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Expression and function of soluble guanylate cyclase in pulmonary arterial hypertension

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ABSTRACT: Alterations of the nitric oxide receptor, soluble guanylate cyclase (sGC) may contribute to the pathophysiology of pulmonary arterial hypertension (PAH). In the present study, the expression of sGC in explanted lung tissue of PAH patients was studied and the effects of the sGC stimulator BAY 63-2521 on enzyme activity, and haemodynamics and vascular remodelling were investigated in two independent animal models of PAH.

Strong upregulation of sGC in pulmonary arterial vessels in the idiopathic PAH lungs compared with healthy donor lungs was demonstrated by immunohistochemistry. Upregulation of sGC was detected, similarly to humans, in the structurally remodelled 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 sensitises it to its physiological stimulator, nitric oxide. Chronic treatment of hypoxic mice and MCT-injected rats, with fully established PAH, with BAY 63-2521 (10 $mg \cdot kg^{-1} \cdot day^{-1}$) partially reversed the PAH, the right heart hypertrophy and the structural remodelling of the lung vasculature.

Upregulation of soluble guanylate cyclase in pulmonary arterial smooth muscle cells was noted in human idiopathic pulmonary arterial hypertension lungs and lungs from animal models of pulmonary arterial hypertension. Stimulation of soluble guanylate cyclase reversed right heart hypertrophy and structural lung vascular remodelling. Soluble guanylate cyclase may thus offer a new target for therapeutic intervention in pulmonary arterial hypertension.

KEYWORDS: BAY 63-2521, cardiovascular diseases, nitric oxide, pharmacology, pulmonary arterial hypertension, smooth muscle

Pulmonary arterial hypertension (PAH) is a disabling disease, with high mortality, characterised by sustained elevation in pulmonary arterial pressure (P_{pa}) and pulmonary vascular remodelling due to proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) [1]. Imbalance of vasodilatory and vasoconstrictive mediators has been implicated in these changes. Reduced urinary excretion of prostaglandin (PG)I_2 and augmented excretion of thromboxane metabolites were found in patients with idiopathic PAH (IPAH) [2], and immunohistological studies have shown reduced expression of PGI_2 synthase in the pulmonary vessels originating from those patients [3]. Another important mediator in the regulation of vascular tone is nitric oxide (NO), which is synthesised by NO synthases. Local NO production from endothelium and epithelium regulates pulmonary perfusion, depending on alveolar ventilation to assure optimised ventilation/perfusion distribution [4–6]. In patients with IPAH, it has been reported that the expression of endothelial NO 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 a receptor for NO. Typically, sGC is 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 cyclic guanosine

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monophosphate (cGMP). Furthermore, cGMP activates cGMP-dependent protein kinases (protein kinase G) leading to reduction in cytosolic Ca^{2+} concentration and desensitisation of the actin–myosin contractile system. Recently, an increase of sGC expression was described in experimental hypoxia-induced PAH [9, 10]. Therefore, pharmacological stimulation of sGC is an appealing strategy to treat PAH 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 PAH in experimental models of PAH [12]. The aim of the present study was to investigate the expression of sGC in IPAH and experimental models of PAH. Furthermore, the present authors wanted to investigate the *in vitro* profile of the new sGC stimulator, BAY 63-2521, which is currently in clinical development for treatment of PAH [13]. In addition, 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 five donors and five 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 supplementary data. 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 deparaffinised in xylene and rehydrated in a graded ethanol series to PBS (pH 7.2). Antigen retrieval was performed by autoclaving in citrate buffer (pH 6.0) for 15 min. Immunohistochemical staining was performed using rabbit anti-sGCα1 (diluted 1:400; A. Friebe, Bochum, Germany) and anti-sGCβ1 (diluted 1:800; ab50333; Abcam, Cambridge, CA, USA) antibodies [14, 15] in conjunction with an avidin–biotin-peroxidase kit following the manufacturer's instructions (Histostain-SP kit; Zymed Lab Inc., San Francisco, CA, USA). Development of the dye was carried out with AEC substrate for 1–3 min. Finally, sections were counterstained with haematoxylin and coverslipped using mounting medium. In order to demonstrate the specificity and localisation, sequential lung sections were stained without a primary antibody (negative control) or with α-smooth muscle actin.

Effects of BAY 63-2521 on sGC activity

A detailed description of the *in vitro* assays is provided in the online supplementary data. BAY 63-2521 (methyl-4,6-diamino-2-(1-(2-fluorobenzyl)-1H-pyrazolo[3,4-*b*]pyridin-3-yl)-pyrimidin-5-ylmethylcarbamate) was synthesised as described previously [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 provided in supplemental figure 1. A possible inhibition of phosphodiesterases (PDE) was investigated in a PDE activity assay as described previously [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% O_2) in a ventilated chamber, as described previously [18, 19]. Rats were injected with 60 $mg \cdot kg^{-1}$ monocrotaline (MCT) subcutaneously [18–20]. A detailed description of the animal models is provided in the online supplementary data.

Haemodynamic measurements

Animals were anaesthetised with a mixture of ketamine and xylazine. The trachea was cannulated and the lungs were ventilated with room air. Systemic arterial pressure (P_{sa}) was determined by catheterisation of the carotid artery. For measurement of right ventricular systolic pressure (P_{rvs}) a catheter was inserted into the right ventricle *via* the right vena jugularis, as described previously [12, 18].

Radiotelemetric measurements of P_{rvs} and cardiac frequency

The radiotelemetric technique has previously been described in detail [12, 18] (refer to the online supplementary data).

Isolated mouse-lung experiments

The isolated perfused lung model has previously been described in detail [19] (refer to the online supplementary data).

Quantification of mRNA expression

The hearts, lungs and PASMCs were homogenised and RNA was extracted according to the manufacturer's protocol (AGS, Heidelburg, Germany) in order to obtain total cellular RNA as described previously [21]. Aliquots (1 μg) were used for real-time PCR using the I-cycler (Biorad, Munich, 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* mRNAs were normalised to a housekeeping gene for loading control. To determine the relative changes in the gene expression of sGCα1, sGCα2, sGCβ1 and sGCβ2 the ΔCT method was used for the calculation of the regulation factor. In brief, ΔΔCT is determined by subtracting the average reference gene porphobilinogen deaminase Ct value from the average target gene Ct value. ΔΔCT is calculated by subtracting ΔCT of the experiment from ΔCT of the control. The primers used in the present study have been mentioned previously [21, 22] and are listed in the online supplementary data.

Pharmacological treatment

Acute intervention

For acute intervention studies, a sustained increase of P_{pa} 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 $pmol \cdot kg^{-1} \cdot min^{-1}$ as described previously. Individual titration was performed. This level of PAH was then maintained for at least 150 min with variations in P_{pa} of

<2 mmHg. The animals were then randomised into five groups, as follows. Group 1: U46619 was continuously infused for 150 min in order to provoke an increase in P_{pa} to ~24 mmHg (n=5); group 2: oral application of the PDE5 inhibitor sildenafil (10 mg·kg^{-1}), 30 min after onset of U46619 infusion (n=8); groups 3 and 4: oral application of the sGC activator BAY 63-2521 (1 mg·kg^{-1} or 10 mg·kg^{-1}, respectively), 30 min after onset of U46619 infusion (n=5 each); and group 5: combined application of sildenafil (10 mg·kg^{-1}) and BAY 63-2521 (1 mg·kg^{-1}), 30 min after onset of U46619 infusion (n=5).

Chronic intervention

For chronic intervention studies four groups of mice were used: 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 and who received the vehicle (2% methylcellulose solution) from day 21 to day 35 (n=10); and mice exposed for 35 days to hypoxic gas and who received BAY 63-2521 (10 mg·kg^{-1}) once a day by oral application (n=10) from day 21 to day 35. For continuous measurement of P_{rvs} and cardiac frequency by radiotelemetry, a separate group of mice was exposed for 35 days to hypoxic gas and received BAY 63-2521 (10 mg·kg^{-1}) once a day by oral application from day 21 to day 35. In order to investigate vascular reactivity in isolated mouse lungs, an additional two groups of animals were investigated: control mice (n=12) and animals exposed for 21 days to hypoxic conditions (n=12).

