Abstract

Introduction

Current treatment with vasodilators for pulmonary hypertension associated with respiratory diseases is limited by their inhibitory effect on hypoxic pulmonary vasoconstriction (HPV) and uncoupling effects on ventilation-perfusion (V'/Q'). Hypoxia is also a well-known modulator of the nitric oxide (NO) pathway, and may therefore differentially affect the responses to phosphodiesterase 5 (PDE5) inhibitors and soluble guanylyl cyclase (sGC) stimulators. So far, the effects of the sGC stimulator riociguat on HPV have been poorly characterized.

Materials and methods

Contraction was recorded in pulmonary arteries (PA) in a wire myograph. Anesthetized rats were catheterized to record PA pressure. Ventilation and perfusion were analyzed by micro-CT-SPECT images in rats with pulmonary fibrosis induced by bleomycin.

Results

The PDE5 inhibitor sildenafil and the sGC stimulator riociguat similarly inhibited HPV in vitro and in vivo. Riociguat was more effective as vasodilator in isolated rat and human PA than sildenafil. Riociguat was ∼3-fold more potent under hypoxic conditions and it markedly inhibited HPV in vivo at a dose that barely affected the thromboxane A₂ (TXA₂) mimetic U46619-induced pressor responses. Pulmonary fibrosis was associated with V'/Q' uncoupling and riociguat did not affect the V'/Q' ratio.
Conclusion

PDE5 inhibitors and sGC stimulators show a different vasodilator profile. Riociguat was highly effective and potentiated by hypoxia in rat and human PA. In vivo, riociguat preferentially inhibited hypoxic than non-hypoxic vasoconstriction. However, it did not worsen V'/Q’ coupling in a rat model of pulmonary fibrosis.

Introduction

Pulmonary hypertension (PH) is a life-threatening progressive disorder of various aetiologies, exhibiting a complex pathophysiology characterized by vasoconstriction, vascular remodelling and thrombosis that lead to reduced pulmonary arterial lumen and elevated pulmonary arterial pressure (PAP) [1–3]. The World Health Organization (WHO) classifies PH into five groups. Currently therapies approved for pulmonary arterial hypertension (PAH; i.e group 1 of PH) are directed mainly to reduce the vasoconstrictor component [1]. Recently, the soluble guanylyl cyclase (sGC) stimulator riociguat has been approved for both PAH and chronic thromboembolic pulmonary hypertension (CTEPH, i.e group 4 of PH) [4]. Unfortunately, there are no approved specific therapies for the most common forms of PH: PH due to left heart disease (group 2) and associated to lung diseases and hypoxia (group 3, e.g. associated to chronic obstructive pulmonary disease [COPD] or idiopathic pulmonary fibrosis [IPF]). PH is a frequent complication of COPD [5] and IPF [6], present in more than half of the patients with severe disease. Despite the only moderate increase in PAP in these patients, the presence of PH is associated to worse prognosis [6–8].

HPV is a highly conserved adaptive physiological mechanism that increases pulmonary vascular resistance in poorly aerated lung regions, thereby reducing blood flow to the hypoxic alveoli, redirecting it to the best ventilated ones [9]. The sensor, transduction, and effector mechanisms involved in HPV reside essentially in the pulmonary arterial smooth muscle cells [9, 10]. A limitation of currently available vasodilators systemically administered is the uncoupling effects on ventilation-perfusion (V'/Q’) due to the inhibition of hypoxic pulmonary vasoconstriction (HPV) as described below [11].

Mild, moderate or severe hypoxemia is a common feature of PH associated to COPD, IPF or sleep apnea. In this scenario, HPV represents a crucial protective mechanism that redistributes blood flow away from diseased (hypoxic) lung tissue to the best oxygenated alveoli, reducing shunt flow to optimize oxygen saturation [9] at the expense of an elevated PAP. Systemic administration of vasodilators in PH associated with lung diseases might reduce PAP and improve the outcomes of these patients in terms of reducing right ventricular load and increasing exercise tolerance [8]. However, these drugs, by inhibiting HPV, may also increase blood flow to poorly-ventilated or non-ventilated areas of the lung, worsening preexisting V’/Q’ mismatch and hypoxemia. Therefore, the use of vasodilator therapy in COPD and IPF patients with PH is not currently recommended [1]. However, due to the poor prognosis of patients presenting PH associated to COPD/IPF and the lack of alternative treatments, some researchers have suggested the cautious use of vasodilators [12–17]. The phosphodiesterase 5 (PDE5) inhibitor sildenafil has demonstrated a significant reduction in pulmonary vascular resistance in patients with lung fibrosis [15] or COPD [14]. Larger studies in IPF patients have also shown a significant positive effect on V’/Q’ mismatch and arterial oxygenation while the effects on exercise capacity are inconsistent [16, 17]. In contrast, sildenafil worsened V’/Q’ coupling and impaired arterial oxygenation in patients with COPD [14]. In an open-label, uncontrolled pilot trial in patients with interstitial lung disease and PH, riociguat improved pulmonary
vascular resistance, cardiac output and exercise capacity [12]. However, a subsequent randomized controlled trial in patients with PH associated with idiopathic interstitial pneumonias was prematurely terminated due to an apparent increase in death and serious adverse events (NCT02138825, Clinicaltrials.org). In addition, a small pilot study showed that acute riociguat reduced pulmonary pressure and vascular resistance and had no significant effect on arterial oxygenation [13]. Therefore, the treatment of group 3 PH with vasodilators would require further basic and clinical research.

