

Therapeutic Efficacy Of Azaindole-1 In Experimental Pulmonary Hypertension

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ABSTRACT: Accumulating body of evidence incriminate Rho-kinase (ROCK) in the pathogenesis of pulmonary hypertension (PH). Therapeutic efficacy of azaindole-1, a novel highly selective and orally active ROCK inhibitor, has not yet been investigated in PH.

This study aimed to investigate the effects of azaindole-1 on 1) acute hypoxic pulmonary vasoconstriction (HPV), 2) proliferation of pulmonary arterial smooth muscle cells (PASMCs) and 3) animal models of PH.

Azaindole-1 significantly inhibited HPV in isolated, ventilated and buffer-perfused murine lungs and proliferation of primary rat PASMCs *in vitro*. Azaindole-1 was administered orally from 21 to 35 days after monocrotaline injection in rats (MCT-rats) and hypoxic exposure in mice. Azaindole-1 (10 and 30 mg/kg body weight/day in rats and mice, respectively) significantly improved hemodynamics and right ventricular hypertrophy. Moreover, the medial wall thickness and muscularization of peripheral pulmonary arteries were ameliorated. Azaindole-1 treatment resulted in a decreased immunoreactivity for phospho-myosin phosphatase target subunit 1 (p-MYPT1) and proliferating cell nuclear antigen (PCNA) in pulmonary vessels of MCT-rats, suggesting an impaired ROCK activity and reduced proliferating cells.

Azaindole-1 provided therapeutic benefit in experimental PH, and this may be attributable to its potent vasorelaxant and antiproliferative effects. Azaindole-1 may offer a useful approach for treatment of PH.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a chronic fatal disease characterized by sustained elevation of pulmonary artery pressure and reduced exercise tolerance. As a consequence the right ventricular (RV) afterload increases and culminates in RV failure. PAH has a complex pulmonary vascular pathophysiology including vasoconstriction, vascular remodeling and *in situ* thrombosis. The progressive vascular remodeling, the hallmark of PAH pathology, is attributable to abnormalities in vascular cells such as increased proliferation and resistance to apoptosis (1-3). However, the precise molecular mechanism is incompletely understood, and the therapeutic approach to cure the disease is yet most desirable.

The small GTPase RhoA is one of the members of the Rho 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 (4). Two isoforms of the serine/threonine kinase ROCK have been identified: ROCK-1 and ROCK-2 (5-7). ROCKs are ubiquitously expressed in tissues including vasculature and heart. Due to its role in key cell functions a hyperactive ROCK signaling results in cardiovascular disorders associated with sustained abnormal vasoconstriction and promotion of vascular remodeling (8, 9).

Studies on animal models of pulmonary hypertension (PH) such as chronic hypoxia-, monocrotaline (MCT)-, VEGF receptor inhibition and chronic hypoxia-, pneumonectomy and MCT- and bleomycin-induced PH suggest that increased ROCK is involved in the pathogenesis (10-16). Moreover, data are emerging that implicate ROCK signaling in clinical PH and that Rho kinase inhibition provides beneficial acute effects in patients (17-23).

Fasudil and Y-27632 are the two most commonly investigated ROCK inhibitors (24, 25).

Fasudil has been approved for human use in Japan for the treatment of cerebral vasospasm after subarachnoid hemorrhage and is also a relatively potent inhibitor of other kinases such

as protein kinase C (26, 27). These inhibitors have been instrumental in elucidating the role of ROCK in the pathobiology of PH. The ROCK inhibitors show beneficial effects on rodent models of PH; however, discrepancy in their efficacy has also been observed depending on the dose, route of administration and animal model (9). Recently, a highly selective and orally active azaindole-based ROCK inhibitor has been reported (28, 29). This novel ROCK inhibitor, azaindole-1 functions in ATP-competitive manner with activity in the low nanomolar range (IC₅₀ of 0.6nM and 1.1nM for human ROCK1 and ROCK2, respectively). 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 (28). However, the therapeutic potential of azaindole-1 in animal models of PH has not yet been explored. In this study, we have therefore investigated the effects of azaindole-1 on 1) acute hypoxic pulmonary vasoconstriction (HPV) in isolated, ventilated and buffer-perfused murine lungs and proliferation of rat PASMC *in vitro*, 2) hemodynamics, right ventricular hypertrophy and pulmonary vascular remodeling in experimental PH induced by monocrotaline injection in rats and by chronic hypoxic exposure in mice, 3) ROCK activity and pulmonary vascular cell proliferation by immunohistochemistry of the lung tissues of monocrotaline-injected rats for phospho-myosin phosphatase target subunit 1 (p-MYPT1) and proliferating cell nuclear antigen (PCNA).

METHODS

Animals

Adult male Sprague-Dawley rats (300-350 g BW) and C57BL/6 mice (20-22 g BW) were obtained from Charles River Laboratories, Sulzfeld, Germany. Pulmonary hypertension (PH) was induced in rats by monocrotaline (Sigma-Aldrich) injection and in mice by exposing to chronic hypoxia (10%) as described (30). All studies were performed according to the

guidelines of the University of Giessen and were approved by the local authorities (Regierungs praesidium Giessen, GI20/10-Nr.48/2009; GI 20/10 Nr.39/2009).

Experimental design

Radio-telemetry study

Monocrotaline-injected rats (MCT-rats) were randomized into two groups and they received either azaindole-1 (Bayer Health Care AG) or placebo from day 21 for two weeks.

Azaindole-1 was prepared daily in transcutol-based vehicle as described (28) and rats were treated by oral gavage at the dose of 10 mg/kg BW/day. The placebo group received only vehicle. The dose of azaindole-1 was selected based on literature (28) and our own pilot experiments (data not shown). The right ventricular systolic pressure (RVSP) and heart rate (HR) were monitored online with an implanted radio-telemetry system (Dataquest A.R.T. 2.1; Data Sciences Inc.) as described (30). Briefly, a catheter connected to a fluid filled sensor was inserted into the jugular vein and forwarded to the RV of rats under anaesthesia. The signal from the transmitter (model TA11PA-C40) was transferred to a remote receiver and a data-exchange matrix connected to a computer. The waveform was displayed on the computer and used to ensure correct positioning of the catheter. Animals were allowed to recover and were housed individually in standard rat cages. RVSP and HR were recorded once per day over the next 35 days from the time of MCT-injection.

Chronic treatment study

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

were treated daily with azaindole-1 (30mg/kg BW/day) or placebo from 21 to 35 days. As mentioned, the dose of azaindole-1 was selected based on literature (28) and our own pilot experiments (data not shown) and prepared daily for oral application. Control mice remained under normoxia (21% O₂). 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.