Rats were randomised for chronic BAY 63-2521 treatment, 21 days after MCT injection. The experimental groups included rats that received BAY 63-2521 (10 mg·kg^{-1}) or vehicle (2% methylcellulose solution) by oral application, once per day. Rats were examined daily and subjected to haemodynamic measurements and histological assessment at day 35.

Data analysis

Data are presented as mean±SEM. Differences between groups were assessed by ANOVA and Newman–Keuls *post hoc* test for multiple comparisons. A p-value <0.05 was considered to be significant.

RESULTS

Expression of sGC in 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 (fig. 1a). Similarly, Western-blot analysis of lung homogenate also demonstrated equal expression of sGCβ1 in healthy donor tissue and lung tissue from IPAH patients (fig. 1b). Immunohistochemistry demonstrated an extensive expression of sGCα1 and sGCβ1 in the medial wall of pulmonary arteries from IPAH patients, as shown by co-localisation with α-smooth muscle actin in sequential sections. The specificity of the antibodies was shown by the negative control sections (fig. 2a). Furthermore, a significant increase in sGCα2 ($p<0.05$) and a tendency to increase sGCβ1 expression was confirmed in PASMCs from IPAH patients compared with PASMCs from healthy donors (fig. 3).

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 to 100 μM with a two-fold to 73-fold effect. 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 synergised 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 (fig. 4). The sGC stimulation induced by BAY 63-2521 could be almost completely blocked by the sGC inhibitor, ODQ, oxidising the prosthetic haem group at the sGC (fig. 4). Using haem-free preparations of sGC and UV–visual spectra of purified sGC under unstimulated and NO-stimulated conditions, it was shown that BAY 63-2521 activates sGC by an NO-independent but haem-dependent mechanism (data not shown). BAY 63-2521 had virtually no effect on a broad range of cyclic nucleotide-metabolising enzymes (PDE1-9 and PDE11) up to a concentration of 3 μM, indicating that the

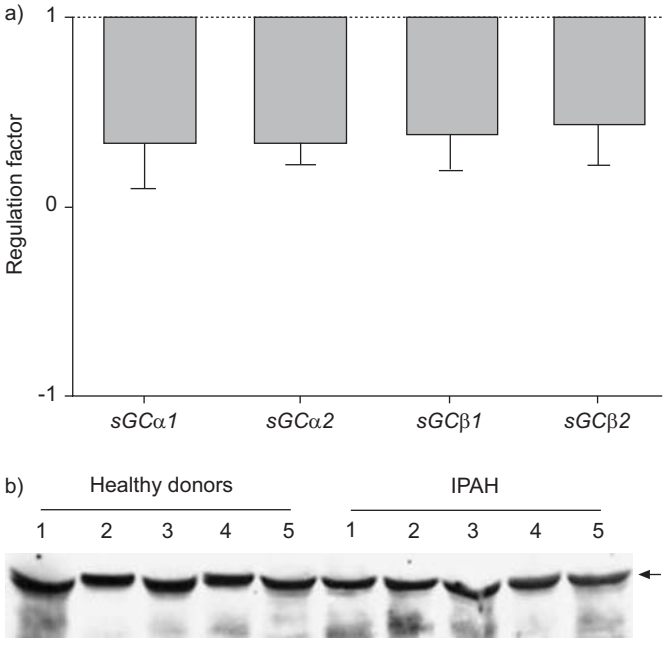

FIGURE 1. Expression levels of soluble guanalate cyclase (sGC) in human lung tissues from healthy donors and idiopathic pulmonary arterial hypertension (IPAH) patients. a) RNA isolated from lung homogenate was analysed for the regulation of *sGCα1*, *sGCα2*, *sGCβ1* and *sGCβ2* by quantitative RT-PCR using the ΔΔCT method (see Methods section) for the calculation of the regulation factor. The CT values of individual genes were normalised to house keeping gene porphobilinogen deaminase. b) Soluble fractions (60 μg of protein) of lung homogenates derived from healthy donors or patients with IPAH (n=5 each) were subjected to Western-blot analysis using anti-sGCβ1 antibody. Immunoreactive sGCβ1 was detectable at 68 kDa (arrow). Loading of equal protein amounts was checked by protein staining. The results shown are representative of five immunoblots performed.

