Inhibition of Phosphodiesterase 2 Augments cGMP and cAMP Signaling to Ameliorate Pulmonary Hypertension

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Background—Pulmonary hypertension (PH) is a life-threatening disorder characterized by increased pulmonary artery pressure, remodeling of the pulmonary vasculature, and right ventricular failure. Loss of endothelium-derived nitric oxide (NO) and prostacyclin contributes to PH pathogenesis, and current therapies are targeted to restore these pathways. Phosphodiesterases (PDEs) are a family of enzymes that break down cGMP and cAMP, which underpin the bioactivity of NO and prostacyclin. PDE5 inhibitors (eg, sildenafil) are licensed for PH, but a role for PDE2 in lung physiology and disease has yet to be established. Herein, we investigated whether PDE2 inhibition modulates pulmonary cyclic nucleotide signaling and ameliorates experimental PH.

Methods and Results—The selective PDE2 inhibitor BAY 60-7550 augmented atrial natriuretic peptide– and treprostinil-evoked pulmonary vascular relaxation in isolated arteries from chronically hypoxic rats. BAY 60-7550 prevented the onset of both hypoxia- and bleomycin-induced PH and produced a significantly greater reduction in disease severity when given in combination with a neutral endopeptidase inhibitor (enhances endogenous natriuretic peptides), treprostinil, inorganic nitrate (NO donor), or a PDE5 inhibitor. Proliferation of pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension was reduced by BAY 60-7550, an effect further enhanced in the presence of atrial natriuretic peptide, NO, and treprostinil.

Conclusions—PDE2 inhibition elicits pulmonary dilation, prevents pulmonary vascular remodeling, and reduces the right ventricular hypertrophy characteristic of PH. This favorable pharmacodynamic profile is dependent on natriuretic peptide bioactivity and is additive with prostacyclin analogues, PDE5 inhibitor, and NO. PDE2 inhibition represents a viable, orally active therapy for PH. (Circulation. 2014;130:496-507.)

Key Words: cyclic nucleotide ■ natriuretic peptide ■ nitric oxide ■ phosphodiesterase inhibitor ■ pulmonary hypertension

Pulmonary hypertension (PH) is a life-threatening, multifactorial disorder characterized by increased pulmonary vascular resistance and remodeling of the small pulmonary arteries, which precipitate right ventricular hypertrophy (RVH) and failure.1,2 Despite therapeutic innovation, including the introduction of prostacyclin (PGI2) analogues,3 endothelin receptor antagonists,4 and phosphodiesterase 5 inhibitors (PDE5i),5 mortality remains unacceptably high.6,7 As such, there is a clear unmet medical need for improved drug efficacy in this disorder.

Clinical Perspective on p 507

Loss of endothelial nitric oxide (NO)– and PGI2-driven cGMP and cAMP signaling is a hallmark of PH, particularly the pulmonary arterial hypertension (PAH) subgroup (World Health Organization Group 1).1 Within the pulmonary circulation, cyclic nucleotides are responsible for mediating endothelium-dependent dilation, thereby maintaining pulmonary vascular homeostasis, but they also have salutary actions on pulmonary vascular remodeling, fibrosis, and right ventricular (RV) function.8,9 Thus, augmenting cyclic nucleotide signaling represents an appealing, proven strategy for improving therapy and underpins the efficacy of PDEi, PGI2 analogues, and soluble guanylate cyclase (sGC) stimulators.9 Moreover, combinations of cGMP (eg, PDE5i) and cAMP-elevating agents (eg, iloprost or epoprostenol) exert an additive, if not synergistic, beneficial effect in PH patients.10

Cyclic nucleotide phosphodiesterases (PDEs) are homologous enzymes that facilitate the breakdown of cAMP and/or cGMP.11 Within the lung, cGMP-hydrolyzing PDE5 is the most abundant isof orm,12 and enzyme expression and activity are upregulated in preclinical models of PH and patients with the disease.13,14 The expression and activity of additional PDE isoforms, including PDE1, PDE3, PDE4, and PDE10, are
also altered in the pulmonary vasculature of PH patients, isofrom-selective inhibitors are effective in preclinical models of PH. One PDE isoform that has received little or no attention in the setting of PH is PDE2. This “cGMP-stimulated” PDE metabolizes cAMP and cGMP and, akin to PDE5, possesses a GAF domain within its N-terminus that acts as a positive feedback loop to expedite cyclic nucleotide hydrolysis in the presence of cGMP. PDE2 (and splice variants thereof) is expressed in a wide variety of cells and tissues in the cardiovascular system (eg, myocardium, platelets, endothelium) and is also found in the lung, including pulmonary artery smooth muscle cells from patients with PH. Indeed, the enzyme is functionally active in the pulmonary circulation because inhibition of this isoform has been shown to modulate microvascular permeability and acute hypoxic vasorestrictive in vitro.

We therefore hypothesized that in PH, pulmonary PDE2 activity curtails cytoprotective cGMP and cAMP signaling (because it metabolizes both cyclic nucleotides) and exacerbates pathology. In accord, we investigated the effects of the selective PDE2i BAY 60-7550 (in vitro IC50 = 4.7 nmol/L; >50-fold selectivity over PDE1 and >100-fold selectivity over other PDE isozymes) on pulmonary vascular dynamics and pulmonary vascular smooth muscle proliferation in vitro, and etiologically distinct preclinical models of PH, to identify beneficial activity of the molecule per se, interactions with endogenous pulmonary protective mediators, and additive effects with existing therapies.

Methods

All studies conformed to the UK Animals (Scientific Procedures) Act of 1986 and had approval from a local ethics committee within Barts and The London School of Medicine. Treatment groups, doses, and route of administration for in vivo studies are outlined in Table I in the online-only Data Supplement. Male mice were randomly assigned to each drug treatment.

