various concentrations of the Rho-kinase inhibitors (500, 1.000 and 5.000nM) on the thymidine incorporation into PASMCs derived from healthy rats.

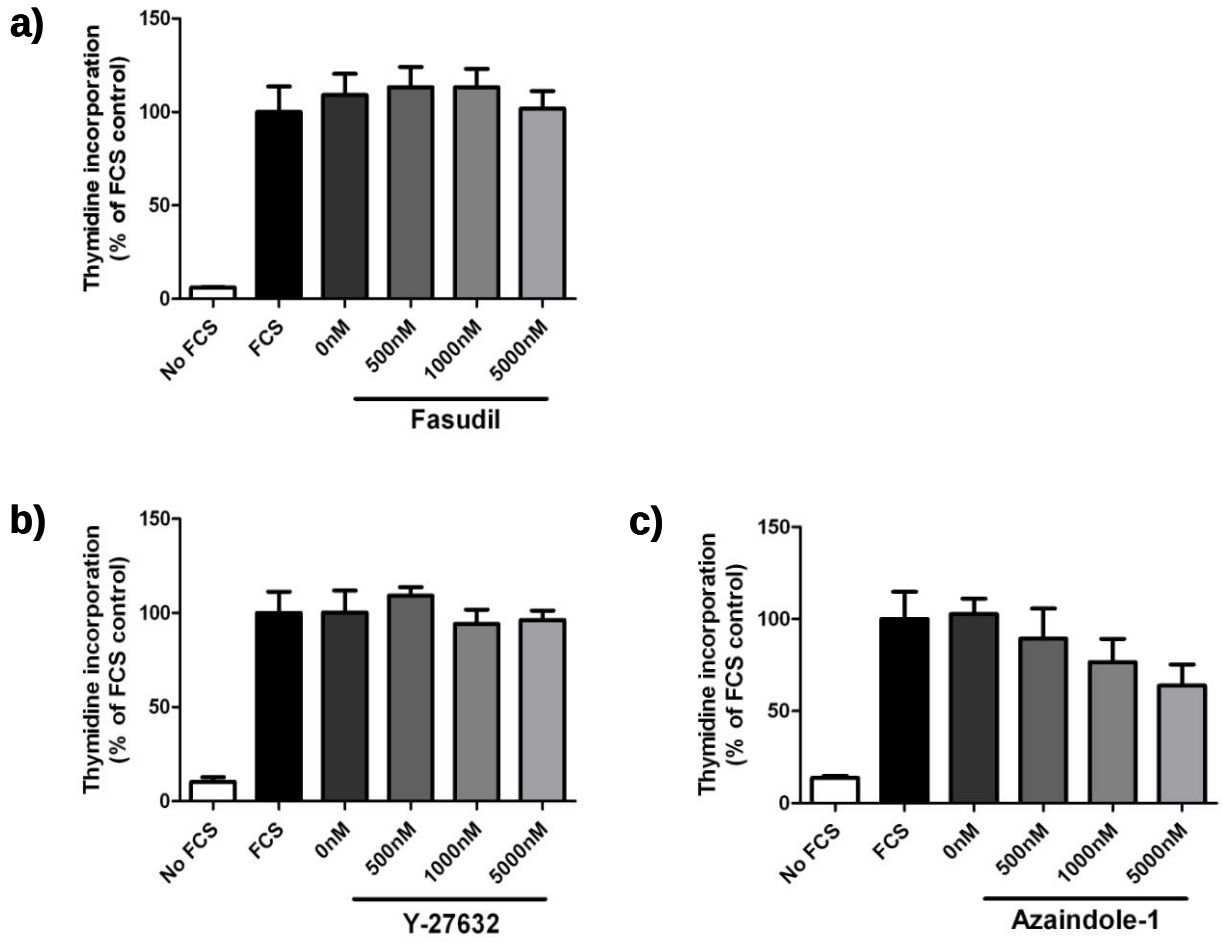


Figure 3. Effects of fasudil, Y-27632 and azaindole-1 on proliferation of pulmonary arterial smooth muscle cells (PASMCs) isolated from healthy rats. The proliferation assays were performed as described in methods. Effects of various concentrations of (**a**) fasudil, (**b**) Y-27632 and (**c**) azaindole-1 on thymidine incorporation into primary PASMCs isolated from healthy rats (n=3) are shown. Data are presented as % of FCS control. Bars represent mean ± SEM.

3.4. Effects of Rho-kinase inhibitors on proliferation of PASMCs isolated from MCT-injected rats

As PASMCs are phenotypically changed in disease conditions, we investigated the effect of azaindole-1 on proliferation of PASMCs derived from MCT-injected rats by thymidine incorporation assay. In each assay, fasudil and Y-27632 were also included. In PASMCs derived from MCT-rats, there was a tendency towards reduction in the thymidine incorporation by fasudil and Y-27632 at their higher concentrations; however, the reduction was significant only by 5000nM of Y-27632 ($p<0.05$ versus FCS control, figure 4b). Azaindole-1 reduced the thymidine incorporation significantly at all concentrations tested ($p<0.001$ versus FCS control, figure 4c). The findings suggest that azaindole-1 has more potent inhibitory effect on PASMC proliferation than other inhibitors.

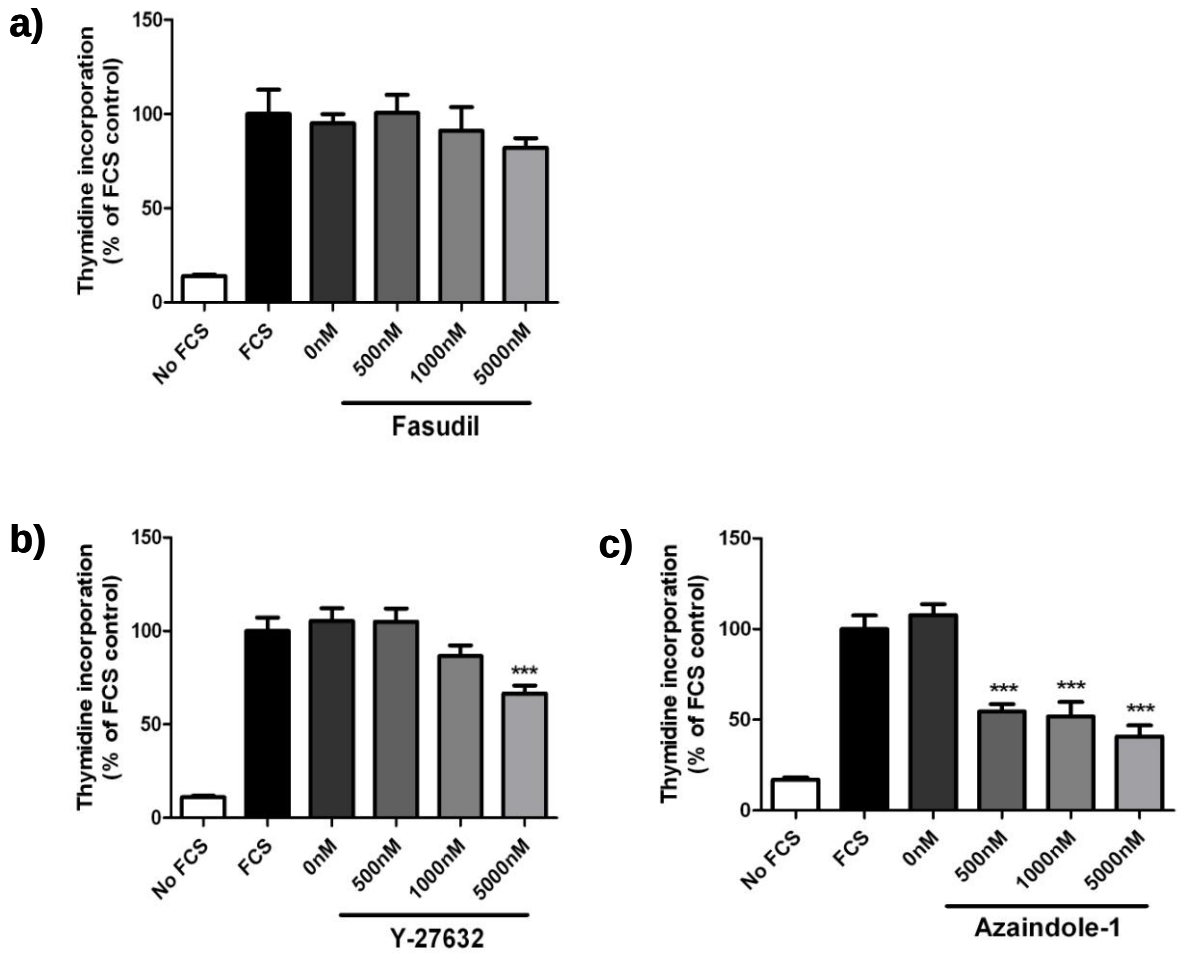


Figure 4. Effects of fasudil, Y-27632 and azaindole-1 on proliferation of pulmonary arterial smooth muscle cells (PASMCs) isolated from MCT-injected rats. The proliferation assays were performed as described in methods. Effects of various concentrations of (**a**) fasudil, (**b**) Y-27632 and (**c**) azaindole-1 on thymidine incorporation into primary PASMCs (n=3) are shown. Data are presented as % of FCS control. Bars represent mean ± SEM. ***$p<0.001$ versus FCS.

3.5. Effect of azaindole-1 on MCT-induced progressive elevation of RVSP

To investigate the *in vivo* efficacy of azaindole-1, progressive elevation of RVSP was monitored online by telemetry technique in MCT-injected rats treated with azaindole-1. The RVSP at day 1 (26.5 ± 1.7 mmHg) was significantly elevated at day 21 (37.8 ± 0.9 mmHg) and further at day 35 (67.9 ± 4.3 mmHg) in rats receiving placebo (figure 5a). Treatment with azaindole-1 daily from day 21 to 35 significantly reduced RVSP (38.8 ± 2.9 mmHg at day 35). To check if azaindole-1 induced any reflex tachycardia, we monitored the heart rate of the rats. We observed that the heart rates of placebo and azaindole-1 treated rats were comparable (figure 5b). The findings suggest that azaindole-1 has potent pulmonary vasorelaxant effect *in vivo*.

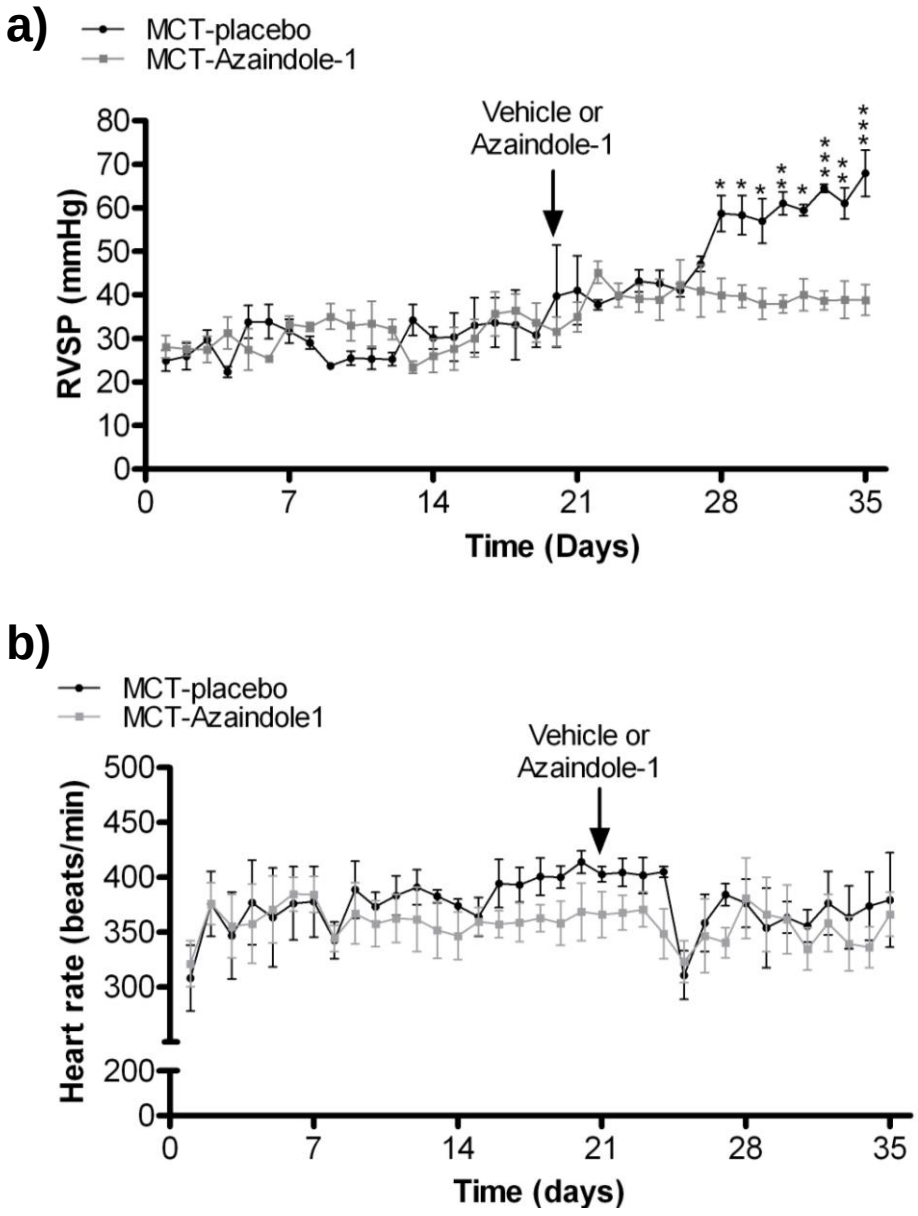


Figure 5. Effect of azaindole-1 on progressive elevation of right ventricular systolic pressure (RVSP). The effects of azaindole-1 on progressive increase of RVSP induced by MCT injection in rats were investigated by radio-telemetry technique and RVSP and heart rate were monitored online, as described in methods. (**a**) RVSP and (**b**) heart rate are given. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.6. Effects of azaindole-1 on hemodynamics in MCT-induced pulmonary hypertension

We investigated therapeutic efficacy of azaindole-1 in MCT-induced pulmonary hypertension (PH) as described in methods. MCT induced a robust PH in rats receiving placebo as reflected by the significant increase of RVSP (72.3 ± 2.6 versus 27.2 ± 1.3 mmHg in healthy control) at day 35 of MCT-injection (figure 6a). Rats receiving azaindole-1 resulted in a significant decrease of RVSP (49.5 ± 3.7 mmHg) as compared to the placebo rats. There was no significant change in systemic arterial pressure (SAP) among the experimental groups (figure 6b).

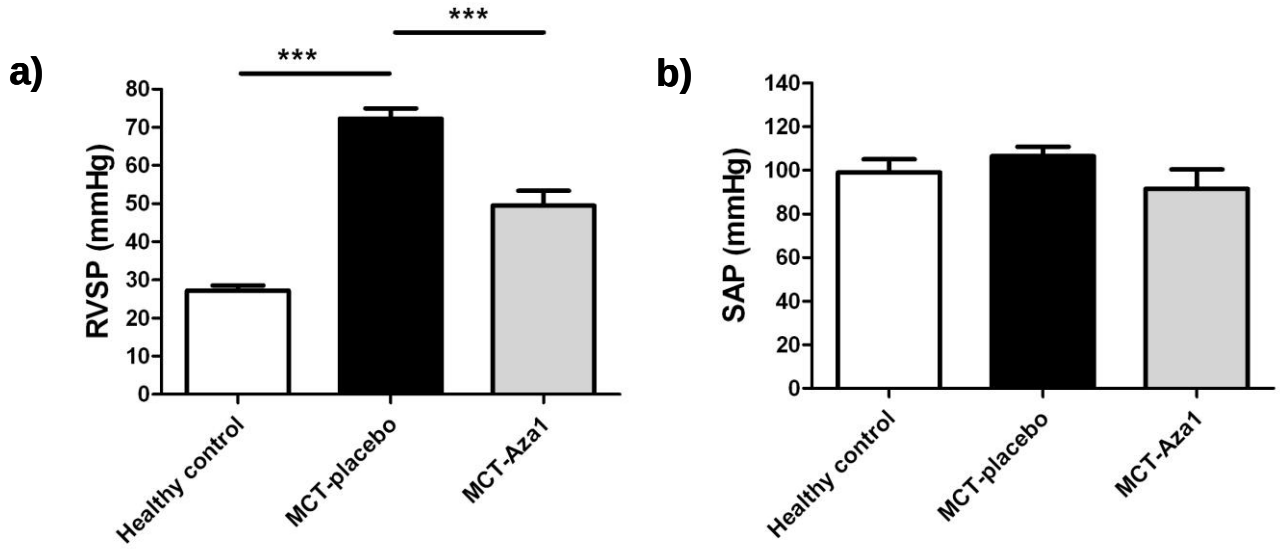


Figure 6. Effect of azaindole-1 on hemodynamics in MCT-induced pulmonary hypertension in rats. Rats were injected with saline (healthy controls) or MCT. MCT-injected rats were treated with azaindole-1 (MCT-Aza1) or placebo from day 21 to 35 after MCT injection, followed by hemodynamic measurement as described in methods. (**a**) Right ventricular systolic pressure (RVSP) and (**b**) systemic arterial pressure (SAP) are shown. Data are presented as mean ± SEM (n=10-15). ***p<0.001.