Hypoxia is also a well-known modulator of the nitric oxide (NO)-cyclic GMP (cGMP) pathway. Because sGC stimulators and PDE5 inhibitors activate the NO-cGMP pathway in a different manner, we hypothesized that the two drugs may behave differently depending on the oxygen concentration. Thus, while PDE5 inhibitors prolong the action of cGMP, potentiating the effect of endogenous NO, stimulators of sGC such as riociguat may increase cGMP synthesis independently of NO. The effects of stimulators of sGC such as riociguat on HPV have hardly been investigated. In the present study, we analysed the effects of the sGC stimulator riociguat and the PDE5 inhibitor sildenafil on HPV in human and rat arteries in vitro and in vivo.

Methods
Forty one pathogen-free male Wistar rats (12 weeks of age) were obtained from Harlan Iberica (Barcelona, Spain). All rats were kept with free access to food and water throughout the whole experiment period and on standard rat chow and maintained at 24°C under a 12h light/12 h dark cycle. No animal died as a result of the experiments. Animals were sacrificed by an overdose of the anesthetic. All experimental procedures utilizing animals were carried out according to the Spanish Royal Decree 1201/2005 and 53/2013 on the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the University of Valencia (Valencia, Spain). After written informed consent, human neoplasia-free samples of lung tissue discarded by the pathologist were obtained from 14 adult patients of both sexes (12 men and 2 women, 66 ± 3 years) undergoing lung carcinoma surgery. The procedures were approved by the Human Research Ethics Committee of the Hospital Universitario de Getafe and the regional Committee for Laboratory Animals Welfare.

Vascular reactivity in vitro
Rat or human pulmonary artery (PA) and rat mesenteric artery (MA) rings (0.4–0.8 mm of internal diameter) were mounted in Krebs solution in a myograph [18, 19]. Arteries were exposed to KCl (80 mM), then 5-HT (10 μM) and Ach (1 μM) was added to test endothelial function. In some experiments arteries were mounted in 21% O₂ and then exposed to hypoxia 0% O₂ (2–3% final concentration of O₂ in the Krebs solution) to elicit HPV and after returning to 21% O₂, the TXA₂ mimetic U46619 was added to the bath. Some arteries, incubated either in 0, 21 or 95% O₂, were exposed a cocktail of three vasoconstrictors at submaximally effective concentrations (~50% of their individual maximal response), i.e. U46619 at 30 nM, ET-1 at 3 nM and 5-HT at 3 μM and then a concentration response curve was constructed by cumulative addition of the sGC stimulators riociguat and BAY412272 and the PDE5 inhibitors sildenafil and tadalafil (all from 10⁻⁹ M to 10⁻⁵ M).

Hemodynamic measurements
Rats were anesthetized (80 mg/kg ketamine and 8 mg/kg xylacine i.p.), tracheostomyzed and ventilated with room air (tidal volume 9 mL/kg, 60 breaths/min, and a positive end-expiratory
pressure of 2 cm H₂O, Nemi Scientific Inc, Medway, USA). Arterial saturation (SpO₂) was continuously recorded using a pulsioxymeter (StarrOx) placed in the lower extremity of the animal. The carotid artery was cannulated for systemic arterial pressure (SAP) recording. After sternotomy, a catheter was placed in the PA through the right ventricle for PA pressure recording. Drugs were administered intravenously. Hypoxia was induced by ventilating the animals with a FIO₂ of 0.1 for 5 min.

The experimental design for the acute in vivo study is shown in Fig 1. After a 10 minutes stabilization period under normoxic (room air) ventilation, rats were exposed to global hypoxia (FIO₂ of 0.1, I, GH) for 5 minutes followed by a 20 minutes recovery period of normoxia. Then vehicle (dimethyl sulfoxide, DMSO, 500 μl, n = 6), riociguat (0.1 mg/kg, n = 6) or sildenafil (0.5 mg/kg, n = 6) were infused manually over a period of 5 minutes through the jugular vein, and after 40 minutes rats were again exposed to GH (II). Finally rats were treated with U46619 (U4) (0.003 mg/kg) for 5 minutes. At the end the animals were euthanized by stopping the ventilator.

**Bleomycin model of pulmonary fibrosis**

Rats were anaesthetized with ketamine/medetomidine and then a single dose of bleomycin at 3.75 U/kg (dissolved in 200 μL of saline) was administered intratracheally via the endotracheal route [20]. This dose of bleomycin reproducibly generated pulmonary fibrosis in previous experiments [21]. Sham control treated rats received the identical volume of intratracheal saline instead of bleomycin. The rats were weighed and observed daily for any signs of severe suffering or distress (e.g. inability to rise or ambulate, weight loss >20%, labored breathing, distended abdomen, hunched posture and ruffled fur), which did not happen in any animal and there was no need for euthanasia.

