Expression and Function of Soluble Guanylate Cyclase in Pulmonary Arterial Hypertension

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sGC activation in pulmonary hypertension

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ABSTRACT

Background – Alterations of the nitric oxide receptor soluble guanylate cyclase (sGC) may contribute to the pathophysiology of pulmonary arterial hypertension (PAH). We studied the expression of sGC in explanted lung tissue of PAH patients and investigated the effects of the sGC stimulator BAY 63-2521 on enzyme activity and on hemodynamics and vascular remodeling in two independent animal models of pulmonary hypertension.

Methods and Results – Strong upregulation of sGC in pulmonary arterial vessels in the idiopathic PAH lungs as compared to healthy donor lungs was demonstrated by immunohistochemistry. Similar to human, upregulation of sGC was detected in the structurally remodeled smooth muscle layer in chronic hypoxic mouse lungs and lungs from monocrotaline (MCT)-injected rats. BAY 63-2521 is a novel orally available compound that directly stimulates sGC and sensitizes it to its physiological stimulator, nitric oxide (NO). Chronic treatment of hypoxic mice and MCT rats with fully established pulmonary hypertension with BAY 63-2521 (10 mg/kg day) partially reversed the pulmonary hypertension, the right heart hypertrophy and the structural remodeling of the lung vasculature.

Conclusions – Upregulation of sGC in pulmonary arterial smooth muscle cells was noted in human IPAH lungs and lungs from animal models of PAH. Stimulation of sGC reversed right heart hypertrophy and structural lung vascular remodeling. Soluble guanylate cyclase may thus offer as new target for therapeutic intervention in pulmonary hypertension.

Key Words: cardiovascular diseases, hypertension, pulmonary, muscle, smooth, nitric oxide, pharmacology, BAY 63-2521
INTRODUCTION

Pulmonary hypertension (PH) is a disabling disease with high mortality characterized by sustained elevation in pulmonary artery pressure and pulmonary vascular remodeling due to proliferation and migration of pulmonary artery smooth muscle cells (1). Imbalance of vasodilatory and vasoconstrictive mediators have been implicated in these changes. Reduced urinary excretion of prostaglandin (PG)I2- and augmented excretion of thromboxane metabolites were found in patients with idiopathic pulmonary arterial hypertension (IPAH) (2), and immunohistological studies showed reduced expression of PGI2 synthase in the pulmonary vessels originating from these patients (3). Another important mediator in the regulation of vascular tone is nitric oxide (NO) which is synthesized by NO synthases. Local NO production from endothelium and epithelium regulates pulmonary perfusion depending on alveolar ventilation to assure optimized ventilation/perfusion distribution (4-6). In patients with idiopathic pulmonary hypertension it has been reported that the expression of endothelial nitric oxide synthase is downregulated (7), while other reports show an upregulation in plexiform lesions of IPAH patients (8). However, little is known about the expression and regulation of soluble guanylate cyclase (sGC) which operates as receptor for nitric oxide. Soluble GC is typically found as a heterodimer, consisting of a larger α-subunit and a smaller haem-binding β-subunit. The binding of NO to sGC results in activation and synthesis of the second messenger cGMP. Further, cGMP activates cGMP-dependent protein kinases (PKGs) leading to reduction in cytosolic Ca^{2+} concentration and desensitization of the actin-myosin contractile system. Recently, an increase of sGC protein expression was described in experimental hypoxia-induced pulmonary hypertension (9;10). Therefore, pharmacological stimulation of sGC is an appealing strategy to treat pulmonary hypertension and discovery of pharmacological tools, such as sGC stimulators is of high interest. Recently, the sGC stimulator BAY 41-2272 was shown to be a systemic and pulmonary vasodilator (11) and to
improve pulmonary arterial hypertension in experimental models of pulmonary hypertension (12). The aim of the present study was to investigate the expression of sGC in idiopathic pulmonary arterial hypertension and experimental models of pulmonary hypertension. Furthermore, we wanted to investigate the in vitro profile of the new sGC stimulator BAY 63-2521 which is in currently in clinical development for treatment of pulmonary arterial hypertension (13). Lastly, the in vivo efficacy of BAY 63-2521 was investigated in two independent models of PAH.
METHODS

Patient characteristics and measurements

Human lung tissue was obtained from 5 donors and 5 IPAH patients undergoing lung transplantation. Lung tissue was snap-frozen directly after explantation for mRNA and protein extraction. The study protocol for tissue donation was approved by the "Ethik-Kommission am Fachbereich Humanmedizin der Justus-Liebig-Universitaet Giessen" of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with Good Clinical Practice/International Conference on Harmonisation guidelines. Written informed consent was obtained from each individual patient or the patient’s next of kin.