3.7. Effect of azaindole-1 on cardiac index and total pulmonary and systemic resistance in MCT-induced pulmonary hypertension (PH)

The total pulmonary resistance (TPR) was significantly increased in MCT-injected rats receiving placebo (2.96 ± 0.24 mmHg·min·ml^{-1}·100g BW) as compared to the healthy control (1.51 ± 0.15 mmHg·min·ml^{-1}·100g BW) and azaindole-1 treatment reduced the TPR (2.04 ± 0.22 mmHg·min·ml^{-1}·100g BW, $p<0.05$ versus placebo) (figure 7a). There was no significant change in total systemic resistance (TSR) among the experimental groups (figures 7b). In addition, analysis of cardiac output showed a comparable cardiac index (CI) among the experimental groups (24.9 ± 4.2, 23.8 ± 1.9 and 28.8 ± 3.5 ml·min^{-1}·100g BW for healthy control, MCT-placebo and azaindole-1 treated rats respectively) (figure 7c).

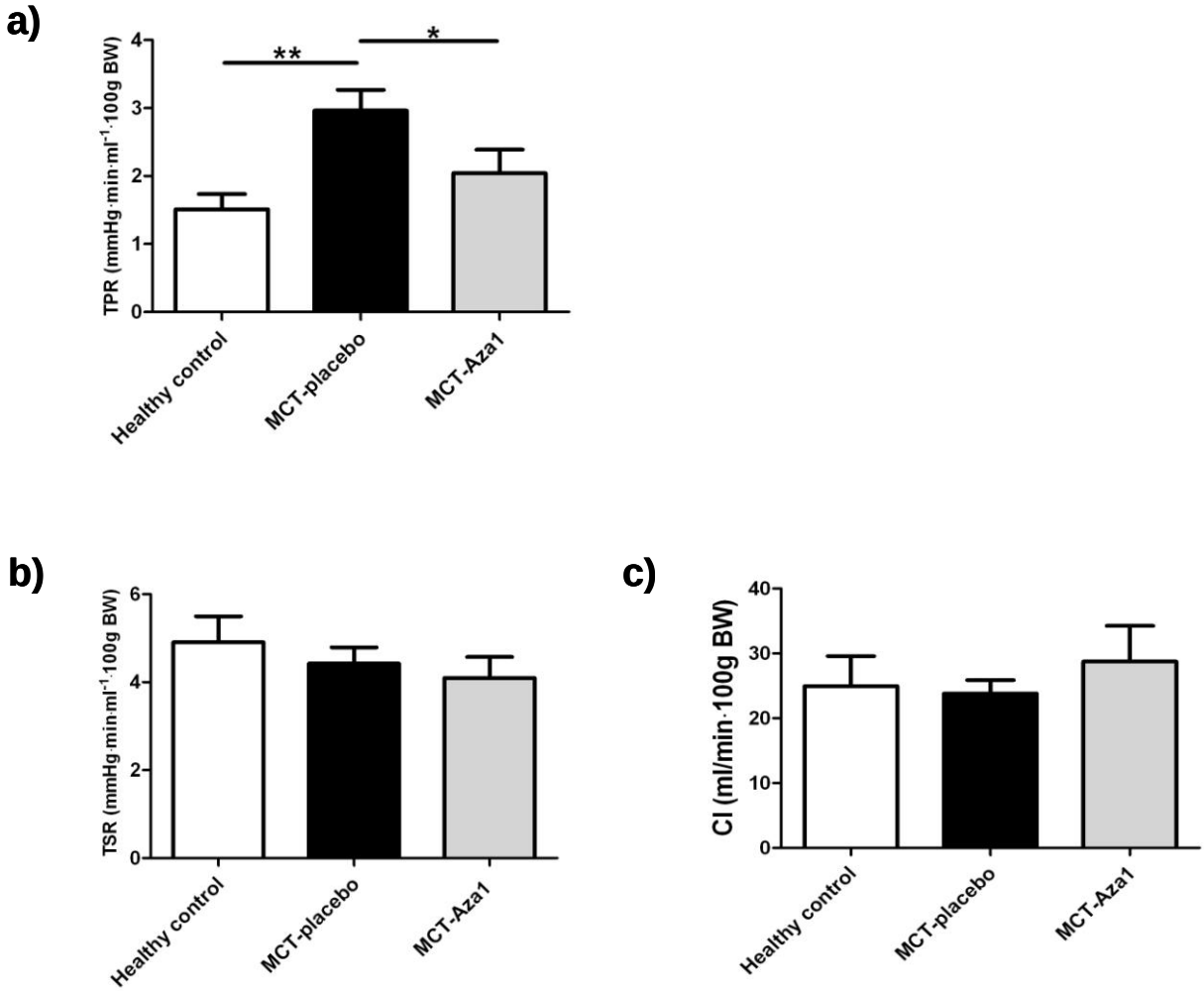


Figure 7. Effect of azaindole-1 on cardiac index and total pulmonary and systemic resistance in MCT-induced pulmonary hypertension in rats. Rats were injected with saline (healthy controls) or MCT. MCT-injected rats were treated with azaindole-1 (MCT-Aza1) or placebo from day 21 to 35 after MCT injection. (**a**) Total pulmonary resistance (TPR), (**b**) total systemic resistance (TSR) and (**c**) cardiac index (CI) are shown. Data are presented as mean ± SEM (n=10-15). *$p<0.05$, **$p<0.01$.

3.8. Effects of azaindole-1 on hemodynamics in chronic hypoxia-induced pulmonary hypertension (PH) in mice.

Chronic hypoxia induced a PH in mice receiving placebo as reflected by significant increase in RVSP (33.3 ± 1.2 versus 23.3 ± 1.1 mmHg under normoxia) (figure 8a). Treatment with azaindole-1 significantly decreased RVSP (26.7 ± 0.8 mmHg) as compared to the hypoxic placebo mice. Hypoxic mice tended to have slightly decreased SAP; however, no significant difference was observed in SAP between placebo and azaindole-1 treated mice (68.1 ± 1.7 and 61.0 ± 3.5 mmHg respectively) (figure 8b).

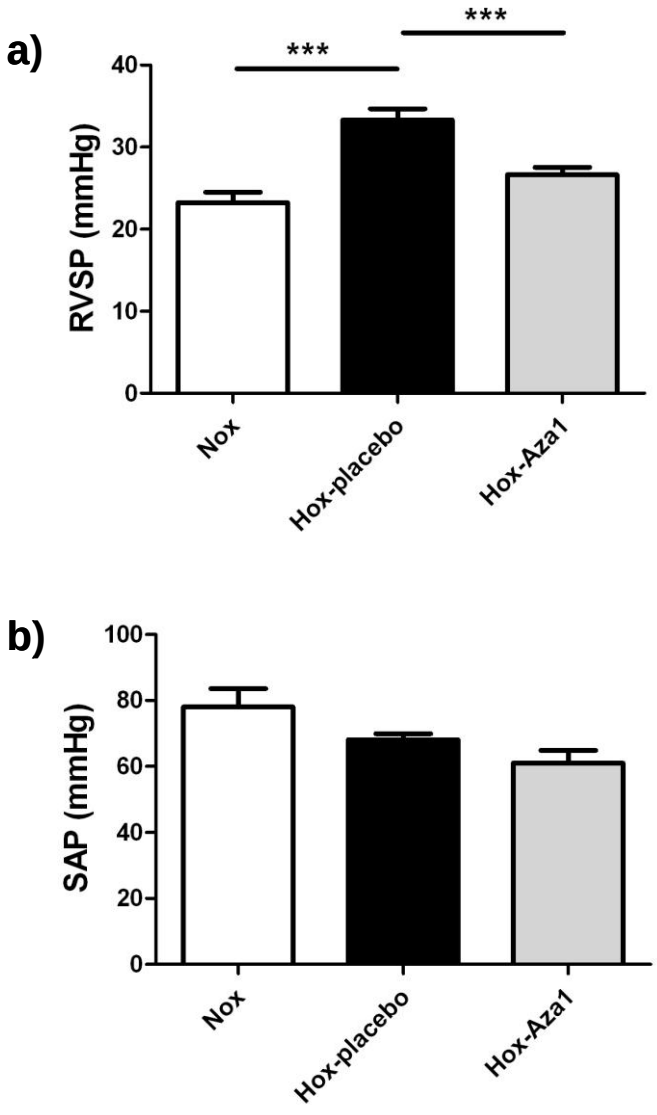


Figure 8. Effect of azaindole-1 on hemodynamics in chronic hypoxia-induced pulmonary hypertension (PH) in mice. Mice were exposed to normoxia (Nox) or chronic hypoxia (Hox). Hypoxic mice were treated with azaindole-1 (Hox-Aza1) or placebo and hemodynamic measurement was done at day 35, as described in methods. (**a**) Right ventricular systolic pressure (RVSP) and (**b**) systemic arterial pressure (SAP) of different experimental groups are given. Data are presented as mean ± SEM (n=6-10). ***p<0.001.

3.9. Effects of azaindole-1 on right ventricular (RV) hypertrophy in MCT- and chronic hypoxia-induced pulmonary hypertension (PH)

We investigated RV hypertrophy by measuring RV/(LV+S) ratio and found that the increased RVSP was accompanied by RV hypertrophy in both MCT- and chronic hypoxia-induced PH. The RV/(LV+S) ratio was significantly increased in MCT-injected rats receiving placebo (0.48 ± 0.01) as compared to the healthy rats (0.22 ± 0.01). Treatment with azaindole-1 significantly reduced the RV/(LV+S) ratio (0.38 ± 0.02 versus placebo) (figure 9a). Mice under chronic hypoxia revealed significantly higher RV/(LV+S) ratio (0.34 ± 0.01) as compared to the normoxic mice (0.24 ± 0.01). Treatment with azaindole-1 improved the chronic hypoxia-induced RV hypertrophy as reflected by significantly reduced RV/(LV+S) ratio (0.310 ± 0.001) (figure 9b).

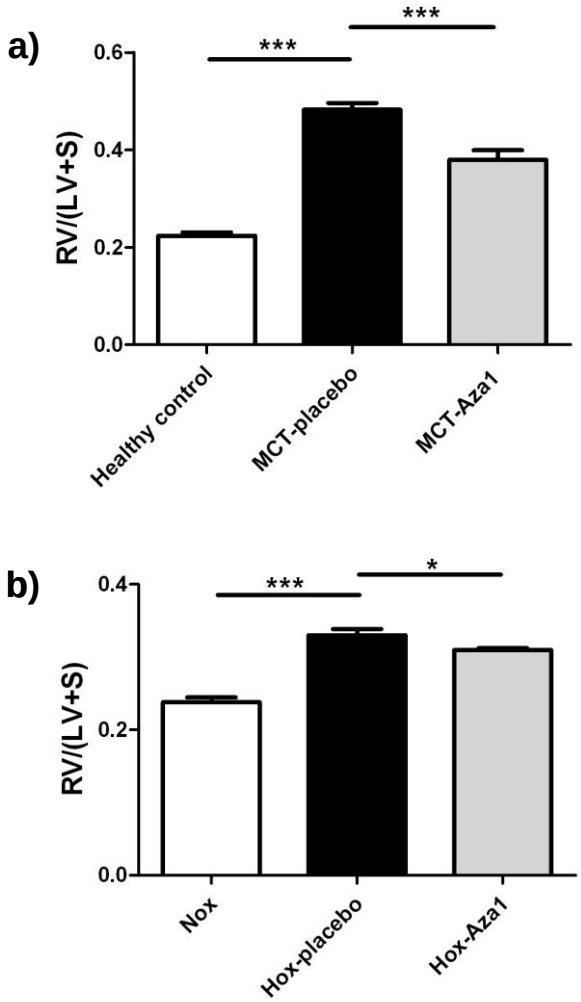
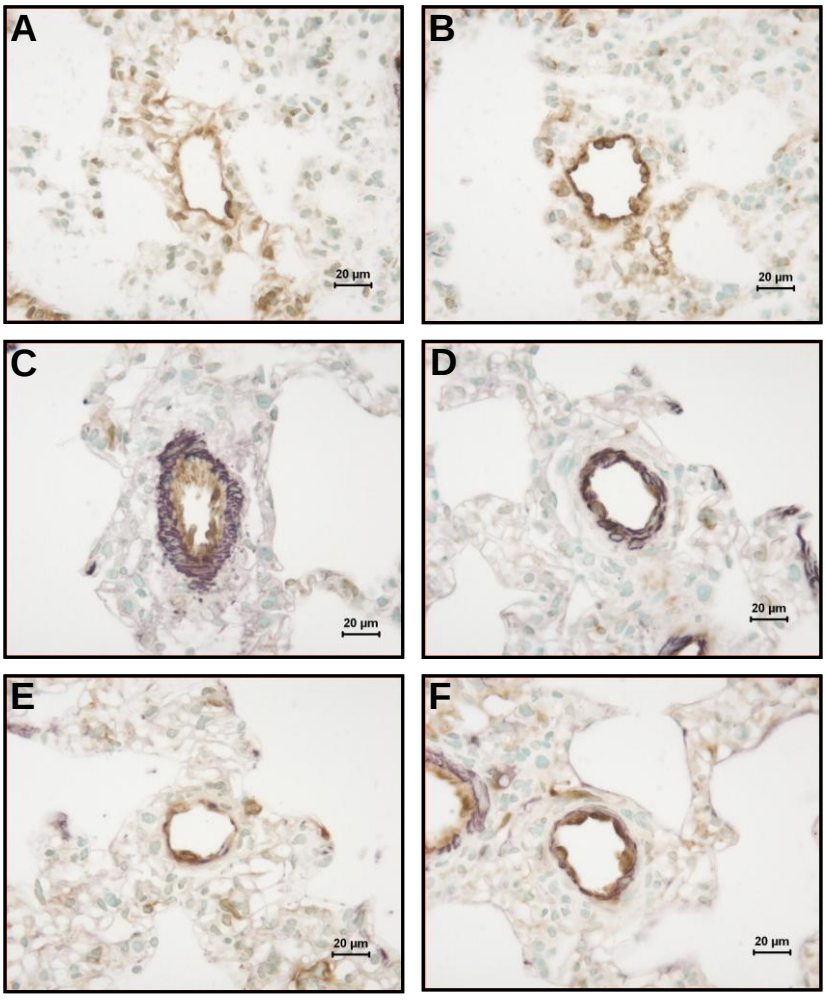


Figure 9. Effect of azaindole-1 on right ventricular hypertrophy in MCT- and chronic hypoxia-induced pulmonary hypertension. Rats were injected with saline (healthy control) or MCT. Mice were exposed to normoxia (Nox) or hypoxia (Hox). Both animal models were treated with azaindole-1 (MCT-Aza1 and Hox-Aza1) or placebo from day 21 for 2 weeks followed by right ventricular hypertrophy measurement, as described in methods. Right to left ventricular plus septum weight ratio (RV/(LV+S)) of (**a**) MCT-injected rats (n=10-15) and (**b**) hypoxic mice (n=6-10) are given. Data are presented as mean ± SEM. *p<0.05, ***p<0.001.

3.10. Effects of azaindole-1 on pulmonary vascular remodelling in MCT-induced pulmonary hypertension – Degree of muscularization

The effects of azaindole-1 on pulmonary vascular remodelling were assessed by determining the degree of muscularization of the peripheral pulmonary arteries. MCT injection in rats resulted in an enhanced pulmonary artery muscularization as evident from the enhanced immunoreactivity for α-smooth muscle cell actin (figure 10a). Pulmonary vascular morphometry revealed significantly increased fully muscularized vessels (57.0 ± 1.5%) and decreased non-muscularized vessels (2.4 ± 0.3%) in MCT-injected rats receiving placebo compared with healthy controls (2.2 ± 0.8% and 42.2 ± 3.7%, respectively). Azaindole-1 treatment significantly decreased fully muscularized vessels (18.5 ± 2.4%) (figure 10b). There was significantly higher proportion of partially muscularized vessels in azaindole-1-treated rats (73.3 ± 2.2% versus 40.6 ± 1.4% in placebo), suggesting that the treatment impaired the progressive muscularization by preventing the shift from partial towards full muscularization of the pulmonary vessels.

a)



b)