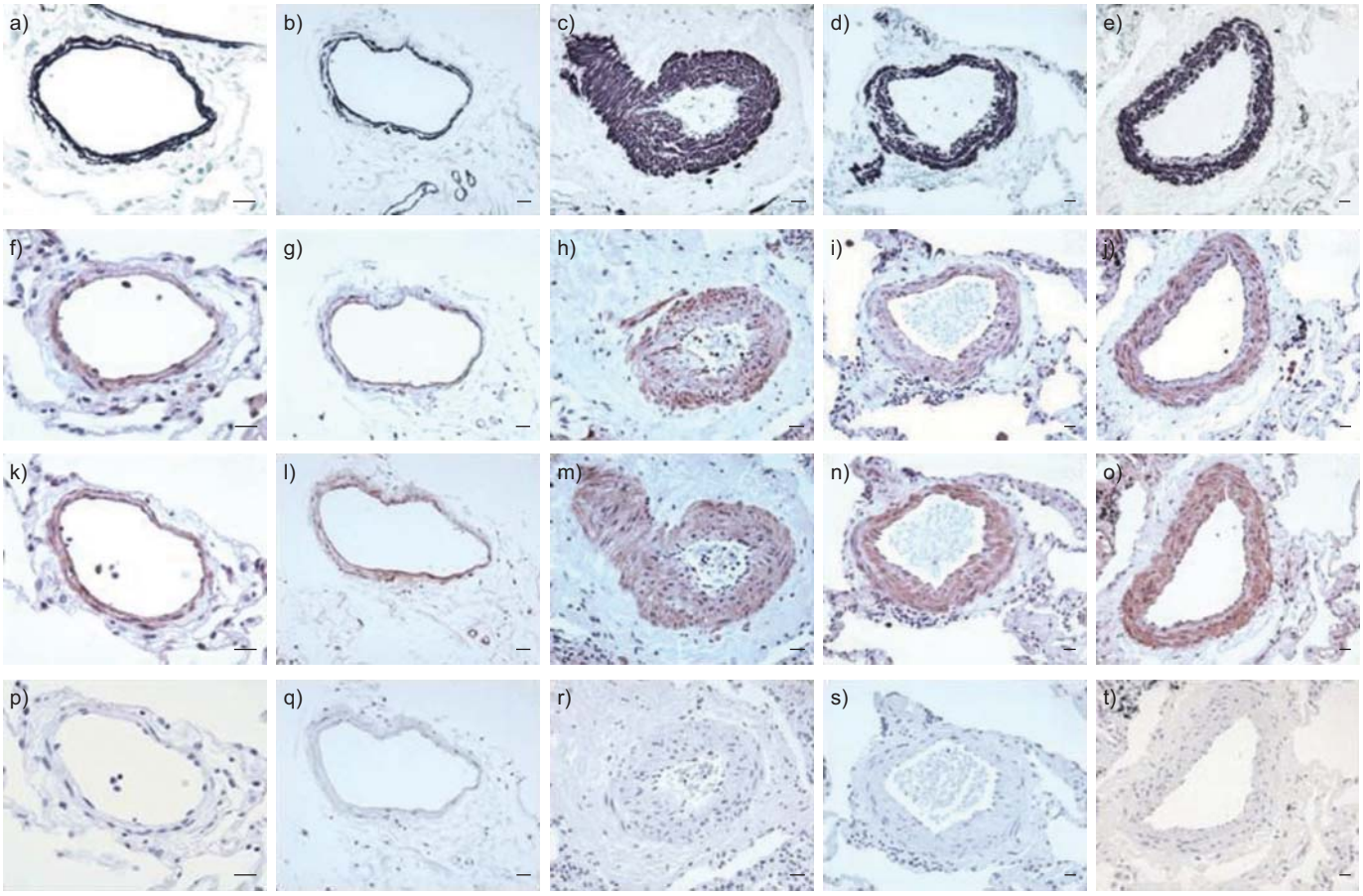

FIGURE 2. Vascular muscularisation and soluble guanalate cyclase (sGC) expression in lungs and pulmonary arterial smooth muscle cells from healthy donors (donor 1: a, f, k and p; donor 2: b, g, l and q) and idiopathic pulmonary arterial hypertension (IPAH) patients (patient 1: c, h, m and r; patient 2: d, i, n and s; patient 3: e, j, o and t). Muscularisation is demonstrated by α-smooth muscle actin staining for identifying vascular smooth muscle cells (a–e). Expression of sGCα1 (f–j) and sGCβ1 (k–o) is demonstrated by positive staining in the medial layer of small pulmonary arteries. Specificity of antibodies is demonstrated in negative control (absence of primary antibody) sections (p–t). Scale bars=20 μm.

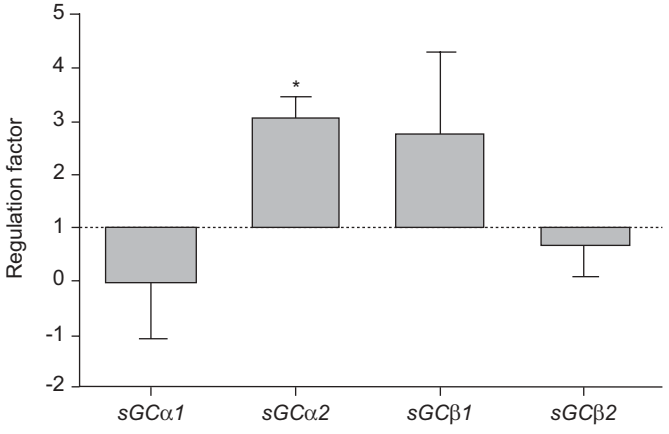

FIGURE 3. Pulmonary arterial smooth muscle cells isolated from healthy donors and idiopathic pulmonary arterial hypertension patients were analysed for the regulation of soluble guanalate cyclase (*sGC*)*α1*, *sGCα2*, *sGCβ1* and *sGCβ2* RNA by quantitative RT-PCR using the ΔΔCT method (see Methods section) for the calculation of the regulation factor. The CT values of individual genes were normalised to house keeping gene porphobilinogen deaminase. *: $p<0.05$ *versus* healthy donors.

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 (supplementary figure 2). Notably, when investigating lungs isolated from mice that were kept for 21 days under hypoxic conditions (10% O_2), slightly enhanced sensitivity to BAY 63-2521 was noted (supplementary figure 2).

Acute effects of sildenafil and BAY 63-2521 on U46619-induced PAH in intact animals

Continuous infusion of U46619 provoked an increase in P_{pa} to 23.4 ± 0.9 mmHg within 30 min, with a subsequent plateau of the PAH. This level of PAH was then maintained for $\geqslant$300 min with variations in P_{pa} of <2 mmHg. As shown in figure 5, subsequent oral application of 10 mg·kg^{-1} sildenafil resulted in a selective pulmonary vasodilatation, while BAY 63-2521 at oral doses of 1 and 10 mg·kg^{-1} decreased both P_{pa} and P_{sa}, dose dependently, to a similar extent. The combination of the

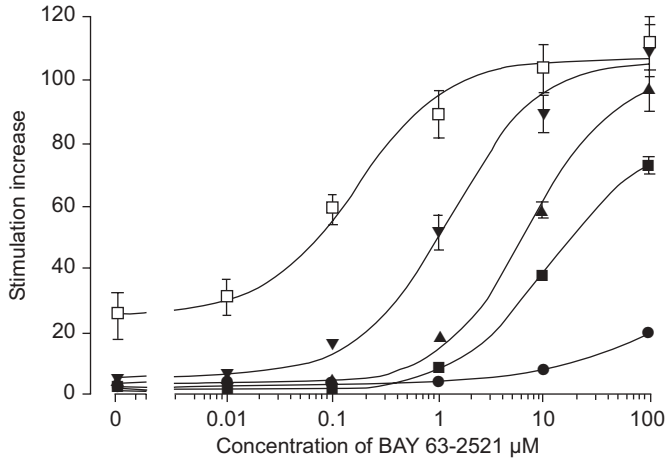

FIGURE 4. Pharmacological profile of BAY 63-2521. Stimulation of purified native soluble guanalate cyclase (sGC) by BAY 63-2521 (0.01—100 μM) in the absence (□) and presence of DEA/NO (▲: 0.001 μM; ▼: 0.01 μM; and ■: 0.1 μM) or ODQ (●). The specific activity of sGC is expressed as x-fold stimulation of specific basal activity (239±7 and 178±14 nmol·mg^{-1}·min^{-1} for the ODQ group). Data are presented as mean±SEM from four to eight independent experiments performed in duplicate.

effective sildenafil dose (10 mg·kg^{-1}) with the sub-maximal dose of BAY 63-2521 (1 mg·kg^{-1}) was operative in an additive manner and increased pulmonary selectivity of sildenafil, while the reduction in P_{sa} was still moderate (~10%).

Chronic effects of BAY 63-2521 on P_{rsv}, P_{sa} and cardiac frequency in mice with hypoxia-induced PAH

Upon chronic hypoxic exposure, P_{rvs} increased significantly from 23.0±0.4 to 29.8±1.9 and 34.8±1.9 mmHg (for control, 21 and 35 days hypoxia, respectively; fig. 6a). No significant changes in P_{sa} were observed (fig. 6b). In the chronic treatment group, BAY 63-2521 was orally applied at a dose of 10 mg·kg^{-1}·day^{-1} from day 21 to 35. BAY 63-2521 significantly decreased P_{rvs} to 29.0±0.6 mmHg ($p<0.05$ *versus* hypoxia at day 35). In order to further investigate haemodynamics in chronic hypoxic mice, a telemetric approach was performed. Continuous telemetric measurement of P_{rvs} in conscious mice under chronic hypoxic exposure revealed a continuous increase in the P_{rvs} curve, which 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^{-1}·day^{-1} from day 21 to 35 reduced hypoxia-induced PAH (fig. 7a). During the development of PAH, cardiac frequency increased from 380±30 to 496±14 bpm (fig. 7b). When comparing the pre- and post-treatment values, no significant decrease was noted by chronic BAY 63-2521 application.

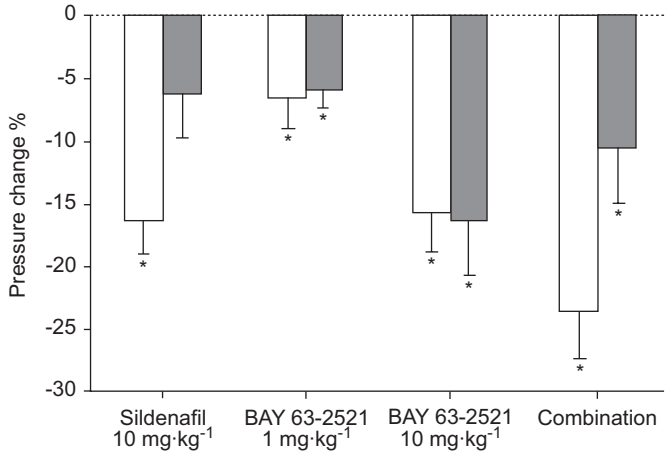

FIGURE 5. Effects of sildenafil, BAY 63-2521 and combination of sildenafil/BAY 63-2521 on U46619-induced pulmonary arterial pressure (□) and systemic arterial pressure (■). Immediate vasodilatory effects of phosphodiesterase 5 inhibitor sildenafil (10 mg·kg^{-1}), soluble guanylate cyclase stimulator BAY 63-2521 (1 mg·kg^{-1} and 10 mg·kg^{-1}) and combination of sildenafil and BAY 63-2521 (10 mg·kg^{-1} and 1 mg·kg^{-1}, respectively) are given. These were orally applied in intact rabbits that had developed pulmonary arterial hypertension in response to U46619. Data are presented as mean±SEM. *: $p<0.05$ *versus* control.