Hypoxia-Induced PH

Male mice (C57BLK/6J; Charles River, UK), or wild-type (WT) and natriuretic peptide receptor (NPR)-A knockout (KO) littermates (male, 20–25 g; C57BLK/6J background) were placed inside a normobaric chamber with 10% oxygen for either 3 weeks with drug treatment from day 1 (groups 1–6; Table I in the online-only Data Supplement) or 5 weeks of hypoxia with drug treatment from day 1 (groups 7–14; Table I in the online-only Data Supplement). Age-matched normoxic control mice were housed in room air.

Bleomycin-Induced PH

A second, etiologically distinct model of PH was used to validate the efficacy of BAY 60-7550 in reducing disease severity. Male mice (C57BLK/6J; Charles River, UK) were exposed to bleomycin (2 mg/kg, 1 mL/kg volume) once by oropharyngeal instillation under light isoflurane-induced anesthesia (1.5% isoflurane, 0.2 mL/min oxygen). Controls were similarly instilled with sterile saline (1 mL/kg). Drug treatments were administered daily over a 3-week period, starting on the day of bleomycin administration.

Mouse Hemodynamics

Mice were anesthetized with isoflurane (1.5%, 0.2 mL/min oxygen) and maintained at 37°C. The RV systolic pressure (RVSP) and mean arterial blood pressure (MABP) were measured with the use of a Mikrotip pressure catheter (size 1F; SPR-1000, Millar Instruments, Houston, TX), and RVH was calculated by weight of RV to left ventricle + septum ratio (RV/LV+S). Plasma was obtained from centrifugation of whole blood (10000g, 2 minutes) and assayed for cGMP (cGMP Direct Biotrak EIA, GE Healthcare, Buckinghamshire, UK) and cAMP (cAMP enzyme-linked immunosorbent assay, Enzo Life Sciences, Exeter, UK).

Immunohistochemistry

Serial sections (4 μm) were used for trichrome blue staining and α-smooth muscle actin immunohistochemistry. For the latter, sections were stained with mouse monoclonal anti-α-smooth muscle actin antibody (DAKO, UK; 1:1000 dilution), followed by biotinylated anti-mouse secondary antibody. Immunoreactivity was detected with the use of the ABC-peroxide-based system (DAKO, UK). Stained slides were imaged with Nanozoomer Virtual Microscopy (Hamamatsu, Welwyn Garden City, UK).

Morphological Analysis

Transverse formalin-fixed lung sections were stained with the van Gieson elastic method. Pulmonary arterial muscularization was then assessed as we have described previously (see Methods in the online-only Data Supplement).

Vascular Function

Aortic and pulmonary artery (third-order) rings, isolated from hypoxic (2 weeks, 10% O2) or normoxic (control) rats (male, Sprague-Dawley, 225–275 g), were set up for isometric tension measurement, as we have described previously. For this set of experiments, rat vessels were used to permit concomitant analysis of aorta and pulmonary arteries from the same animals. Vessels were precontracted with an EC50 concentration of U46619, and endothelial function determined by relaxation responses to acetylcholine (10 μmol/L). Relaxation curves were then constructed for either atrial natriuretic peptide (ANP; 0.01 nmol/L to 0.3 μmol/L), the NO donor spermine NONOate (1 nmol/L to 30 μmol/L), or the PGI2 analogue treprostinil (1 nmol/L to 3 μmol/L) in the presence or absence of the PDE1i vinpocetine (30 μmol/L), PDE2i BAY 60-7550 (0.1 μmol/L), PDE3i milrinone (10 μmol/L), or PDE5i sildenafil (3 μmol/L). Relaxation in response to BAY 60-7550 (1 nmol/L to 3 μmol/L) per se was also assessed.

Cell Proliferation

Growth of human distal pulmonary artery smooth muscle cells isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) or control cells from adults undergoing transplantation or lung resection for suspected malignancy were monitored as we have described previously after treatment with BAY 60-7550 (1 μmol/L), ANP (1 μmol/L), DETNA-NONOate (10 μmol/L), or treprostinil (1 μmol/L), alone or in combination.

Reverse Transcription Polymerase Chain Reaction and Immunoblotting

cDNA was prepared from pulmonary arteries from normoxic and hypoxic rats and pulmonary artery smooth muscle cells isolated from patients with IPAH and control cells (as above) and analyzed for PDE2A expression with the use of quantitative real-time polymerase chain reaction over 40 cycles (see Methods in the online-only Data Supplement for primer sequence and polymerase chain reaction conditions). PDE2A protein expression was determined by immunoblot with the use of primary anti-PDE2A antibody (Santa Cruz Biotechnology, CA; 1:500) and secondary horseradish peroxidase conjugated anti-goat IgG antibody (Santa Cruz Biotechnology; 1:10000). Bands were quantified by densitometry with the use of ImageJ and normalized to the loading control (anti-actin, 1:20000, Millipore, Watford, UK; secondary antibody horseradish peroxidase conjugated anti-mouse IgG, Dako, Cambridge, UK).
PDE2 Activity and NO Production

PDE2 activity in cytosolic extracts from rat pulmonary arteries and human pulmonary artery smooth muscle cells was determined by the production of 5′-GMP with the use of a commercially available kit (Enzo Life Sciences, Exeter, UK). Total PDE activity was determined with the nonselective PDEi 3-isobutyl-1-methylxanthine (300 μmol/L), and specific PDE2 activity was calculated as the reduction in 5′-GMP formation in the presence of BAY 60-7550 (1 μmol/L).

Plasma nitrite (NO₂⁻) levels, as an index of vascular endothelial nitric oxide synthase activity, were determined by ozone chemiluminescence as we have described previously.²⁶

Data Analysis

Results are expressed as mean±SEM, and *P<0.05 denotes significance. The n value denotes the number of animals used in each group. Statistical analyses were performed with the use of GraphPad Prism version 5, as described in each figure legend.