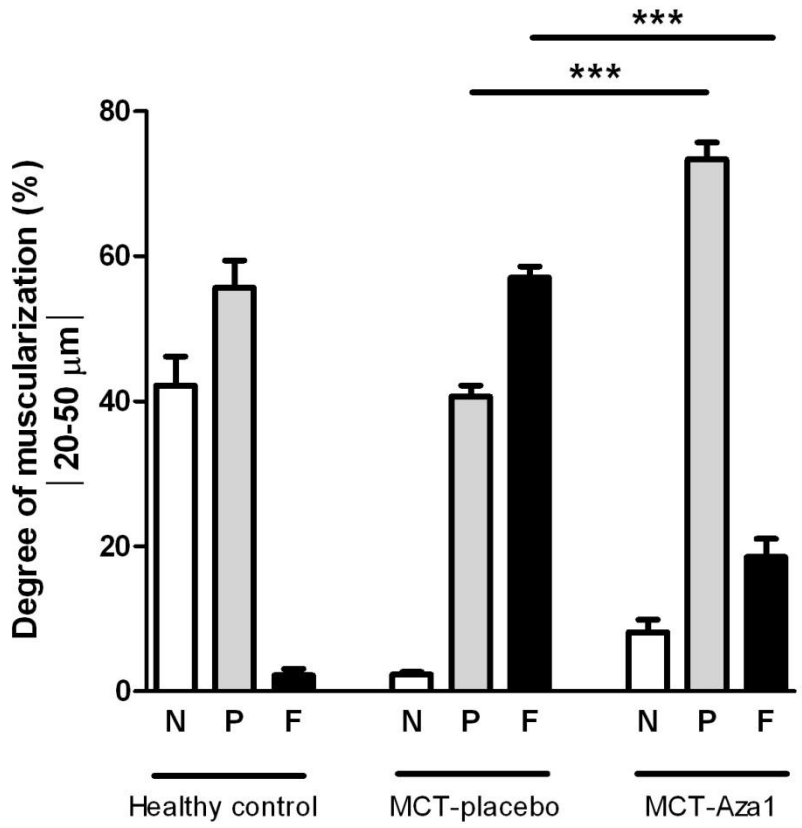


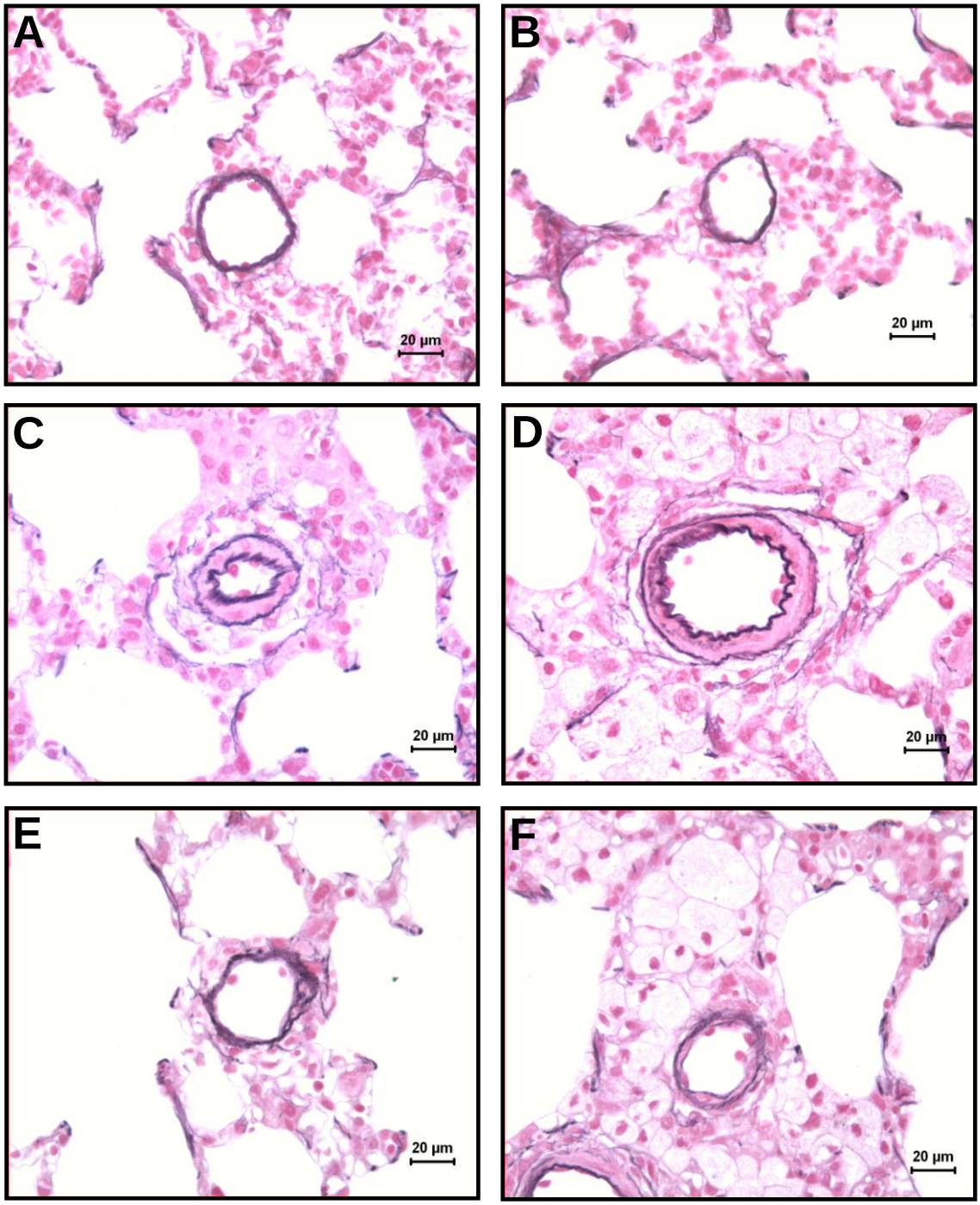
Figure 10. Effect of azaindole-1 on degree of muscularization in MCT-induced pulmonary hypertension. The rat lung sections were immunostained for von Willebrand factor and α-smooth muscle actin and pulmonary vascular morphometry was performed as described in methods. Representative photomicrographs are shown (**a**) (healthy controls (**A**, **B**), MCT-placebo (**C**, **D**) and azaindole-1 treated group (MCT-Aza1, **E** and **F**). (**b**) Proportion of non-(N), partially (P) or fully (F) muscularized vessels, as a percentage of total pulmonary vessel cross-section (sized 20-50µm), is given for healthy controls and MCT-injected rats receiving placebo and azaindole-1 (MCT-Aza1). Data are presented as mean ± SEM (n=10). Scale bars=20µm. ***p<0.001.

3.11. Effects of azaindole-1 on pulmonary vascular remodelling in MCT-induced pulmonary hypertension – Medial wall thickness

The effects of azaindole-1 on pulmonary vascular remodelling were assessed also by determining the medial wall thickness of the peripheral pulmonary arteries. Prior to

determination of the medial wall thickness, the rat lungs were stained with elastica (figure 11a). There was significantly increased medial wall thickness in placebo (22.3 ± 0.9% versus 9.7 ± 0.4% in healthy controls). Corroborating the decreased fully muscularized vessels, azaindole-1 significantly reduced the medial wall thickness (14.0 ± 0.6 %) (figure 11b).

a)



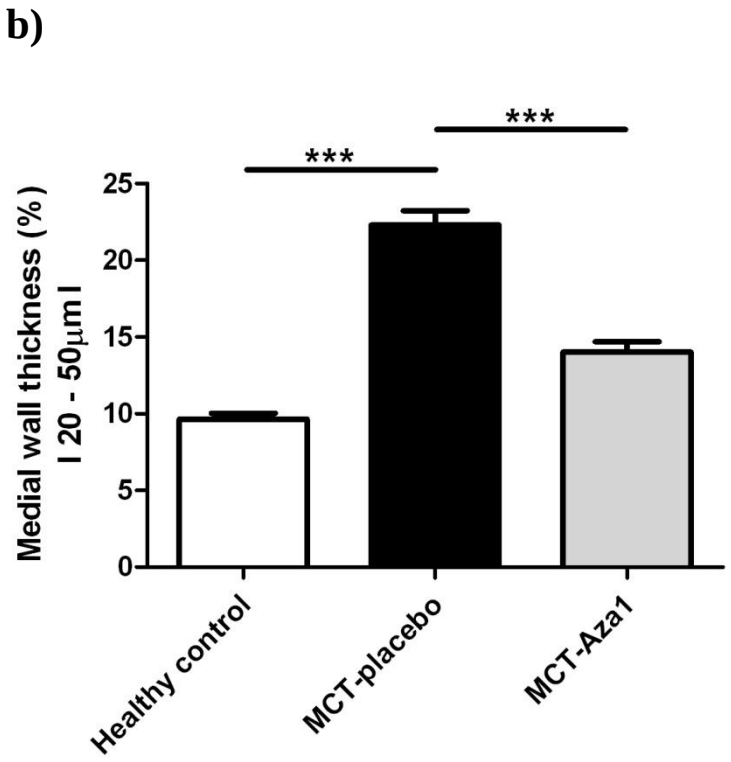


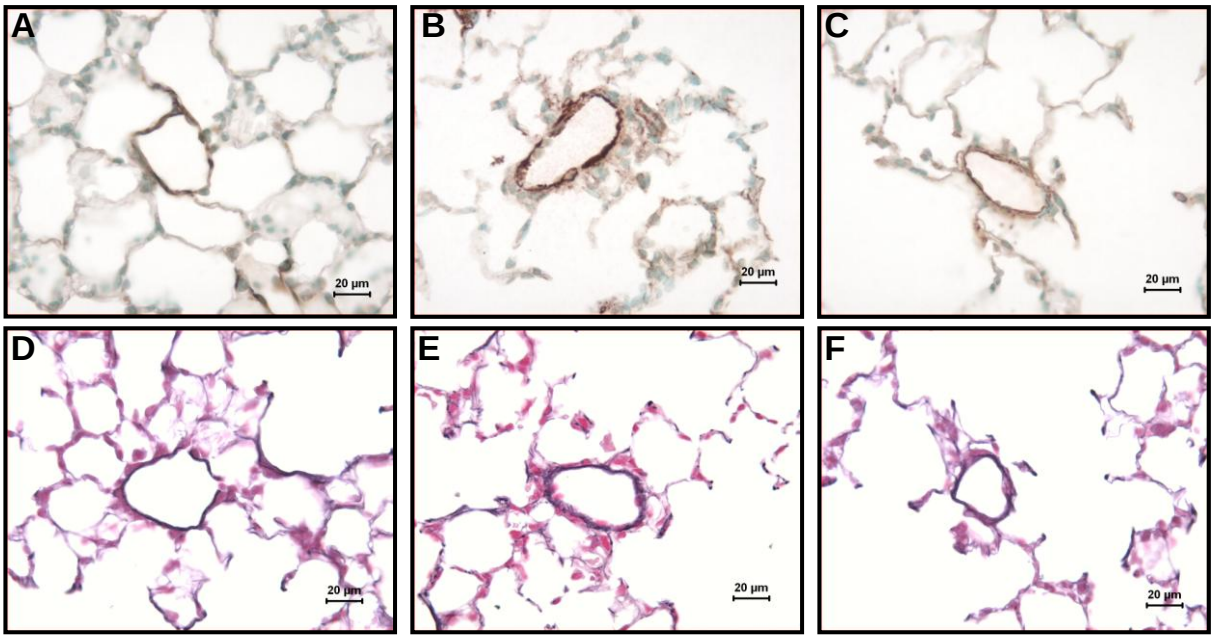
Figure 11. Effect of azaindole-1 on medial wall thickness in MCT-induced pulmonary hypertension. The rat lung sections were stained with elastica and pulmonary vascular morphometry was performed as described in methods. Representative photomicrographs are shown (**a**) (healthy controls (**A**, **B**), MCT-placebo (**C**, **D**) and azaindole-1 treated group (MCT-Aza1, **E** and **F**). (**b**) Medial wall thickness (%) of the pulmonary vessels (sized 20-50µm) is given for healthy controls and MCT-injected rats receiving placebo and azaindole-1 (MCT-Aza1). Data are presented as mean ± SEM (n=10). Scale bars=20µm. ***p<0.001.

3.12. Effects of azaindole-1 on pulmonary vascular remodelling in chronic hypoxia-induced pulmonary hypertension (PH) in mice

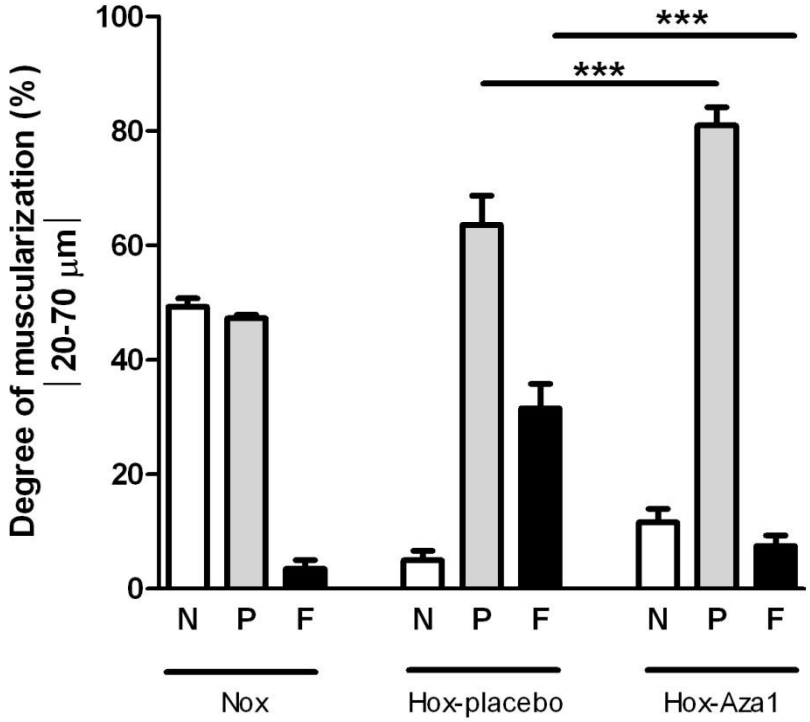
The effects of azaindole-1 on pulmonary vascular remodelling were assessed by determining the degree of muscularization (figure 12a, A, B, C) and medial wall thickness (figure 12a, D, E, F) of the peripheral pulmonary arteries. In chronic hypoxic mice, the non-muscularized vessels were significantly decreased (4.9 ± 1.5% versus 49.3 ± 1.3% in normoxic mice), whereas the partially and fully muscularized vessels were significantly increased (63.5 ± 4.7% and 31.5 ± 3.9% versus 47.3 ± 0.5% and 3.5 ± 1.3% in normoxic mice, respectively). Treatment with azaindole-1 resulted in significant reduction of fully muscularized vessels (7.5 ± 1.7%) (figure 12b). As it was observed in MCT-injected rats, the proportion of partially muscularized arteries was higher in mice receiving azaindole-1 (80.9 ± 2.9%). Chronic

hypoxia resulted in significantly increased medial wall thickness (17.8 ± 0.9%) compared with the normoxic control mice (10.1 ± 0.3%) (figure 12c). Corroborating the decrease in fully muscularized vessels, the medial wall thickness was significantly reduced in azaindole-1-treated mice (12.4 ± 0.4 %).

a)



b)