Chronic effects of BAY 63-2521 on right heart hypertrophy, gene expression and vascular remodelling in mice with hypoxia-induced PAH

Exposure of the mice to chronic hypoxia resulted in an increase in the ratio of right ventricle to left ventricle plus septum weight from 0.30±0.01 to 0.38±0.01 and 0.41±0.01 for controls, 21 and 35 days hypoxia, respectively (both hypoxic conditions $p<0.05$ *versus* controls; fig. 8a). The ratio decreased to 0.37±0.01 in the BAY 63-2521 group ($p<0.05$ *versus* hypoxia at day 35). In addition, the expression of the *TGF-β* gene and *ANF* gene was significantly elevated in the right ventricle of chronic hypoxic mice (fig. 8b and 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 muscularisation of small pulmonary arteries at a size between 20 and 70 μm, mice chronically exposed to hypoxia showed a reduction in non-muscular vessels (given as percentage of total vessel count) from 48.9±7.8% at baseline to 3.4±0.9 and 1.0±0.5% at 21 and 35 days, respectively (fig. 8d). 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 muscularised vessels in chronic hypoxic mice.

Expression of sGC increased in 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 with control rat lung homogenate (fig. 9a). Interestingly, the upregulation of *sGCβ1* was confined to PASMCs that were freshly isolated from distal pulmonary arteries of MCT-induced PAH rats (fig. 9b). In corroboration, immunohistochemistry demonstrated an extensive expression of sGCβ1 in the medial wall of pulmonary arteries from MCT-induced PAH rats, as shown by co-localisation with α-smooth muscle actin in sequential sections (fig. 9c).

Chronic effects of BAY 63-2521 on P_{rvs}, pulmonary resistance and P_{sa} in rats with MCT-induced PAH

MCT injection in rats induced severe PAH with a marked increase in P_{rvs} from 25.8±1.9 to 51.7±5.4 mmHg at 21 days,

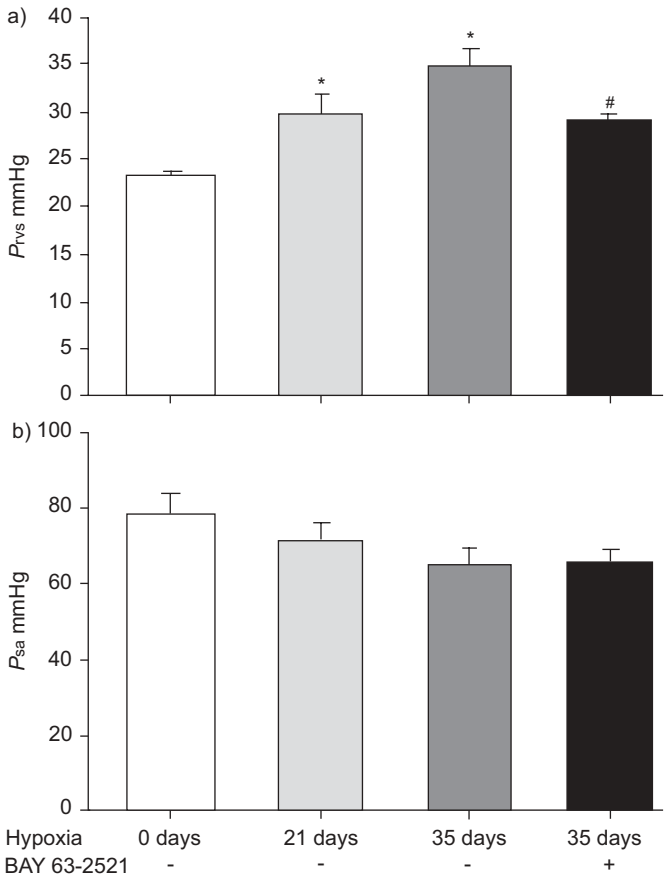

FIGURE 6. Impact of chronic BAY 63-2521 treatment on haemodynamics in hypoxia-induced pulmonary arterial hypertension. Mice were exposed to hypoxic conditions for 21 or 35 days, or remained in normoxia throughout. The soluble guanylate cyclase stimulator BAY 63-2521 or vehicle (2% methylcellulose solution) were orally applied from day 21 to day 35 in hypoxic mice (n=10) at a dose of 10 mg·kg^{-1}. a) Right ventricular systolic pressure (P_{rvs}) and b) systemic arterial pressure (P_{sa}) are given. *: p<0.05 *versus* control; #: p<0.05 *versus* hypoxia at day 35.

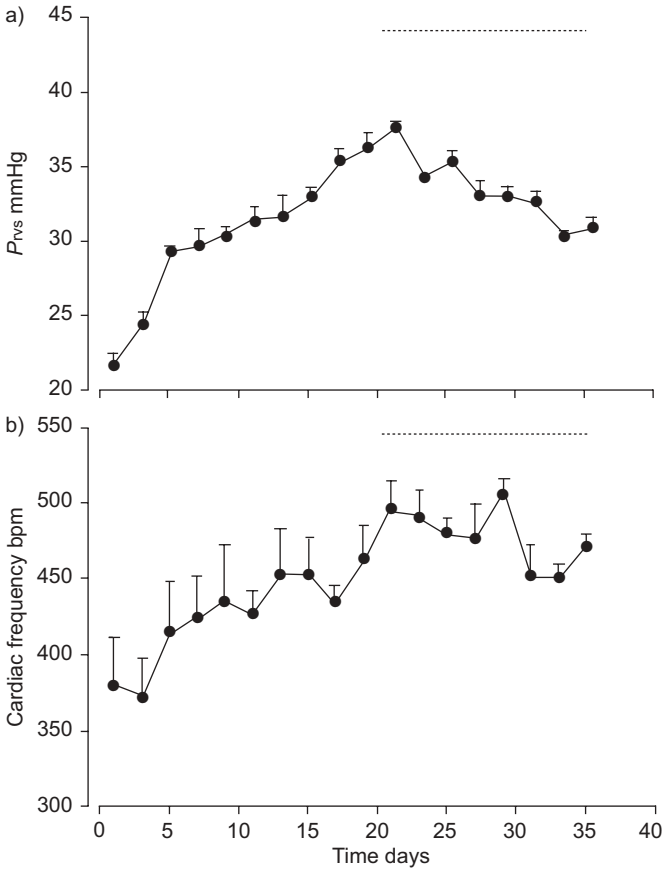

FIGURE 7. Telemetric data of a) right ventricular systolic pressure (P_{rvs}) and b) cardiac frequency in chronic hypoxic mice treated with BAY 63-2521. Mice were exposed to hypoxic conditions (10% O_2) for 35 days and treated orally with BAY 63-2521 from day 21 to day 35 (·····). Effect of BAY 63-2521 on the course of P_{rvs} and cardiac frequency in hypoxia-induced pulmonary arterial hypertension was assessed by telemetry in conscious mice (n=5).

and which increased further to 84.1±0.6 mmHg at day 35 (fig. 10a). Consequently, these animals developed significant increase in total pulmonary vascular resistance from 0.91±0.05 to 3.60±0.04 and 6.13±0.60 mmHg min·mL^{-1} 100 g bodyweight for control, 21 and 35 days, respectively (fig. 10b). No significant changes in systemic arterial pressure were noted (fig. 10c). Daily treatment of MCT-injected rats with BAY 63-2521 at a dose of 10 mg·kg^{-1} from day 21 to 35, significantly decreased P_{rvs} 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^{-1}·mL^{-1} 100 g bodyweight (p<0.05 *versus* MCT at day 35). P_{sa} did not change in response to the treatment.