Results

PDE2 Plays a Key Role in Regulating the Vasoreactivity of Pulmonary Arteries

Rats exposed to 2 weeks of hypoxia exhibited substantial RVH (Figure 1A in the online-only Data Supplement) and pulmonary artery (Figure 1B in the online-only Data Supplement), but not aortic (Figure 1C in the online-only Data Supplement), endothelial dysfunction compared with normoxic animals, confirming the induction of a PH phenotype. Incubation with BAY 60-7550 sensitized pulmonary arteries from both normoxic (Figure 1A) and hypoxic (Figure 1B) rats to ANP.

Spermine-NONOate–evoked responses were also increased in the presence of BAY 60-7550 (Figure 1C), yet this effect was abolished in hypoxic animals (Figure 1D). PDE2 inhibition increased the potency of treprostinil in pulmonary vessels from hypoxic (Figure 1F) but not normoxic (Figure 1E) rats.

In the presence of BAY 60-7550, there were no differences in ANP-evoked relaxation (Figure 2A and 2B) in aortas from normoxic or hypoxic rats. However, in aortas from normoxic rats, BAY 60-7550 enhanced relaxation by spermine-NONOate (Figure 2C), an effect absent in arterial of hypoxic rats. Interestingly, treprostinil did not induce relaxation in the aorta of normoxic rats in the absence or presence of BAY 60-7550 (Figure 2E). In hypoxic rat aorta, treprostinil had little or no relaxant activity per se, but its vasorelaxant potency was markedly enhanced by BAY 60-7550 (Figure 2F).

In contrast to PDE2i (Figure 1A through 1D), inhibition of PDE1 or PDE3 did not alter ANP- or spermine-NONOate–induced relaxation (Table II in the online-only Data Supplement).

PDE2 Inhibition Decreases RV Pressure and Hypertrophy in 2 Murine Models of PH

Hypoxic vehicle-treated mice developed augmented RVH (Figure 3A and 3C), both of which remained commensurate with control values. Hypoxic mice had more than twice the number of muscularized small pulmonary arteries compared

Figure 1. Concentration–response curves to atrial natriuretic peptide (ANP), spermine-NONOate (S-NO), and treprostinil in pulmonary arteries in the absence or presence of BAY 60-7550 (0.1 μmol/L) isolated from normoxic (A, C, E; n=4–6) and hypoxic (B, D, F; 2 weeks of 10% O₂; n=3–8) rats. Data are presented as mean±SEM. Curves are compared with 2-way ANOVA with repeated measures. *P<0.05, ***P<0.001, BAY 60-7550 vs control.
with normoxic controls; PDE2 inhibition prevented this morphological pathology (Figure 3G and 3H).

Bleomycin-treated mice had increased RVSP (Figure 3B) and RV/(LV+S) ratio (Figure 3D) compared with saline-treated animals. Akin to the hypoxia model, both RVSP (Figure 3B) and RV/(LV+S) ratio (Figure 3D) were lower in BAY 60-7550–treated mice. Importantly, in both models, MABP was similar in hypoxic mice in the absence and presence of BAY 60-7550 (Figure 3E and 3F), suggesting that this drug exhibits a degree of pulmonary selectivity.

**Obligatory Role of Natriuretic Peptide Bioactivity in the Beneficial Effects of PDE2 Inhibition in PH**

In WT mice, the RVSP was lower in BAY 60-7550–treated hypoxic mice compared with vehicle-treated WT animals (Figure 4A), but this effect was abolished in natriuretic peptide receptor A (NPR-A) KO mice (Figure 4A). Likewise, the ability of BAY 60-7550 to prevent the RVH in hypoxic WT mice was lost in NPR-A KO animals (Figure 4C), suggesting that natriuretic peptide–generated cGMP is regulated by PDE2. Vehicle-treated NPR-A KO mice had elevated MABP compared with vehicle-treated WT animals (Figure 4E), but PDE2 inhibition per se did not alter MABP.

To assess the effect of the NO pathway on the beneficial activity of BAY 60-7550, WT mice were treated with the NO synthase inhibitor Nω-nitro-l-arginine methyl ester (L-NAME) for the duration of hypoxia. The ability of BAY 60-7550 to prevent the increased RVSP was maintained in L-NAME–treated animals (Figure 4B), suggesting that an intact NO pathway is not necessary for PDE2 inhibition to be effective. BAY 60-7550 also caused a similar reduction in RVH in L-NAME–treated mice, although the NO synthase inhibitor per se caused an apparent reduction in the RV/(LV+S) ratio, at least in part because of a modest LV hypertrophy resulting from blockade of systemic NO production11 (Figure 4D). L-NAME treatment caused elevated MABP and a significant reduction in the plasma nitrite (NO2−) concentrations (index of vascular NO production16; control=1.04±0.06 μmol/L, L-NAME=0.66±0.04 μmol/L; P<0.001; n=8), confirming effective inhibition of endothelial NO synthase activity, but BAY 60-7550 treatment did not alter MABP in these mice (Figure 4F).

**Interaction Between PDE2 Inhibition and Natriuretic Peptides in PH**

The preceding experiments ascertained a pivotal role for natriuretic peptides in the beneficial activity of PDE2i in experimental models of PH. We recently reported that augmentation of endogenous natriuretic peptide levels with the use of the neutral endopeptidase (an enzyme that hydrolyses and terminates the biological activity of endogenous natriuretic peptide) inhibitor ecadotril synergistically interacts with the PDE5i sildenafil to ameliorate PH.35 Therefore, we investigated whether increasing endogenous natriuretic peptides with ecadotril would also increase the efficacy of BAY 60-7550.