**Analysis of ventilation and perfusion by micro-CT-SPECT images**

*In vivo* imaging and quantitative analysis were performed using computer tomography (micro-CT) and single photon emission computed tomography (Albira micro-CT-SPECT-PET Imaging System (Oncovision®, Spain) using pinhole collimators and a radius of rotation of 3.5 cm [22]. Perfusion lung imaging depends on the embolization of 99ᵐTc-labelled 10–40 μm human albumin macroaggregates (MAA-Tc99ᵐ) and ventilation is monitored using diethylene-triamine-pentaacetate (DTPA-Tc99ᵐ) (Molipharma, Valencia, Spain) (10 mCi) [23]. Twenty-one days after the administration of bleomycin or saline, a single 0.1 mg·kg⁻¹ dose of riociguat or vehicle was administered intraperitoneally. Rats were tracheally intubated through the oral cavity after anaesthetization with ketamine and xylazine (90 and 6 mg/kg, respectively). The animals were then ventilated (0.02 L/min, 125 strokes/min) on a rodent ventilator (model 683; Harvard Apparatus) with DTPA-Tc99ᵐ for 15 min. After DTPA-Tc99ᵐ delivery, the animals were removed from the ventilator and allowed to breathe freely. After the ventilation SPECT scan, rats were injected with 0.5–1 mCi of MAA-Tc99ᵐ via the tail vein. The relationship between ventilation and perfusion data was determined with PMOD™ software analyzing the intensity of radiation (arbitrary units) of each volume of interest (VOI) of the whole lung region selected of 256 image sections for each animal study corrected by the maximal activity. Corrected radiation intensities of ventilation (V) and perfusion (Q) studies were represented.

**Drugs**

All drugs were from Sigma-Aldrich (Spain) except tadalafil (SantaCruz Biotechnology, USA) and riociguat (MedChem Express, China).
Fig 1. Riociguat and sildenafil inhibit hypoxic pulmonary vasoconstriction and U46619-induced vasoconstriction in isolated rat pulmonary arteries (PA). PA bubbled with 21% O₂, 74% N₂ and 5% CO₂ were initially exposed to hypoxia (95% N₂ and 5% CO₂), then treated with the drugs (both at 100 nM), exposed again to hypoxia and finally treated with U46619 (100 nM). Panel A shows the study protocol and representative traces. Panels B and C show the results (means ± SEM, n = 12, 9 and 8 for vehicle, riociguat and sildenafil, respectively) for the effects of hypoxia and U46619, respectively. *P<0.05 and **P < 0.01 versus vehicle.

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Statistical analysis
Results are expressed as means ± SEM. N indicates the number of animals or arteries tested. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect was interpolated from the fitted concentration-response curves for each ring and expressed as negative log molar concentration (pIC$_{50}$). Statistical analysis was performed using a one-way ANOVA followed by Bonferroni post hoc test or paired Student’s t test as appropriate. Differences were considered statistically significant when P < 0.05.

Results
Effects on hypoxia- and U46619-induced PA contractions in vitro
In isolated PA, bubbling the myograph chamber with nominally O$_2$-free (95% N$_2$-5% CO$_2$) yields a 2–3% concentration of O$_2$ in the Krebs solution. In the absence of any other vasoconstrictor stimuli, shifting from normoxia (21% O$_2$) to hypoxia induced a contractile response (HPV). As shown in Fig 1A, this contraction was small (i.e. ≈5–10% of the maximal response induced by 80 mM KCl), sustained for 10 min, reversible after returning to normoxia and reasonably well reproduced after a period of 20 min of normoxia in the presence of vehicle (86 ±8% of the first response). Stronger responses to hypoxia can be achieved if arteries are primed with a pre-tone induced by a vasoconstrictor, but then the effects of any subsequent intervention can be attributed to either the hypoxic response or the pre-tone. After returning again to normoxic conditions for 10 min, exposure to the thromboxane A$_2$ mimetic U46619 (100 nM) leads to a sustained contractile response. Riociguat and sildenafil inhibited HPV (Fig 1). At a concentration (100 nM for both drugs) selected to produce a similar inhibitory effects on HPV (50±15% and 58±9% of the response in vehicle-treated arteries, respectively, Fig 1B), the response to U46619 was more strongly inhibited by riociguat (21±4% and 43±8%, respectively, p < 0.05 sildenafil vs riociguat, Fig 1C).

Vasodilator responses in pulmonary and mesenteric arteries. Effects of oxygen
In order to compare the vasodilator responses in vitro under different conditions of oxygenation and for a wide range of concentrations of the drugs, we continuously exposed rat and human PA to nominally 0, 21 or 95% oxygen. The endothelial function was tested with 1 μM acetylcholine (ACh) in arteries pre-contracted by 5-HT (10 μM). Because vasodilator responses are known to vary widely depending on the vasoconstrictor stimuli, arteries were contracted with a mixture of three vasoconstrictors, or its analogs, which are known to be elevated in PH (the thromboxane A$_2$ analog U46619, ET-1 and 5-HT). This pan-constrictor cocktail induces a strong and sustained vasoconstrictor response (similar to that induced by 80 mM KCl). The potency of the vasodilator drugs under the different conditions was calculated as the concentration producing 50% relaxation (expressed as the negative log molar, pIC$_{50}$) and the efficacy as the maximal relaxation ($E_{max}$, %) induced by the highest concentration of the drug tested (Table 1).