Immunoblot analyses

A description of the immunoblotting is provided in the online Data Supplement. Briefly, protein samples were probed with an antibody directed against sGCβ1 (diluted 1:5,000, Alexis, San Diego, CA, USA).

Immunohistochemical staining

Paraffin-embedded lung tissue sectioned at 3 μm thickness was deparaffinized in xylene and rehydrated in a graded ethanol series to phosphate-buffered saline (PBS, pH 7.2). Antigen retrieval was performed by pressure cooking in citrate buffer (pH 6.0) for 15 min. Immunohistochemical staining was performed using rabbit anti-sGCα1 (1:400, A. Friebe, Bochum, Germany) and anti-sGCβ1 (1:800, ab50333, Abcam, USA) antibodies in conjunction with an avidin-biotin-peroxidase kit as per manufacturer’s instructions (Histostain-SP kit, Zymed Lab Inc., USA). Development of the dye was carried out with AEC substrate for 1-3 min. Finally, sections were counterstained with hematoxylin and coverslipped using mounting medium. To demonstrate the specificity and the localization,
lung sequential sections were stained without a primary antibody (negative control) or with α-smooth muscle actin.

**Effects of BAY 63-2521 on soluble guanylate cyclase activity**

A detailed description of the in vitro assays is given in the supplement. BAY 63-2521 (methyl 4,6-diamino-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-5-ylmethylcarbamate) was synthesized as described (16). BAY 63-2521 is structurally similar to BAY 41-2272 and belongs to the family of sGC stimulators (13). The structure of BAY 63-2521 is given in supplemental Figure 1. A possible inhibition of phosphodiesterases (PDE) was investigated in a PDE activity assay as described (17).

**In vivo experiments**

All animal experiments were performed according to the institutional guidelines that comply with national and international regulations. Mice were exposed to chronic hypoxia (10% O₂) in a ventilated chamber, as described previously (18;19). Rats were injected with 60 mg/kg Monocrotaline (MCT) subcutaneously (18-20). A detailed description of the animal models is given in the online Data Supplement.

**Hemodynamic measurements**

Animals were anaesthetized with a mixture of ketamine and xylazine. The trachea was cannulated, and the lungs were ventilated with room air. Systemic arterial pressure was determined by catheterization of the carotid artery. For measurement of right ventricular systolic pressure (RVSP) a catheter was inserted into the right ventricle via the right vena jugularis, as described (12;18).
Radiotelemetric measurements of right ventricular pressure and heart rate
The radiotelemetric technique has previously been described in detail (12;18). See the online Data Supplement.

Isolated mouse lung experiments
The isolated perfused lung model has previously been described in detail (19). See the online Data Supplement.

Quantification of mRNA expression
The hearts, lungs and pulmonary arterial smooth muscle cells (PASMC) were homogenized and RNA was extracted according to the manufacturer’s protocol to obtain total cellular RNA as described (21). Aliquots (1 µg) were used for Real-time polymerase chain reactions (PCRs) using the I-cycler (Biorad, Germany) and Syber-green as the fluorescence signal. The expressions of atrial natriuretic factor (ANF), transforming growth factor (TGF)-β1, sGCα1, sGCα2, sGCβ1 and sGCβ2 were normalized to housekeeping gene for loading control. The primers used in this study have been mentioned before (21;22) or in the online Data Supplement.

Pharmacological treatment (Acute intervention)
For acute intervention studies, a sustained increase of PAP from ≈6 to ≈24 mmHg was achieved by continuous infusion of the thromboxane-mimetic U46619 in rabbit lungs with a dose range of 70–160 pmol/kg/min as described previously. Individual titration was performed. This level of pulmonary hypertension was then maintained for at least 150 min with variations in pulmonary arterial pressure of less than 2 mmHg. The animals were then randomized into five groups. Group 1: U46619 was continuously infused for 150 min to
provoke an increase in pulmonary arterial pressure to ~24 mmHg (n=5); Group 2: Oral application of the PDE5 inhibitor sildenafil (10mg/kg body weight) 30 min after onset of U46619 infusion (n=8); Group 3 and 4: Oral application of the sGC activator BAY 63-2521 (1mg/kg or 10mg/kg body weight) 30 min after onset of U46619 infusion (n=5 each) and Group 5: combined application of sildenafil (10mg/kg body weight) and BAY 63-2521 (1mg/kg body weight) 30 min after onset of U46619 infusion (n=5).