Mice treated with ecadotril alone had RVSP (Figure 5A) and RV/(LV+S) (Figure 5C) similar to those of hypoxic vehicle-treated mice. In combination with BAY 60-7550, the hypoxia-induced increases in RVSP and RVH were attenuated, essentially back to control levels (Figure 5A and 5C). MABP was not altered by ecadotril or the ecadotril/BAY
60-7550 combination (Figure 5E). When administered alone, the effect of BAY 60-7550 on established PH was smaller than that achieved when given prophylactically (Figure 5B and 5H), with a significantly reduced RVSP only achieved at a dose of 100 mg/kg per day (Figure 5H), 10-fold higher than that necessary to prevent the onset of PH (Figure 3A). However, in the presence of ecadotril, BAY 60-7550 reversed the RVSP (Figure 5B) and RVH (Figure 5D) to a greater extent than either drug alone. This dual therapy did not alter MABP (Figure 5F).

PDE2 Inhibition Promotes the Salutary Effects of NO and PGI2 in PH
Experiments in l-NAME–treated mice suggested that the attenuation of RVSP with PDE2 inhibition is not dependent on endogenous NO. However, we investigated the possibility that a pharmacological interaction between PDE2 inhibition and NO bioactivity may be evident in reversing hypoxia-induced PH. In support of this concept, treatment with inorganic nitrate (which we have shown previously to ameliorate hypoxia-induced PH28), at a dose that was ineffective per se, significantly attenuated the RVSP in combination with PDE2 inhibition (Figure 6A). However, this treatment effect was less pronounced against the corresponding RVH (Figure 6C).

In parallel experiments, because PDE2 hydrolyzes both cGMP and cAMP, and PDE2i augmented the vasorelaxant activity of treprostinil in vitro, we explored whether BAY 60-7550 could potentiate the pharmacodynamic effect of treprostinil and reverse established PH. A very similar pattern of activity was observed such that in combination, BAY 60-7550 and treprostinil were able to reverse the hypoxia-induced increase in RVSP, whereas neither intervention as monotherapy produced a significant effect (Figure 6B). Again, the benefit of dual therapy
was less evident against RVH (Figure 6D). Neither combination resulted in a significant decrease in MABP (Figure 6E and 6F). The efficacy of the PDE2i/treprostinil combination appeared to be linked to cAMP accumulation because the dual therapy resulted in a doubling in plasma cAMP levels that was greater than either drug alone (Figure 6G).

PDE2 Expression and Activity in Rodent and Human PH
PDE2 mRNA and protein expression were significantly reduced in the pulmonary arteries of hypoxic animals compared with normoxic controls (Figure 7A and 7B). Enzyme activity trended toward a lower level but was not significantly different between normoxic and hypoxic arteries (Figure 7C). Expression of PDE2 mRNA was also downregulated in cells from patients with IPAH compared with cells from normal controls (Figure 7D). PDE2 activity in cells from IPAH patients was commensurate with that seen in the pulmonary arteries of animals with hypoxia-induced PH (Figure 7E); however, the PDE2 activity observed in pulmonary vascular smooth muscle cells from normal individuals was markedly lower than that in the pulmonary arteries from normoxic control animals, entailing that overall there is little or no reduction in PDE2 activity in PH patients. At the concentration used for in vitro evaluation, BAY 60-7550 caused a significant reduction in PDE activity in both rat pulmonary arteries and pulmonary vascular smooth muscle cells, confirming effective inhibition of PDE2 (Figure 7C and 7E).

PDE2 Inhibition Regulates the Proliferation of Pulmonary Artery Smooth Muscle Cells From Patients With IPAH
Because PH is characterized by dysregulated proliferation of pulmonary artery smooth muscle cells and to provide proof of concept in the human disease, we assessed the effect of PDE2 inhibition on growth of pulmonary artery smooth muscle cells from patients with IPAH. Compared with untreated control cells, proliferation of pulmonary artery smooth muscle cells from IPAH patients was significantly reduced by BAY 60-7550 (Figure 7F through 7H). This antiproliferative effect was enhanced by combination with ANP (Figure 7F), DETA-NONOate (Figure 7G), and treprostinil (Figure 7H). The effect of dual therapy was additive, if not synergistic, because treatment of cells with PDE2i/ANP or PDE2i/treprostinil caused a greater reduction in cell growth than PDE2 inhibition alone (Figure 7F and 7H).

Additive Beneficial Effect of PDE2i and PDE5i in PH
Because PDE5i are first-line therapy for PH, we investigated whether PDE2 inhibition sustains a therapeutic effect in the presence of PDE5i. In established PH, sildenafil alone did not cause a significant reduction in RVSP (Figure 8A), RVH...
Discussion

The strategy of promoting pulmonary cyclic nucleotide signaling, by both cGMP (eg, PDE5 inhibition, sGC stimulation) and cAMP (PGI analogues), is clinically effective in PH. Moreover, drug combinations that target both cyclic nucleotide systems have additive or synergistic effects to diminish disease severity. In addition to the clinical efficacy of PDE5i, blockade of additional PDEs has shown promise in experimental models of PH, including PDE1, PDE3, PDE4, and PDE10. In distinct contrast, there is a paucity of information regarding a role for PDE2 in pulmonary physiology and PH. As a therapeutic target in PH, PDE2 is particularly attractive because it metabolizes both cGMP and cAMP, implying that blockade of this enzyme will concomitantly promote signaling by both cyclic nucleotides. In accord with this hypothesis, herein we demonstrate that the selective PDE2 inhibitor BAY 60-7550 produces a prophylactic salutary activity in 2 preclinical models of PH and reverses multiple aspects of pathology in hypoxia-induced PH, affecting pulmonary vasoconstriction, remodeling, and RVH. These positive outcomes were augmented in the presence of interventions, including approved therapies for PH, that bolster cGMP (ie, sildenafil, inorganic nitrate) and cAMP signaling (ie, treprostinil).
Finally, inhibition of PDE2 prevents the hyperproliferative phenotype of pulmonary artery smooth muscle cells from patients with PAH.