Hemodynamic and right ventricular hypertrophy (RVH) measurements

RVSP was measured by a catheter inserted into the RV via the right jugular vein, and for systemic arterial pressure (SAP) the left carotid artery cannulation was performed as described (30). Cardiac output was calculated using the Fick principle, by employing the mixed venous oxygen and the arterial oxygen content as previously described (31). The total pulmonary and systemic vascular resistance indexed to body weight (TPR and TSR respectively) were determined as reported (32). The heart was dissected to separate right ventricle (RV) from left ventricle plus septum (LV+S), and the ratio RV/(LV+S) was calculated as a measurement for RVH.

Histology and pulmonary vascular morphometry

Lung tissue preparation, sectioning, staining and vascular morphometry were done as described (33). 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. All analyses were done in a blinded fashion.

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

Paraffin-embedded lung tissue sections with thickness of 3 μm were deparaffinized in xylene and rehydrated in a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4). 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. PCNA positive pulmonary vascular cells were counted throughout the entire section and the index of proliferation (IOP) was determined as the number of PCNA positive cells per pulmonary vessel. The IOP (in %) for placebo and azaindole-1 treated groups was calculated by assuming the average IOP of healthy control lungs as 100 %.

Isolated murine lungs

The technique of successive hypoxic maneuvers was employed in isolated, ventilated and buffer-perfused mice lungs to investigate the effect of azaindole-1, fasudil (H139, Sigma) and Y-27632 (Y0503, Sigma) on acute hypoxic pulmonary vasoconstriction (HPV) as described (34). The ROCK inhibitors were prepared 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% O₂) 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: 0.1–30.0 μM).

Controls received the vehicle (DMSO) only.

MTT and thymidine incorporation assays

The isolation, culture and proliferation assay of primary pulmonary artery smooth muscle cells (PASMCs) were done as described previously (33). Briefly, 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 ($\sim 4 \times 10^4$ cells/well) were seeded and the following day the medium was substituted with DMEM/F12 containing 0.1% FBS to render the cells quiescent. After 24h serum starvation, cells were induced to cell cycle reentry by FCS (10 %) together with different concentrations of fasudil, Y-27632 and azaindole-1 (500, 1000 and 5000 nM in DMSO) for 24 h, including in the last 12h the incorporation of [^3H]-thymidine (1.5 $\mu\text{Ci}/\text{ml}$, Amersham Biosciences, Munich, Germany). Cells were then washed twice with 500 μl chilled HBSS, fixed with 250 μl ice-cold methanol and precipitated by 250 μl 10% trichloroacetic acid (TCA). Finally samples were lysed in 0.1 M NaOH and transferred to 4 ml scintillation solution and counted by a beta-counter. The values are expressed as disintegration per minute (dpm). All the experiments were done in triplicate.

Cell viability/ cytotoxicity were assessed by the MTT assay using a CellTiter 96AQ kit (Promega) according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates and allowed to attach for 6 h, and then cultured under serum-free conditions with various concentrations of fasudil, Y-27632 and azaindole-1 for 48 h. The number of surviving cells was determined by measuring the absorbance at 560 nm ($A_{560\text{ nm}}$) of the dissolved

formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt for 1 hour. All of the experiments were carried out in triplicate.

Data analysis

All data are expressed as Mean \pm 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.

RESULTS

Effects of azaindole-1 on acute hypoxic pulmonary vasoconstriction (HPV)

The pulmonary vasorelaxant potency of azaindole-1 and the other commonly used ROCK inhibitors fasudil and Y-27632 were investigated in isolated, ventilated and buffer-perfused murine lungs. All the ROCK inhibitors significantly reduced the HPV in a dose dependent manner ($P < 0.05$ versus vehicle control). The maximum inhibitory effects on HPV were ~ 75 % for fasudil and Y-27632 and ~ 90 % for azaindole-1 at the highest concentration, 30 μM (figure 1a). 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 30 μM) 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.

Effects of azaindole-1 on PASMC proliferation

The effect of azaindole-1 on PASMC proliferation was investigated by thymidine incorporation assay. In each assay, fasudil and Y-27632 were also included. There were no significant effects of various concentrations of the ROCK inhibitors (500, 1000 and 5000 nM) on the thymidine incorporation into PASMCs derived from healthy rats (Data supplement, figure 1a, 1b and 1c). 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 by 5000 nM of Y-27632 ($P < 0.05$ versus FCS control, figure 1b, 1c). Azaindole-1 reduced the thymidine incorporation significantly at all concentrations tested ($P < 0.05$ versus FCS control, figure 1d). We did not observe cytotoxic effects of all the ROCK inhibitors at the concentrations tested in our study, as determined by MTT assay (Data supplement, figure 2a and 2b). The findings suggest that azaindole-1 has potent inhibitory effect on PASMC proliferation.

Effect of azaindole-1 on monocrotaline-induced progressive elevation of RVSP

To investigate the *in vivo* efficacy, progressive elevation of RVSP was monitored online by telemetry technique in MCT-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 2a). 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 2b). The findings suggest that azaindole-1 has potent pulmonary vasorelaxant effect *in vivo*.

Effects of azaindole-1 on hemodynamics in MCT- and chronic hypoxia-induced PH

We investigated therapeutic efficacy of azaindole-1 in two independent animal models of PH as described in methods. MCT induced 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 3a). Rats receiving azaindole-1 resulted in a significant decrease of RVSP (49.5 ± 3.7 mmHg) as compared to the placebo rats. The total pulmonary resistance (TPR) was significantly increased in 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 3b). There was no significant change in systemic arterial pressure (SAP) and total systemic resistance (TSR) among the experimental groups (Figures 3c, 3d). 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 3e).

Chronic hypoxia induced 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 4a). 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 4b). There was no significant effect on body weight of the animals associated with azaindole-1 treatment (Data supplement, figure 3a and 3b).

Effects of azaindole-1 on right ventricular (RV) hypertrophy in MCT- and chronic hypoxia-induced 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 5a). 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 5b).