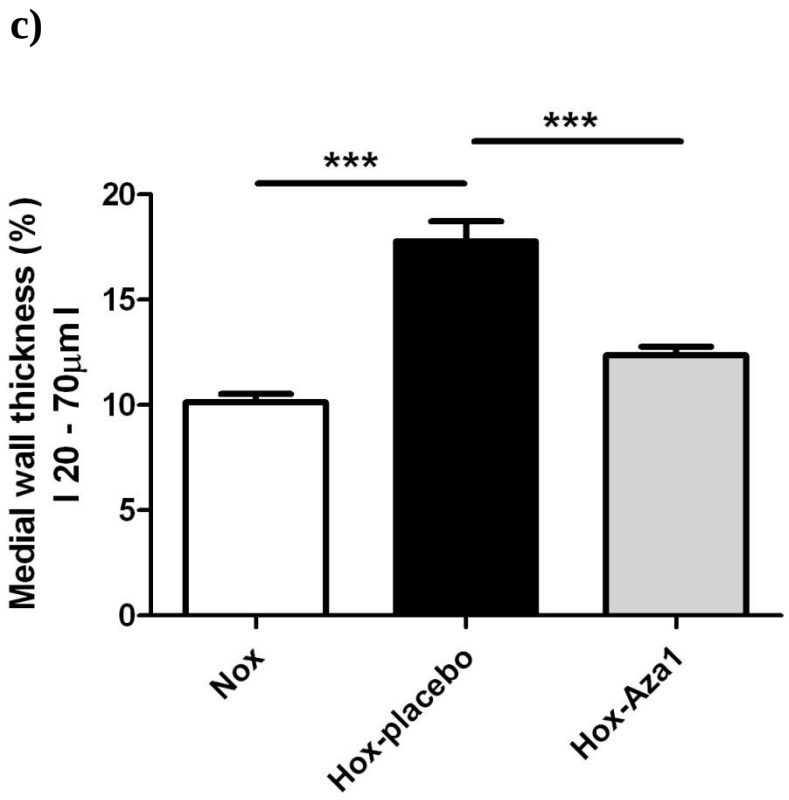


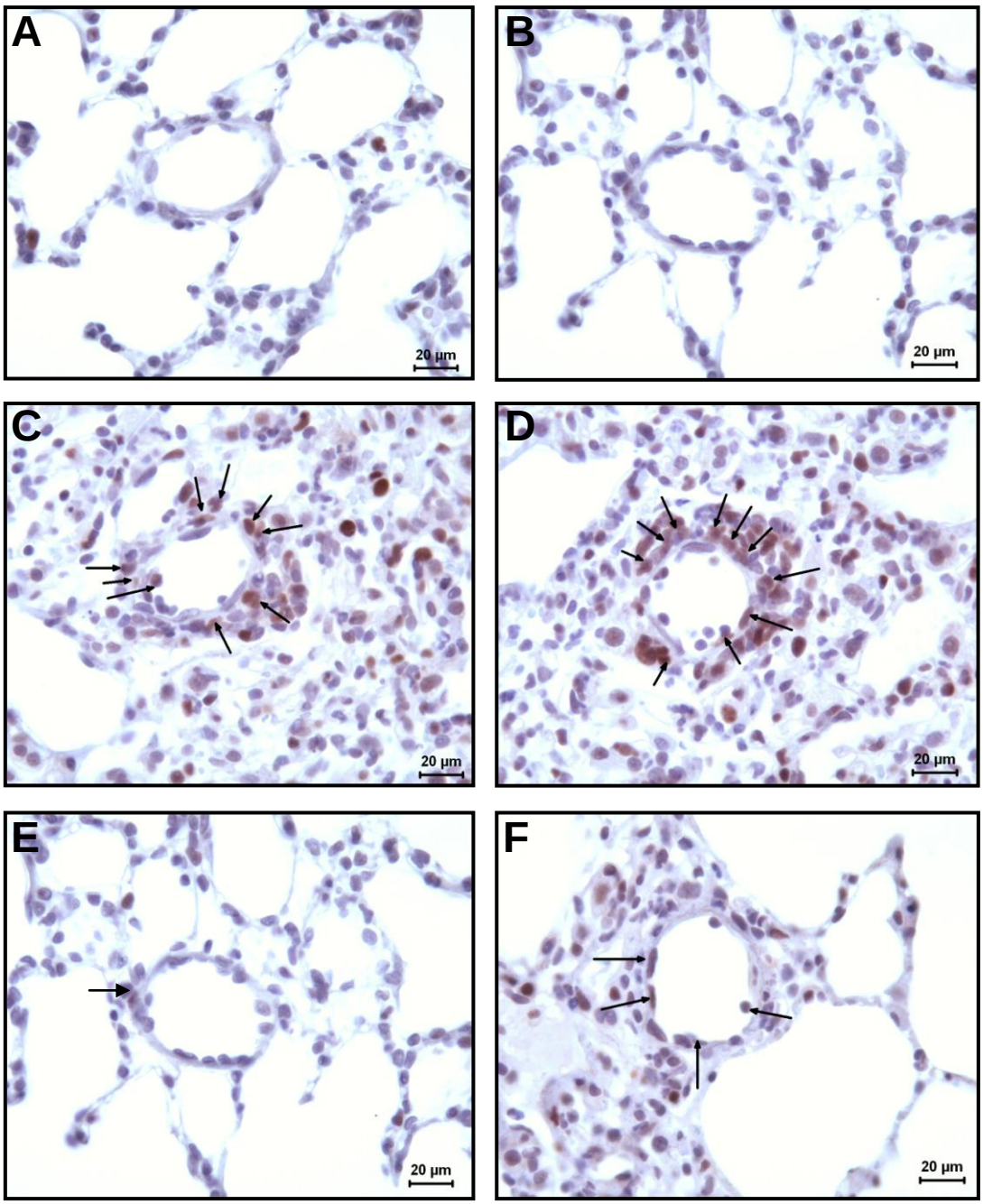
Figure 12. Effect of azaindole-1 on pulmonary vascular remodelling in hypoxia-induced pulmonary hypertension in mice. The lung sections exposed to normoxia and hypoxia were immunostained for von Willebrand factor and α-smooth muscle actin followed by pulmonary vascular morphometry as described in the methods section. (**a**) Representative photomicrographs of normoxic (Nox, **A**) and hypoxic mice receiving placebo (Hox, **B**) and azaindole-1 (Hox-Aza1, **C**) are shown. (**b**) Proportion of non- (N), partially (P) or fully (F) muscularized vessels, as a percentage of total pulmonary vessel cross-section (sized 20–70µm) is given. The lung sections were stained with elastica and the medial wall thickness (%) was determined as described in the methods section. (**a**) Representative photomicrographs of normoxic control (**D**) and hypoxic mice treated with placebo (**E**) and azaindole-1 (**F**) are given. (**c**) The medial wall thickness (%) of pulmonary vessels is shown. Data are presented as mean ± SEM (n=6–10). Scale bars=20µm. ***p<0.001.

3.13. Effect of azaindole-1 on pulmonary vascular cell proliferation

To confirm if the observed *in vitro* effect of azaindole-1 on cell proliferation was also present *in vivo*, we performed immunostaining for proliferating cell nuclear antigen (PCNA). We observed that immunoreactivity for PCNA was significantly increased in lung tissues from MCT-injected rats compared with that in healthy control rats (figure 13a). We analyzed the same size vessels as was used for vascular morphometry (20-50µm) to quantify the PCNA-

positive vascular cells and express the result as Index of Proliferation (IOP). The findings revealed that there was higher IOP in MCT-injected rats receiving placebo (431.9 ± 7.2% versus 100.0 ± 25.8% in healthy control rats) (figure 13b). Corroborating the *in vitro* data, the IOP was significantly reduced in MCT-injected rats receiving azaindole-1 (184.1 ± 10.9 %).

a)



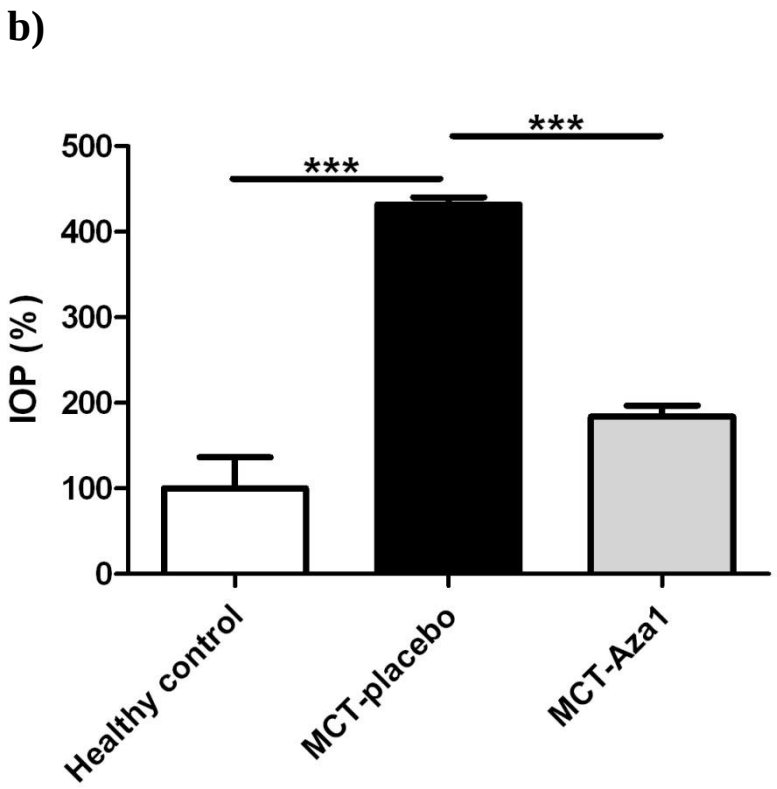


Figure 13. Effect of azaindole-1 on pulmonary vascular cell proliferation. The pulmonary vascular cell proliferation was investigated in the rat lung sections by immunostaining of proliferating cell nuclear antigen (PCNA), as described in the methods section. Representative photomicrographs of PCNA (**a**) staining (healthy controls (**A**, **B**), monocrotaline (MCT)–placebo (**C**, **D**) and MCT–azaindole-1 (**E**, **F**)) are shown. The PCNA-positive vascular cells were counted and the index of proliferation (IOP) was calculated as described in the methods section. (**b**) IOP (%) for healthy controls, MCT–placebo and MCT–azaindole-1 (MCT-Aza1) is given. Data are presented as mean ± SEM (n=6–8). Positive immunoreactivity is indicated by an arrow (brown staining); scale bar=20µm. ***p<0.001.

3.14. Effects of azaindole-1 on Rho-kinase activity

We investigated the effects of azaindole-1 on Rho-kinase activity by employing immunohistochemistry for p-MYPT1. The immunoreactivity was localized in the media of the vessels and was strongly enhanced in the lung tissues of MCT-injected rats receiving placebo. There was decreased immunoreactivity in MCT-injected rats treated with azaindole-1 (figure 14).

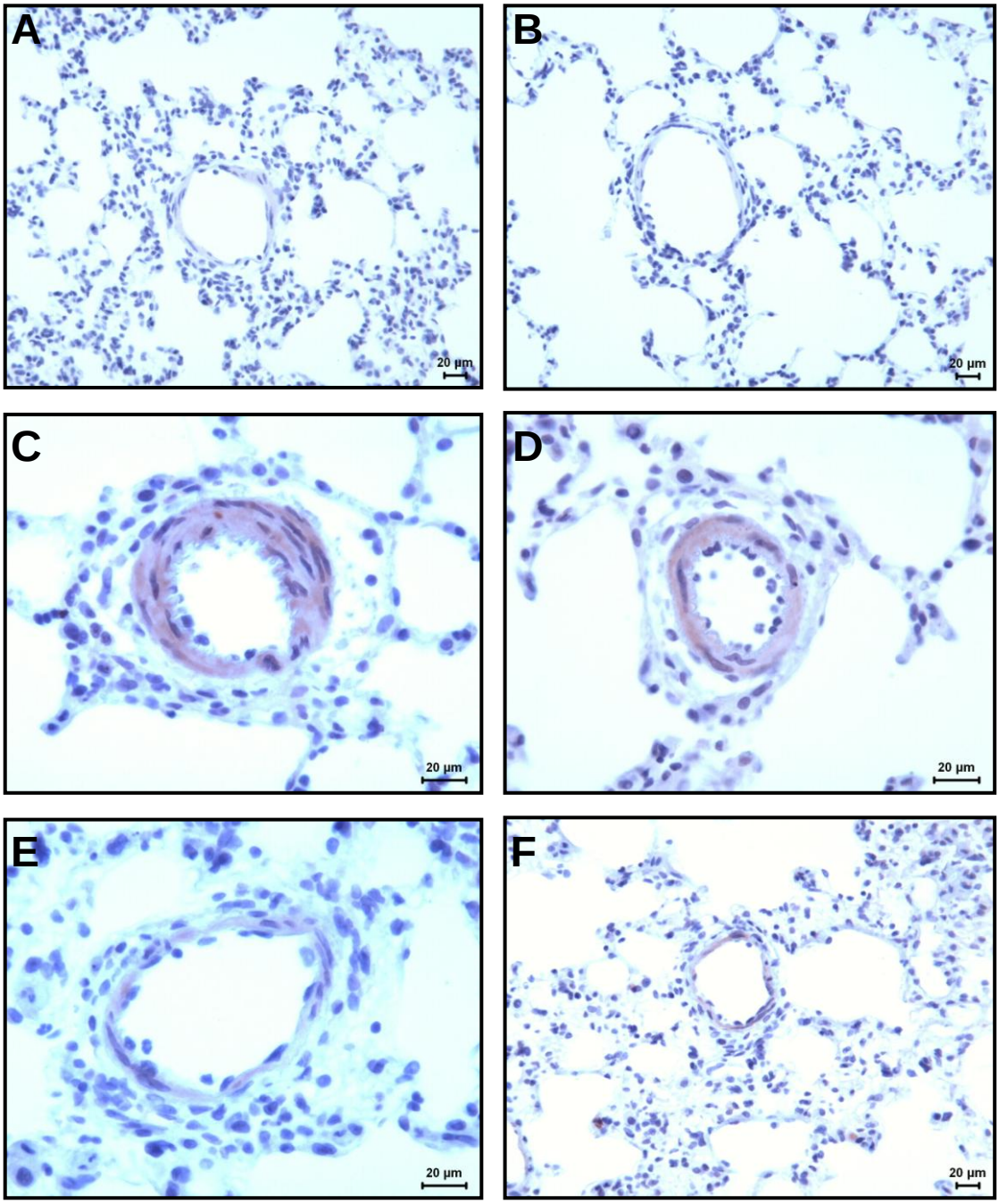


Figure 14. Effect of azaindole-1 on Rho-kinase activity. The ROCK activity was investigated in the rat lung sections by immunostaining phospho-myosin phosphatase target subunit 1 (p-MYPT1), as described in the methods section. Representative photomicrographs of p-MYPT1 staining (healthy controls (**A**, **B**), monocrotaline (MCT)–placebo (**C**, **D**) and MCT–azaindole-1 (**E**, **F**)) are shown. Positive immunoreactivity appears as reddish-brown staining; scale bars=20µm.

3.15. Effect of azaindole-1 on body weight of animals in hypoxia- and MCT-induced pulmonary hypertension

Body weights of rats and mice were measured upon completion of the experiments at day 35. There was no significant effect on body weight of the animals associated with azaindole-1 treatment (figure 15).

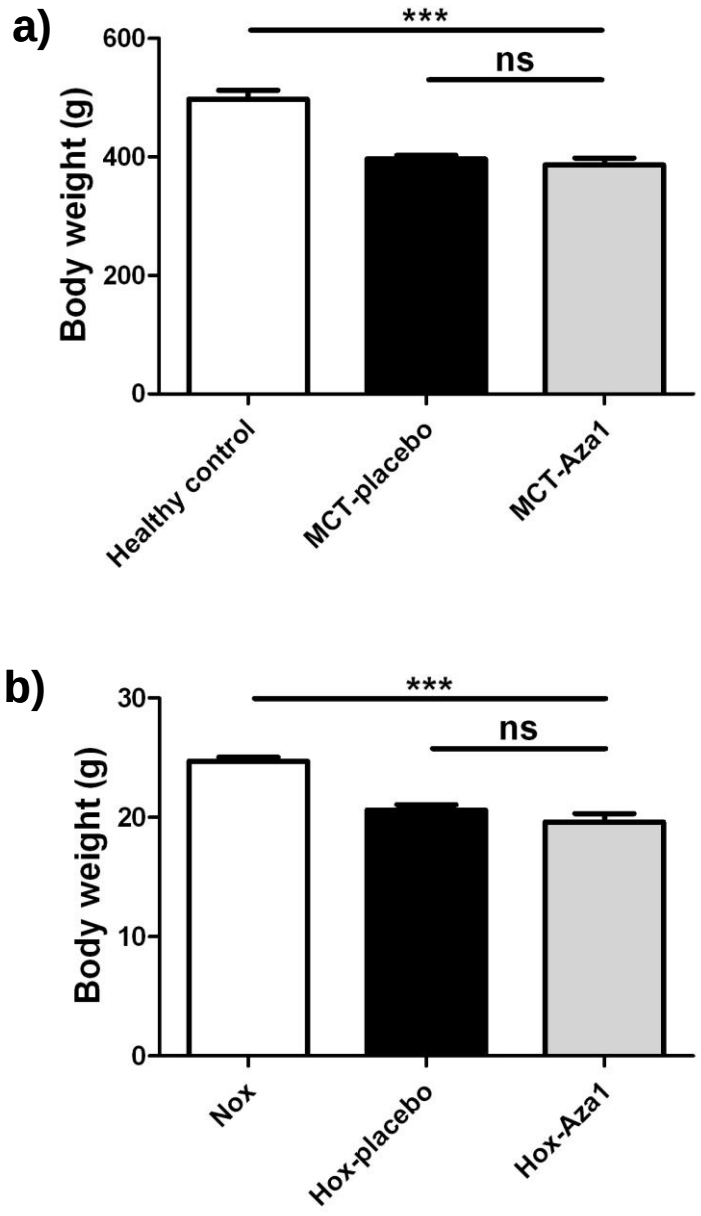


Figure 15. Effect of azaindole-1 on body weight of animals in hypoxia- and MCT-induced pulmonary hypertension. The effects of azaindole-1 treatment on body weights (g) of (**a**) MCT-injected rats and (**b**) hypoxic mice are shown. Bars represent mean ± SEM. **Nox** – normoxia; **Hox** – hypoxia; **Aza1** – azaindole-1. ***p<0.001.

3.16. Effect of azaindole-1 on survival in MCT-induced pulmonary hypertension

The survival of rats was measured from day 22 until 35 day after saline (healthy control) and MCT-injection for all experimental groups. The survival of rats treated with azaindole-1 (~86%) was slightly improved in comparison with MCT-placebo (75%) group (figure 16).