Chronic effects of BAY 63-2521 on right heart hypertrophy and degree of muscularisation of pulmonary arteries in rats with MCT-induced PAH

The MCT-injected rats developed significant right heart hypertrophy which had increased from 0.28±0.01 to 0.40±0.03 and 0.60±0.03 at 21 and 35 days, respectively (fig. 11a). Chronic treatment of the rats by BAY 63-2521 decreased the ratio of right ventricle to left ventricle plus septum weight ratio to 0.42±0.04 (p<0.05 *versus* MCT at day 35). The degree of muscularisation of pulmonary arteries with a diameter of 25–50 μm was quantitatively assessed. In controls, the majority of vessels of this diameter are usually non-muscularised. In the MCT group, both at day 21 and day 35, a dramatic decrease in non-muscularised pulmonary arteries occurred (fig. 11b) with a concomitant increase in fully muscularised pulmonary arteries. Treatment with BAY 63-2521 at 10 mg·kg^{-1} per day resulted in a significant reduction of fully muscularised arteries compared with both MCT groups, and increased the percentage of non-muscularised pulmonary arteries.

DISCUSSION

The novel finding of the present study was that the receptor for NO, sGC, is highly expressed in vascular smooth muscle cells of small pulmonary arteries from patients with IPAH. Furthermore, the *in vitro* efficacy of the novel sGC stimulator BAY 63-2521 was demonstrated, 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-dependent mechanism. It was demonstrated, *in vivo*, that BAY 63-2521 is an effective treatment in two well-established experimental models of severe PAH. Treatment effects showed

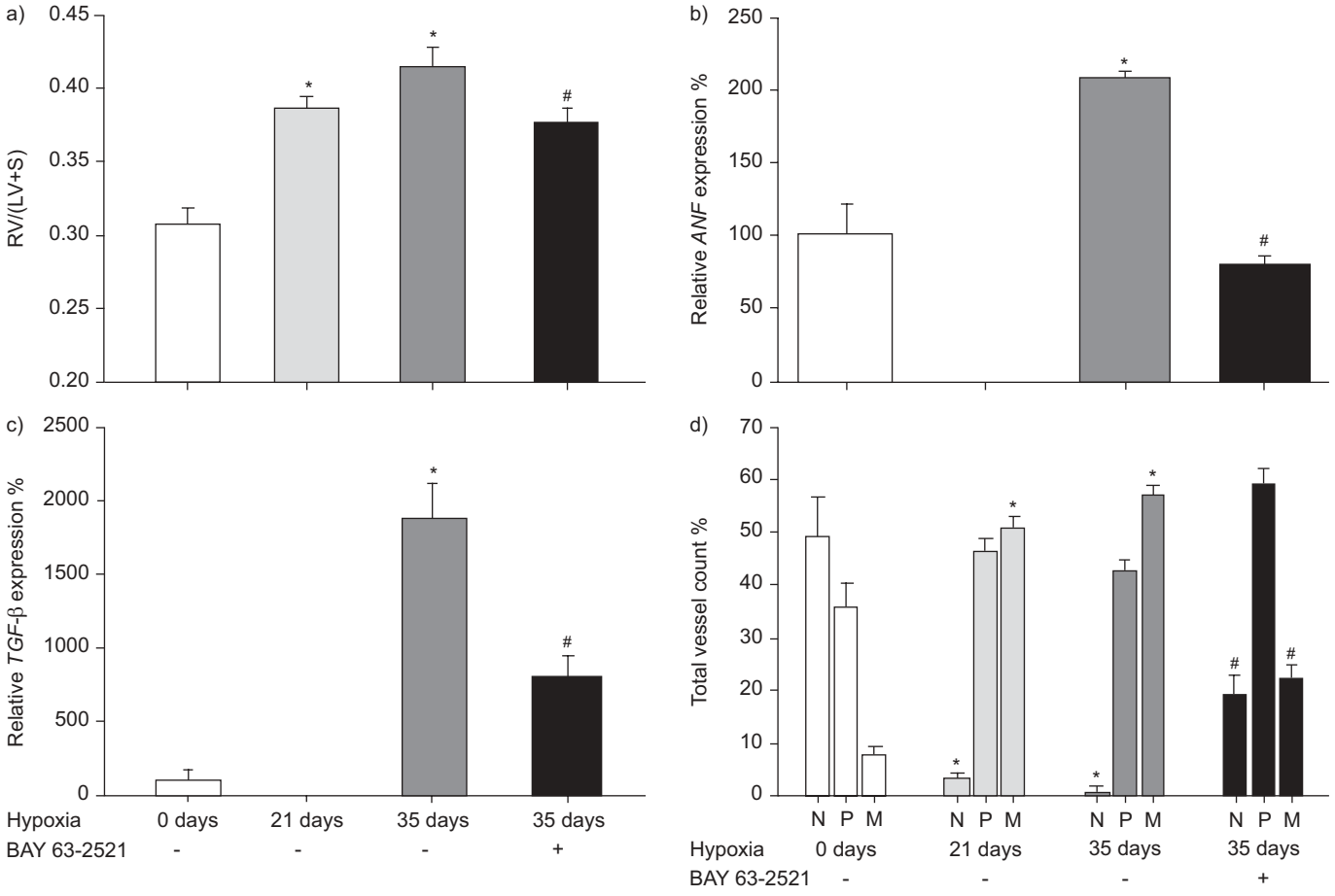

FIGURE 8. Effect of BAY 63-2521 on right heart hypertrophy, atrial natriuretic factor gene (*ANF*) and transforming growth factor beta gene (*TGF-β*) expression in heart and degree of muscularisation of small pulmonary arteries. Mice were exposed to hypoxic conditions for 21 or 35 days, or remained normoxic throughout. The soluble guanylate cyclise 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^{-1}. a) The ratio of right ventricle to left ventricle plus septum weight (RV/(LV+S)) is given. Expression of b) *ANF* and c) *TGF-β* as a percentage of normoxic mice in right ventricular tissue. d) Proportions of non- (N), partially- (P) or fully- (M) muscularised pulmonary arteries as percentage of total vessel count (vessels sized 20–70 μm), are given for hypoxic mice. A total of 80–100 intra-acinar vessels were analysed in each lung. *: $p<0.05$ *versus* control; #: $p<0.05$ *versus* hypoxia 35 days.

a partial reversal of PAH, a reduction in right heart hypertrophy and reversal of pulmonary vessel remodelling.