Effects of azaindole-1 on vascular remodeling in MCT- and chronic hypoxia-induced PH

The effects of azaindole-1 on pulmonary vascular remodeling were assessed by determining the degree of muscularization and medial wall thickness of the peripheral pulmonary arteries. MCT injection in rats and chronic hypoxia in mice resulted in an enhanced pulmonary artery muscularization as evident from the enhanced immunoreactivity for α -SMC actin (Figures 6a, 7a). 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 as compared to healthy control (2.2 ± 0.8 and 42.2 ± 3.7 % respectively). Azaindole-1 treatment significantly decreased fully muscularized vessels (18.5 ± 2.4 %) (Figure 6b). 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 vessels. Moreover, we measured the medial wall thickness of the same size vessels. There was significantly increased medial wall thickness in placebo (22.3 ± 0.9 versus 9.7 ± 0.4 % in healthy control). Corroborating the decreased fully

muscularized vessels, azaindole-1 significantly reduced the medial wall thickness (14.0 ± 0.6 %) (Figure 6c, 6d).

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 7b). As 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 %) as compared to the normoxic control mice (10.1 ± 0.3 %) (Figure 7c, 7d). Corroborating the decreased fully muscularized vessels, the medial wall thickness was significantly reduced in azaindole-1 treated mice (12.4 ± 0.4 %) (Figure 7d).

Effects of azaindole-1 on ROCK activity and pulmonary vascular cell proliferation

We investigated the effects of azaindole-1 on ROCK activity by employing immunohistochemistry for the phospho-myosin phosphatase target subunit 1 (p-MYPT1). The immunoreactivity was localized in the media of the vessels and was enhanced in the lung tissues of MCT-rats receiving placebo. There was decreased immunoreactivity in MCT-rats treated with azaindole-1 (Figure 8a).

To confirm if the effect of azaindole-1 on cell proliferation as was observed *in vitro* 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 as compared to that in healthy control rats (Figure 8b). We analyzed the same size vessels as was used for vascular morphometry to quantify the PCNA positive

vascular cells and expressed as index of proliferation (IOP). The findings revealed that there was higher IOP in MCT-rats receiving placebo (431.9 ± 7.2 versus 100.0 ± 25.8 % in healthy control rats) (Figure 8b). Corroborating the *in vitro* data, the IOP was significantly reduced in MCT-rats receiving azaindole-1 (184.1 ± 10.9 %) (Figure 8c).

DISCUSSION

The major findings of this study are a) Azaindole-1 significantly inhibited acute hypoxic pulmonary vasoconstriction *ex vivo* and the proliferation of primary rat PASMC *in vitro*, b) Azaindole-1 treatment significantly improved hemodynamics, right ventricular hypertrophy and pulmonary vascular remodeling in MCT-rats and chronically hypoxic mice, and c) The improvement in hemodynamics and vascular remodeling in rats was accompanied by an impaired ROCK activity and a decreased proliferating cells in pulmonary vessels as evident from reduced immunoreactivity for p-MYPT1 and PCNA.

Exploring promising targets and developing effective therapeutic approaches for pulmonary hypertension has been an actively pursued research focus over the past years. As a consequence prolonged survival of the patients and improvements in their quality of life have been achieved with the therapeutic options available for PH such as endothelin receptor inhibitor, prostacyclin and PDE5 inhibitor, and other therapeutic approaches are under clinical investigation (35, 36).

The role of Rho kinase (ROCK) signaling in cardiovascular physiology and pathophysiology is rapidly unfolding in the recent years and the ROCK inhibitors, fasudil and Y-27632 have served as a useful tool (8). Recently, Kast *et al.* reported a novel ROCK inhibitor azaindole-1 that dose-dependently inhibited human ROCK-1 and ROCK-2 in a low nanomolar range with IC₅₀ values of 0.6nM and 1.1nM respectively (28). Azaindole-1 is more potent than fasudil

and Y-27632 that have the IC_{50} values 158 and 162 nM respectively for ROCK-2 (37). In addition, azaindole-1 in a nanomolar range inhibits the phenylephrine-induced contraction of rabbit saphenous artery in a concentration-dependent manner (28). In agreement with the previous data we found that azaindole-1 (IC_{50} of 79.2 nM) potently inhibited phenylephrine-induced contraction of the rabbit saphenous artery as compared to fasudil and Y-27632 (IC_{50} of 9313 and 793 nM, respectively) (data not shown). Extending the findings, we showed in the current study that azaindole-1 significantly inhibited acute hypoxic pulmonary vasoconstriction in isolated, ventilated and buffer-perfused murine lungs. Moreover, we observed that effect of azaindole was more potent than fasudil and Y-27632. Taken together, the data suggest that azaindole-1 is not only a highly selective and potent inhibitor of ROCK but also a stronger vasorelaxant as compared to fasudil and Y-27632.

It is well established that hyperactivation of ROCK, by inhibiting myosin phosphatase (MLCP) activity and increasing myosin light chain phosphorylation, leads to contraction of vascular smooth muscle cells (VSMC) and thus to vasoconstriction (4, 38). In experimental PH, acute inhibition of ROCK has been demonstrated to result in pulmonary vasorelaxation and thus in reduced pulmonary arterial pressure (14, 16, 39). In this line, we observed that azaindole-1 significantly impaired the monocrotaline induced progressive elevation of RVSP in rats as monitored online by radio-telemetry technique. However, no significant effect was observed on heart rates, suggesting that azaindole-1 treatment did not result in reflex tachycardia. Our finding is in agreement with the studies that describe the involvement of ROCK-mediated sustained vasoconstriction in the PH pathogenesis (14, 39-41). Moreover, as inhibition of ROCK has been shown to impair pulmonary vascular remodeling (10, 42-44), we sought to test the response of proliferating PASMC to azaindole-1 *in vitro*. We observed that azaindole-1 significantly inhibited the thymidine incorporation into the primary rat

PASMC at high nanomolar to low micromolar range, and the effect was more potent than fasudil and Y-27632. Taken together, the findings of our *in vivo*, *ex vivo* and *in vitro* studies attribute potent vasorelaxant and antiproliferative properties to azaindole-1 and thus substantiate its therapeutic potential in PH.