Cumulative addition of riociguat to rat PA (Fig 2A) produced a concentration-dependent relaxation which was 3.1 fold less potent (p < 0.05) under 21 or 95% oxygen than under hypoxic conditions. Riociguat was highly effective, at 10 μM it induced a near maximal relaxation under high oxygen or hypoxic conditions. Riociguat also induced a highly effective relaxation in rat MA, being 3.6 more potent (p < 0.05) than in PA (both at 95% O$_2$). A qualitatively similar pattern in PA was observed for BAY412272, another sGC stimulator (Fig 2B), but the 2.2 fold
The relaxant effect of BAY412272 in MA was very similar to that in PA under high oxygen. The PDE5 inhibitors sildenafil and tadalafil also produced concentration-dependent relaxations (Fig 2C and 2D) which were not significantly different under 0, 21 or 95% oxygen in rat PA. When compared under the same conditions of high oxygen, the maximal relaxations of PDE inhibitors were stronger in MA than in PA. Notably, the two PDE5 inhibitors were much less effective than the sGC stimulators in PA, with the maximal response achieved at the highest concentration tested (10 μM) being around 50% in all conditions and, therefore, pIC<sub>50</sub> could not be properly calculated. In addition, the curves for the PDE5 inhibitors had a milder slope.

The insets in Fig 2 plot the correlation between the vasodilator response to ACh (i.e. the endothelial function) and the relaxation induced in each ring by the concentration of the drugs producing 50% relaxation under all conditions studied. While no correlation was observed for riociguat, the relaxant response to ACh in each arterial ring predicted the vasodilator effect of sildenafil (slope = 0.49 ± 0.12, R<sup>2</sup> = 0.38, p < 0.05).

In human PA (Fig 3 vs Fig 2), riociguat was 5–10 fold less potent (i.e. lower pIC<sub>50</sub>, p< 0.05) and less effective (i.e. lower E<sub>max</sub>, p<0.05) in human PA.

### Pulmonary and systemic effects in vivo

In order to analyze the effects in vivo, we employed a protocol with a repeated 5 min challenge to hypoxia (FlO<sub>2</sub> = 0.1) before and after the exposure to the drugs. Baseline parameters were not significantly different among groups despite a trend for higher SAP in the experimental series of riociguat. Hypoxia led to an increase in PAP (≈8–12 mmHg, i.e. HPV), a decrease in SAP and a decrease in oxygen saturation with minimal and inconsistent effects on heart rate (Fig 4 shows the absolute values and Fig 5 the changes induced by each intervention). The administration of vehicle (DMSO) had no significant effect on PAP or SAP and did not modify the changes induced by hypoxia (Figs 4 and 5). Riociguat and sildenafil were administered at doses selected in preliminary experiments to produce similar inhibitory effects on HPV (0.1 and 0.5 mg/kg, respectively). This doses produced minimal changes in PAP but riociguat

### Table 1. Potency and efficacy of sGC stimulators in rat and human arteries (calculated from data shown in Figs 2 and 3).

|                        | PA 21% oxygen | PA hypoxia | PA 95% oxygen | MA 95% oxygen |
|------------------------|---------------|------------|---------------|---------------|
|                        | pIC<sub>50</sub> | E<sub>max</sub> (%) | n | pIC<sub>50</sub> | E<sub>max</sub> (%) | n | pIC<sub>50</sub> | E<sub>max</sub> (%) | n | pIC<sub>50</sub> | E<sub>max</sub> (%) | n |
| Riociguat (rat)        | 6.88 ± 0.24   | 82 ± 2     | 7 | 7.39 ± 0.09   | 95 ± 4       | 6 | 6.86 ± 0.04   | 89 ± 2       | 6 | 7.45 ± 0.08   | 108 ± 6      | 6 |
| Riociguat (human)      | n.d.          | n.d.       | - | 6.60 ± 0.19   | 77 ± 5       | 11| 6.07 ± 0.25   | 65 ± 9       | 10| n.d.          | n.d.        | - |
| BAY412272 (rat)        | n.d.          | n.d.       | - | 7.87 ± 0.16   | 111 ± 4      | 5 | 7.52 ± 0.21   | 94 ± 9       | 6 | 7.59 ± 0.19   | 98 ± 4       | 5 |

PA, pulmonary arteries; MA, mesenteric arteries. pIC<sub>50</sub>, drug concentration inducing 50% relaxation (expressed as negative log molar), E<sub>max</sub> maximal relaxation achieved. Results are means ± SEM

* p < 0.05 and

** p < 0.01 vs PA 95% oxygen

n.d., not determined.

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Fig 2. Oxygen and pulmonary selectivity of the vasodilator effects in isolated rat arteries. The sGC stimulators riociguat (A) and BAY412272 (B) and the PDE5 inhibitors sildenafil (C) and tadalafil (D) relax rat pulmonary (PA) and mesenteric arteries (MA) bubbled with 21% O$_2$-74% N$_2$-5% CO$_2$, 95% O$_2$-5% CO$_2$ or with 95% N$_2$-5% CO$_2$ (hypoxia). Arteries were initially stimulated with a cocktail of U46619 (30 nM), 5-HT (3 μM) and endothelin (3 nM) and, thereafter, the drugs were added in a cumulative fashion (all from 10$^{-9}$ M to 10$^{-5}$ M). The insets show the correlation between the initial response to ACh (1 μM) and the subsequent relaxation induced by riociguat (0.1 μM) or sildenafil (1 μM) in each artery (data from MA and PA under all conditions). Results are means ± SEM (n = 7, 6, 6 and 6 for riociguat 7, 9, 9 and 5 for sildenafil, in PA at 21, 95, and 0% O$_2$ or MA, respectively, and 4, 4 and 4 for tadalafil and 6, 5 and 5 for BAY412272 in PA at 95, and 0% O$_2$ or MA, respectively). The parameters calculated from these curves and their statistical analysis are shown in Table 1.