Previous work exploring the contribution of PDE isoforms to pulmonary vascular physiology and PH-centric pathology has not advocated a major role for PDE2 based on expression levels and cyclic nucleotide turnover. This study took a functional approach to evaluate the capacity of "classic" cGMP-hydrolyzing PDEs (ie, PDEs 1–3 and PDE5) to modulate the reactivity of aorta and pulmonary arteries from healthy (normoxic) animals and in vitro studies using isolated vessels. As established in preclinical models, PDE5 inhibition augmented responses to NO and ANP in the pulmonary artery, a capacity that plays a role in the therapeutic efficacy of this drug class in PH. Notably, neither PDE1 nor PDE3 inhibition was able to recapitulate the vasodilator capacity of sildenafil, indicating that these PDEs do not play a functional role in regulating vascular cGMP turnover (at least in this model system). However, evidence to support our hypothesis that PDE2 plays an active role in pulmonary physiology and PH was provided by the observation that BAY 60-7550 enhanced the vasorelaxant responses to ANP in pulmonary vessels in tissues from both normoxic and hypoxic animals and increased responses to NO in tissues from normoxic rats. These data raise the possibility that in the hypoxic environment of PH, PDE2i increases pulmonary sensitivity to natriuretic peptides without promoting the vasorelaxant activity of cGMP in the systemic circulation, thereby bringing about a pulmonary-specific vasodilator activity. This observation dovetails well with previous reports intimating a role for PDE2 in acute hypoxic vasoconstriction but not in regulating peripheral vascular tone. Vascular reactivity studies also demonstrated that pulmonary PDE2 is involved in modulating cAMP signaling mechanisms, as we hypothesized on the basis of the dual substrate utilization of this enzyme. Augmentation of the vasorelaxant activity of treprostinil by BAY 60-7550 was only apparent under hypoxic conditions, implying that akin to cGMP signaling, PDE2i will selectively promote cAMP signaling (in the lung) in PH.

Having determined that PDE2i produced a pulmonary-selective effect of vascular function, we examined whether BAY 60-7550 is effective in 2 well-validated experimental models: hypoxia- and bleomycin-induced PH. Prophylactic administration of BAY 60-7550 resulted in a significantly less severe phenotype, with lower RVSP, reduced RVH, and fewer muscularized pulmonary arteries. Indeed, treatment with BAY 60-7550 returned these indices of disease severity to near normal values. Importantly, the MABP in animals receiving BAY 60-7550 was not altered, mirroring the pulmonary-selective profile of PDE2 inhibition in isolated arteries. Notably, BAY 60-7550 alone produced a less pronounced effect on the altered pulmonary hemodynamics and RVH in established PH, with a dose-response relationship shifted 10-fold. This reduced efficacy matches that of sildenafil in preclinical models of PH (shown herein and in Reference 26) and is commensurate with the small reduction in pulmonary artery pressure that sildenafil produces in PH patients. Only with the use of combined therapies, most effectively BAY 60-7550 plus aciditrol but also BAY 60-7550 plus sildenafil, inorganic nitrate, or treprostinil, was a significant salutary effect on RVSP and RVH evident. This advocates the use of such dual approaches in PH to optimize cyclic nucleotide signaling as a treatment strategy and improve on existing therapeutics on the basis of these mechanisms.

We determined that the efficacy of BAY 60-7550 was dependent on intact natriuretic peptide bioactivity, but not on NO-dependent signaling, because the salutary effects of PDE2 inhibition were absent in mice lacking NPR-A (the cognate receptor for ANP and B-type natriuretic peptide) but maintained in animals treated chronically with L-NAME. As a logical extension, we also established that pharmacological augmentation of natriuretic peptide levels, using the neutral endopeptidase inhibitor ecadotril, in combination with BAY 60-7550 caused an additive if not synergistic effect on both
the prevention and reversal of hypoxia-induced PH. The efficacy of the combination was dependent on cGMP production and was pulmonary selective, providing further evidence that PDE2 inhibition has its most pronounced effect on natriuretic peptides (ANP and BNP), and was pulmonary selective, providing further evidence that pulmonary vascular smooth muscle cells from normal individuals and patients with pulmonary arterial hypertension (n=5; D and E). Proliferation of human pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension in the absence (control; n=9) or presence of BAY 60-7550 (BAY; 1 µmol/L; n=9), atrial natriuretic peptide (ANP; 1 µmol/L; n=4), DETA-NONOate (DETA-NO; 10 µmol/L; n=9), treprostinil (T; 3 µmol/L; n=4), or combinations thereof (at the same concentrations; F through H). Data are shown as mean±SEM. Statistical analysis by unpaired Student t test (A through E) or 2-way ANOVA (F through H). *P<0.05, **P<0.01, ***P<0.001 vs control/normoxia/normal; #P<0.05, ##P<0.01 vs BAY 60-7550; #P<0.05 vs treprostinil. +ve indicates positive control.

Figure 7. Phosphodiesterase 2A (PDE2A) mRNA (A and D) and protein (B) expression and activity (defined as 5'-GMP formation inhibitable by BAY 60-7550 [1 µmol/L]; C and E) in isolated pulmonary arteries from normoxic (Nx) and hypoxic (Hx) rats (2 weeks of 10% O2; n=3–8; A through C) and pulmonary vascular smooth muscle cells from normal individuals and patients with pulmonary arterial hypertension (n=5; D and E). Proliferation of human pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension in the absence (control; n=9) or presence of BAY 60-7550 (BAY; 1 µmol/L; n=9), atrial natriuretic peptide (ANP; 1 µmol/L; n=4), DETA-NONOate (DETA-NO; 10 µmol/L; n=9), treprostinil (T; 3 µmol/L; n=4), or combinations thereof (at the same concentrations; F through H). Data are shown as mean±SEM. Statistical analysis by unpaired Student t test (A through E) or 2-way ANOVA (F through H). *P<0.05, **P<0.01, ***P<0.001 vs control/normoxia/normal; #P<0.05, ##P<0.01 vs BAY 60-7550; #P<0.05 vs treprostinil. +ve indicates positive control.