We therefore performed the chronic treatment studies and investigated the therapeutic efficacy of azaindole-1 in experimental PH induced by monocrotaline injection in rats and by chronic hypoxia in mice. We found that azaindole-1 improved PH, right ventricular hypertrophy (RVH) and pulmonary vascular remodeling as evident from significantly reduced RVSP, TPR, RV/(LV+S) and muscularization and medial wall thickness of peripheral pulmonary vessels. Moreover, the systemic arterial pressure did not change significantly in the treated animals. Although at first glance it seems contradictory to the findings by Kast *et al.*, who observed reduction of blood pressure in spontaneously hypertensive (SH) rats receiving azaindole-1 (28). However, the discrepancy could be explained at least in part by the differences in animal models and duration of treatment. In SH rats the ROCK activity is enhanced in systemic vasculature (45); furthermore, Kast *et al* treated the normotensive rats only once and SH rats for 4 days and noted in the SH rats that the blood pressure lowering effect was gradually decreasing. In the current study, we used different animal models of PH and treated the animals for two weeks. Our data therefore may not be directly comparable to that of Kast *et al*. Notably, the sustained pulmonary ROCK activation in animal models of PH (9) may underlie the pulmonary specific effects of selective ROCK inhibitors. An accumulating body of literature describes beneficial effects of ROCK inhibition on the development of MCT- and chronic hypoxia-induced PH in rodents (11, 15, 42, 44, 46-48); and our results extend these findings demonstrating that azaindole-1 is beneficial even when the treatment commences at the time the disease is already rapidly

progressing. Moreover, we found that azaindole-1 treatment resulted in an impairment of ROCK activity as determined by a reduction in p-MYPT1 immunoreactivity and a diminution of proliferating pulmonary vascular cells as determined by PCNA staining. Taken together, our data suggest that the therapeutic efficacy of ROCK inhibition by azaindole-1 may be associated with its vasorelaxant and antiproliferative potency. In this line, Abe *et al.* have reported an improvement of MCT-induced PH in rats by long-term treatment with fasudil (10). However, despite that fasudil shows therapeutic efficacy, Oka *et al.* have discussed in their recent review the discrepancy in the findings depending on the dose, route of administration and animal models of PH (9). Fasudil has been used in previous studies at the dose range of 30 - 100 mg/kg BW, whereas azaindole-1 shows the therapeutic benefit at considerably lower dose of 10 mg/kg BW, suggesting that not only the *in vitro* but also the *in vivo* effects of azaindole-1 is potent. Our findings indicate that the therapeutic efficacy of azaindole-1 is independent of the cause of the disease. This may be attributable to the involvement of ROCK in the pathogenesis of experimental PH induced by different stimuli (10, 13, 15, 16). Moreover, ROCK signaling is implicated in the beneficial effects of therapeutic approaches targeting signaling cascades other than ROCK (12, 13, 19, 47-49). It seems therefore likely that ROCK may be the convergent point for various signaling cascades implicated in the pathogenesis of PH.

The monocrotaline-induced PH in rats and chronic hypoxia-induced PH in mice share many features of clinical PH and are well accepted animal models to investigate pathomechanism and therapeutics of PH; they, however, are not without shortcomings with regard to mimicking human PH pathology. Extensive studies on various animal models in the recent years have resulted in the discovery of promising therapeutic targets including Rho kinase (35). Moreover, activation of ROCK has been demonstrated in animal models of severe PH

characterized by the presence of occlusive pulmonary vascular lesions (13, 16). Azaindole-1 can thus be anticipated to yield therapeutic benefit; however, studies are warranted to investigate if the antiproliferative and vasorelaxant potency of azaindole-1 is adequate to improve the severely remodeled pulmonary vessels with occlusive lesion that is commonly observed in human PH. Regarding human PH, literature is emerging that implicates Rho kinase signaling in the disease pathogenesis and thus this signaling pathway represents a potential therapeutic target (17, 19, 21, 23). Moreover, 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 PH (17, 18, 20, 22). The data thus support that inhibition of Rho kinase with a potent and highly selective inhibitor may offer a potential therapeutic strategy.

In summary, we demonstrate that daily oral application of azaindole-1 improves the hemodynamics, right ventricular hypertrophy and pulmonary vascular remodeling in experimental PH. This study, to our knowledge, is the first to investigate the therapeutic efficacy of a novel potent and orally available ROCK inhibitor azaindole-1 in two independent animal models of PH. Moreover, we show that azaindole-1 potently inhibits the hypoxic pulmonary vasoconstriction in isolated, ventilated and buffer-perfused murine lungs, significantly inhibits proliferation of primary rat PASMC *in vitro* and reduces the number of proliferating pulmonary vascular cells in MCT-injected rats, suggesting that the therapeutic benefit of azaindole-1 may be associated with its vasorelaxant and antiproliferative potency. Taken together, the findings suggest that azaindole-1 may represent a novel therapeutic approach for the treatment of pulmonary hypertension.

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FIGURE LEGENDS.

Figure 1. *Effects of fasudil, Y-27632 and azaindole-1 on hypoxic pulmonary vasoconstriction (HPV) and pulmonary artery smooth muscle cell (PASMC) proliferation.*

The effects of various concentrations of azaindole-1, fasudil and Y-27632 on acute HPV in isolated, ventilated and buffer-perfused murine lungs (n=5), and on proliferation of primary PASMCs isolated from monocrotaline-injected rats (n=3) were investigated as described in methods. (a) Dose-response curve of azaindole-1, fasudil and Y-27632 on acute HPV is shown. The effects of (b) fasudil, (c) Y-27632 and (d) azaindole-1 on thymidine incorporation into PASMCs (in % of FCS control) are shown. Bars represent mean \pm SEM.

p<0.01, *p<0.001 versus FCS/vehicle; †p<0.05, ††p<0.01 versus fasudil/Y-27632.

Figure 1.