The NO-signalling pathway is a major target for the treatment of PAH. The inhalation of NO has been shown to reduce P_{pa} and to improve gas exchange in patients with acute and chronic PAH. However, this approach was limited by the short half-life of NO and the rebound phenomenon after withdrawal. Another possibility to manipulate this signalling pathway is the enhancement of endogenous NO signalling by inhibition of PDE5, the enzyme that hydrolyses the secondary messenger cGMP. Recent studies have shown that the cGMP-specific PDE5 is highly expressed in lung tissue [23, 24]. Moreover, further upregulation of PDE5 may occur under conditions of PAH, 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 haemodynamics, gas exchange and exercise capacity [28–32]. Based on a positive clinical phase-III study, sildenafil has been approved for the treatment of PAH [33, 34]. However, there is increasing evidence that modification of sGC under pathophysiological conditions, either by tyrosine phosphorylation at the β1 subunit [35] or haem oxidation [36, 37], results in an altered NO response followed by reduced local cGMP production. This may explain, in part, limitations of current therapies addressing the NO pathway in cardiopulmonary diseases. The pharmacological activation of sGC is therefore attractive to restore NO signalling. However, several cardiovascular disease states are accompanied by downregulation of the activity and expression of the haem-containing sGC [38–40]. The present authors were, therefore, interested in the role of sGC in PAH and performed studies on explanted tissue of patients with 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 co-localises with the smooth muscle marker, α-smooth muscle actin. In

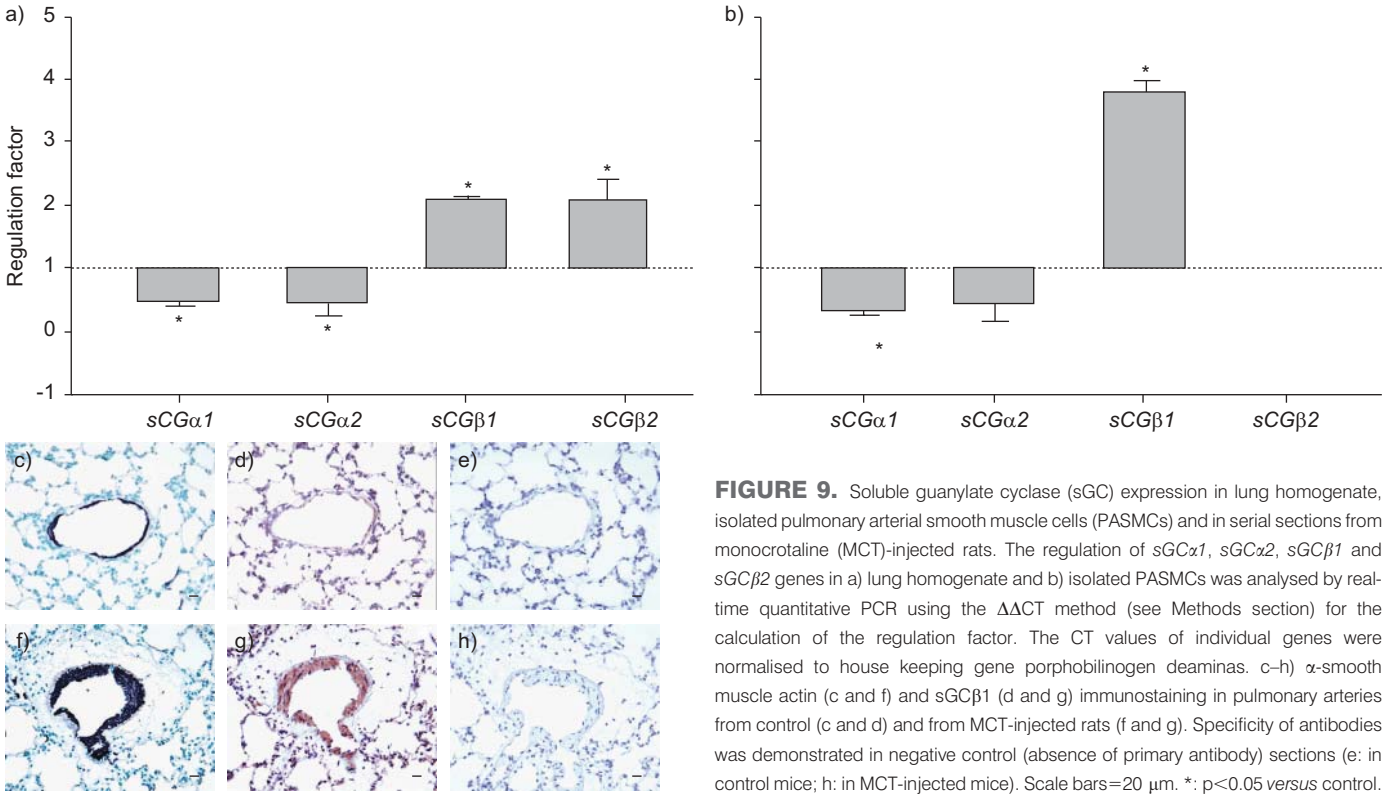

FIGURE 9. Soluble guanylate cyclase (sGC) expression in lung homogenate, isolated pulmonary arterial smooth muscle cells (PASMCs) and in serial sections from monocrotaline (MCT)-injected rats. The regulation of *sGCα1*, *sGCα2*, *sGCβ1* and *sGCβ2* genes in a) lung homogenate and b) isolated PASMCs was analysed by real-time quantitative PCR using the ΔΔCT method (see Methods section) for the calculation of the regulation factor. The CT values of individual genes were normalised to house keeping gene porphobilinogen deaminas. c–h) α-smooth muscle actin (c and f) and sGCβ1 (d and g) immunostaining in pulmonary arteries from control (c and d) and from MCT-injected rats (f and g). Specificity of antibodies was demonstrated in negative control (absence of primary antibody) sections (e: in control mice; h: in MCT-injected mice). Scale bars=20 μm. *: $p<0.05$ *versus* control.

contrast, as shown in figure 9, quantitative mRNA assessment of pulmonary hypertensive lung tissue resulted in possible conflicting data with opposite changes in *sGCα1* levels in lung homogenates and isolated PASMCs. The only consistent finding with regard to the human tissues appeared to be the increase in sGCβ1 expression. In line with these results, an upregulation *sGCβ1* expression was reported in mice chronically exposed to hypoxic conditions. In contrast, no significant changes were reported in hypoxic rat lungs, suggesting species-specific differences, with regard to sGCβ1 expression in the hypoxia-induced PAH [9, 10].

There may also 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 and sodium nitroprusside [41]. In *in vitro* studies on purified sGC in haem-containing and haem-free preparations were investigated. The present study describes, for the first time, the NO-independent, but haem-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 PAH, the chronic hypoxic mouse model and MCT-induced PAH in rats. Pathological characteristics of both models include elevated P_{pa}, right ventricular hypertrophy and anatomical structural changes of lung vasculature including *de novo* muscularisation of normally non-muscularised small pulmonary arteries. In order to mimic the clinical situation, the protective effects of BAY 63-2521 on the development of PAH were not investigated, but treatment was initiated 3 weeks after chronic hypoxia exposure/MCT injection when elevated P_{pa}, vascular remodelling and right heart hypertrophy were fully established. In both animal models, partial reversal of the haemodynamic and structural changes was noted. Most impressively, continuous telemetric measurement of P_{rvs} right ventricular pressure in mice demonstrated anti-remodelling of BAY 63-2521. This technique has recently been presented in rats [18, 44] but the present study is the first which employs on-line measurement of right heart pressure in mice. Interestingly, an increase in P_{rvs} shoulder around day 5, which was followed by a progressive increase in pressure thereafter was observed in chronic hypoxic mice (fig. 7). As compared with the present data, which were determined in mice, SEBKHI *et al.* [44] recently demonstrated increased pulmonary pressures (measured by telemetry) in chronic hypoxic rats, although the differences between the pressure curves in rats and mice are comparable.