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significantly reduced SAP. However, it should be noted that there is a mild but progressive decrease in SAP along the experimental period in all groups which is more prominent in the riociguat group, probably due to higher baseline levels. Upon exposure to hypoxia, both drugs inhibited the increase in PAP to a similar extent but they did not modify the desaturation. Riociguat and sildenafil had no significant effect on heart rate, pulmonary arterial $dp/dt_{max}$ or the systemic hypotension induced by hypoxia. Finally, sildenafil strongly inhibited the pulmonary pressor response to U46619, while the partial inhibition by riociguat was mild and borderline significant ($p = 0.06$).

**Effects on ventilation-perfusion in rats with pulmonary fibrosis**

In order to analyze the effects of the drugs on ventilation and perfusion we used control rats and a model of bleomycin-induced fibrosis in which $V'/Q'$ is strongly unpaired. Three weeks after bleomycin or vehicle administration, ventilation and perfusion were analyzed by micro-CT-SPECT imaging. In control animals, acute administration of riociguat had no significant effects on ventilation, perfusion or the $V'/Q'$ ratio (Fig 6). Fibrosis induced a strong decrease in both ventilation and perfusion resulting also in reduced $V'/Q'$. Acute administration of riociguat had no significant effect on ventilation, produced a modest but significant increase in perfusion and again did not modify $V'/Q'$. These data resemble those recently reported for sildenafil in the same model and identical conditions, in which sildenafil had no significant effect on ventilation, perfusion or $V'/Q'$ [22].

**Discussion**

Herein we show that PDE5 inhibitors and sGC stimulators show a different vasodilator profile. Riociguat was highly effective, not correlated with endothelial function and potentiated by hypoxia in rat and human PA. *In vivo*, riociguat preferentially inhibited hypoxic than non-hypoxic vasoconstriction. However, it did not worsen $V'/Q'$ coupling in rat model of pulmonary fibrosis.

NO exerts most of its actions, including vasodilation, by binding to and activating sGC, resulting in increased intracellular cGMP levels. In order to increase the defective NO signaling pathway in PH, two different therapeutic approaches have been developed: to increase cGMP synthesis by increasing sGC activity and to prevent cGMP degradation by inhibition of PDE5. In addition to NO and NO releasing compounds, two classes of drugs have been developed to increase cGMP synthesis, sGC stimulators and sGC activators [24]. sGC stimulators, such as riociguat, sensitize sGC to low levels of bioavailable NO but can also increase sGC activity in the absence of NO. In contrast, sGC activators preferentially activate sGC when it is in an oxidized NO-insensitive form, but its therapeutic use has not been approved yet. Thus, stimulation of sGC may have a theoretical advantage over PDE5 inhibition because of its NO-independent mode of action [4]. Therefore, sGC stimulators and activators could still be effective when NO levels are impaired, as occurs in PH. On the contrary, PDE5 inhibitors may be more effective when PDE5 is overexpressed as it has been shown in patients with IPF and PH [22]. The present experiments in isolated arteries clearly demonstrate this concept. In PA the...
Fig 4. Effects of riociguat and sildenafil on systemic and pulmonary arterial pressure (SAP and PAP) and oxygen arterial saturation (SpO₂). After stabilization, rats were ventilated with FIO₂ = 0.1 (Hyp), then vehicle, riociguat (0.1 mg/kg) or sildenafil (0.5 mg/kg) were injected IV and after 20 min again ventilated with FIO₂ = 0.1 and finally U46619 (0.003 mg/kg, U4) was injected IV. (A) Heart rate (HR), (B) SpO₂, (C) SAP and (D) PAP. Results are means ± SEM (n = 6 for vehicle and 5 for riociguat and sildenafil). The graphs show absolute values. Relative changes and the statistical analysis of these results are shown in Fig 5.

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maximal relaxant response of sildenafil was only 50% (Fig 2C) while riociguat was able to
induce a full relaxant response (Fig 2A). This difference was reproduced in human PA (Fig 3)
and in rat PA by the sGC stimulator BAY412272 and the PDE5 inhibitor tadalafil (Fig 2B and
2D), demonstrating that it is a characteristic feature of these drug classes and conserved
between species. This difference presumably reflects a low basal NO activity under these condi-
tions so that even after a strong inhibition of PDE5 there is insufficient cGMP to induce a full

Fig 5. Changes in systemic and pulmonary arterial pressure and oxygen arterial saturation. Changes in mean PAP (panel A, HPV) and in saturation
(panel B, SpO₂) in response to hypoxia before (baseline) or after the exposure to the drugs. Changes in mean pulmonary (C) and systemic (D) pressure
induced by the drugs. Effects on the vasopressor response to U46619 (E). Data are calculated from experiments shown in Fig 4, *p<0.05 and **p < 0.01
versus baseline (paired t test), #p< 0.01 vs vehicle (one-way Anova followed by Bonferroni's test).

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Fig 6. Effects of riociguat on ventilation-perfusion coupling (V'/Q'). Effects of riociguat on (A) ventilation (V'), (B) perfusion (Q') and (C) ventilation-perfusion coupling (V'/Q') in control and bleomycin-induced fibrotic rats. (D) Representative images of ventilation and perfusion were obtained by micro-CT-SPECT. A single dose of riociguat (0.1 mg/kg) or vehicle was administered 21 days after the administration of bleomycin or saline. The relationship between the ventilation and perfusion data was determined using PMOD™ software to analyze the intensity of radiation (arbitrary units) of each volume of interest. Results are means ± SEM (n = 8). *P < 0.05 vs vehicle control and #P < 0.05 versus vehicle bleomycin.