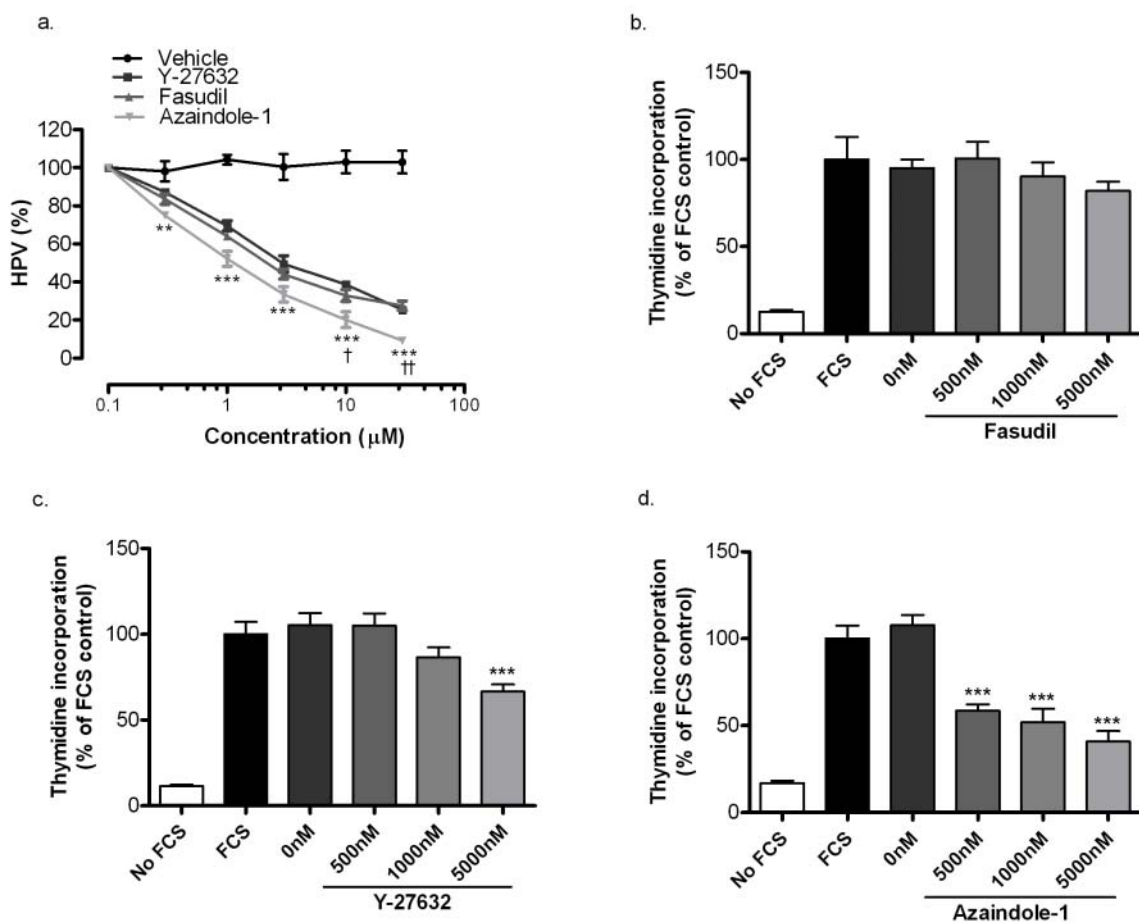


Figure 2. *Effect of azaindole-1 on progressive elevation of right ventricular systolic pressure (RVSP).*

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 (HR) were monitored online as described in methods. **(a)** RVSP and **(b)** HR are given. Each dot represents mean \pm SEM (n=6). *p<0.05, **p<0.01 and ***p<0.001.

Figure 2

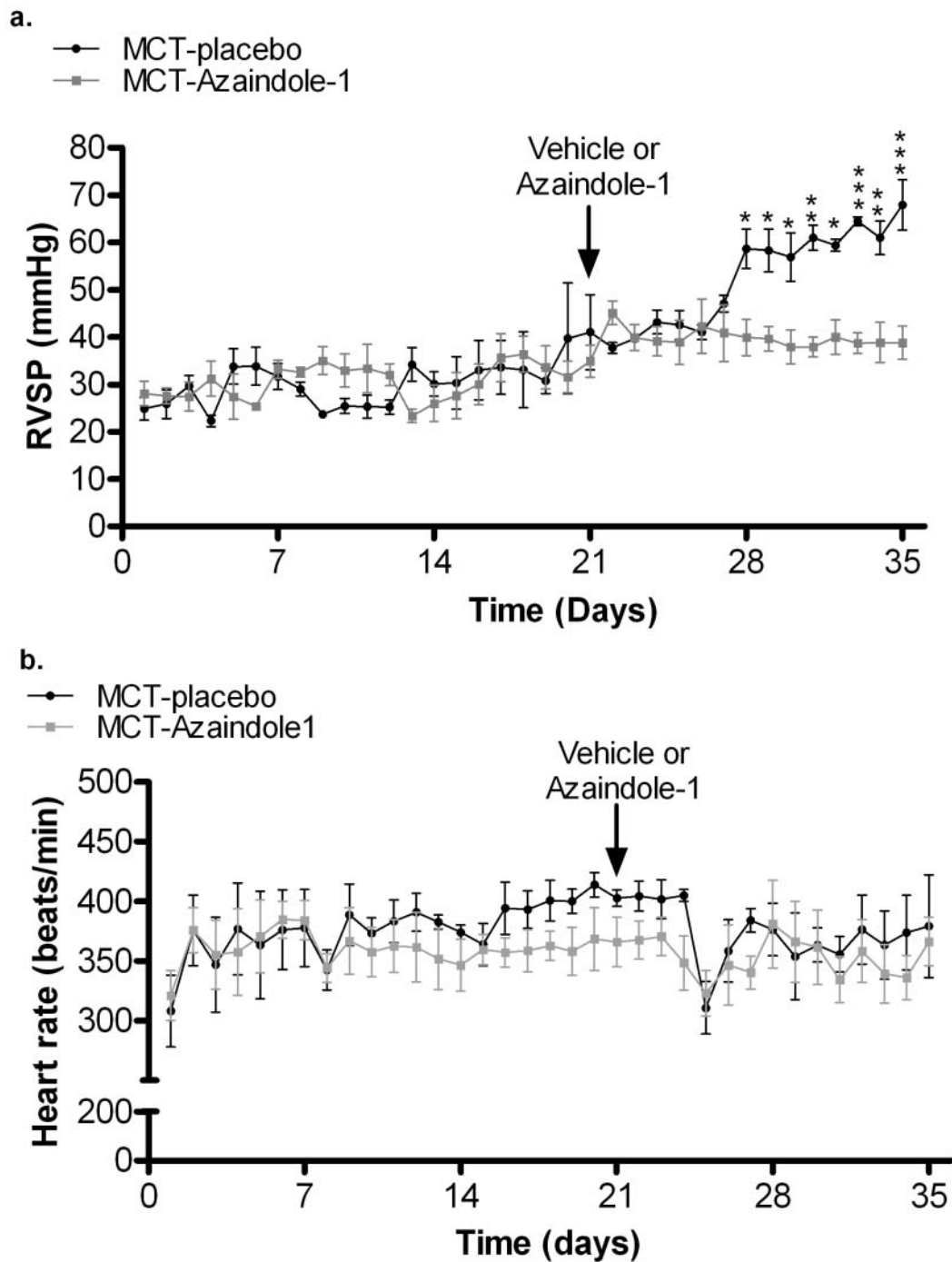


Figure 3. Effect of azaindole-1 on hemodynamics in monocrotaline-induced PH in rats.

Rats were injected with saline (healthy control) or MCT. The MCT-injected rats were treated with azaindole-1 or placebo from day 21 to 35 after MCT-injection followed by

hemodynamic measurement at day 35 as described in methods. (a) Right ventricular systolic pressure (RVSP), (b) total pulmonary resistance (TPR), (c) systemic arterial pressure (SAP), (d) total systemic resistance (TSR) and (e) cardiac index (CI) of different experimental groups are given. Bars represent mean \pm SEM (n=10-15). *p<0.05, **p<0.01 and ***p<0.001.