In addition to the reduction of pressure, histomorphometric analysis revealed strong anti-remodelling potency of this compound in both animal models. The former sGC stimulator BAY 41-2272 has demonstrated similar efficacy in experimental PAH 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 P_{pa} in an additive manner and increased pulmonary selectivity of sildenafil. Although, the long-term effects of this combination therapy on haemodynamics and vascular remodelling need to be demonstrated in the future; the present study suggests the beneficial

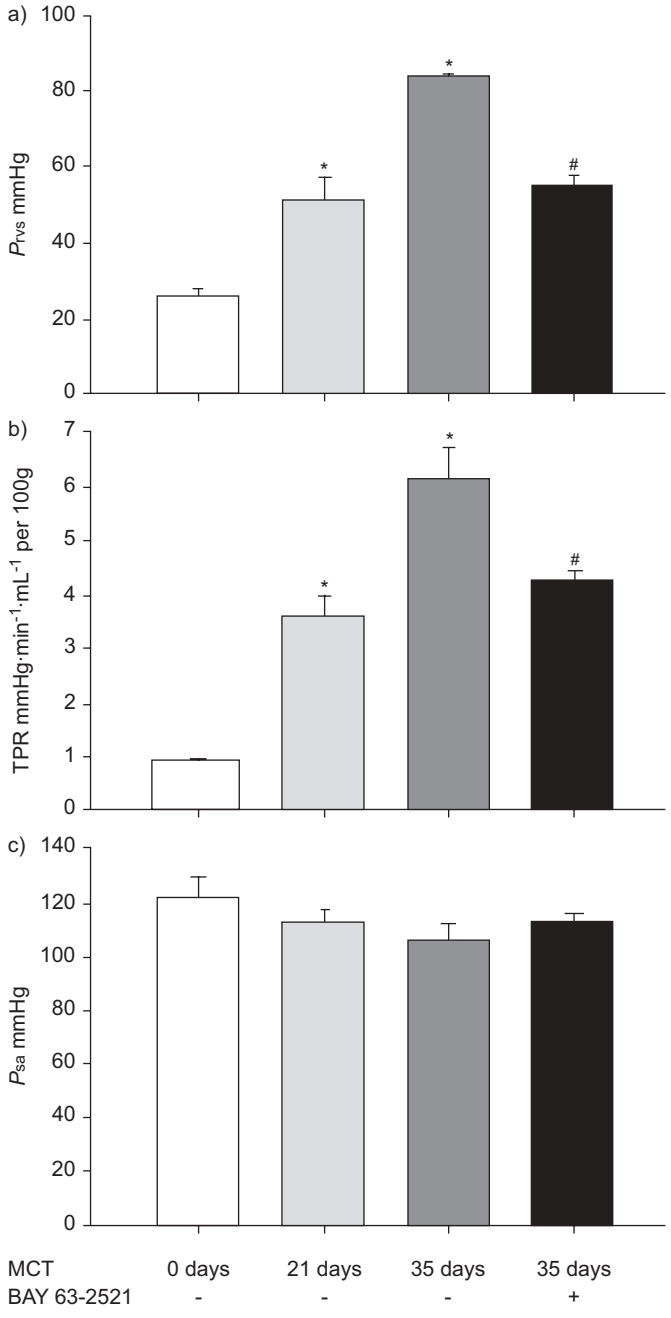

FIGURE 10. Effects of BAY 63-2521 on haemodynamics in monocrotaline (MCT)-induced pulmonary arterial hypertension. Rats were subcutaneously injected with MCT or saline and investigated on day 21 or 35. The soluble guanylate cyclase stimulator BAY 63-2521 was orally applied from day 21 to day 35 in MCT rats (n=8) at a dose of 10 mg·kg^{-1}. a) Right ventricular systolic pressure (P_{rvs}), b) total pulmonary resistance and c) systemic arterial pressure (P_{sa}). *: $p<0.05$ *versus* control; #: $p<0.05$ *versus* MCT at day 35.

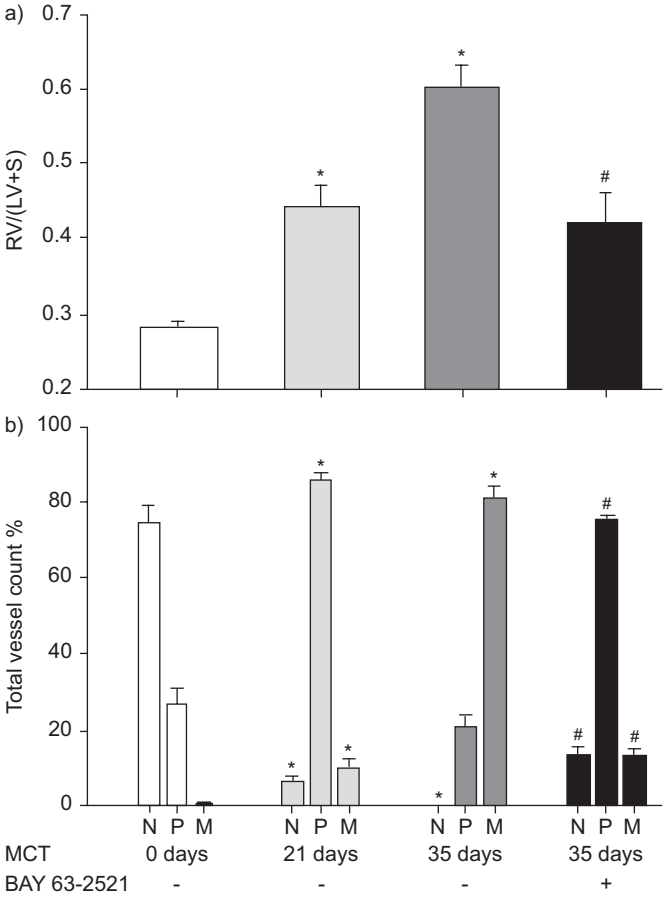

FIGURE 11. Effect of BAY 63-2521 on right heart hypertrophy and degree of muscularisation of small pulmonary arteries in monocrotaline (MCT)-induced pulmonary arterial hypertension. Rats were subcutaneously injected with MCT or saline and investigated on day 21 or 35. The soluble guanylate cyclase stimulator BAY 63-2521 was orally applied from day 21 to day 35 in MCT rats (n=8) at a dose of 10 mg·kg^{-1}. a) Right ventricular hypertrophy was assessed by the right to left ventricle and septum (RV/(LV+S)) ratio. b) Degree of muscularisation is given as percentage of non- (N), partially- (P) or fully- (M) muscularised pulmonary arteries from total vessel count (vessels sized 20–70 μm) in MCT-injected rats. A total of 80–100 intra-acinar vessels were analysed in each lung. *: $p<0.05$ *versus* control; #: $p<0.05$ *versus* MCT at day 35.

clinical effects of BAY 63-2521 alone or in combination with approved targeted therapies (*i.e.* sildenafil) of PAH.

The present study was the first to describe the expression of sGC in IPAH and the successful therapeutic use of the sGC stimulator BAY 63-2521 in two well-accepted animal models of PAH. The present study demonstrated that: 1) sGCα1 and β1 proteins 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 synergises with NO; and 3) treatment with BAY 63-2521 reversed haemodynamic and structural changes provoked by hypoxia in mice and MCT in rats.

Limitation of the study

Neither hypoxia- nor monocrotaline-induced pulmonary arterial hypertension in rodents fully mimics human pulmonary arterial hypertension associated with chronic lung disease or idiopathic pulmonary arterial hypertension. Therefore, the effectiveness of BAY 63-2521 in experimental models of pulmonary arterial hypertension does not predict beneficial effects in patients with pulmonary arterial hypertension. However, the anti-remodelling potential of BAY 63-2521 in

two independent models of pulmonary arterial hypertension (hypoxia-induced pulmonary arterial hypertension in mice and monocrotaline-induced pulmonary arterial hypertension in rats) and the demonstration of the strong soluble guanylate cyclase expression in human explanted pulmonary arterial hypertension 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.