**Pharmacological treatment (Chronic intervention)**

For chronic intervention studies four groups of mice were used: normoxic control mice exposed for 35 days to normoxic gas (n=10), mice exposed for 21 days to hypoxic gas (n=10), mice exposed for 35 days to hypoxic gas which received the vehicle from day 21 to day 35 (n=10), and mice exposed for 35 days to hypoxic gas which received from day 21 to day 35 Bay 63-2521 in a dose of 10 mg/kg body weight once a day by oral application (n=10). For continuous measurement of right ventricular systolic pressure and heart rate by radiotelemetry, a separate group of mice was exposed for 35 days to hypoxic gas and received from day 21 to day 35 Bay 63-2521 as described above. To investigate vascular reactivity in isolated mouse lungs additional two groups of animals were investigated: control mice (n=12) and animals exposed for 21 days to hypoxia (n=12).

Rats were randomized for chronic Bay 63-2521 treatment 21 days after MCT injection. The experimental groups included rats that received once per day Bay 63-2521 (10 mg/kg body weight) or vehicle (2% methylcellulose solution) by oral application. Rats were daily examined and subjected to hemodynamic measurements and histological assessment at day 35.
Data analysis

All data are given as means ± SEM. Differences between groups were assessed by analysis of variance and Student-Newman-Keuls post-hoc test for multiple comparisons with a p value < 0.05 regarded to be significant.
RESULTS

Expression of soluble guanylate cyclase (sGC) in idiopathic pulmonary hypertension (IPAH)

The mRNA expression of sGCα1, sGCα2, sGCβ1 and sGCβ2 was investigated by real-time RT-PCR in lung tissue homogenate from healthy donor and IPAH patients. There were no significant changes in any of these isoforms between healthy donor and IPAH patient lungs (Figure 1A). Similarly, western blot analysis of lung homogenate also demonstrated equal expression of sGCβ1 in healthy donor tissue and lung tissue from IPAH patients (Figure 1B). Immunohistochemistry demonstrated an extensive expression of sGCα1 and sGCβ1 in the medial wall of pulmonary arteries from IPAH patients, as shown by colocalization with α-smooth muscle actin in sequential sections. The specificity of the antibodies was shown by the negative control sections (Figure 2A). Further, a significant increase in sGCα2 (p<0.05) and a tendency to increase in sGCβ1 expression is confirmed in PASMCs from IPAH patients as compared to PASMCs from healthy donors (Figure 2B).

Pharmacological profile of BAY 63-2521

The effects of BAY 63-2521 and NO on stimulation of the highly purified sGC and the blocking effects of the sGC inhibitor ODQ were studied. BAY 63-2521 stimulated the recombinant sGC concentration dependently from 0.1 μM to 100 μM with an effect of 2-fold to 73-fold. In addition the sGC stimulatory effects of BAY 63-2521 and the NO releasing drug DEA/NO alone and in combination were investigated. DEA/NO induced a maximal increase in the sGC activity of 25-fold at a concentration of 0.1 μM. In combination, BAY 63-2521 and DEA/NO synergized over a wide range of concentrations. At highest concentrations of BAY 63-2521 (100 μM) and DEA/NO (0.1 μM) the specific activity of sGC was 112-fold above the baseline (Figure 3). The sGC stimulation induced by BAY 63-2521 could be nearly completely blocked by the sGC inhibitor ODQ oxidizing the prosthetic haem
group at the sGC (Figure 3). Using haem-free preparations of the sGC and UV-visual spectra of the purified sGC under unstimulated and NO-stimulated conditions, it could be shown that BAY 63-2521 activates sGC by an NO-independent, but haem-dependent mechanism (not shown in detail). BAY 63-2521 had virtually no effect on a broad range of cyclic nucleotide-metabolizing enzymes (PDE1-9 and PDE11) up to a concentration of 3 µM, indicating that the effect of BAY 63-2521 must be due to an effect on cGMP synthesis rather than cGMP degradation.

**Effect of Bay 63-2521 on acute hypoxic pulmonary vasoconstriction in isolated mouse lungs.**

The sGC stimulator BAY 63-2521 dose dependently reversed acute pulmonary vasoconstriction in isolated lungs from mice that were kept under normoxic conditions (Figure 2 in supplement, closed circles). Notably, when investigating lungs isolated from mice that were kept for 21 days under hypoxic conditions (10% O₂), slightly enhanced sensitivity to BAY 63-2521 was noted (Figure 2 in supplement, open circles).