a PDE2-regulated pool. This also brings into the spotlight the additive activity between BAY 60-7550 and sildenafil revealed in the present study. This cooperative action was apparent at many different levels: pulmonary artery vasodilatation, RVH, and the proliferation of pulmonary vascular smooth muscle cells from PAH patients. However, a compounded effect was not observed in the periphery because MABP remained unchanged in the presence of both inhibitors. This cross talk between PDE2 and PDE5 therefore appears to be specific to the heart and pulmonary circulation. We conclude that increasing cellular cGMP levels by pharmacological blockade of either PDE2 or PDE5 results in activation of the alternate isozyme as a result of cGMP binding to the analogous N-terminal GAF domains both enzymes possess. Only with combined blockade of both PDE2 and PDE5 is it possible to optimize the beneficial effects of cGMP signaling. This finding is important from a therapeutic standpoint because sildenafil is a first-line treatment for PAH, and drug efficacy is likely to be limited by activation of PDE2. Thus, evidence that PDE2 inhibition can produce additional activity above and beyond PDE5 inhibition alone is encouraging and might explain why a significant
cohort of PAH patients do not respond well to sildenafil or experience a diminution of efficacy over time; coadministration of a PDE2 inhibitor to this population may be superior.

Despite similarities between the RV and LV with respect to spatially constrained cGMP signaling, an interesting dichotomy exists in terms of the pharmacodynamic effect of PDE2 inhibition. Recent work has revealed PDE2 to change its substrate profile in the LV on the basis of the dynamic levels of cGMP and cAMP. Thus, under physiological circumstances, PDE2 hydrolyzes natriuretic peptide-generated cGMP almost exclusively, whereas in the presence of β-adrenergic activation, PDE2 metabolizes predominantly cAMP, thereby augmenting adrenergic signaling. In the failing heart, PDE2 expression and activity are upregulated, and enzyme inhibition appears to be detrimental because the inotropic and chronotropic activity driven by cAMP is exacerbated. This is in distinct contrast to the present study, in which PDE2 inhibition is clearly beneficial in RVH associated with PH. The mechanisms underlying this RV-LV difference remain to be determined but might reside with the bioactivity of cAMP. Long-term potentiation of sympathetic, cAMP-dependent pathways (eg, β-agonists, PDE3 inhibitors) increases mortality in patients with left heart failure, whereas pharmacologically targeting cAMP signaling via the use of PGI₂ analogues offers a survival advantage in PAH patients. Therefore, it is possible that PDE2 inhibition is beneficial in PH because it slows the breakdown of natriuretic peptide–driven cytoprotective cGMP first and foremost but additionally reverses the cAMP signaling deficit (resulting from endothelial dysfunction and loss of PGI₂ functionality) to reduce pulmonary vascular resistance and remodeling, thereby exerting an indirect, beneficial effect on the RV. In addition, the RV possesses an inherently greater capacity, compared with the LV, to recover structure and function in the face of substantially reduced afterload (eg, pulmonary thromboendarterectomy in chronic thromboembolic PH patients versus valve replacement in individuals with aortic stenosis). Thus, despite the fact that β-blockers slow RV deterioration in PH, the clinical outcome of augmenting cAMP likely depends on the relative impact of the pharmacological intervention on the pulmonary circulation versus the RV. Interestingly, the effects of BAY 60-7550 in combination with treprostinil were more pronounced on RVSP compared with RVH in the preclinical model used herein, supporting a greater impact of cAMP elevation on the pulmonary circulation. This thesis also dovetails well with beneficial activity of inhibitors of PDE4, a cAMP-specific PDE, in preclinical models of PH and on the proliferation of pulmonary vascular smooth muscle cells from PH patients. Finally, the reported involvement of PDE2 in maintaining endothelial barrier function intimates that PDE2 inhibition is beneficial in PH because it slows the breakdown of natriuretic peptide–driven cytoprotective cGMP first and foremost but additionally reverses the cAMP signaling deficit (resulting from endothelial dysfunction and loss of PGI₂ functionality) to reduce pulmonary vascular resistance and remodeling, thereby exerting an indirect, beneficial effect on the RV.

This study also gleaned proof-of-concept data in human disease by establishing a potent antiproliferative effect of BAY 60-7550 in pulmonary vascular smooth muscle cells from patients with IPAH. In agreement with the in vitro vessel studies and experimental models of PH, the favorable activity of BAY 60-7550 was additive, if not synergistic, with NO.
natriuretic peptides, and treprostinil. Furthermore, we demonstrate that PDE2A mRNA and protein expression are reduced in pulmonary artery smooth muscle cells from PAH patients and pulmonary arteries from rats with hypoxia-induced PH. Importantly, however, a commensurate drop in PDE2 activity was not apparent. We conclude that this is indicative of an innate defense mechanism that helps to preserve cytoprotective cyclic nucleotide signaling in PH by reducing PDE2-mediated hydrolysis of cGMP (and cAMP) and maximizing the beneficial activity of NO, natriuretic peptides, and PGI2. The sharp reduction in mRNA and protein expression only results in a subtle drop in enzyme activity, probably the result of the higher tissue cGMP background (resulting from the increased natriuretic peptide expression and bioactivity associated with PH) that activates PDE2 via interaction with its GAF-B domain. Regardless, this is in marked contrast to other PDE isoforms (e.g., PDE1, PDE3, PDE5, PDE10),15–20 which have been reported to be upregulated in PH.