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REFERENCES

1 Humbert M, Morrell NW, Archer SL, *et al.* Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004; 43: Suppl. 12, 13S–24S.
2 Christman BW, McPherson CD, Newman JH, *et al.* An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med* 1992; 327: 70–75.
3 Tuder RM, Cool CD, Geraci MW, *et al.* Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med* 1999; 159: 1925–1932.
4 Ghofrani HA, Pepke-Zaba J, Barbera JA, *et al.* Nitric oxide pathway and phosphodiesterase inhibitors in pulmonary arterial hypertension. *J Am Coll Cardiol* 2004; 43: Suppl. 12, 68S–72S.
5 Grimminger F, Spriestersbach R, Weissmann N, Walmrath D, Seeger W. Nitric oxide generation and hypoxic vasoconstriction in buffer-perfused rabbit lungs. *J Appl Physiol* 1995; 78: 1509–1515.
6 Ide H, Nakano H, Ogasa T, *et al.* Regulation of pulmonary circulation by alveolar oxygen tension *via* airway nitric oxide. *J Appl Physiol* 1999; 87: 1629–1636.
7 Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1995; 333: 214–221.
8 Berger RM, Geiger R, Hess J, Bogers AJ, Mooi WJ. Altered arterial expression patterns of inducible and endothelial nitric oxide synthase in pulmonary plexogenic arteriopathy caused by congenital heart disease. *Am J Respir Crit Care Med* 2001; 163: 1493–1499.
9 Li D, Zhou N, Johns RA. Soluble guanylate cyclase gene expression and localization in rat lung after exposure to hypoxia. *Am J Physiol* 1999; 277: L841–L847.
10 Li D, Laubach VE, Johns RA. Upregulation of lung soluble guanylate cyclase during chronic hypoxia is prevented by deletion of eNOS. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L369–L376.
11 Evgenov OV, Ichinose F, Evgenov NV, *et al.* Soluble guanylate cyclase activator reverses acute pulmonary hypertension and augments the pulmonary vasodilator response to inhaled nitric oxide in awake lambs. *Circulation* 2004; 110: 2253–2259.
12 Dumitrascu R, Weissmann N, Ghofrani HA, *et al.* Activation of soluble guanylate cyclase reverses experimental pulmonary hypertension and vascular remodeling. *Circulation* 2006; 113: 286–295.
13 Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 2006; 5: 755–768.
14 Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF, Muller D. Progenitor cells of the testosterone-producing Leydig cells revealed. *J Cell Biol* 2004; 167: 935–944.
15 Davidoff MS, Schulze W, Middendorff R, Holstein AF. The Leydig cell of the human testis--a new member of the diffuse neuroendocrine system. *Cell Tissue Res* 1993; 271: 429–439.
16 Alonso-Alija C, Bischoff E, Münter K, *et al.* Carbamate substituted pyrazolopyridines. Patent WO-03/095451-A1 2003.11.20. March 20, 2003.
17 Wunder F, Tersteegen A, Rebmann A, Erb C, Fahrig T, Hendrix M. Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. *Mol Pharmacol* 2005; 68: 1775–1781.
18 Schermuly RT, Dony E, Ghofrani HA, *et al.* Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005; 115: 2811–2821.
19 Weissmann N, Dietrich A, Fuchs B, *et al.* Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci USA* 2006; 103: 19093–19098.
20 Schermuly RT, Kreisselmeier KP, Ghofrani HA, *et al.* Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension. *Circ Res* 2004; 94: 1101–1108.
21 Wenzel S, Schorr K, Degenhardt H, *et al.* TGF-β(1) downregulates PTHrP in coronary endothelial cells. *J Mol Cell Cardiol* 2001; 33: 1181–1190.
22 van Eickels M, Schreckenberg R, Doevendans PA, Meyer R, Grohe C, Schluter KD. The influence of oestrogen-deficiency and ACE inhibition on the progression of myocardial hypertrophy in spontaneously hypertensive rats. *Eur J Heart Fail* 2005; 7: 1079–1084.
23 Giordano D, De Stefano ME, Citro G, Modica A, Giorgi M. Expression of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in mouse tissues and cell lines using an antibody against the enzyme amino-terminal domain. *Biochim Biophys Acta* 2001; 1539: 16–27.
24 Hanson KA, Burns F, Rybalkin SD, Miller JW, Beavo J, Clarke WR. Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. *Am J Respir Crit Care Med* 1998; 158: 279–288.
25 MacLean MR, Johnston ED, Mcculloch KM, Pooley L, Houslay MD, Sweeney G. Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J Pharmacol Exp Ther* 1997; 283: 619–624.
26 Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR. sGC and PDE5 are elevated in

lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L1051–L1057.

27 Wharton J, Strange JW, Moller GM, *et al.* Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med* 2005; 172: 105–113.

28 Ghofrani HA, Voswinckel R, Reichenberger F, *et al.* Differences in hemodynamic and oxygenation responses to three different phosphodiesterase-5 inhibitors in patients with pulmonary arterial hypertension: a randomized prospective study. *J Am Coll Cardiol* 2004; 44: 1488–1496.

29 Ghofrani HA, Reichenberger F, Kohstall MG, *et al.* Sildenafil increased exercise capacity during hypoxia at low altitudes and at Mount Everest base camp: a randomized, double-blind, placebo-controlled crossover trial. *Ann Intern Med* 2004; 141: 169–177.

30 Ghofrani HA, Schermuly RT, Rose F, *et al.* Sildenafil for long-term treatment of nonoperable chronic thromboembolic pulmonary hypertension. *Am J Respir Crit Care Med* 2003; 167: 1139–1141.

31 Ghofrani HA, Wiedemann R, Rose F, *et al.* Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial. *Lancet* 2002; 360: 895–900.

32 Ghofrani HA, Wiedemann R, Rose F, *et al.* Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. *Ann Intern Med* 2002; 136: 515–522.

33 Ghofrani HA, Osterloh IH, Grimminger F. Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nat Rev Drug Discov* 2006; 5: 689–702.

34 Galie N, Ghofrani HA, Torbicki A, *et al.* Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 2005; 353: 2148–2157.

35 Meurer S, Pioch S, Gross S, Muller-Esterl W. Reactive oxygen species induce tyrosine phosphorylation of and Src kinase recruitment to NO-sensitive guanylyl cyclase. *J Biol Chem* 2005; 280: 33149–33156.

36 Stasch JP, Schmidt PM, Nedvetsky PI, *et al.* Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 2006; 116: 2552–2561.

37 Kalk P, Godes M, Relle K, *et al.* NO-independent activation of soluble guanylate cyclase prevents disease progression in rats with 5/6 nephrectomy. *Br J Pharmacol* 2006; 148: 853–859.

38 Ruetten H, Zabel U, Linz W, Schmidt HH. Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats. *Circ Res* 1999; 85: 534–541.

39 Kloss S, Bouloumie A, Mulsch A. Aging and chronic hypertension decrease expression of rat aortic soluble guanylyl cyclase. *Hypertension* 2000; 35: 43–47.

40 Bauersachs J, Bouloumie A, Mulsch A, Wiemer G, Fleming I, Busse R. Vasodilator dysfunction in aged spontaneously hypertensive rats: changes in NO synthase III and soluble guanylyl cyclase expression, and in superoxide anion production. *Cardiovasc Res* 1998; 37: 772–779.

41 Crawley DE, Zhao L, Giembycz MA, *et al.* Chronic hypoxia impairs soluble guanylyl cyclase-mediated pulmonary arterial relaxation in the rat. *Am J Physiol* 1992; 263: L325–L332.

42 Stasch JP, Schmidt P, Alonso-Alija C, *et al.* NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. *Br J Pharmacol* 2002; 136: 773–783.

43 Stasch JP, Becker EM, Alonso-Alija C, *et al.* NO-independent regulatory site on soluble guanylate cyclase. *Nature* 2001; 410: 212–215.

44 Sebkhi A, Strange JW, Phillips SC, Wharton J, Wilkins MR. Phosphodiesterase type 5 as a target for the treatment of hypoxia-induced pulmonary hypertension. *Circulation* 2003; 107: 3230–3235.

45 Deruelle P, Balasubramaniam V, Kunig AM, Seedorf GJ, Markham NE, Abman SH. BAY 41-2272, a direct activator of soluble guanylate cyclase, reduces right ventricular hypertrophy and prevents pulmonary vascular remodeling during chronic hypoxia in neonatal rats. *Biol Neonate* 2006; 90: 135–144.

46 Deruelle P, Grover TR, Abman SH. Pulmonary vascular effects of nitric oxide-cGMP augmentation in a model of chronic pulmonary hypertension in fetal and neonatal sheep. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L798–L806.