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relaxation. In fact, we found that the NO-dependent endothelial function (relaxation to ACh) predicts well the response to PDE5 inhibitors but does not affect the response to sGC stimulators.

The systemic effects of oral and intravenous vasodilators leading to severe hypotension may preclude the use of effective doses of these drugs. The development of pulmonary selective drugs is a long-lasting unmet need [25, 26]. The results of the present study show that both PDE5 inhibitors and sGC stimulators display no pulmonary selectivity in vitro, showing similar or stronger effects in MA than in PA. In vivo, sildenafil and riociguat had weak effects on basal PAP, but reduced basal systemic arterial pressure, which was particularly evident for riociguat as previously reported [27]. In contrast, the pressor effects of U46619 were inhibited by sildenafil in the pulmonary circulation but unaffected in the systemic one, showing a clear pulmonary selectivity under these conditions which is consistent with previous studies with the drug in acute pulmonary hypertension induced by U46619 [26]. These differences in the systemic and pulmonary circulation reflect a different role of the NO-cGMP-PDE5 signaling pathway in the systemic and pulmonary circulation and a different regulation by thromboxane receptors in both vascular beds.

Hypoxia is also a well-known modulator of the NO pathway [28] and may therefore differentially affect the responses to PDE5 inhibitors and sGC stimulators. We have compared the effects of sildenafil with the novel sGC stimulator riociguat under well controlled conditions in vitro in rat and human arteries. Several studies have described the inhibitory effects of PDE5 inhibitors on HPV [29–32] while the effects of riociguat have been hardly analyzed [33]. Herein we show that the sGC stimulator riociguat shows a similar profile to sildenafil in inhibiting HPV in vitro, i.e., in isolated pulmonary arteries, and in vivo, i.e., the pulmonary pressor response to hypoxia. We compared their inhibitory effects on HPV to those on the vasoconstrictor response to the thromboxane A2 analog U46619. An ideal drug should inhibit U46619-induced responses at doses that preserve HPV. In vitro, both drugs were at least as effective on U46619-induced contractions as on HPV. However, in vivo, riociguat, at a dose which markedly inhibited HPV or induced systemic hypotension, hardly affected the U46619-induced pressor responses while sildenafil strongly inhibited it.

Thus, we analyzed the influence of oxygen on the vasodilator responses over a wide range of concentrations of the drugs. We chose a cocktail of vasoconstrictors, a thromboxane analog, ET-1 and 5-HT, inducing a strong contractile response under high, normal or low oxygen concentrations. The rationale for this protocol is that these are the main vasoactive factors known to be involved in PH [3] and that the possible differences in the potency or efficacy of the vasodilators depending on the vasoconstrictor stimuli can be minimized. We found that riociguat induced a 3-fold stronger vasodilator effects under hypoxic vs normal or high oxygen conditions in both human and rat PA, while these differences were smaller for the other sGC stimulator studied BAY412272 (2-fold). In contrast, the effects of sildenafil or tadalafil were similar in high, normal or low oxygen.

A limitation of our in vitro study is that we evaluated the effects of the drugs in healthy rat or human arteries or in vivo in healthy rats. To analyze a possible impact in a more clinically relevant context of V’/Q’ mismatch, we studied the effects of the drugs in a rat model of IPF associated with PH induced by bleomycin. This model is characterized by elevated right ventricular systolic pressure (≈ 45 mm Hg), right ventricular hypertrophy and vascular remodelling [34]. Ventilation and perfusion analysis, as monitored by the analysis of the inhaled DTPA-99mTc signal and perfused MAA-99mTc [22, 35]. Rats showed a markedly impaired V’/Q’ SPECT ratio three weeks after bleomycin-induced pulmonary fibrosis. Acute administration of sildenafil [22] and riociguat (present data) had no effect on ventilation in the bleomycin-induced pulmonary fibrosis model associated with PH, riociguat (but not sildenafil)
induced a modest increase in lung perfusion but there were no significant changes in the V′/Q′ ratio. Several other limitations should be mentioned in our study. First, the hemodynamic study was conducted in open chest ventilated rats under anesthesia. Second, in the in vivo models, we have only tested a single drug dose. Third, despite the frequent use of the bleomycin model of pulmonary fibrosis, it has significant limitations in its ability to mimic human IPF since many treatments successful in the bleomycin model were not transferable to human IPF.

Oxygen-selective drugs which exert vasodilator effects preferentially in high ventilated/oxygenated lung regions when administered systemically are currently lacking but they could represent a significant therapeutic advance, particularly in patients with PH associated to hypoxia and/or V/Q mismatch.

In conclusion, as a result of different mechanisms of action, PDE5 inhibitors and sGC stimulators show a different vasodilator profile. Both sildenafil and riociguat effectively inhibit HPV in vitro. In vivo, riociguat, at a dose which markedly inhibited HPV hardly affected the U46619-induced pressor responses while sildenafil similarly inhibited hypoxic and non-hypoxic vasoconstriction. This may be due to a 3-fold stronger vasodilator potency of riociguat under hypoxic conditions in both human and rat PA. However, the two drugs did not worsen V′/Q′ coupling in rat model of IPF.

**Author Contributions**

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**References**

1. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). Eur Respir J. 2015; 46 (4):903–75. https://doi.org/10.1183/13993003.01032-2015 PMID: 26318161.