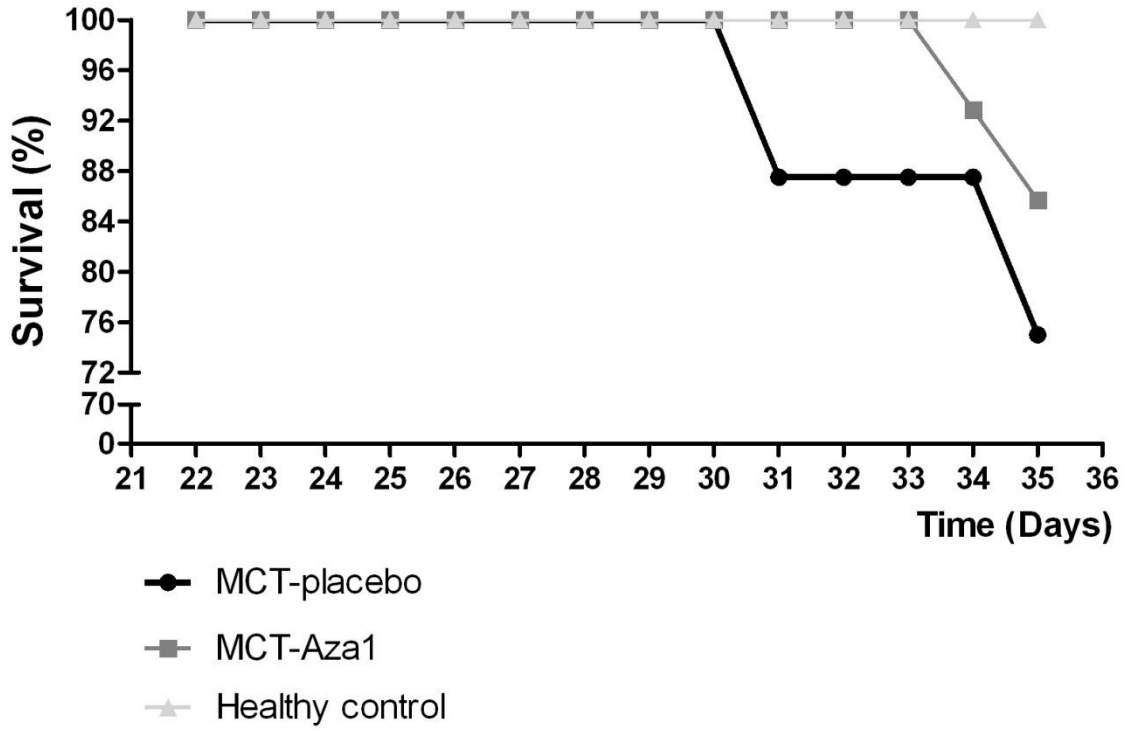
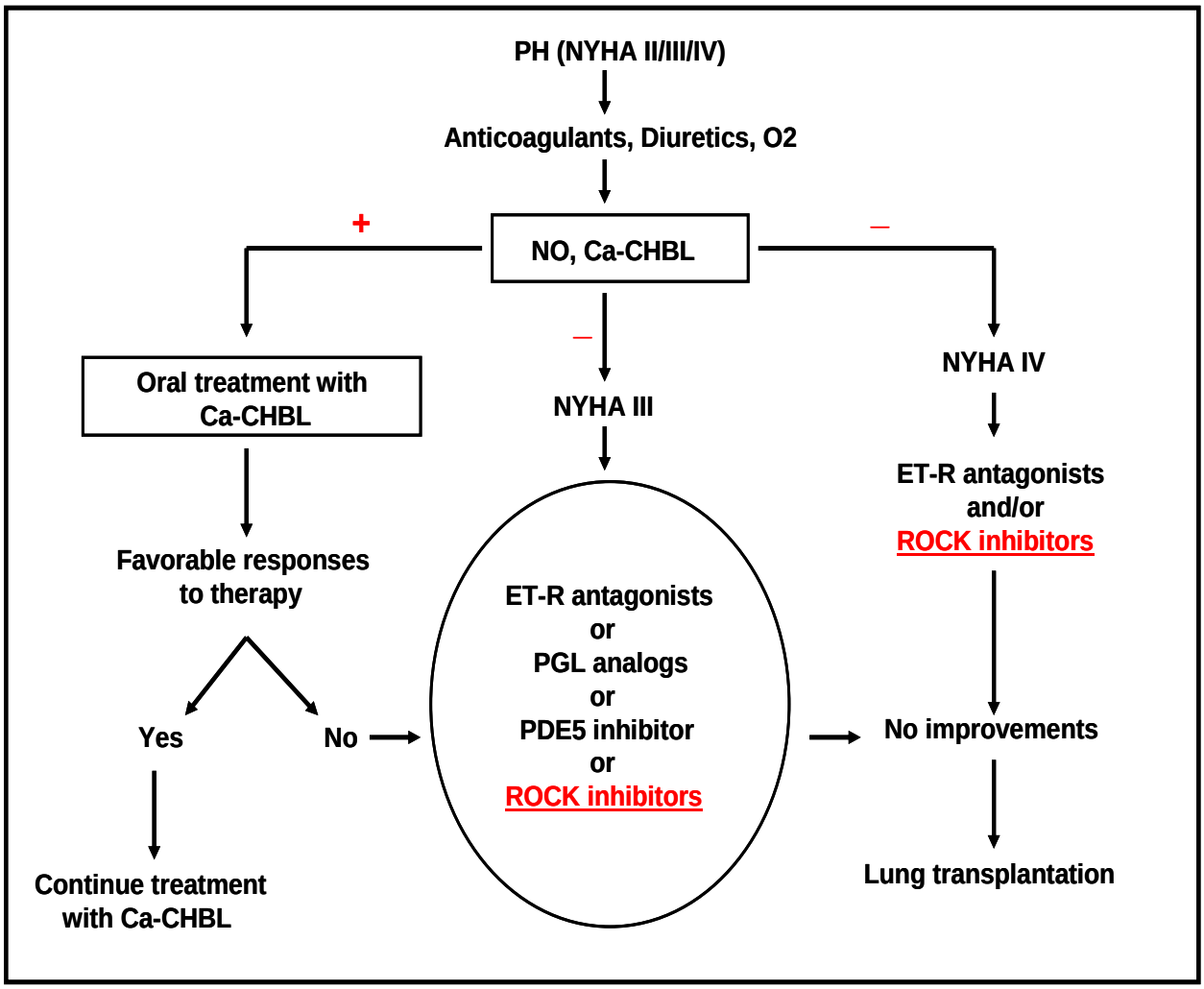


Figure 16. Effect of azaindole-1 on survival in MCT-induced pulmonary hypertension. The survival was monitored each day for last two weeks of experiments and the mean values on day 22 were considered as 100%. The survival curves (%) and different time points for healthy control and MCT-injected rats receiving placebo and azaindole-1 (MCT-Aza1) are given.

4. Discussion

Investigation of a promising targets and development of a novel and effective therapeutic strategies and approaches for pulmonary hypertension has been an actively pursued research focus over the past 10 years. The therapeutic options available for pulmonary hypertension, such as phosphodiesterase (PDE)-5 inhibitors, endothelin receptor antagonists and prostacyclin analogs, as well as other approaches, significantly prolonged survival of the patients and improved the quality of their life (scheme 10)[27;28].



Scheme 10. Diagnostic and treatment algorithm for pulmonary hypertension and potential application of ROCK inhibitors. Legend: **PH** – pulmonary hypertension; **NYHA** – New York Heart Association Functional Classification; **NO** – nitric oxide; **Ca-CHBL** – calcium channel blockers; **ET-R** – endothelin receptor; **PGL** – prostaglandin; **PDE5** – phosphodiesterase-5; + - positive response; - - negative response. Modified from Fukumoto Y. *et al*, 2007.

For the treatment of pulmonary hypertension, usually the first step is to examine the acute pulmonary vascular response to conventional medical approach that includes O_2 and NO inhalation, as well as calcium channel blockers (scheme 10)[51]. The positive response to NO inhalation is defined as more than 30% reduction in pulmonary vascular resistance[51]. According to positive or negative response to acute vasoreactivity tests and the severity of the disease (NYHA classification) the further strategy is determined. Currently the patients are treated with anticoagulants/vasodilators (prostacyclin, sildenafil, bosentan) as a monotherapeutic approach or combination of different compounds and in advanced stages of the disease the lung transplantation is often performed[51].

Despite the enormous progressions and advances in pulmonary hypertension therapy, there are still no successful options to finally cure the disease. A specific therapy for the majority of pulmonary hypertension patients is still lacking. Therefore, the discovery of a new therapeutic targets and strategies are more than needed.

Animal studies suggested that Rho-kinase pathway plays an important role in the development and progression of pulmonary hypertension and the chronic treatment with Rho-kinase inhibitors significantly improved this severe disorder. Additionally, the recent clinical investigations demonstrated the involvement of Rho-kinase in human disease and moreover, the acute beneficial effects of intravenous ROCK inhibitor fasudil have been shown in patients[77]. Therefore, the literature clearly implicates the ROCK signalling as promising target for the treatment of pulmonary hypertension and Fukumoto Y. *et al* suggested the potential application of Rho-kinase inhibitors in diagnostic and treatment algorithm for pulmonary hypertension (scheme 10)[51]. Recently, a novel azaindole-based ROCK inhibitor has been described[87]. Azaindole-1 acts in an ATP-competitive manner with activity in the lower nanomolar range, suggesting this inhibitor as a very potent compound. Importantly, although ATP-competitive, azaindole-1 was inactive against 89 of different kinases and exhibited only a weak activity against 21 kinases, suggesting this compound as a very selective ROCK inhibitor.

However, the therapeutic efficacy of azaindole-1 has not yet been investigated in pulmonary hypertension. Therefore, we aimed to explore the potential therapeutic effects of azaindole-1 in experimental models of pulmonary hypertension.

In general, the major findings of the present study are:

1) azaindole-1 significantly inhibited acute hypoxic pulmonary vasoconstriction *ex vivo* and the proliferation of primary rat pulmonary arterial smooth muscle cells (PASMCs) *in vitro*;

2) azaindole-1 treatment significantly improved hemodynamics, right ventricular hypertrophy and pulmonary vascular remodelling in MCT-injected rats and chronically hypoxic mice and

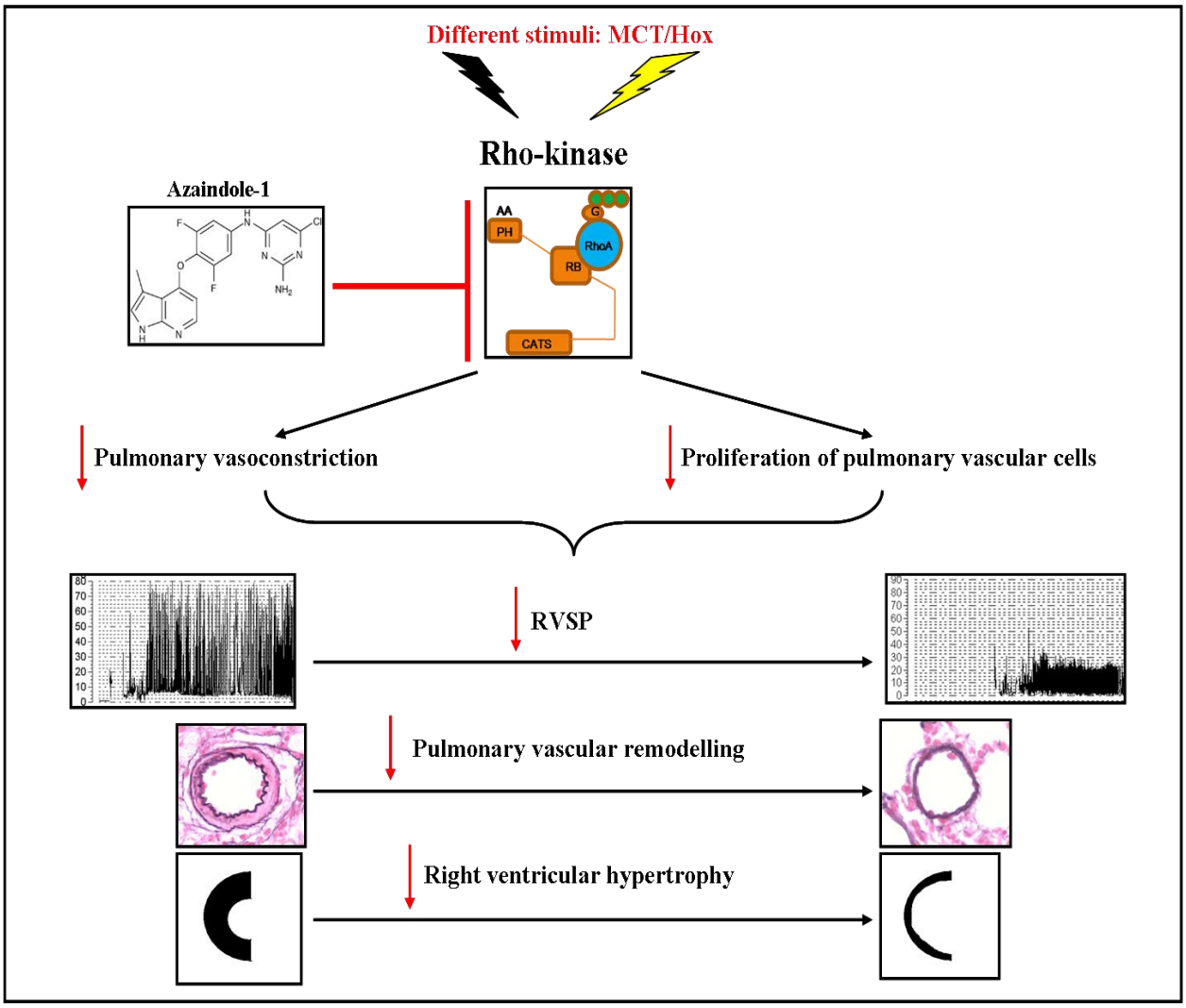
3) the improvement in hemodynamics and vascular remodelling in rats was accompanied by an impaired ROCK activity and a decrease in proliferating cells in pulmonary vessels, as evident from reduced immunoreactivity for phospho-myosin phosphatase subunit 1 (p-MYPT1) and proliferating cell nuclear antigen (PCNA)[70;101].

4.1. Rho-kinase inhibition in MCT-induced pulmonary arterial hypertension

Hyperactivation of ROCKs, by inhibition of MYPT activity and increase of MLC phosphorylation, leads to contraction of vascular smooth muscle cells and subsequently to vasoconstriction[43;102]. In experimental models of pulmonary hypertension, acute inhibition of ROCK caused the pulmonary vasorelaxation and reduced pulmonary arterial pressure[73;74;103]. In agreement with literature we successfully demonstrated that azaindole-1 significantly impaired the MCT-induced progressive increase of RVSP in rats as monitored by online telemetry system. Importantly, no significant effect was observed on heart rates, suggesting that azaindole-1 treatment did not cause the reflex tachycardia. This finding is in line with the studies that investigated the role of ROCK-mediated sustained vasoconstriction in the pathogenesis of pulmonary hypertension[73;103-105]. As it has been shown that the ROCK inhibition reduced pulmonary vascular remodelling, we performed the experiments to test the effect of azaindole-1 on PASMCs proliferation *in vitro*[106-109]. We found that azaindole-1 significantly inhibited the thymidine incorporation into primary PASMCs isolated form MCT-injected rats, at high nanomolar to low micromolar range. In addition, the effect of azaindole-1 was more potent in comparison with fasudil and Y-27632. Before the proliferation assays were performed we tested the possible toxicity of azaindole-1 by MTT assay and observed that this compound was not toxic on PASMCs at the tested doses. This clearly indicates that the reduced number of the cells was only because of the inhibited proliferation. Taken together, these *in vivo* and *in vitro* findings attribute potent vasorelaxant and antiproliferative

properties of azaindole-1 and clearly substantiate its therapeutic potential in pulmonary hypertension.

To investigate the potential therapeutic efficacy of azainodole-1, we performed the chronic treatment studies in experimental pulmonary hypertension induced by MCT-injection in rats. We successfully demonstrated that azaindole-1 improved pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling as evident from significantly reduced RVSP, TPR and RV/(LV+S) and degree of muscularization and medial wall thickness of peripheral pulmonary vessels (scheme 11).



Scheme 11. Therapeutic effects of azaindole-1 in experimental pulmonary hypertension.
Legend: **MCT** – monocrotaline; **Hox** – hypoxia; **RVSP** – right ventricular systolic pressure.

Importantly, the SAP did not change significantly in the treated animals. As Kast *et al*. observed the significant reduction of blood pressure in spontaneously hypertensive rats by azaindole-1, our findings at first glance may look contradictory to their findings[87]. First, the discrepancy in the findings could be explained at least in part by the differences in animal models used for experiments and duration of the treatment with ROCK inhibitor. The ROCK activity is enhanced in the systemic vasculature of spontaneously hypertensive rats[110]. Additionally, Kast *et al*. treated the normotensive rats only once and spontaneously hypertensive rats for 4 days and also noted that the blood pressure lowering effect in spontaneously hypertensive rats was gradually decreasing[87]. In our study we used another animal model and treated the animals for 2 weeks. As a conclusion, our results may not be directly comparable with data of Kast *et al*[87]. Notably, the sustained pulmonary ROCKs activity in animal models of pulmonary hypertension may underlie the pulmonary specific effects of a very selective ROCK inhibitors, like azaindole-1[48]. The literature sources suggest beneficial effects of Rho-kinase inhibition on the development of MCT-induced pulmonary hypertension in rats[105;109]. Our results extend these findings, demonstrating that azaindole-1 is therapeutically beneficial even when the treatment commences at the time the disease is already established and rapidly progressing.

As a further support to our data, we found that azaindole-1 treatment resulted in an impaired ROCK activity, as determined by a significant reduction in p-MYPT1 immunoreactivity in MCT rat model. Additionally, in the line with our data from *in vitro* proliferation experiments, we demonstrated that azaindole-1 treatment significantly decreased the index of proliferation (IOP) of vascular cells in MCT-injected rat lungs, suggesting the antiproliferative effects of the compound not only *in vitro*, but *in situ* as well. Furthermore, azaindole-1 treatment of MCT-injected rats showed the improved survival as compared to the MCT rats receiving just a vehicle.

As a summary, our results suggest that the therapeutic efficacy of ROCK inhibition by azaindole-1 may be associated with its vasorelaxant and antiproliferative potency. In the line with our study, Abe *et al*. have described an improvement of MCT-induced pulmonary hypertension in rats by chronic treatment with well-known ROCK inhibitor fasudil[68]. Although the fasudil exhibited the therapeutic efficacy in MCT rat model, Oka *et al*. suggested that there is a noticeable discrepancy in the findings depending on the dose, route of administration and animal models of pulmonary hypertension[48]. Importantly, fasudil was

used in previous studies at the doses from 30 to 100 mg/kg of body weight, while azaindole-1 showed the successful therapeutic potency at lower dose of 10mg/kg of body weight, suggesting that not only the *in vitro* antiproliferative effects, but also the *in vivo* therapeutic effects of azaindole-1 are more potent in comparison with fasudil.

The literature implicates the involvement of ROCKs in the pathology of experimental pulmonary hypertension induced by different stimuli[68;72;74;75]. Furthermore, Rho-kinase signalling plays a role in other signalling cascades than ROCK and accumulating body of literature suggests the beneficial effects of therapeutic strategies that involve other targets[71;72;78;111-113]. According to this, ROCK system may be involved in other signalling pathways that are implicated in the pathogenesis of pulmonary hypertension.

To investigate if the ROCK inhibition by azaindole-1 may demonstrate the beneficial effects independently of the cause of the disease, we included in our study the hypoxia-induced model of pulmonary hypertension in mice.