Figure 3.

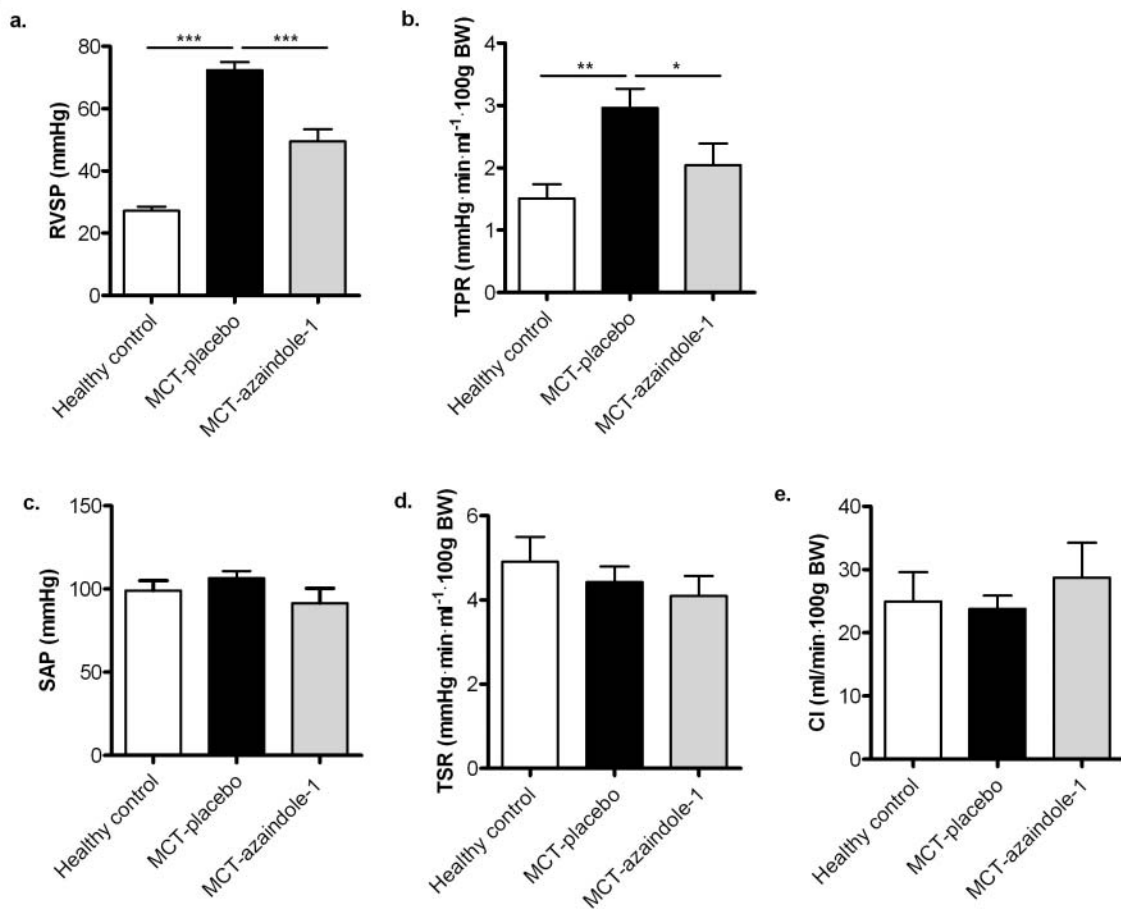


Figure 4. *Effect of azaindole-1 on hemodynamics in chronic hypoxia-induced PH in mice.*

Mice were exposed to normoxia (Nox) or chronic hypoxia (Hox). Hypoxic mice were treated with azaindole-1 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. Bars represent mean \pm SEM (n=6-10).

***p<0.001.

Figure 4.

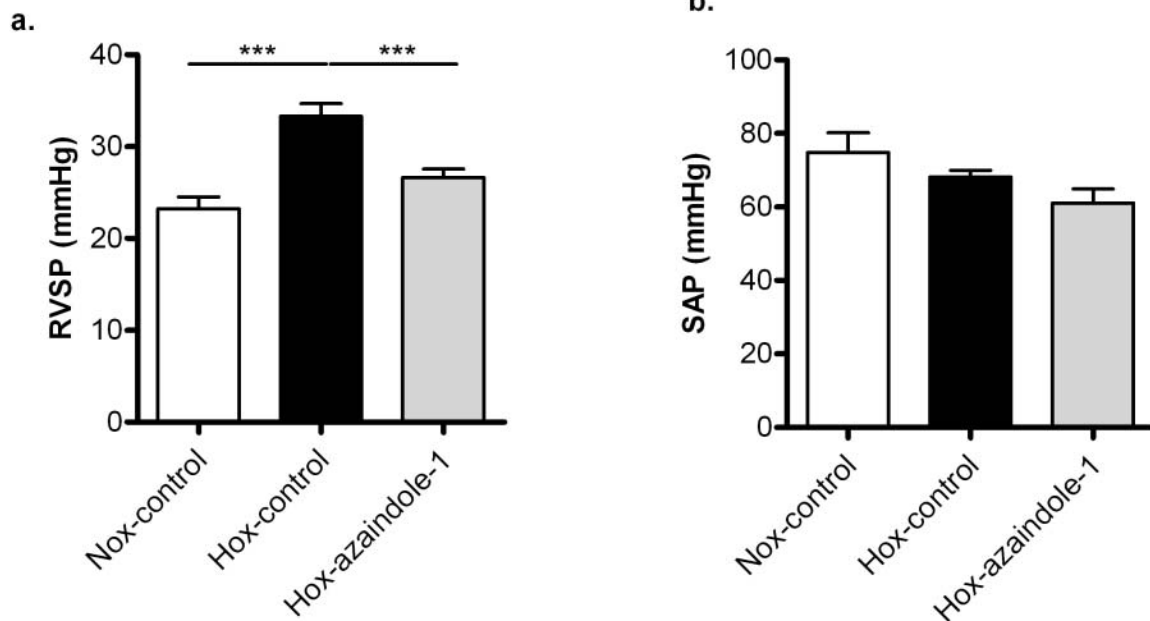


Figure 5. Effect of azaindole-1 on right ventricular hypertrophy in MCT- and chronic hypoxia-induced PH.