2. Tuder RM, Archer SL, Dorfmuller P, Erzurum SC, Guignabert C, Michelakis E, et al. Relevant issues in the pathology and pathobiology of pulmonary hypertension. J Am Coll Cardiol. 2013; 62(25 Suppl):D4–12. https://doi.org/10.1016/j.jacc.2013.10.025 PMID: 24355640; PubMed Central PMCID: PMCPMC3970402.

3. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest. 2012; 122 (12):4306–13. https://doi.org/10.1172/JCI60658 PMID: 23202738; PubMed Central PMCID: PMCPMC3533531.

4. Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, et al. Riociguat for the treatment of pulmonary arterial hypertension. N Engl J Med. 2013; 369(4):330–40. https://doi.org/10.1056/NEJMoa1209655 PMID: 23983378.

5. Thabut G, Dauriat G, Stern JB, Logeart D, Levy A, Marrash-Chahla R, et al. Pulmonary hemodynamics in advanced COPD candidates for lung volume reduction surgery or lung transplantation. Chest. 2005; 127(5):1531–6. https://doi.org/10.1378/chest.127.5.1531 PMID: 15888824.
6. Lettieri CJ, Nathan SD, Barnett SD, Ahmad S, Shorr AF. Prevalence and outcomes of pulmonary arterial hypertension in advanced idiopathic pulmonary fibrosis. Chest. 2006; 129(3):746–52. https://doi.org/10.1378/chest.129.3.746 PMID: 16537877.

7. Kessler R, Faller M, Fourgaut G, Memencier B, Weitzenblum E. Predictive factors of hospitalization for acute exacerbation in a series of 64 patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1999; 159(1):158–64. https://doi.org/10.1164/ajrccm.159.1.9830117 PMID: 9872834.

8. Seeger W, Adir Y, Barbera JA, Champion H, Coghlan JG, Cottin V, et al. Pulmonary hypertension in chronic lung diseases. J Am Coll Cardiol. 2013; 62(25 Suppl):D109–16. https://doi.org/10.1016/j.jacc.2013.10.036 PMID: 24355635.

9. Sylvester JT, Shimoda LA, Aaronson PI, Ward JP. Hypoxic pulmonary vasoconstriction. Physiol Rev. 2012; 92(1):367–520. https://doi.org/10.1152/physrev.00041.2010 PMID: 22298659.

10. Cogolludo A, Moreno L, Frazziano G, Moral-Sanz J, Menendez C, Castaneda J, et al. Activation of neutral sphingomyelinase is involved in acute hypoxic pulmonary vasoconstriction. Cardiovasc Res. 2009; 82(2):296–302. https://doi.org/10.1093/cvr/cvn349 PMID: 19088082; PubMed Central PMCID: PMCPMC2675929.

11. Agusti AG, Barbera JA, Roca J, Wagner PD, Guitard R, Rodriguez-Roisin R. Hypoxic pulmonary vasoconstriction and gas exchange during exercise in chronic obstructive pulmonary disease. Chest. 1990; 97(2):268–75. PMID: 2298050.

12. Hoeper MM, Halank M, Wilkens H, Gunther A, Weimann G, Gebert I, et al. Riociguat for interstitial lung disease and pulmonary hypertension: a pilot trial. Eur Respir J. 2013; 41(4):853–60. https://doi.org/10.1183/09031936.00213911 PMID: 22936711.

13. Ghofrani HA, Staehler G, Grunig E, Halank M, Mitrovic V, Unger S, et al. Acute effects of riociguat in borderline or manifest pulmonary hypertension associated with chronic obstructive pulmonary disease. Pulm Circ. 2015; 5(2):296–304. https://doi.org/10.1086/680214 PMID: 26064454; PubMed Central PMCID: PMCPMC4449240.

14. Blanco I, Gimeno E, Munoz PA, Pizarro S, Gustau C, Rodriguez-Roisin R, et al. Hemodynamic and gas exchange effects of sildenafil in patients with chronic obstructive pulmonary disease and pulmonary hypertension. Am J Respir Crit Care Med. 2010; 181(3):270–8. https://doi.org/10.1164/rccm.200907-0988OC PMID: 19875684.

15. Ghofrani HA, Wiedemann R, Rose F, Schermuly RT, Olschewski H, Weissmann N, et al. Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial. Lancet. 2002; 360(9337):895–900. https://doi.org/10.1016/S0140-6736(02)11024-5 PMID: 12354470.

16. Zisman DA, Schwarz M, Anstrom KJ, Collard HR, Flaherty KR, Humphreys GW. A controlled trial of sildenafil in advanced idiopathic pulmonary fibrosis. N Engl J Med. 2010; 363(7):620–8. https://doi.org/10.1056/NEJMoa1002110 PMID: 20484178; PubMed Central PMCID: PMCPMC3587293.

17. Han MK, Bach DS, Hagan PG, Yow E, Flaherty KR, Toews GB, et al. Sildenafil preserves exercise capacity in patients with idiopathic pulmonary fibrosis and right-sided ventricular dysfunction. Chest. 2013; 143(6):1699–708. https://doi.org/10.1378/chest.12-1594 PMID: 23732584; PubMed Central PMCID: PMCPMC3673665.

18. Cogolludo A, Moreno L, Lodi F, Tamargo J, Perez-Vizcaino F. Postnatal maturational shift from PKCzeta and voltage-gated K+ channels to RhoA/Rho kinase in pulmonary vasocostriction. Cardiovasc Res. 2005; 66(1):84–93. https://doi.org/10.1016/j.cardiores.2004.12.019 PMID: 15769451.