4.2. Rho-kinase inhibition in hypoxia-induced pulmonary hypertension

Kast *et al*. found that azaindole-1 dose-dependently inhibited human ROCK-1 and ROCK-2 in a low nanomolar range with IC_{50} values of 0.6nM and 1.1nM, respectively[87]. Therefore, azaindole-1 is more potent compound than fasudil and Y-27632, which have IC_{50} values in higher nanomolar range, of 158nM and 162nM, respectively[114]. Additionally, it has been shown that azaindole-1 inhibits the phenylephrine-induced contraction of rabbit saphenous artery in a concentration-dependent manner, suggesting the vasorelaxant efficacy of the compound[87]. To extend the finding to pulmonary circulation, that is more relevant for the studies of pulmonary hypertension, we performed the experiment with isolated, ventilated and buffer-perfused mice lungs. In our study, all three ROCK inhibitors (azaindole-1, fasudil and Y-27632) significantly reduced the acute hypoxic pulmonary vasoconstriction in isolated murine lungs. But importantly, the observed effect of azaindole-1 was more potent than the effects of fasudil and Y-27632. Taken together, our data and results from Kast *et al*., suggest that azaindole-1 is not only a highly selective and potent inhibitor of Rho-kinase, but also a stronger vasorelaxant than fasudil and Y-27632[87].

The finding that azaindole-1 shows a vasorelaxant properties on acute hypoxia-induced pulmonary vasoconstriction and that the effect was more prominent than with other two ROCK inhibitors, prompted us to investigate the therapeutic efficacy of azaindole-1 on chronic hypoxia-induced pulmonary hypertension in mice.

ROCK system plays a role in hypoxia induced pulmonary hypertension and ROCK inhibition demonstrated the beneficial therapeutic effects in this model, as suggested by the augmented literature sources[103;104;107;111-113]. In the line with literature, we found that oral treatment of hypoxic mice with azaindole-1 improved pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling, as evident from significantly reduced RVSP, RV/(LV+S) and degree of muscularization and medial wall thickness of peripheral pulmonary vessels.

Therefore, our findings indicate that the therapeutic efficacy of azaindole-1 is independent of the cause of the disease.

4.3. Future experimental and clinical perspective of azaindole-based Rho-kinase inhibition

Although MCT-induced pulmonary hypertension in rats and hypoxia-induced pulmonary hypertension in mice are well accepted animal models for the research of the pathological mechanisms and therapeutic strategies of this disease, they still have a certain limitations with regard to mimicking the human pathological events and manifestations. The improved animal models that more closely share some human features (neointima and plexiform lesions) were reported in recent years, such as combination of MCT and one-sided pneumonectomy and hypoxia in combination with vascular endothelial growth factor receptor (VEGFR)-2 inhibitor SU5416[16;35;36]. Azaindole-1 can thus be anticipated to yield therapeutic benefit in these animal models and can be used as a useful tool to further investigate the development of these complex pathohistological manifestations.

Azaindole-1 with its advantages (table 5) in comparison with previously developed ROCK inhibitors, such as fasudil and Y-27632 may offer a potential therapeutic strategy to cure this severe human disease.

ROCK inhibitor	Azaindole-1	Fasudil	Y-27632
IC_{50} values (nM) (ROCK)	0.6/1.1	158	162
Effect on HPV (%) (dose: 30µM)	~90	~75	~75
Effect on PASMCs proliferation (% FCS)	~41	~82	~67

Table 5. The advantages of azaindole-1 in comparison with fasudil and Y-27632. Legend: **IC_{50}** - half maximum inhibitory concentration; **HPV** – hypoxic pulmonary vasoconstriction; **PASMCs** – pulmonary artery smooth muscle cells; **FCS** - Fetal calf serum.

Regarding human pulmonary hypertension, the literature sources are emerging that implicate ROCK signalling in the disease pathogenesis and therefore this signalling pathway represents a potential therapeutic target[76;78;80;82]. More importantly, acute inhibition of ROCK by fasudil has revealed beneficial effects, as evident from reduction of pulmonary vascular resistance and pulmonary arterial pressure in patients with severe pulmonary hypertension[76;77;79;81].

Taken together, this study strongly suggests that azaindole-1 may represent a novel therapeutic approach for the treatment of pulmonary hypertension.

5. Abbreviations and acronyms

PH	Pulmonary hypertension
PAH	Pulmonary arterial hypertension
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PDE	Phosphodiesterase
HIF 1α	Hypoxia-inducible factor 1 alpha
VEGFR-2	Vascular endothelial growth factor receptor-2
SU5416	Sugen 5416
MCT	Monocrotaline
GTP	Guanosine triphosphate
Rho	**R**as **ho**mologous
ROCK	Rho-kinase
GDP	Guanosine diphosphate
CATS	Catalytic subunit
RB	Rho-binding domain
PH	Pleckstrin-homology domain
SMC	Smooth muscle cell
MLC	Myosin-light chain
MYPT	Myosin-phosphatase
pMYPT1	Phospho-myosin phosphatase subunit 1
MBS	Myosin-binding subunit
VSMC	Vascular smooth muscle cell
ERK	Extracellular-regulated kinase
$P27^{Kip1}$	Cyclin-dependent kinase inhibitor
UTP	Uridine triphosphate
eNOS	Endothelial nitric oxide synthase
ATP	Adenosine triphosphate

PASMC	Pulmonary artery smooth muscle cell
PCNA	Proliferating cell nuclear antigen
BW	Body weight
HCL	Hydrogen chloride
NaOH	Sodium hydroxide
Hox	Hypoxia
Nox	Normoxia
HPV	Hypoxic pulmonary vasoconstriction
DMSO	Dimethyl sulfoxide
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
FCS	Fetal calf serum
HBSS	Hank's Balanced Salt Solution
TCA	Trichloroacetic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RVSP	Right ventricular systolic pressure
HR	Heart rate
SAP	Systemic arterial pressure
PEEP	Positive end expiratory pressure
RV	Right ventricle
LV+S	Left ventricle plus septum
RVH	Right ventricular hypertrophy
CO	Cardiac output
CI	Cardiac index
TPR	Total pulmonary resistance
TSR	Total systemic resistance
Hb	Hemoglobin
MWT	Medial wall thickness
vWF	von Willebrand factor
H_2O_2	Hydrogen peroxide

BSA	Bovine serum albumin
PBS	Phosphate buffered saline
IOP	Index of proliferation
SEM	Standard error of the mean
ANOVA	Analysis of variance
αSMA	Alpha smooth muscle actin
IC_{50}	Half maximum inhibitory concentration
DAB	3,3' Diaminobenzidine
VIP	Peroxidase Substrate kit
Thr	Threonine

6. Summary

Pulmonary hypertension (PH) is a severe chronic and life-threatening disease characterized by progressive augmentation of pulmonary arterial pressure that finally leads to right ventricle failure and death. PH has a multi-complex pathology which includes a combination of pulmonary vascular remodelling, vasoconstriction and *in situ* thrombosis. Key to the severity of the disease is the progressive pulmonary vascular remodelling. The progressive pulmonary vascular remodelling is the attribute of PH pathology and is characterized by abnormalities of vascular cells such as increased proliferation, migration and resistance to apoptosis. Although the PH pathology is the subject of intensive research the precise molecular mechanisms are still not fully understood and successful therapeutic strategy to cure the disease is still needed.

An accumulating body of evidence incriminates Rho-kinase (ROCK) in the pathogenesis of PH and suggests the Rho-kinase as a promising therapeutic target. In the line with literature, our study aimed to investigate the therapeutic effects of a novel highly selective and orally active ROCK inhibitor, azaindole-1 in animal models of PH. We successfully demonstrated that azaindole-1 significantly inhibited hypoxic pulmonary vasoconstriction in isolated, ventilated and buffer-perfused murine lungs and proliferation of primary rat pulmonary artery smooth muscle cells *in vitro*. Furthermore, azaindole-1 chronic treatment improved hemodynamics and right ventricular hypertrophy in both experimental models of PH. Moreover, the medial wall thickness and muscularization of peripheral pulmonary arteries, as measures of pulmonary vascular remodelling, were significantly ameliorated. Finally, azaindole-1 treatment resulted in a decreased immunoreactivity for phospho-myosin phosphatase target subunit-1 (p-MYPT-1) and proliferating cell nuclear antigen (PCNA) in pulmonary vessels of monocrotaline-injected rats, suggesting an impaired ROCK activity and reduced proliferating vascular cells.

In conclusion, azaindole-1 provided therapeutic benefit in experimental PH and this may be attributable to its potent vasorelaxant and antiproliferative effects. Thus, azaindole-1 may offer a useful approach for the treatment of pulmonary hypertension and may have a potential clinical application.

7. Zusammenfassung

Pulmonaler Hochdruck (PH) ist eine schwere chronische und lebensbedrohliche Erkrankung, charakterisiert duch progressive Zunahme des pulmonal arteriellen Drucks, welche letztlich in Rechtsherzversagen und Tod endet. PH hat besitzt eine komplexe Pathologie mit der Kombination eines Remodelling der Pulmonalgefäße, Vasokonstriktion und *in situ* Thrombose. Der Schlüssel zu der Schwere dieser Erkrankung ist das progressiv verlaufende Gefäßremodelling. Dieses progressive pulmonale Gefässremodelling ist das Attribut der PH Pathologie und wird charakterisiert durch Abnormalitäten der Gefäßzellen, wie zB. verstärkte Proliferation, Migration, und Unempfindlichkeit gegenüber Apoptose. Obwohl die PH Pathologie Gegenstand intensiver Forschung ist, sind die genauen molekularen Mechanismen noch nicht vollständig verstanden. Eine erfolgreiche Therapie zur Heilung dieser schweren Krankheit ist noch nicht verfügbar.

Rho-kinasen (ROCK) scheinen in der Pathologie der PH eine wichtige Rolle zu spielen und lassen vermuten, hier einen erfolgsversprechenden Angriffspunkt für eine mögliche Therapie darzustellen.

Im Einklang mit dementsprechender Literatur, erforschen unsere Studien im Tiermodell den therapeutischen Effekt des hochselektiven, oral verfügbaren ROCK Inhibitors, Azaindole-1.

Wir konnten erfolgreich zeigen, dass Azaindole-1 die Vasokonstriktion in isolierter, ventilierter und Puffer-perfundierter Mauslunge sowie die Proliferation von arteriellen glatten Muskelzellen der Ratte *in vitro* verminderte. Weiterhin verbesserte eine Behandlung mit Azaindole-1 die Hämodynamik und Rechtsherzhypertrophie in zwei Tiermodellen der PH. Ebenfalls kam es zu einer signifikanten Verbesserung der medialen Wandstärke sowie dem Muskularisierungsgrad der Pulmonalgefäße, welche als Paramater für das vaskuläre Remodelling gelten. Letztlich führte eine Behandlung mit Azaindole-1 zu einer Abnahme der Immunreaktivität der Phospho-Myosin Phosphatase Target Subunit-1 (p-MYPT-1) und PCNA (prolferating cell nuklear antigen) in Pulmonalgefäßen der mit Monocrotalin injizierten Ratten, was auf eine geminderte ROCK Aktivität und reduzierte Anzahl proliferierender Gefäßzellen schließen lässt.

Zusammenfassend lässt sich sagen, dass Azaindole-1 ein vielversprechender Ansatz für die Behandlung experimenteller PH darstellt, was sich wahrscheinlich auf seine potenten vasorelaxierenden und antiproliferierenden Effekte zurückführen lässt. Dadurch könnte Azaindole-1 einen guten Therapieansatz für die Behandlung des pulmonalen Hochdrucks darstellen und das Potential für eine klinische Anwendung besitzen.

8. Reference List

1. Rosenkranz, S. 2007. Pulmonary hypertension: current diagnosis and treatment. *Clin.Res.Cardiol.* 96:527-541.

2. Simonneau, G., I. M. Robbins, M. Beghetti, R. N. Channick, M. Delcroix, C. P. Denton, C. G. Elliott, S. P. Gaine, M. T. Gladwin, Z. C. Jing, et al. 2009. Updated clinical classification of pulmonary hypertension. *J.Am.Coll.Cardiol.* 54:S43-S54.

3. Jain, S., H. Ventura, and B. deBoisblanc. 2007. Pathophysiology of pulmonary arterial hypertension. *Semin.Cardiothorac.Vasc.Anesth.* 11:104-109.

4. Ghofrani, H. A., R. J. Barst, R. L. Benza, H. C. Champion, K. A. Fagan, F. Grimminger, M. Humbert, G. Simonneau, D. J. Stewart, C. Ventura, et al. 2009. Future perspectives for the treatment of pulmonary arterial hypertension. *J.Am.Coll.Cardiol.* 54:S108-S117.

5. Humbert, M., N. W. Morrell, S. L. Archer, K. R. Stenmark, M. R. MacLean, I. M. Lang, B. W. Christman, E. K. Weir, O. Eickelberg, N. F. Voelkel, et al. 2004. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J.Am.Coll.Cardiol.* 43:13S-24S.

6. Stenmark, K. R., K. A. Fagan, and M. G. Frid. 2006. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ.Res.* 99:675-691.

7. Tuder, R. M., J. H. Yun, A. Bhunia, and I. Fijalkowska. 2007. Hypoxia and chronic lung disease. *J.Mol.Med.* 85:1317-1324.

8. Rich, S., D. R. Dantzker, S. M. Ayres, E. H. Bergofsky, B. H. Brundage, K. M. Detre, A. P. Fishman, R. M. Goldring, B. M. Groves, S. K. Koerner, et al. 1987. Primary pulmonary hypertension. A national prospective study. *Ann.Intern.Med.* 107:216-223.

9. Nauser, T. D. and S. W. Stites. 2001. Diagnosis and treatment of pulmonary hypertension. *Am.Fam.Physician* 63:1789-1798.

10. Rabinovitch, M. 2008. Molecular pathogenesis of pulmonary arterial hypertension. *J.Clin.Invest* 118:2372-2379.

11. Budhiraja, R., R. M. Tuder, and P. M. Hassoun. 2004. Endothelial dysfunction in pulmonary hypertension. *Circulation* 109:159-165.

12. Christman, B. W., C. D. McPherson, J. H. Newman, G. A. King, G. R. Bernard, B. M. Groves, and J. E. Loyd. 1992. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N.Engl.J.Med.* 327:70-75.

13. Giaid, A. and D. Saleh. 1995. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N.Engl.J.Med.* 333:214-221.

14. Morrell, N. W., S. Adnot, S. L. Archer, J. Dupuis, P. L. Jones, M. R. MacLean, I. F. McMurtry, K. R. Stenmark, P. A. Thistlethwaite, N. Weissmann, et al. 2009. Cellular and molecular basis of pulmonary arterial hypertension. *J.Am.Coll.Cardiol.* 54:S20-S31.

15. Nicod, L. P. 2007. The endothelium and genetics in pulmonary arterial hypertension. *Swiss.Med.Wkly.* 137:437-442.

16. Yi, E. S., H. Kim, H. Ahn, J. Strother, T. Morris, E. Masliah, L. A. Hansen, K. Park, and P. J. Friedman. 2000. Distribution of obstructive intimal lesions and their cellular

phenotypes in chronic pulmonary hypertension. A morphometric and immunohistochemical study. *Am.J.Respir.Crit Care Med.* 162:1577-1586.

17. Meyrick, B. and L. Reid. 1980. Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am.J.Pathol.* 100:151-178.

18. Jones, P. L., K. N. Cowan, and M. Rabinovitch. 1997. Tenascin-C, proliferation and subendothelial fibronectin in progressive pulmonary vascular disease. *Am.J.Pathol.* 150:1349-1360.

19. Stenmark, K. R., E. Gerasimovskaya, R. A. Nemenoff, and M. Das. 2002. Hypoxic activation of adventitial fibroblasts: role in vascular remodeling. *Chest* 122:326S-334S.

20. Frid, M. G., V. A. Kale, and K. R. Stenmark. 2002. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ.Res.* 90:1189-1196.

21. Yamashita, J., H. Itoh, M. Hirashima, M. Ogawa, S. Nishikawa, T. Yurugi, M. Naito, K. Nakao, and S. Nishikawa. 2000. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 408:92-96.

22. Berger, G., Z. S. Azzam, R. Hoffman, and M. Yigla. 2009. Coagulation and anticoagulation in pulmonary arterial hypertension. *Isr.Med.Assoc.J.* 11:376-379.

23. Welsh, C. H., K. L. Hassell, D. B. Badesch, D. C. Kressin, and R. A. Marlar. 1996. Coagulation and fibrinolytic profiles in patients with severe pulmonary hypertension. *Chest* 110:710-717.

24. Tuder, R. M. and N. F. Voelkel. 1998. Pulmonary hypertension and inflammation. *J.Lab Clin.Med.* 132:16-24.

25. Hassoun, P. M., L. Mouthon, J. A. Barbera, S. Eddahibi, S. C. Flores, F. Grimminger, P. L. Jones, M. L. Maitland, E. D. Michelakis, N. W. Morrell, et al. 2009. Inflammation, growth factors, and pulmonary vascular remodeling. *J.Am.Coll.Cardiol.* 54:S10-S19.

26. Dahal, B. K., D. Kosanovic, R. K. Savai, H. A. Ghofrani, N. Weissmann, W. M. Kuebler, F. Grimminger, W. Seeger, and R. Schermuly. Role of mast cells in experimental pulmonary hypertension. Am.J.Respir.Crit Care Med.1812010:A6330 . 2010.

Ref Type: Abstract

27. Boutet, K., D. Montani, X. Jais, A. Yaici, O. Sitbon, G. Simonneau, and M. Humbert. 2008. Therapeutic advances in pulmonary arterial hypertension. *Ther.Adv.Respir.Dis.* 2:249-265.

28. Chin, K. M. and L. J. Rubin. 2008. Pulmonary arterial hypertension. *J.Am.Coll.Cardiol.* 51:1527-1538.

29. Barst, R. J., D. Langleben, D. Badesch, A. Frost, E. C. Lawrence, S. Shapiro, R. Naeije, and N. Galie. 2006. Treatment of pulmonary arterial hypertension with the selective endothelin-A receptor antagonist sitaxsentan. *J.Am.Coll.Cardiol.* 47:2049-2056.