Rats were injected with saline (healthy control) or MCT. Mice were exposed to normoxia (Nox) and chronic hypoxia (Hox). The MCT-injected rats and hypoxic mice were treated with azaindole-1 or placebo from day 21 for 2 weeks followed by hemodynamic and right ventricular hypertrophy measurements at day 35 as described in methods. Right to left ventricular plus septum weight ratio [RV/(LV+S)] of (a) MCT-rats (n = 10-15) and (b) hypoxic mice (n = 6-10) are given. Each bar represents Mean \pm SEM. *p<0.05, ***p<0.001.

Figure 5.

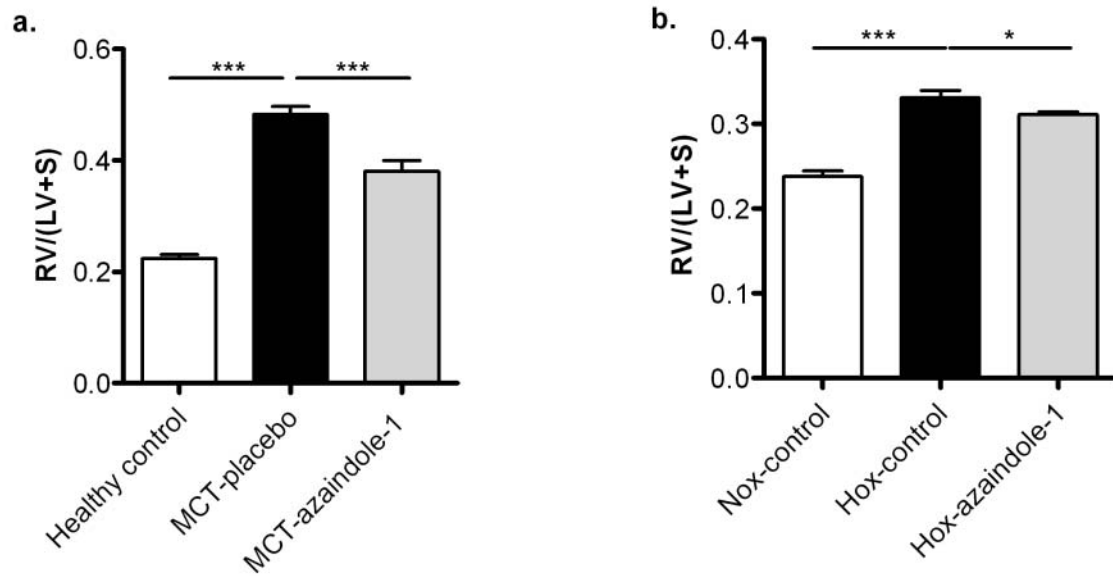


Figure 6. Effect of azaindole-1 on vascular remodeling in MCT-induced PH.

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. (a) Representative photomicrographs are shown (A - healthy control, B - MCT-placebo and C - MCT-azaindole-1). (b) Proportion of non (N), partially (P) or fully (M) muscularized vessels, as a percentage of total pulmonary vessel cross-section (sized 20-50 μ m) is given for healthy control and MCT-rats receiving placebo and azaindole-1. The rat lung sections were stained with elastica followed by determination of the media wall thickness (%) as described in methods. (c) Representative photomicrographs are shown (A - healthy control, B - MCT-placebo and C- MCT-azaindole-1) (d) The medial wall thickness of pulmonary vessels (%) is shown. Each bar represents mean \pm SEM (n=10). Scale = 20 μ m, ***p<0.001.

Figure 6.

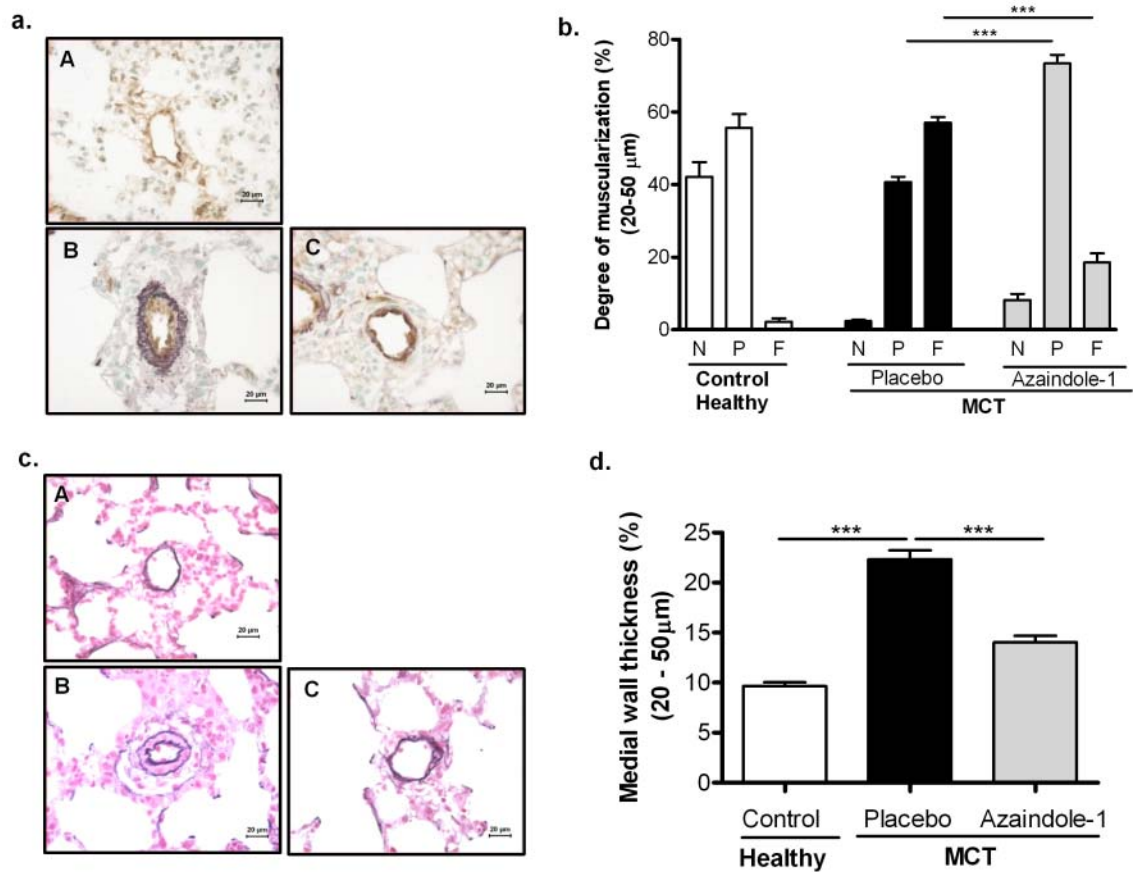


Figure 7. *Effect of azaindole-1 on vascular remodeling in chronic hypoxia-induced PH.*