**Acute effects of sildenafil and BAY 63-2521 on U46619-induced pulmonary hypertension in intact animals**

Continuous infusion of U46619 provoked an increase in pulmonary arterial pressure (PAP) to 23.4 ± 0.9 mmHg within 30 min, with subsequent plateau of the pulmonary hypertension. This level of pulmonary hypertension was then maintained for at least 300 min with variations in PAP of less than 2 mmHg. As shown in Figure 4, subsequent oral application of 10mg/kg sildenafil resulted in a selective pulmonary vasodilatation while BAY 63-2521 at oral doses of 1 and 10mg/kg decreased dose dependently both pulmonary and systemic arterial pressure to a similar extent. The combination of the effective sildenafil dose (10mg/kg) with the sub-maximal dose of BAY 63-2521 (1mg/kg) was operative in an additive manner and increased
pulmonary selectivity of sildenafil while the reduction in systemic arterial pressure was still moderate (~10%).

_Chronic effects of BAY 63-2521 on right ventricular pressure, systemic arterial pressure and heart rate in mice with hypoxia-induced pulmonary hypertension._

Upon chronic hypoxic exposure, right ventricular systolic pressure (RVSP) increased significantly from 23.0 ± 0.4 mmHg (controls) to 29.8 ± 1.9 mmHg (21 d hypoxia) and 34.8 ± 1.9 mmHg (35 d hypoxia) (Figure 5A). No significant changes in systemic arterial pressure were noted (Figure 5B). In the chronic treatment group, BAY 63-2521 was orally applied at a dose of 10 mg/kg body weight/day from day 21 to 35. BAY 63-2521 significantly decreased RVSP to 29.0 ± 0.6 mmHg (p<0.05 versus hypoxia at day 35). To further investigate hemodynamics in chronic hypoxic mice, a telemetric approach was performed. Continuous telemetric measurement of RVSP in conscious mice under chronic hypoxic exposure revealed continuous increase in RVSP pressure curve that was shouldered around day 5 and followed by a progressive increase thereafter. Oral treatment with BAY 63-2521 at a dose of 10 mg/kg day from day 21 to day 35 reduced hypoxia-induced pulmonary hypertension (Figure 6A). During the development of pulmonary hypertension heart rate increased from 380±30 to 496±14 beats per minute (bpm) (Figure 6B). When comparing the pre- and post treatment values, no significant decrease were noted by chronic BAY 63-2521 application.

_Chronic effects of BAY 63-2521 on right heart hypertrophy, gene expression and vascular remodeling in mice with hypoxia-induced pulmonary hypertension._

Exposure of the mice to chronic hypoxia resulted in an increase in the ratio of right ventricle to left ventricle plus septum weight (RV/(LV+S)) from 0.30 ± 0.01 (controls) to 0.38 ± 0.01 (21 d hypoxia) and 0.41 ± 0.01 (35 d hypoxia), respectively (both p<0.05 versus controls)
(Figure 7A). The RV/(LV+S) ratio decreased to 0.37 ± 0.01 in the BAY 63-2521 group (p<0.05 versus hypoxia at day 35). In addition, the gene expression of transforming growth factor beta (TGF-β) and atrial natriuretic factor (ANF) were significantly elevated in the right ventricle of chronic hypoxic mice (Figure 7B, C) and reversed to normal level under BAY 63-2521 treatment. No significant changes were noted in the left ventricle (not shown in detail).

When quantitatively assessing the degree of muscularization of small pulmonary arteries at a size between 20 and 70 µm, mice exposed chronically to hypoxia showed a reduction in non-muscular vessels (given as percent of total vessel count) from 48.9 ± 7.8% to 3.4 ± 0.9% at 21 days and to 1.0 ± 0.5% at 35 days, respectively (Figure 7D). Oral treatment with BAY 63-2521 increased the percentage of non-muscular vessels to 18.9 ± 3.9%. In parallel, BAY 63-2521 treatment also significantly reduced the percentage of muscularized vessels in chronic hypoxic mice.

Expression of soluble guanylate cyclase (sGC) increased in monocrotaline (MCT) induced PAH

The mRNA expression of sGCα1, sGCα2, sGCβ1 and sGCβ2 was investigated by real-time RT-PCR in lung tissue homogenate from control and MCT induced PAH rats. A significant upregulation of sGCβ1 and sGCβ2 was observed in MCT induced PAH rat lung homogenate as compared to control rat lung homogenate (Figure 8A). Interestingly, the upregulation of sGCβ1 was confined to PASMCs that were freshly isolated from distal pulmonary arteries of MCT induced PAH rats (Figure 8B). In corroboration, immunohistochemistry demonstrated an extensive expression of sGCβ1 in the medial wall of pulmonary arteries from MCT induced PAH rats, as shown by colocalization with α-smooth muscle actin in sequential sections (Figure 8C).
Chronic effects of BAY 63-2521 on right ventricular pressure, pulmonary resistance and systemic arterial pressure in rats with monocrotaline-induced pulmonary hypertension.