30. Langleben, D., T. Brock, R. Dixon, and R. Barst. 2004. STRIDE 1: effects of the selective ET(A) receptor antagonist, sitaxsentan sodium, in a patient population with

pulmonary arterial hypertension that meets traditional inclusion criteria of previous pulmonary arterial hypertension trials. *J.Cardiovasc.Pharmacol.* 44 Suppl 1:S80-S84.

31. Hoeper, M. M., E. Spiekerkoetter, V. Westerkamp, R. Gatzke, and H. Fabel. 2002. Intravenous iloprost for treatment failure of aerosolised iloprost in pulmonary arterial hypertension. *Eur.Respir.J.* 20:339-343.

32. Campian, M. E., M. Hardziyenka, M. C. Michel, and H. L. Tan. 2006. How valid are animal models to evaluate treatments for pulmonary hypertension? *Naunyn Schmiedebergs Arch.Pharmacol.* 373:391-400.

33. Meyrick, B. and L. Reid. 1980. Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am.J.Pathol.* 100:151-178.

34. Stenmark, K. R., E. Gerasimovskaya, R. A. Nemenoff, and M. Das. 2002. Hypoxic activation of adventitial fibroblasts: role in vascular remodeling. *Chest* 122:326S-334S.

35. Sakao, S., L. Taraseviciene-Stewart, J. D. Lee, K. Wood, C. D. Cool, and N. F. Voelkel. 2005. Vascular endothelial growth factor receptor blockade by SU5416 combined with pulsatile shear stress causes apoptosis and subsequent proliferation of apoptosis-resistant endothelial cells. *Chest* 128:610S-611S.

36. Taraseviciene-Stewart, L., Y. Kasahara, L. Alger, P. Hirth, M. G. Mc, J. Waltenberger, N. F. Voelkel, and R. M. Tuder. 2001. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J.* 15:427-438.

37. LALICH, J. J. and L. MERKOW. 1961. Pulmonary arteritis produced in rat by feeding Crotalaria spectabilis. *Lab Invest* 10:744-750.

38. Reid, M. J., M. W. Lame, D. Morin, D. W. Wilson, and H. J. Segall. 1997. Monocrotaline metabolism and distribution in Fisher 344 and Sprague-Dawley rats. *Comp Biochem.Physiol B Biochem.Mol.Biol.* 117:115-123.

39. Reid, M. J., M. W. Lame, D. Morin, D. W. Wilson, and H. J. Segall. 1998. Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. *J.Biochem.Mol.Toxicol.* 12:157-166.

40. Loscalzo, J. 1992. Endothelial dysfunction in pulmonary hypertension. *N.Engl.J.Med.* 327:117-119.

41. Stenmark, K. R., B. Meyrick, N. Galie, W. J. Mooi, and I. F. McMurtry. 2009. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am.J.Physiol Lung Cell Mol.Physiol* 297:L1013-L1032.

42. Dumitrascu, R., S. Koebrich, E. Dony, N. Weissmann, R. Savai, S. S. Pullamsetti, H. A. Ghofrani, A. Samidurai, H. Traupe, W. Seeger, et al. 2008. Characterization of a murine model of monocrotaline pyrrole-induced acute lung injury. *BMC.Pulm.Med.* 8:25.

43. Etienne-Manneville, S. and A. Hall. 2002. Rho GTPases in cell biology. *Nature* 420:629-635.

44. Loirand, G., P. Guerin, and P. Pacaud. 2006. Rho kinases in cardiovascular physiology and pathophysiology. *Circ.Res.* 98:322-334.

45. Leung, T., E. Manser, L. Tan, and L. Lim. 1995. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J.Biol.Chem.* 270:29051-29054.

46. Matsui, T., M. Amano, T. Yamamoto, K. Chihara, M. Nakafuku, M. Ito, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* 15:2208-2216.

47. Nakagawa, O., K. Fujisawa, T. Ishizaki, Y. Saito, K. Nakao, and S. Narumiya. 1996. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* 392:189-193.

48. Oka, M., K. A. Fagan, P. L. Jones, and I. F. McMurtry. 2008. Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. *Br.J.Pharmacol.* 155:444-454.

49. Amano, M., Y. Fukata, and K. Kaibuchi. 2000. Regulation and functions of Rho-associated kinase. *Exp.Cell Res.* 261:44-51.

50. Wibberley, A., Z. Chen, E. Hu, J. P. Hieble, and T. D. Westfall. 2003. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. *Br.J.Pharmacol.* 138:757-766.

51. Fukumoto, Y., S. Tawara, and H. Shimokawa. 2007. Recent progress in the treatment of pulmonary arterial hypertension: expectation for rho-kinase inhibitors. *Tohoku J.Exp.Med.* 211:309-320.

52. Ratz, P. H., K. M. Berg, N. H. Urban, and A. S. Miner. 2005. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am.J.Physiol Cell Physiol* 288:C769-C783.

53. Somlyo, A. P. and A. V. Somlyo. 2003. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev.* 83:1325-1358.

54. Pfitzer, G. 2001. Invited review: regulation of myosin phosphorylation in smooth muscle. *J.Appl.Physiol* 91:497-503.

55. Kamiyama, M., K. Utsunomiya, K. Taniguchi, T. Yokota, H. Kurata, N. Tajima, and K. Kondo. 2003. Contribution of Rho A and Rho kinase to platelet-derived growth factor-BB-induced proliferation of vascular smooth muscle cells. *J.Atheroscler.Thromb.* 10:117-123.

56. Sauzeau, V., M. E. Le, J. Bertoglio, E. Scalbert, P. Pacaud, and G. Loirand. 2001. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ.Res.* 88:1102-1104.

57. Seasholtz, T. M., M. Majumdar, D. D. Kaplan, and J. H. Brown. 1999. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. *Circ.Res.* 84:1186-1193.

58. Laufs, U., D. Marra, K. Node, and J. K. Liao. 1999. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing rho GTPase-induced down-regulation of p27(Kip1). *J.Biol.Chem.* 274:21926-21931.

59. Sawada, N., H. Itoh, K. Ueyama, J. Yamashita, K. Doi, T. H. Chun, M. Inoue, K. Masatsugu, T. Saito, Y. Fukunaga, et al. 2000. Inhibition of rho-associated kinase

results in suppression of neointimal formation of balloon-injured arteries. *Circulation* 101:2030-2033.

60. Ai, S., M. Kuzuya, T. Koike, T. Asai, S. Kanda, K. Maeda, T. Shibata, and A. Iguchi. 2001. Rho-Rho kinase is involved in smooth muscle cell migration through myosin light chain phosphorylation-dependent and independent pathways. *Atherosclerosis* 155:321-327.

61. Chaulet, H., C. Desgranges, M. A. Renault, F. Dupuch, G. Ezan, F. Peiretti, G. Loirand, P. Pacaud, and A. P. Gadeau. 2001. Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin. *Circ.Res.* 89:772-778.

62. Seasholtz, T. M., T. Zhang, M. R. Morissette, A. L. Howes, A. H. Yang, and J. H. Brown. 2001. Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. *Circ.Res.* 89:488-495.

63. Birukova, A. A., K. Smurova, K. G. Birukov, P. Usatyuk, F. Liu, K. Kaibuchi, A. Ricks-Cord, V. Natarajan, I. Alieva, J. G. Garcia, et al. 2004. Microtubule disassembly induces cytoskeletal remodeling and lung vascular barrier dysfunction: role of Rho-dependent mechanisms. *J.Cell Physiol* 201:55-70.

64. Gorovoy, M., J. Niu, O. Bernard, J. Profirovic, R. Minshall, R. Neamu, and T. Voyno-Yasenetskaya. 2005. LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. *J.Biol.Chem.* 280:26533-26542.

65. Eto, M., C. Barandier, L. Rathgeb, T. Kozai, H. Joch, Z. Yang, and T. F. Luscher. 2001. Thrombin suppresses endothelial nitric oxide synthase and upregulates

endothelin-converting enzyme-1 expression by distinct pathways: role of Rho/ROCK and mitogen-activated protein kinase. *Circ.Res.* 89:583-590.

66. Laufs, U., F. La, V, J. Plutzky, and J. K. Liao. 1998. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 97:1129-1135.

67. Ming, X. F., C. Barandier, H. Viswambharan, B. R. Kwak, F. Mach, L. Mazzolai, D. Hayoz, J. Ruffieux, S. Rusconi, J. P. Montani, et al. 2004. Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction. *Circulation* 110:3708-3714.

68. Abe, K., H. Shimokawa, K. Morikawa, T. Uwatoku, K. Oi, Y. Matsumoto, T. Hattori, Y. Nakashima, K. Kaibuchi, K. Sueishi, et al. 2004. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ.Res.* 94:385-393.

69. Abe, K., S. Tawara, K. Oi, T. Hizume, T. Uwatoku, Y. Fukumoto, K. Kaibuchi, and H. Shimokawa. 2006. Long-term inhibition of Rho-kinase ameliorates hypoxia-induced pulmonary hypertension in mice. *J.Cardiovasc.Pharmacol.* 48:280-285.

70. Dahal, B. K., D. Kosanovic, P. K. Pamarthi, A. Sydykov, Y. J. Lai, R. Kast, H. Schirok, J. P. Stasch, H. A. Ghofrani, N. Weissmann, et al. 2010. Therapeutic efficacy of azaindole-1 in experimental pulmonary hypertension. *Eur.Respir.J.* 36:808-818.

71. Hemnes, A. R., A. Zaiman, and H. C. Champion. 2008. PDE5A inhibition attenuates bleomycin-induced pulmonary fibrosis and pulmonary hypertension through inhibition of ROS generation and RhoA/Rho kinase activation. *Am.J.Physiol Lung Cell Mol.Physiol* 294:L24-L33.

72. Homma, N., T. Nagaoka, V. Karoor, M. Imamura, L. Taraseviciene-Stewart, L. A. Walker, K. A. Fagan, I. F. McMurtry, and M. Oka. 2008. Involvement of RhoA/Rho kinase signaling in protection against monocrotaline-induced pulmonary hypertension in pneumonectomized rats by dehydroepiandrosterone. *Am.J.Physiol Lung Cell Mol.Physiol* 295:L71-L78.

73. McNamara, P. J., P. Murthy, C. Kantores, L. Teixeira, D. Engelberts, V. T. van, B. P. Kavanagh, and R. P. Jankov. 2008. Acute vasodilator effects of Rho-kinase inhibitors in neonatal rats with pulmonary hypertension unresponsive to nitric oxide. *Am.J.Physiol Lung Cell Mol.Physiol* 294:L205-L213.

74. Nagaoka, T., S. A. Gebb, V. Karoor, N. Homma, K. G. Morris, I. F. McMurtry, and M. Oka. 2006. Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat. *J.Appl.Physiol* 100:996-1002.

75. Oka, M., N. Homma, L. Taraseviciene-Stewart, K. G. Morris, D. Kraskauskas, N. Burns, N. F. Voelkel, and I. F. McMurtry. 2007. Rho kinase-mediated vasoconstriction is important in severe occlusive pulmonary arterial hypertension in rats. *Circ.Res.* 100:923-929.

76. Do e Z, Y. Fukumoto, A. Takaki, S. Tawara, J. Ohashi, M. Nakano, T. Tada, K. Saji, K. Sugimura, H. Fujita, et al. 2009. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ.J.* 73:1731-1739.

77. Fukumoto, Y., T. Matoba, A. Ito, H. Tanaka, T. Kishi, S. Hayashidani, K. Abe, A. Takeshita, and H. Shimokawa. 2005. Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. *Heart* 91:391-392.

78. Guilluy, C., S. Eddahibi, C. Agard, C. Guignabert, M. Izikki, L. Tu, L. Savale, M. Humbert, E. Fadel, S. Adnot, et al. 2009. RhoA and Rho kinase activation in human pulmonary hypertension: role of 5-HT signaling. *Am.J.Respir.Crit Care Med.* 179:1151-1158.

79. Ishikura, K., N. Yamada, M. Ito, S. Ota, M. Nakamura, N. Isaka, and T. Nakano. 2006. Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. *Circ.J.* 70:174-178.

80. Laumanns, I. P., L. Fink, J. Wilhelm, J. C. Wolff, R. Mitnacht-Kraus, S. Graef-Hoechst, M. M. Stein, R. M. Bohle, W. Klepetko, M. A. Hoda, et al. 2009. The noncanonical WNT pathway is operative in idiopathic pulmonary arterial hypertension. *Am.J.Respir.Cell Mol.Biol.* 40:683-691.

81. Li, F., W. Xia, S. Yuan, and R. Sun. 2009. Acute inhibition of Rho-kinase attenuates pulmonary hypertension in patients with congenital heart disease. *Pediatr.Cardiol.* 30:363-366.

82. Liu, Y., W. Ren, R. Warburton, D. Toksoz, and B. L. Fanburg. 2009. Serotonin induces Rho/ROCK-dependent activation of Smads 1/5/8 in pulmonary artery smooth muscle cells. *FASEB J.* 23:2299-2306.

83. Ono-Saito, N., I. Niki, and H. Hidaka. 1999. H-series protein kinase inhibitors and potential clinical applications. *Pharmacol.Ther.* 82:123-131.

84. Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, et al. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389:990-994.

85. Casey, D. B., A. M. Badejo, J. S. Dhaliwal, J. L. Sikora, A. Fokin, N. H. Golwala, A. J. Greco, S. N. Murthy, B. D. Nossaman, A. L. Hyman, et al. 2010. Analysis of responses to the Rho-kinase inhibitor Y-27632 in the pulmonary and systemic vascular bed of the rat. *Am.J.Physiol Heart Circ.Physiol* 299:H184-H192.

86. Asano, T., T. Suzuki, M. Tsuchiya, S. Satoh, I. Ikegaki, M. Shibuya, Y. Suzuki, and H. Hidaka. 1989. Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. *Br.J.Pharmacol.* 98:1091-1100.

87. Kast, R., H. Schirok, S. Figueroa-Perez, J. Mittendorf, M. J. Gnoth, H. Apeler, J. Lenz, J. K. Franz, A. Knorr, J. Hutter, et al. 2007. Cardiovascular effects of a novel potent and highly selective azaindole-based inhibitor of Rho-kinase. *Br.J.Pharmacol.* 152:1070-1080.

88. Schirok, H., R. Kast, S. Figueroa-Perez, S. Bennabi, M. J. Gnoth, A. Feurer, H. Heckroth, M. Thutewohl, H. Paulsen, A. Knorr, et al. 2008. Design and synthesis of potent and selective azaindole-based Rho kinase (ROCK) inhibitors. *ChemMedChem.* 3:1893-1904.

89. Dahal, B. K., T. Cornitescu, A. Tretyn, S. S. Pullamsetti, D. Kosanovic, R. Dumitrascu, H. A. Ghofrani, N. Weissmann, R. Voswinckel, G. A. Banat, et al. 2010. Role of epidermal growth factor inhibition in experimental pulmonary hypertension. *Am.J.Respir.Crit Care Med.* 181:158-167.

90. Schermuly, R. T., E. Dony, H. A. Ghofrani, S. Pullamsetti, R. Savai, M. Roth, A. Sydykov, Y. J. Lai, N. Weissmann, W. Seeger, et al. 2005. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J.Clin.Invest* 115:2811-2821.

91. Weissmann, N., M. Nollen, B. Gerigk, G. H. Ardeschir, R. T. Schermuly, A. Gunther, K. Quanz, L. Fink, J. Hanze, F. Rose, et al. 2003. Downregulation of hypoxic vasoconstriction by chronic hypoxia in rabbits: effects of nitric oxide. *Am.J.Physiol Heart Circ.Physiol* 284:H931-H938.

92. Weissmann, N., S. Hackemack, B. K. Dahal, S. S. Pullamsetti, R. Savai, M. Mittal, B. Fuchs, T. Medebach, R. Dumitrascu, M. Eickels, et al. 2009. The soluble guanylate cyclase activator HMR1766 reverses hypoxia-induced experimental pulmonary hypertension in mice. *Am.J.Physiol Lung Cell Mol.Physiol* 297:L658-L665.

93. Weissmann, N., E. Akkayagil, K. Quanz, R. T. Schermuly, H. A. Ghofrani, L. Fink, J. Hanze, F. Rose, W. Seeger, and F. Grimminger. 2004. Basic features of hypoxic pulmonary vasoconstriction in mice. *Respir.Physiol Neurobiol.* 139:191-202.

94. Seeger, W., D. Walmrath, F. Grimminger, S. Rosseau, H. Schutte, H. J. Kramer, L. Ermert, and L. Kiss. 1994. Adult respiratory distress syndrome: model systems using isolated perfused rabbit lungs. *Methods Enzymol.* 233:549-584.

95. Akylbek Sydykov. Characterization of the ROS production in Ischemia/Reperfusion-induced lung injury. 2009.

Ref Type: Thesis/Dissertation

96. Handoko, M. L., I. Schalij, K. Kramer, A. Sebkhi, P. E. Postmus, W. J. van der Laarse, W. J. Paulus, and A. Vonk-Noordegraaf. 2008. A refined radio-telemetry technique to monitor right ventricle or pulmonary artery pressures in rats: a useful tool in pulmonary hypertension research. *Pflugers Arch.* 455:951-959.

97. Hess, P., M. Clozel, and J. P. Clozel. 1996. Telemetry monitoring of pulmonary arterial pressure in freely moving rats. *J.Appl.Physiol* 81:1027-1032.