19. Frazziano G, Moreno L, Moral-Sanz J, Menendez C, Escolano L, Gonzalez C, et al. Neutral sphingomyelinase, NADPH oxidase and reactive oxygen species. Role in acute hypoxic pulmonary vasoconstriction. J Cell Physiol. 2011; 226(10):2633–40. https://doi.org/10.1002/jcp.22611 PMID: 21792922.

20. Bivas-Benita M, Zwier R, Junginger HE, Borchard G. Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. Eur J Pharm Biopharm. 2005; 61(3):214–8. https://doi.org/10.1016/j.ejpb.2005.04.009 PMID: 16039104.

21. Mata M, Ruiz A, Carda M, Martinez-Losa M, Cortijo J, Santangelo F, et al. Oral N-acetylcycteine reduces bleomycin-induced lung damage and mucoi mucosac expression in rats. Eur Respir J. 2003; 22(6):900–5. Epub 2003/12/19. PMID: 14680076.

22. Milara J, Escriva J, Ortiz JL, Juan G, Artigues E, Morcillo E, et al. Vascular effects of sildenafil in patients with pulmonary fibrosis and pulmonary hypertension: an ex vivo/in vitro study. Eur Respir J. 2016; 47(6):1737–49. https://doi.org/10.1183/13993003.01259-2015 PMID: 27009174.

23. Oyen WJ, van Oort AM, Tanke RB, van Mill GJ, Aengevaeren WR, Corstens FH. Pulmonary perfusion after endovascular stenting of pulmonary artery stenosis. J Nucl Med. 1995; 36(11):2006–8. Epub 1995/11/01. PMID: 7472589.

24. Stasch JP, Pacher P, Evgenov OV. Soluble Guanylate Cyclase as an Emerging Therapeutic Target in Cardiopulmonary Disease. Circulation. 2011; 123(20):2263–73. doi: 10.1161/CIRCULATIONAHA.110.981738. WOS:000290852200021. PMID: 21606405.
25. Wanstall JC, Jeffery TK. Recognition and management of pulmonary hypertension. Drugs. 1998;56(6):989–1007. PMID: 9878988.

26. Ghofrani HA, Pepke-Zaba J, Barbera JA, Channick R, Keogh AM, Gomez-Sanchez MA, et al. Nitric oxide pathway and phosphodiesterase inhibitors in pulmonary arterial hypertension. J Am Coll Cardiol. 2004;43(12 Suppl S):685–72S. https://doi.org/10.1016/j.jacc.2004.02.031 PMID: 15194181.

27. Schermuly RT, Stasch JP, Pullamsetti SS, Middendorff R, Muller D, Schluter KD, et al. Expression and function of soluble guanylate cyclase in pulmonary arterial hypertension. Eur Respir J. 2008;32(4):881–91. https://doi.org/10.1183/09031936.00114407 PMID: 18550612.

28. Ho JJ, Man HS, Marsden PA. Nitric oxide signaling in hypoxia. J Mol Med (Berl). 2012;90(3):217–31. https://doi.org/10.1007/s00109-012-0880-5 PMID: 22349396.

29. Fesler P, Pagnamenta A, Rondelet B, Kerbau F, Naeije R. Effects of sildenafil on hypoxic pulmonary vascular function in dogs. J Appl Physiol (1985). 2006;101(4):1085–90. https://doi.org/10.1152/japplphysiol.00332.2006 PMID: 16778005.

30. Preston IR, Hill NS, Gambardella LS, Warburton RR, Klinger JR. Synergistic effects of ANP and sildenafil on cGMP levels and amelioration of acute hypoxic pulmonary hypertension. Exp Biol Med (Maywood). 2004;229(9):920–5. PMID: 15388887.

31. Tessler R, Wu S, Fiori R, Macgowan CK, Beil J. Sildenafil acutely reverses the hypoxic pulmonary vasoconstriction response of the newborn pig. Pediatr Res. 2008;64(3):251–5. https://doi.org/10.1203/PDR.0b013e31817d9b93 PMID: 18458659.

32. Zhao L, Mason NA, Morrell NW, Kojonazarov B, Sadykov A, Maripov A, et al. Sildenafil inhibits hypoxia-induced pulmonary hypertension. Circulation. 2001;104(4):424–8. PMID: 11468204.

33. Lang M, Kojonazarov B, Tian X, Kalymbetov A, Weissmann N, Grimminger F, et al. The soluble guanylate cyclase stimulator riociguat ameliorates pulmonary hypertension induced by hypoxia and SU5416 in rats. PLoS One. 2012;7(8):e43433. https://doi.org/10.1371/journal.pone.0043433 PMID: 22912874; PubMed Central PMCID: PMCPMC3422306.

34. Rathinasabapathy A, Bruce E, Espejo A, Horowitz A, Sudhan DR, Nair A, et al. Therapeutic potential of adipose stem cell-derived conditioned medium against pulmonary hypertension and lung fibrosis. Br J Pharmacol. 2016;173(19):2859–79. https://doi.org/10.1111/bjp.13562 PMID: 27448286; PubMed Central PMCID: PMCPMC5275771.

35. Amen EM, Becker EM, Truebel H. Analysis of V/Q-matching—a safety “biomarker” in pulmonary drug development? Biomarkers. 2011;16 Suppl 1:S5–10. https://doi.org/10.3109/1354750X.2011.585243 PMID: 21707444.