In sum, this study provides convincing evidence in vitro and in vivo of the therapeutic potential of PDE2 inhibition in PH. The beneficial effect of PDE2i is dependent on endogenous natriuretic peptide bioactivity but also produces additive effects with existing therapies, including PDE5i, PGI2, and NO. The double-pronged mechanism of action inherent to PDE2 inhibition (ie, promoting cGMP and cAMP signaling) is unique in terms of existing therapy for PH, which targets one or another cyclic nucleotide transduction system (ie, PGI2, analogues, PDE5i) and therefore holds a theoretical advantage in treating the disease.

Sources of Funding
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Disclosures
Dr Clapp has received research grants from United Therapeutics and acted as a consultant. Dr Hobs has acted as a consultant/advisory board member for Bayer AG, Novartis, Merck, and Palatin Technologies. The other authors report no conflicts.

References
PDE2 Inhibition in Pulmonary Hypertension

The cyclic nucleotide second messengers cGMP and cAMP play key roles in maintaining cardiopulmonary hemodynamics and integrity. A family of phosphodiesterase (PDE) enzymes inactivates cGMP and/or cAMP, thereby dynamically regulating signaling in a temporal and spatial manner. Pulmonary hypertension (PH) is characterized by a deficit in cyclic nucleotide signaling, in part caused by loss of the endothelium-derived, cytoprotective mediators nitric oxide and prostacyclin, which utilize cGMP and cAMP, respectively, to preserve pulmonary vascular structure and function. One mechanism underpinning loss of cGMP signaling, in particular, is thought to be dysregulated PDE activity. This is best illustrated by the upregulation of PDE5 expression and activity in PH and the beneficial effects of PDE5 inhibitors (eg, sildenafil) in treating the disease. Herein, we provide evidence that another PDE isozyme, PDE2, is critical to cardiopulmonary homeostasis and that selective pharmacological inhibition of this enzyme reverses the development of PH in preclinical models and dampens the hyperproliferative phenotype of pulmonary vascular smooth muscle cells from PH patients. These data identify PDE2 as a novel therapeutic target to treat PH that appears to possess the advantage (in comparison with PDE5 inhibitors) of augmenting both cGMP and cAMP signaling. Furthermore, inhibition of PDE2 provides additional benefit in conjunction with existing medicines that bolster pulmonary cyclic nucleotide bioactivity (eg, sildenafil, treprostinil), implying that further therapeutic gain is possible by optimizing current medicines enhancing cGMP or cAMP bioactivity.
Inhibition of Phosphodiesterase 2 Augments cGMP and cAMP Signaling to Ameliorate Pulmonary Hypertension
Kristen J. Bubb, Sarah L. Trinder, Reshma S. Baliga, Jigisha Patel, Lucie H. Clapp, Raymond J. MacAllister and Adrian J. Hobbs

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SUPPLEMENTAL MATERIAL
Supplemental Methods

Materials

Spermine-NONOate and DETA-NONOate were purchased from Calbiochem (Nottingham, U.K). N\textomega\textsuperscript{-}nitro-L-arginine methyl ester hydrochloride (L-NAME), potassium nitrate, acetylcholine (ACh), rat ANP, carboxymethylcellulose sodium salt (CMC) and polyethylene glycol (PEG) were obtained from Sigma-Aldrich (Dorset, UK). 9, 11-dideoxy-11\textalpha, \alpha\textsuperscript{-}epoxymethano-prostaglandin F\textsubscript{2\alpha} (U46619) was purchased from Affiniti (Exeter, U.K). Sildenafil was extracted from proprietary tablets obtained from the Pharmacy at University College London Hospital. BAY 60-7550 and ecadotril were the kind gift of Dr. Johannes-Peter Stasch (Bayer AG, Wuppertal, Germany). All drugs used for isolated vessel or cell culture were dissolved in distilled water apart from BAY 60-7550 which was dissolved in dimethyl sulfoxide (DMSO, final concentration no more than 0.01\%). For in vivo experiments, BAY 60-7550 and ecadotril were made into suspension in 0.5\% CMC + 10\% PEG and administered via oral gavage. Nitrate, L-NAME and sildenafil were dissolved in distilled water and were administered to mice in their drinking water. All treatments in the drinking water were made fresh on alternate days to a concentration adjusted for fluctuating water intake. Treprostinil (provided by United Therapeutics, Silver Spring, MD) was dissolved in sterile saline and administered subcutaneously via Azlet\textregistered osmotic minipumps (model 1004, Durect Corporation, CA, USA) at a rate of 0.11 \mu l/hour. Table 1 lists all drug doses.

Morphological analysis

Transverse formalin fixed lung sections were stained with van Gieson’s elastic (EVG) method. Pulmonary arterial muscularisation was then assessed as previously
described \(^1\). Briefly, total vessels $<100$ µm were counted in each lung section, and
defined according to degree of muscularisation; fully muscularised (two distinct and
continuous elastic lamina), partially muscularised (second elastic lamina not
continuous [$<50\%$]) and non muscularised (single elastic lamina). At least 150
vessels were counted per section and the proportion of vessels in each category was
expressed as a percentage of total vessels counted. Morphometric analysis was
carried out by two independent blinded examiners, with an inter-person variability of
$<10\%$. Twenty five muscularised arteries ranging from 25-100 µm from different
fields were then imaged at 400x magnification by light microscopy from
representative animals in each group.