Monocrotaline (MCT) injection in rats induced severe pulmonary hypertension with marked increase in right ventricular systolic pressure from 25.8 ± 1.9 mmHg to 51.7 ± 5.4 mmHg at 21 days and increased further to 84.1 ± 0.6 mmHg at day 35 (Figure 9A). Consequently, these animals developed significant increase in total pulmonary vascular resistance (TPR) from 0.91 ± 0.05 mmHg min/ml 100 g bodyweight to 3.60 ± 0.04 mmHg min/ml 100 g bodyweight (21 days) and 6.13 ± 0.60 mmHg min/ml 100 g bodyweight (35 days)(Figure 9B). No significant changes in systemic arterial pressure were noted (Figure 9C). Daily treatment of MCT-injected rats with BAY 63-2521 at a dose of 10 mg/kg body weight from day 21 to 35 significantly decreased RVSP to 55.4 ± 2.5 mmHg (p<0.05 versus MCT at day 35). Total pulmonary resistance decreased to 4.22 ± 0.08 mmHg min/ml 100 g bodyweight (p<0.05 versus MCT at day 35). Systemic arterial pressure (SAP) did not change in response to the treatment.

Chronic effects of BAY 63-2521 on right heart hypertrophy, degree of muscularization of pulmonary arteries and sGC expression in rats with monocrotaline-induced pulmonary hypertension.

The MCT injected rats developed significant right heart hypertrophy which was increasing from 0.28 ± 0.01 to 0.40 ± 0.03 at 21 days and 0.60 ± 0.03 at 35 days, respectively (Figure 10A). Chronic treatment of the rats by BAY 63-2521 decreased RV/(LV+S) ratio to 0.42 ± 0.04 (p<0.05 versus MCT at day 35). We quantitatively assessed the degree of muscularization of pulmonary arteries with a diameter between 25 to 50 µm. In controls, the majority of vessels of this diameter are usually nonmuscularized. In the MCT group, both at day 21 and day 35, a dramatic decrease in nonmuscularized pulmonary arteries occurred.
(Figure 10B) with a concomitant increase in fully muscularized pulmonary arteries. Treatment with BAY 63-2521 at 10 mg/kg day resulted in a significant reduction of fully muscularized arteries as compared to both MCT groups and increased the percentage of non-muscularized pulmonary arteries.
DISCUSSION

The novel finding of the present study is that the receptor for nitric oxide (NO), soluble guanylate cyclase (sGC), is highly expressed in vascular smooth muscle cells of small pulmonary arteries from patients with idiopathic pulmonary hypertension. Furthermore, we demonstrated the in vitro efficacy of the novel sGC stimulator BAY 63-2521 which acts synergistically with NO on cGMP synthesis by sGC. It could be shown that BAY 63-2521 activates sGC mainly by an NO-independent, but haem-dependant mechanism. In vivo we demonstrated that BAY 63-2521 is an effective treatment in two well established experimental models of severe pulmonary hypertension. Treatment effects showed a partial reversal of pulmonary hypertension, a reduction in right heart hypertrophy and reversal of pulmonary vessel remodeling.