98. Schermuly, R. T., K. P. Kreisselmeier, H. A. Ghofrani, H. Yilmaz, G. Butrous, L. Ermert, M. Ermert, N. Weissmann, F. Rose, A. Guenther, et al. 2004. Chronic sildenafil treatment inhibits monocrotaline-induced pulmonary hypertension in rats. *Am.J.Respir.Crit Care Med.* 169:39-45.

99. Schermuly, R. T., S. S. Pullamsetti, G. Kwapiszewska, R. Dumitrascu, X. Tian, N. Weissmann, H. A. Ghofrani, C. Kaulen, T. Dunkern, C. Schudt, et al. 2007. Phosphodiesterase 1 upregulation in pulmonary arterial hypertension: target for reverse-remodeling therapy. *Circulation* 115:2331-2339.

100. Dahal, B. K., D. Kosanovic, P. K. Pamarthi, A. Sydykov, Y. J. Lai, R. Kast, H. Schirok, J. P. Stasch, H. A. Ghofrani, N. Weissmann, et al. 2010. Therapeutic efficacy of azaindole-1 in experimental pulmonary hypertension. *Eur.Respir.J.* 36:808-818.

101. D.Kosanovic, B.K.Dahal, R.Kast, J.P.Stasch, H.A.Ghofrani, N.Weissmann, F.Grimminger, W.Seeger, and R.T.Schermuly. Effects of a novel azaindole-based Rho-kinase inhibitor on hemodynamics and pulmonary vascular remodelling in experimental pulmonary hypertension. ERS Annual Conference, Vienna, Austria . 2009.

Ref Type: Abstract

102. Wang, Y., X. R. Zheng, N. Riddick, M. Bryden, W. Baur, X. Zhang, and H. K. Surks. 2009. ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. *Circ.Res.* 104:531-540.

103. Nagaoka, T., K. A. Fagan, S. A. Gebb, K. G. Morris, T. Suzuki, H. Shimokawa, I. F. McMurtry, and M. Oka. 2005. Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. *Am.J.Respir.Crit Care Med.* 171:494-499.

104. Hyvelin, J. M., K. Howell, A. Nichol, C. M. Costello, R. J. Preston, and P. McLoughlin. 2005. Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. *Circ.Res.* 97:185-191.

105. Jiang, B. H., S. Tawara, K. Abe, A. Takaki, Y. Fukumoto, and H. Shimokawa. 2007. Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. *J.Cardiovasc.Pharmacol.* 49:85-89.

106. Abe, K., H. Shimokawa, K. Morikawa, T. Uwatoku, K. Oi, Y. Matsumoto, T. Hattori, Y. Nakashima, K. Kaibuchi, K. Sueishi, et al. 2004. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ.Res.* 94:385-393.

107. Fagan, K. A., M. Oka, N. R. Bauer, S. A. Gebb, D. D. Ivy, K. G. Morris, and I. F. McMurtry. 2004. Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am.J.Physiol Lung Cell Mol.Physiol* 287:L656-L664.

108. Li, F., W. Xia, A. Li, C. Zhao, and R. Sun. 2007. Long-term inhibition of Rho kinase with fasudil attenuates high flow induced pulmonary artery remodeling in rats. *Pharmacol.Res.* 55:64-71.

109. Tawara, S., Y. Fukumoto, and H. Shimokawa. 2007. Effects of combined therapy with a Rho-kinase inhibitor and prostacyclin on monocrotaline-induced pulmonary hypertension in rats. *J.Cardiovasc.Pharmacol.* 50:195-200.

110. Mukai, Y., H. Shimokawa, T. Matoba, T. Kandabashi, S. Satoh, J. Hiroki, K. Kaibuchi, and A. Takeshita. 2001. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J.* 15:1062-1064.

111. Girgis, R. E., S. Mozammel, H. C. Champion, D. Li, X. Peng, L. Shimoda, R. M. Tuder, R. A. Johns, and P. M. Hassoun. 2007. Regression of chronic hypoxic pulmonary hypertension by simvastatin. *Am.J.Physiol Lung Cell Mol.Physiol* 292:L1105-L1110.

112. Guilluy, C., V. Sauzeau, M. Rolli-Derkinderen, P. Guerin, C. Sagan, P. Pacaud, and G. Loirand. 2005. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br.J.Pharmacol.* 146:1010-1018.

113. Homma, N., T. Nagaoka, Y. Morio, H. Ota, S. A. Gebb, V. Karoor, I. F. McMurtry, and M. Oka. 2007. Endothelin-1 and serotonin are involved in activation of RhoA/Rho kinase signaling in the chronically hypoxic hypertensive rat pulmonary circulation. *J.Cardiovasc.Pharmacol.* 50:697-702.

114. Tamura, M., H. Nakao, H. Yoshizaki, M. Shiratsuchi, H. Shigyo, H. Yamada, T. Ozawa, J. Totsuka, and H. Hidaka. 2005. Development of specific Rho-kinase inhibitors and their clinical application. *Biochim.Biophys.Acta* 1754:245-252.

9. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

10. Acknowledgments

As the doctoral thesis is never done only by the efforts of the author, it is my pleasure to express the gratitude to the following people for precious support and encouragement during the period of my PhD studies.

First of all, I would like to thank my supervisor Professor Ralph Schermuly for providing me the opportunity to work in his research group. His guidance and unreserved support throughout my doctoral studies made the completion of my PhD degree possible. His concept of scientific work is to give the freedom to the members of his group. This approach is the best way to make the creative scientific environment. Dear Ralph, thanks a lot for everything! *Vielen Dank!*

I am extremely grateful to my Postdoc, Dr. Bhola Kumar Dahal for being the best teacher and for shaping me into excellent scientist. But the most of all, thank you for being a good friend, my dear Bhola. *Dhanyabaad!*

I would like to express a special gratitude to Professor Werner Seeger, as the Head of University Hospital Giessen and to my co-supervisor Professor Ernst Petzinger.

I am deeply thankful to the faculty and administrative members of MBML and GGL graduate programs. Especially thanks to Professor Eveline Baumgart-Vogt and Dr. Lorna Lück from GGL for organizing the highly educational and useful courses that cover many important aspects, from the basic and general to the highly specialized scientific topics. I think that the most of the things necessary for a successful scientific career can be learnt during these 3 years of GGL program.

I also owe a deep gratitude to Ewa Bieniek, Michaela Lang, Akylbek Sydykov, Pavan Kumar Pamarthi, Raimund Kast, Hartmut Schirok, Johannes-Peter Stasch, Hossein Ardeschir Ghofrani, Friedrich Grimminger and Norbert Weissmann for their valuable intellectual and practical assistance.

I would like to thank to Oleg Pak for being the best friend. *Спасибо, мой дорогой друг Олежка!*

I would like to thank particularly to Diya and my dear girls, Christina and Stephie for being good friends. It is my pleasure to know you, my dear people!

Finally, nothing would have been possible without my family. I want to thank my father Djokica, my mother Ljiljana and my sister Marija. Thank you for your love, support and existence. You are the lights of my life. *Ljubi vas i voli najviše na svetu vaš sin i brat!*

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

1997 - 2005 Faculty of Biology, University of Belgrade, Department of Molecular Biology and Physiology, Group of Experimental Biomedicine, Serbia

1985 - 1997 Primary and High school (Natural and mathematics sciences)

Research experience

October 2007 PhD studies at Justus-Liebig University, in the lab of Prof. Dr. Ralph Schermuly. The research field of pathophysiology of chronic pulmonary diseases (pulmonary hypertension, lung fibrosis, COPD)

October 2004 - July 2005 The experimental part of the Master studies at the Institute of Physiology and Biochemistry, Faculty of Biology, Belgrade. The research field of comparative physiology and endocrinology

Languages: Serbian (native), English (advanced), Russian (written and spoken)

Teaching experience: Lecturer of 2 x One-day Workshop **(25th November and 14th December 2010.) – "Introduction into Scientific Thinking and Logic/Brief History of the Philosophy of Science/How to make a valid scientific conclusion"** (GGL program – Doctoral development part)

Master thesis

"The effect of extreme environment temperatures on concentration of ascorbic acid in different rat tissues previously treated with ACTH and dexamethasone" (Belgrade, Serbia, **2005**)

Presentations and published abstracts

I) Oral presentations

1. **The Rho-kinase signaling and experimental models of pulmonary arterial hypertension.** (6th Annual retreat of the MBML (Molecular Biology and Medicine of the Lung), Rauischholzhausen, Germany, **2008**)
2. **The inhibition of Rho-kinase (ROCK) for the treatment of pulmonary hypertension.** (7th Annual retreat of the MBML, Rauischholzhausen, Germany, **2009**)

3. **Roles of c-kit and mast cells in experimental pulmonary hypertension.** (8th Annual retreat of the MBML, Rauischholzhausen, Germany, **2010**)

II) Poster presentations

1. **The effect of a novel Rho-kinase inhibitor (ROCK), Azaindole-1, in the rat model of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH). Kosanovic Dj**., *et al*. (The first GGL (Giessen Graduate School for the Life Sciences) conference, Giessen, Germany, **2008**)
2. **The effects of a novel azaindole-based Rho-kinase inhibitor on hemodynamics and pulmonary vascular remodeling in experimental pulmonary hypertension. Kosanovic Dj.**, *et al*. (The retreat of the ECCPS (Excellence Cluster Cardio-Pulmonary System), Max-Planck-Institute for the Heart and Lung Research, Bad Nauheim, Germany, **2009**)
3. **Therapeutic efficacy of a novel Rho-kinase inhibitor Azaindole-1 in experimental pulmonary hypertension (PH). Kosanovic Dj.**, *et al*. (The second GGL conference, Giessen, Germany, **2009**)
4. **A highly selective endothelin-A receptor antagonist TBC3711 reverses monocrotaline induced pulmonary hypertension. D. Kosanovic**, *et al*. (A6316, American Thoracic Society (ATS) conference **2010**, New Orleans, USA)
5. **Effects of a novel endothelin-A receptor antagonist TBC3711 on hemodynamics and pulmonary vascular remodeling in experimental pulmonary hypertension. Kosanovic Dj.**, *et al*. (The retreat of the ECCPS, Max-Planck-Institute for the Heart and Lung Research, Bad Nauheim, Germany, **2010**)
6. **Mast cells as a potential target for the treatment of pulmonary hypertension. Djuro Kosanovic**, *et al*. (P38, Page 88. The third GGL conference, Giessen, Germany, **2010**)
7. **Einfluss des Rho-Kinase Hemmstoffes Azaindol-1 auf Hämodynamic und Gefäßremodelling bei experimenteller pulmonaler Hypertonie. D. Kosanovic**, *et al*. (Forschungswerkstatt Pulmonale Hypertonie, Bayer HealthCare, Leverkusen, **2011**)
8. **Role of mast cells and chymase in idiopathic pulmonary fibrosis. Djuro Kosanovic** *et al*. (ERS 9th Lung Science Conference, Immune system dysregulation in chronic lung disease, PP173, Estoril, Portugal, **2011**)

9. **Pulmonary Mast Cell Activation And Increased Chymase In Patients With Pulmonary Hypertension And Fibrosis. D. Kosanovic** *et al*. (p.A6427, American Thoracic Society (ATS) conference, Denver, USA, **2011**)
10. **Role of mast cells and chymase in idiopathic pulmonary fibrosis. Kosanovic D.**, *et al.* (21st ERS Annual Conference, **2011**, Amsterdam, The Netherlands)

III) E-communication session

1. **Effects of a novel azaindole-based Rho-kinase inhibitor on hemodynamics and pulmonary vascular remodelling in experimental pulmonary hypertension. D. Kosanovic**, *et al*. (E4335, 19th Annual conference of the European Respiratory Society (ERS), Vienna, Austria, **2009**)

IV) Co-author in published abstracts

1. **Effects of the multikinase inhibitor Sunitinib on right ventricular remodeling in an experimental model of right heart hypertrophy**. S.S. Pullamsetti, B. Kojonazarov, A. Sydykov, H. Luitel, **D. Kosanovic**, *et al*. (A6592, Oral presentation, ATS conference **2010**, New Orleans, USA)
2. **Role of Mast cells in experimental pulmonary hypertension**. B.K. Dahal, **D. Kosanovic**, *et al*. (A6330, Poster presentation, ATS conference **2010**, New Orleans, USA)
3. **Reversal of experimental pulmonary hypertension by the multi-kinase inhibitor Sunitinib**. B. Kojonazarov, **D. Kosanovic**, *et al*. (A6334, Poster presentation, ATS conference **2010**, New Orleans, USA)
4. **Effects of multikinase inhibitors on right ventricular remodeling**. Baktybek Kojonazarov, Akylbek Sydykov, Himal Luitel, **Djuro Kosanovic**, *et al*. (AOS.509.01F, 17755, Oral presentation, American Heart Association conference **2010**, Chicago, USA)
5. **Contribution of progenitor cells in experimental right heart hypertrophy induced by pulmonary artery ligation**. Luitel H., Sydykov A., Kojonazarov B., Egemnazarov B., Wiebke J., Dahal B.K., **Kosanovic D.**, *et al*. (P42, Page 92. The third GGL conference, Giessen, Germany, **2010**)
6. **Effects of multi-kinase inhibitors on pulmonary vascular and right ventricular remodeling.** B. Kojonazarov, A. Sydykov, H. luitel, **D. Kosanovic**, *et al*. (P327, 52.

Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V., Dresden, Germany, **2011**)

7. **Contribution of progenitor cells in experimental right heart hypertrophy induced by pulmonary artery ligation.** H. Luitel, A. Sydykov, B. Kojonazarov, B.K. Dahal, **D. Kosanovic**, et al. (P175; 52. Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V., Dresden, Germany, **2011**)
8. **The Dual Neutral Endopeptidase/Endothelin Converting Enzyme Inhibitor SLV338 Inhibits Experimental Pulmonary Hypertension In Rats.** D.M. Peters, B.K. Dahal, **D. Kosanovic** *et al*. (p.A6439, American Thoracic Society (ATS) conference, Denver, USA, **2011**)
9. **Der Einfluss von Tyrosinkinaseinhibitoren auf den Umbau des rechten Herzens im tierexperimentellen Modell.** M. Majewski, B. Kojonazarov, S. Pullamsetti, A. Sydykov, H. Luitel, **D. Kosanovic** *et al*. (117. Kongress der Deutschen Gesellschaft für Innere Medizin, Wiesbaden, Germany, **2011**)
10. **Contribution Of Progenitor Cells In Experimental Right Heart Hypertrophy Induced By Pulmonary Artery Ligation.** H. Luitel, A. Sydykov, B. Kojonazarov, B.K. Dahal, **D. Kosanovic** *et al*. (p.A4980, American Thoracic Society (ATS) conference, Denver, USA, **2011**)
11. **Role of mast cells and chymase in pulmonary vascular remodeling.** Dahal B.K., **Kosanovic D.**, *et al.* (21st ERS Annual Conference, **2011**, Amsterdam, The Netherlands)

PUBLICATIONS

1. **Role of epidermal growth factor inhibition in experimental pulmonary hypertension.** Dahal BK, Cornitescu T, Tretyn A, Pullamsetti SS, **Kosanovic D**, Dumitrascu R, Ghofrani HA, Weissmann N, Voswinckel R, Banat GA, Seeger W, Grimminger F, Schermuly RT. *Am J Respir Crit Care Med*. **2010** Jan 15;181(2):158-67
2. **Therapeutic efficacy of azaindole-1 in experimental pulmonary hypertension.** Dahal BK*, **Kosanovic D***, Pamarthi PK, Sydykov A, Lai YJ, Kast R, Schirok H, Stasch JP, Ghofrani HA, Weissmann N, Grimminger F, Seeger W, Schermuly RT. *Eur Respir J*. **2010** Oct;36(4):808-18 (* both authors contributed equally to this work)

3. **Involvement of Mast Cells in Monocrotaline-Induced Pulmonary Hypertension in Rats.** Dahal BK, **Kosanovic D**, Kaulen C, Cornitescu T, Savai R, Hoffmann J, Reiss I, Ghofrani HA, Weissmann N, Kuebler WM, Seeger W, Grimminger F, Schermuly RT. *Respir Res*. **2011** May 2;12:60
4. **Therapeutic efficacy of TBC3711 in monocrotaline-induced pulmonary hypertension. Kosanovic D**, Kojonazarov B, Luitel H, Dahal BK, Sydykov A, Cornitescu T, Janssen W, Brandes RP, Davie N, Ghofrani HA, Weissmann N, Grimminger F, Seeger W, Schermuly RT. *Respir Res*. **2011** Jun 23;12:87
5. **Activated Mast cells and increased Chymase in pulmonary hypertension, COPD and lung fibrosis. Kosanovic D**, Dahal BK, Messinger J, Fischer Y, Hoffmann K, Antel J, Husen B, Hanke N, Mayet S, Ghofrani HA, Weissmann N, Grimminger F, Seeger W, Schermuly RT. **2011** (**in preparation**)
6. **Effects of multikinase inhibitors on pulmonary vascular and right ventricular remodeling.** Kojonazarov B, Sydykov A, Pullamsetti SS, Luitel H, Dahal BK, **Kosanovic D**, Tian X, Majewski M, Baumann C, Evans S, Phillips P, Fairman D, Davie N, Wayman C, Kilty I, Weissmann N, Grimminger F, Seeger W, Ghofrani HA, Schermuly RT. **2011** (**submitted**)

Awards/grants

MBML Travel Grant (**2010**)

Forschungswerkstatt Pulmonale Hypertonie Poster Award (**2011**)

ERS Travel bursary (**2011**)

Membership: European Respiratory Society (ERS); American Thoracic Society (ATS); Pulmonary Vascular Research Institute (PVRI)