RT-PCR

Tissue lysate and cells were homogenized using QIAshredder technology and RNA
was extracted using a standard extraction kit (Qiagen, UK). RNA was quantified
using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA) and converted
to cDNA by reverse transcription (Quantitect Reverse Transcription kit, Qiagen, UK).
Primers for PDE2A (forward 5’ ATCTTTGCTTGTATTATTTCTG 3’, reverse 5’
CAGCCACGACAGATTTCG 3’, 300 nmol/L) \(^2\) and housekeeping gene 18S (forward
5’ GTAAACCCTGTGTTGTTTCTTCTG 3’, reverse 5’ CCATCCATCGGTAGTACG 3’,
300 nmol/L) were added to cDNA template and SyBr Green quantitative PCR mix
(Quantitect Sybr green kit, Qiagen, UK). 20 ng of cDNA from each sample was
amplified using quantitative real-time PCR over 40 cycles. mRNA expression was
analyzed by expressing the cycle threshold (Ct) value as $2^{\Delta\Delta Ct}$ \(^3\).
### Supplementary Table 1. In vivo model treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle (0.5% carboxycellulose +10% polyethylene glycol)</td>
<td>10 ml/kg/day</td>
<td>Oral, gavage</td>
</tr>
<tr>
<td>2</td>
<td>BAY 60-7550</td>
<td>10 mg/kg/day</td>
<td>Oral, gavage</td>
</tr>
<tr>
<td>3</td>
<td>Ecadotril</td>
<td>60 mg/kg/day</td>
<td>Oral, gavage</td>
</tr>
<tr>
<td>4</td>
<td>BAY 60-7550 + Ecadotril</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>5</td>
<td>L-NAME</td>
<td>100 mg/kg/day</td>
<td>Oral, drinking water</td>
</tr>
<tr>
<td>6</td>
<td>L-NAME + BAY 60-7550</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>7</td>
<td>Nitrate</td>
<td>150 mg/kg/day</td>
<td>Oral, drinking water</td>
</tr>
<tr>
<td>8</td>
<td>Nitrate + BAY 60-7550</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>9</td>
<td>Sildenafil</td>
<td>30 mg/kg/day</td>
<td>Oral, drinking water</td>
</tr>
<tr>
<td>10</td>
<td>Sildenafil + BAY 60-7550</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>11</td>
<td>Treprostinil</td>
<td>20.18 µg/kg/day</td>
<td>Subcutaneous (osmotic mini-pump)</td>
</tr>
<tr>
<td>12</td>
<td>Treprostinil + BAY 60-7550</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>13</td>
<td>BAY 60-7550</td>
<td>30 mg/kg/day</td>
<td>Oral, gavage</td>
</tr>
<tr>
<td>14</td>
<td>BAY 60-7550</td>
<td>100 mg/kg/day</td>
<td>Oral, gavage</td>
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</table>
**Supplementary Table 2.** The effect of PDE1, PDE2, PDE3 & PDE5 inhibition on vascular function in rodent isolated arteries

<table>
<thead>
<tr>
<th></th>
<th>AORTA</th>
<th>PULMONARY ARTERY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td></td>
<td>( pEC_{50} ) (mol/L)</td>
<td>( E_{\text{max}} ) (%)</td>
</tr>
<tr>
<td><strong>ANP Control</strong></td>
<td>8.56 ±0.24</td>
<td>91.83 ±12.86</td>
</tr>
<tr>
<td><strong>ANP + vinpocetine</strong></td>
<td>8.83 ±0.13</td>
<td>92.42 ±11.78</td>
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<tr>
<td>(30 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANP + BAY 60-7550</strong></td>
<td>8.74 ±0.04</td>
<td>95.29 ±10.90</td>
</tr>
<tr>
<td>(0.1 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANP + milrinone</strong></td>
<td>8.56 ±0.36</td>
<td>85.13 ±10.08</td>
</tr>
<tr>
<td>(10 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANP + sildenafil</strong></td>
<td>8.51 ±0.17</td>
<td>94.77 ±6.19</td>
</tr>
<tr>
<td>(3 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-NO Control</strong></td>
<td>7.00 ±0.18</td>
<td>99.10 ±10.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-NO + vinpocetine</strong></td>
<td>7.45 ±0.13</td>
<td>98.69 ±10.26</td>
</tr>
<tr>
<td>(30 µmol/L)</td>
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<td></td>
</tr>
<tr>
<td><strong>S-NO + BAY 60-7550</strong></td>
<td>***8.48 ±0.71</td>
<td>94.38 ±14.91</td>
</tr>
<tr>
<td>(0.1 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-NO + milrinone</strong></td>
<td>6.54 ±0.33</td>
<td>93.19 ±10.15</td>
</tr>
<tr>
<td>(10 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-NO + sildenafil</strong></td>
<td>***7.61 ±0.10</td>
<td>99.30 ±3.82</td>
</tr>
<tr>
<td>(3 µmol/L)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( n=5\text{-}12 \) vessels for each condition. \( ***P<0.001 \) v Control by one-way analysis of variance with repeated measures.
Supplemental Figure Legends

**Figure S1. Confirmation of a PH phenotype in hypoxic rats.** Right ventricle/left ventricle+septum ratio (RV/(LV+S); A; Nx n=4, Hx n=10), endothelial function in isolated pulmonary arteries (B, Nx n=10, Hx n=10) and aortae (C, Nx n=4, Hx n=7) as assessed by acetylcholine-evoked relaxation (10 μmol/l). Data are shown as mean ± SEM. Statistical analysis by unpaired Student’s t-test. ***P<0.001 versus Nx.

**Figure S2. The effect of PDE5 inhibition on vascular function in rodent isolated arteries.** Concentration-dependent relaxation to atrial natriuretic peptide (ANP; n=4-9) or spermine-NONOate (S-NO; n=4-10) in the absence (Control) or presence of the PDE5 inhibitor, sildenafil (3 μmol/l) in aorta (A, B, E, F) or pulmonary arteries (C, D, G, H) from normoxic (Nx; A, C, E, G) or hypoxic (Hx; 2 weeks 10% O₂; B, D, F, H) rats. Data are shown as mean ± SEM. Statistical analysis by two-way analysis of variance with repeated measures. *P<0.05, **P<0.01, ***P<0.01 Hx vs Nx.
Supplemental References


Supplemental Figure S1
Supplemental Figure S2