The NO-signaling pathway is a major target for treatment of pulmonary hypertension. The inhalation of NO has been shown to reduce pulmonary arterial pressure and to improve gas exchange in patients with acute and chronic pulmonary hypertension. However, this approach is limited by the short half life of NO and the rebound phenomenon after withdrawal. Another possibility to manipulate this signaling pathway is the enhancement of endogenous NO signaling by inhibition of phosphodiesterase (PDE) 5 which is the enzyme that hydrolyzes the second messenger cGMP. Recent studies showed that the cGMP-specific PDE 5 is highly expressed in lung tissue (23;24). Moreover, further upregulation of PDE5 may occur under conditions of pulmonary hypertension, thereby contributing to increased lung vascular resistance under these conditions (25-27). Subsequently it has been shown that the PDE5 inhibitor sildenafil is a potent pulmonary vasodilator which improves hemodynamics, gas exchange and exercise capacity (28-32). Based on a positive clinical phase III study, sildenafil has been approved for the treatment of pulmonary arterial hypertension (33;34). However, there is increasing evidence that modification of sGC under pathophysiological conditions,
either by tyrosine phosphorylation at the β1 subunit (35) or heme oxidization (36;37) results in an altered NO response followed by reduced local cGMP production. This may explain in part limitations of the current therapies addressing the NO pathway in cardiopulmonary diseases. The pharmacological activation of sGC is therefore attractive to restore NO signaling. However, several cardiovascular disease states are accompanied by downregulation of the activity and expression of the heme-containing sGC (38-40). We were therefore interested in the role of sGC in pulmonary hypertension and performed studies on explanted tissue of patients with idiopathic pulmonary arterial hypertension (IPAH) in which no expression data were hitherto published. Quantitative mRNA assessment or immunoblotting of all the four subunits did not reveal significant expression changes. However, the vascular rarefication and loss of vasculature in these patients may limit this approach and immunohistochemical investigation of both subunits (sGCα1 and sGCβ1) confirmed stronger immunoreactivity in the medial layer of small pulmonary arteries which colocalizes with the smooth muscle marker α-smooth muscle actin. In contrary, as shown in Figure 8, quantitative mRNA assessment of pulmonary hypertensive lung tissue resulted in possibly conflicting data with opposite changes in sGCα1 levels in lung homogenates and isolated PASMCs. The only consistent finding with regard to the human tissues appears to be the increase in sGCβ1 expression. In line with these results, an upregulation sGCβ1 expression was reported in mice chronically exposed to hypoxia. In contrary, no significant changes were reported in hypoxic rat lungs, suggesting the species specific differences with regard to sGCβ1 expression in the hypoxia- induced pulmonary hypertension (9;10).

Furthermore, there may be a discrepancy between expression and function of sGC as investigations in pulmonary arteries from chronic hypoxic rats demonstrated impaired vasorelaxation in response to acetylcholine (ACh) and sodium nitroprusside (SNP) (41). In in vitro studies on purified sGC we investigated heme- and heme free preparations and describe
here for the first time the NO-independent, but heme-dependent sGC stimulator BAY 63-2521 which shows an activation profile similar to that of the sGC stimulator BAY 41-2272 (42;43) but an improved pharmacokinetic profile (data not shown).

The in vivo efficacy of this compound was investigated in two independent experimental models of pulmonary hypertension, the chronic hypoxic mouse model and monocrotaline (MCT) induced pulmonary hypertension in rats. Pathological characteristics of both models include elevated pulmonary pressure, right ventricular hypertrophy and anatomical structural changes of lung vasculature including de-novo muscularization of normally non-muscularized small pulmonary arteries. To mimic the clinical situation, we did not investigate the protective effects of BAY 63-2521 on the development of pulmonary hypertension, but initiated treatment 3 weeks after chronic hypoxia exposure/MCT injection when elevated pulmonary pressure, vascular remodelling and right heart hypertrophy were fully established. In both animal models, partial reversal of the hemodynamic and structural changes was noted. Most impressively, continuous telemetric measurement of right ventricular pressure in mice demonstrated anti-remodeling of BAY 63-2521. This technique has recently been presented in rats (18;44) but this is the first study which employs on-line measurement of right heart pressure in mice. Interestingly, an increase in RVSP shoulder around Day 5, which was followed by a progressive increase in pressure there after was observed in chronic hypoxic mice. As compared to our data which were raised in mice, Sebkhi et al, demonstrated recently increased pulmonary pressures (measured by telemetry) in chronic hypoxic rats although the differences between the pressure curves in rats and mice are comparable (44).

In addition to the reduction of pressure, histomorphometric analysis revealed strong antiremodeling potency of this compound in both animal models. The former sGC stimulator BAY 41-2272 has demonstrated similar efficacy in experimental pulmonary hypertension induced by hypoxia in mice (12) and hypoxia in neonatal rats (45). Acute vasoreactivity
testing revealed a strong reduction in pulmonary vascular resistance which was shown in
different species such as mice (12) or sheep (11;46) which indicates strong biological
relevance of sGC in regulating pulmonary vascular tone. Most interestingly, BAY 63-2521 in
combination with sildenafil decreased pulmonary arterial pressure in an additive manner and
increased pulmonary selectivity of sildenafil. Although, the long term effects of this
combination therapy on hemodynamics and vascular remodeling needs to be demonstrated in
future, it suggest the beneficial clinical effects of BAY 63-2521 alone or in combination with
approved targeted therapies (e.g., sildenafil) of PAH.