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 methods. **(a)** Representative photomicrographs of normoxic (A) and hypoxic mice receiving placebo (B) and azaindole-1 (C) are shown. **(b)** Proportion of non (N), partially (P) or fully (M) muscularized vessels, as a percentage of total pulmonary vessel cross-section (sized 20-70 μ m) is given. The lung sections were stained with elastica and the medial wall thickness (%) was determined as described in methods. **(c)** Representative photomicrographs of normoxic control (A) and hypoxic mice treated with placebo (B) and azaindole-1 (C) are given. **(d)** The medial wall thickness (%) of pulmonary vessels is shown. Bars represent mean \pm SEM (n= 6-10). ***p<0.001, scale = 20 μ m.

Figure 7.

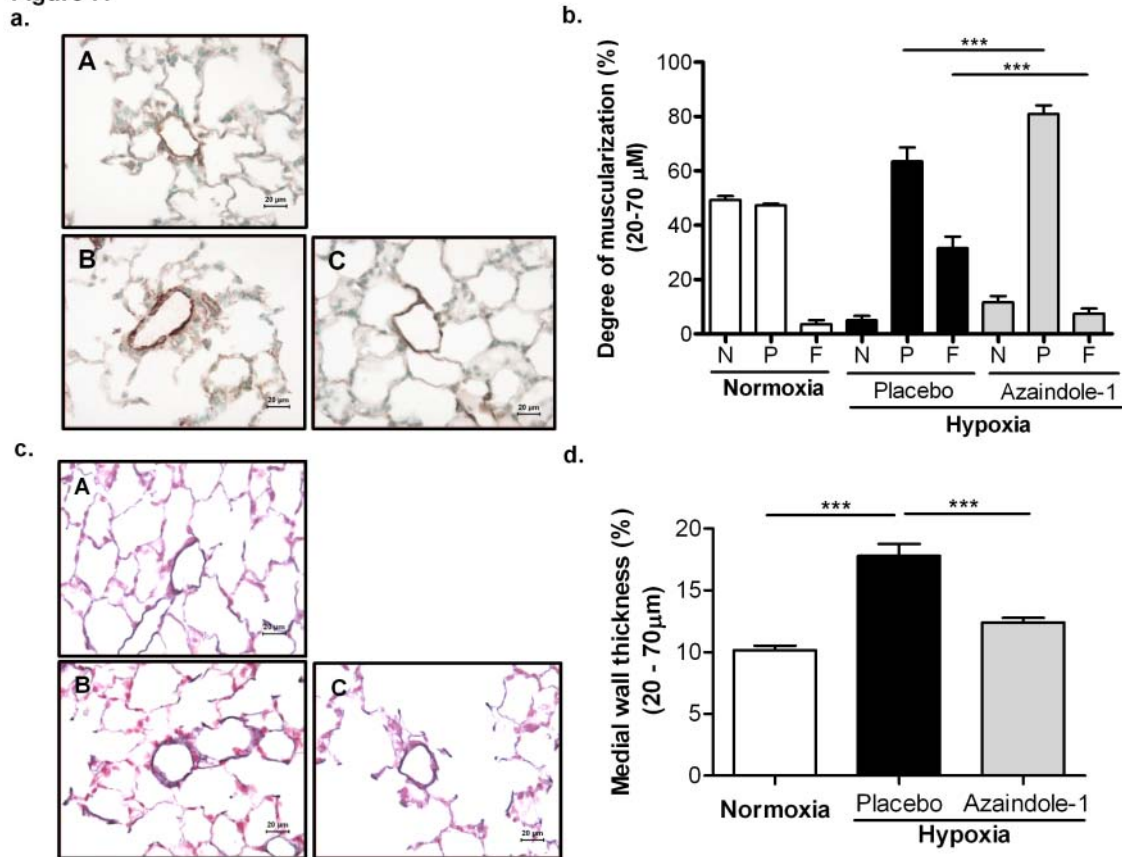
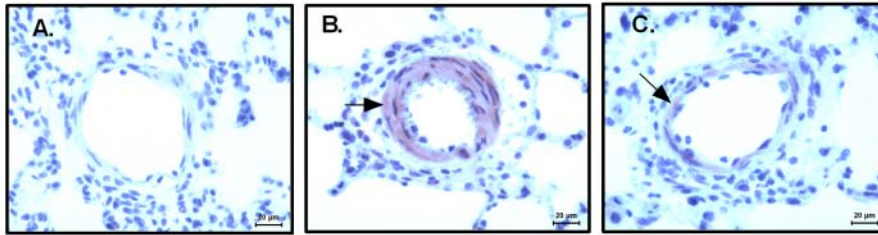


Figure 8. *Effects of azaindole-1 on ROCK activity and pulmonary vascular cell proliferation*

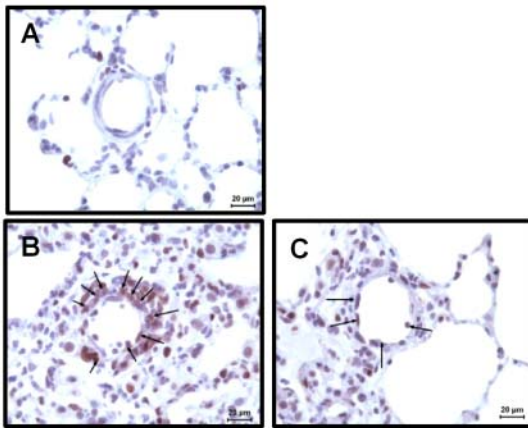
The ROCK activity and pulmonary vascular cell proliferation were investigated in the rat lung sections by immunostaining p-MYPT1 and PCNA respectively as described in methods. Representative photomicrographs of (a) p-MYPT1 and (b) PCNA staining (A- healthy control, B- MCT-placebo and C-MCT-azaindole-1) are shown. The PCNA positive vascular cells were counted and the index of proliferation (IOP) was calculated as described in methods. (c) IOP (%) of healthy control, MCT-placebo and MCT-azaindole-1 is given. Bars represent mean \pm SEM (n = 6-8). ***p<0.001. Positive immunoreactivity is indicated by an arrow (reddish-brown staining), Scale = 20 μ m.

Figure 8

a.



b.



c.