This study is the first to describe the expression of sGC in idiopathic pulmonary hypertension
and the successful therapeutic use of the sGC stimulator BAY 63-2521 in two well accepted
animal models of pulmonary hypertension. We demonstrate that 1) sGCα1 and β1 are
expressed in vascular smooth muscle cells of small pulmonary arteries thus representing a
relevant target for treatment of IPAH, 2) the novel compound BAY 63-2521 stimulates sGC
in a NO independent manner but also synergizes with NO, 3) treatment with BAY 63-2521
reversed hemodynamic and structural changes provoked by hypoxia in mice and MCT in rats.

Limitation of the study
Neither hypoxia- nor monocrotaline-induced pulmonary hypertension in rodents fully mimics
human PH associated with chronic lung disease or idiopathic pulmonary arterial hypertension.
Therefore, the effectiveness of BAY 63-2521 in experimental models of PH does not predict
beneficial effects in patients with PAH. However, the anti-remodeling potential of BAY 63-2521
in two independent models of PH (hypoxia induced PH in mice and MCT induced PH in rats) and the demonstration of the strong sGC expression in human explanted PAH lungs
provide clear evidence that translation of this class of compounds from the bench into the
clinic will be successful. Clinical trials are underway (13) and will address the therapeutic efficiency of BAY 63-2521 in life-threatening advanced pulmonary arterial hypertension.
ACKNOWLEDGEMENTS

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Abbreviations used in this manuscript

RVSP, right ventricular systolic pressure; BPM, beats per minute; SAP, mean arterial pressure; sGC soluble guanylate cyclase.
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FIGURE LEGENDS

Figure 1. sGC expression levels in human lung tissues from healthy and IPAH patients.

(A) RNA isolated from lung homogenate was analyzed for the regulation sGCα1, sGCα2, sGCβ1 and sGCβ2 subunits by quantitative RT-PCR using the ΔΔ CT method for the calculation of the regulation factor (RF). The CT values of individual genes were normalized to house keeping gene PBGD. (B) Soluble fractions (60 µg of protein) of lung homogenates derived from healthy donors or patients with PH (n=5 each) were subjected to Western blot analysis using anti- sGCβ1 antibody. Immunoreactive sGCβ1 is detectable at 68 kDa (arrow). Loading of equal protein amounts was checked by protein staining. The results shown are representative of five immunoblots performed.
Figure 2. Vascular muscularization and sGC expression in lungs and PASMCs from healthy and IPAH patients.

(A) Muscularization is demonstrated in the upper row by $\alpha$-smooth muscle actin staining for identifying vascular smooth muscle cells. sGC$\alpha_1$ and sGC$\beta_1$ expression in human lungs from healthy and IPAH patients is demonstrated in the lower row by positive staining in the medial layer of small pulmonary arteries. Specificity of antibodies is demonstrated in negative control (absence of primary antibody) sections. Scale bars: 20 $\mu$m. (B) PASMCs that were isolated from healthy and IPAH patients were analyzed for the regulation of sGC$\alpha_1$, sGC$\alpha_2$, sGC$\beta_1$ and sGC$\beta_2$ subunits by quantitative RT-PCR using the $\Delta\Delta$ CT method for the calculation of the regulation factor (RF). The CT values of individual genes were normalized to housekeeping gene PBGD. *, p<0.05 versus healthy patients.
Figure 2

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Figure 2

B

![Bar chart showing regulation factor for sGCα1, sGCα2, sGCβ1, and sGCβ2.](chart.png)
Figure 3. Pharmacological Profile of BAY 63-2521.

Stimulation of purified native sGC by BAY 63-2521 (0.01 - 100 µM) in the absence (–□–) and presence of DEA/NO (–▲– 0.001, –▼– 0.01, and –♦– 0.1µM) or ODQ (–○–). The specific activity of sGC is expressed as x-fold stimulation vs. specific basal activity (239 ± 7 and 178 ± 14 nmol/mg/min for the ODQ group) The data presented represent means ± SEM from 4-8 independent experiments performed in duplicate.

Figure 4. Effects of sildenafil, BAY 63-2521 and combination of sildenafil/BAY 63-2521 on U46619-induced pulmonary hypertension.

Immediate vasodilatory effects of soluble guanylate cyclase stimulator BAY 63-2521 (1mg/kg and 10mg/kg body weight), PDE5 inhibitor Sildenafil (10mg/kg body weight) and combination of sildenafil/BAY 63-2521 (10mg/kg and 1mg/kg body weight respectively) are
given. The substances were orally applied in intact rabbits that developed pulmonary hypertension in rabbits in response to U46619. Percent decreases in PAP and SAP in response to the different agents are shown.

*: p < 0.05 versus control

**Figure 4**

![Graph showing effects of different agents on PAP and SAP](image)

**Figure 5.** Impact of chronic BAY 63-2521 treatment on hemodynamics in hypoxia-induced pulmonary hypertension.

Mice were exposed to hypoxia for 21 or 35 days, or remained in normoxia throughout. The soluble guanylate cyclase stimulator BAY 63-2521 or vehicle were orally applied from day 21 to day 35 in hypoxic mice (n = 10) at a dose of 10 mg/kg BW. (A) Right ventricular systolic pressure (RVSP) and (B) systemic arterial pressure (SAP) in mmHg are given.

*, p<0.05 versus control; ‡, p<0.05 versus hypoxia at day 35.
Figure 6. Telemetric data of right ventricular systolic pressure (RVSP) and heart rate in chronic hypoxic mice treated with BAY 63-2521.

Mice were exposed to hypoxia for 35 days and treated orally with BAY 63-2521 from day 21 to day 35. Effect of BAY 63-2521 on the course of RVSP (A) and heart frequency (B) in hypoxia-induced pulmonary hypertension was assessed by telemetry in conscious mice (n=5).
**Figure 7.** Effect of BAY 63-2521 on right heart hypertrophy, ANF and TGF-β expression in heart and degree of muscularization of small pulmonary arteries.

Mice were exposed to hypoxia for 21 or 35 days, or remained in normoxia throughout. The soluble guanylate stimulator BAY 63-2521 was applied from day 21 to day 35 in hypoxia-exposed mice (n = 10) at a dose of 10 mg/kg BW. **(A)** Right to left ventricle + septum (RV/(LV+S)) weight ratio is given. Expression of atrial natriuretic factor (ANF) **(B)** and transforming growth factor (TGF)-β **(C)** as a percentage of normoxic-exposed mice in right ventricular tissue is given. **(D)** Proportions of non- (N), partially- (P) or fully- (M) muscularized pulmonary arteries, as percentage of total vessel count (vessels sized 20-70 μm), are given for hypoxia-exposed mice. A total of 80 to 100 intra-acinar vessels were analyzed in each lung.
*, p<0.05 versus control; ‡, p<0.05 versus hypoxia 35 days.

**Figure 7**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**D**

![Graph D](image)

**Figure 8.** sGC expression in lung homogenate, isolated pulmonary arterial smooth muscle cells (PASMC) and in serial sections from monocrotaline injected rats.

The regulation of sGCα1, sGCα2, sGCβ1 and sGCβ2 subunits in lung homogenate (**A**) and isolated pulmonary arterial smooth muscle cells (PASMC) (**B**) was analyzed by real-time quantitative PCR using the ∆∆CT method for the calculation of the regulation factor (RF). The CT values of individual genes were normalized to house keeping gene PBGD. *, p<0.05 versus control. Panel **C** shows the sGCβ1 and α-smooth muscle actin immunostaining in pulmonary arteries from control and from MCT injected rats. Specificity of antibodies is demonstrated in negative control (absence of primary antibody) sections. Scale bar: 20 µm.
Figure 9. Effects of BAY 63-2521 on hemodynamics in MCT-induced pulmonary hypertension.

Rats were subcutaneously injected with MCT or saline and investigated on day 21 or 35. The soluble guanylate cyclase stimulator BAY 63-2521 was orally applied from day 21 to day 35 in MCT rats (n = 8) at a dose of 10 mg/kg BW. (A) Right ventricular systolic pressure (RVSP), (B) pulmonary vascular resistance index (PVRI) and (C) systemic arterial pressure (SAP) are given.

*, p<0.05 versus control; ‡, p<0.05 versus MCT at day 35.
Figure 10. Effect of BAY 63-2521 on right heart hypertrophy and degree of muscularization of small pulmonary arteries in MCT-induced pulmonary hypertension.

Rats were subcutaneously injected with MCT or saline and investigated on day 21 or 35. The soluble guanylate cyclase stimulator BAY 63-2521 was orally applied from day 21 to day 35 in MCT rats (n = 8) at a dose of 10 mg/kg BW. (A) Right ventricular hypertrophy was assessed by right to left ventricle+septum (RV/(LV+S)) ratio. (B) Degree of muscularization is given as percentage of non- (N), partially- (P) or fully- (M) muscularized pulmonary arteries from total vessel count (vessels sized 20-70 µm) in MCT injected rats. A total of 80 to 100 intra-acinar vessels were analyzed in each lung.

*, p<0.05 versus control; ‡, p<0.05 versus MCT at day 35
Figure 10

A

B