Antiremodeling Effects of Iloprost and the Dual-Selective Phosphodiesterase 3/4 Inhibitor Tolafentrine in Chronic Experimental Pulmonary Hypertension

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Abstract—Severe pulmonary hypertension is a disabling disease with high mortality. We investigated acute and chronic effects of iloprost, a long-acting prostacyclin analogue, and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in monocrotaline-induced pulmonary hypertension in rats. Twenty-eight and 42 days after administration of the alkaloid, right ventricular systolic pressure increased from 25.8 ± 2.0 to 62.9 ± 3.4 and 70.5 ± 7.4 mm Hg, with concomitant decline in cardiac index, central venous oxygen saturation, and arterial oxygenation. Marked right heart hypertrophy was demonstrated by the strongly elevated ratio of right ventricle/left ventricle plus septum weight, and massive thickening of the precapillary artery smooth muscle layer was shown histologically. Western blot analysis demonstrated increased levels of matrix metalloproteinases (MMPs) -2 and -9 and increased gelatinolytic activities in isolated pulmonary arteries. In these animals, both intravenous iloprost and tolafentrine displayed characteristic features of pulmonary vasodilators. When chronically infused from days 14 to 28, both agents significantly attenuated all monocrotaline-induced hemodynamic and gas exchange abnormalities as well as right heart hypertrophy. Full normalization of all variables including right ventricle size was achieved on combined administration of both agents during this period. This was also true for MMP-2 and MMP-9 expression and activity. Moreover, when iloprost plus tolafentrine was used for late therapeutic intervention, with infusion from days 28 to 42 after full establishment of severe pulmonary hypertension and cor pulmonale, hemodynamic, gas exchange, and cardiac and pulmonary vascular remodeling changes were significantly reversed. We conclude that the combined administration of iloprost and a dual-selective phosphodiesterase 3/4 inhibitor prevents and reverses the development of pulmonary hypertension and cor pulmonale in response to monocrotaline in rats. This regimen may therefore offer a possible antiremodeling therapy in severe pulmonary hypertension. (Circ Res. 2004;94:1101-1108.)

Key Words: iloprost ■ pulmonary hypertension ■ monocrotaline ■ phosphodiesterase ■ tolafentrine

Pulmonary arterial hypertension (PAH) is a severe disabling disease characterized by elevation of pulmonary artery pressure and death attributable to circulatory failure.1,2 Predominant features of the pathology of PAH include intimal lesions, medial hypertrophy, and adventitial thickening of precapillary pulmonary arteries and right ventricular hypertrophy. Imbalances of vasodilatory and vasoconstrictor agents have been implicated in both the predominance of increased vasomotor tone and the chronic remodeling of resistance vessels, including vascular smooth muscle cell growth. In patients with primary pulmonary hypertension, a reduced excretion of prostaglandin and an enhanced excretion of thromboxane metabolites has been noted.3 Moreover, enhanced activities of phosphodiesterases (PDEs), which hydrolyze the prostaglandin- and NO-induced second messengers cAMP and cGMP, were observed in experimental conditions of pulmonary hypertension.4

Recently, we showed that PDE3 and PDE4 inhibitors promote acute pulmonary vasodilation in experimental models of pulmonary hypertension.5–8 When combined with iloprost, a stable prostacyclin analogue, an augmented pulmonary vasodilator response was noted both in these experimental models and on catheter testing in patients with severe pulmonary hypertension.9 The dual-selective PDE3/4 inhibitor tolafentrine was particularly potent in this respect.

We now expand this concept to address the question of whether suitably combined iloprost and PDE3/4 inhibition might not only cause acute vasodilation but exert antiremodeling effects on long-term use in chronic experimental hypertension. To this end, the model of monocrotaline (MCT)-
induced pulmonary hypertension was used. MCT is a toxin derived from plants of the Crotalaria species, which, after one injection in rats, causes pulmonary arterial endothelial cell injury and subsequent pulmonary artery smooth muscle hypertrophy with persistent severe pulmonary hypertension. We examined (1) the acute pulmonary vasodilatory efficacy of iloprost and tolafentrine in this model, (2) the antiremodeling effects of long-term infusion of iloprost, tolafentrine, or both agents, and (3) the expression and activity of matrix metalloproteinases (MMPs), reflecting vascular wall remodeling, in nontreated and treated lung tissue. To mimic clinical conditions, infusion of these agents was commenced during development of pulmonary hypertension and after pulmonary hypertension had already been fully established. In essence, antiremodeling effects were demonstrated for both iloprost and tolafentrine, being particular impressive for the combined administration of the prostanooid and the dual-selective PDE3/4 inhibitor.

Materials and Methods

MCT Treatment

The alkaloid MCT (Sigma) was dissolved in 0.5 N HCl, and the pH was adjusted to 7.4 with 0.5 N NaOH. The MCT solution was given as a single subcutaneous injection (60 mg/kg) to male Sprague-Dawley rats (Charles River, Sulzfeld, Germany). Control rats received an equal volume of isotonic saline. The experiments were performed according to institutional guidelines for the use and care of animals.

Surgical Preparation and Tissue Preparation

Two or 4 weeks after MCT injection, rats were subjected to the implantation of osmotic minipumps (Alzet Model 2ML2, Durect). Animals were anesthetized with fentanyl and medetomidine (300 and 300 µg/kg IP), and the minipump was implanted in the dorsal subcutaneous region under sterile techniques. A tunneled catheter (PE 50 tubing) was inserted into the left jugular vein, and the wounds were closed with sutures. Anesthesia was antagonized by an intra-artificial, Harvard Apparatus GmbH). Briefly, a thermistor (1.5F) was referenced at the hilum. Cardiac output (CO) was measured by a transpulmonary thermodilution CO. A 0.15-mL bolus of room-temperature saline was injected into the right ventricle for measurement of right ventricular systolic pressure (RVPsys, in mm Hg), systemic arterial pressure (SAP, in mm Hg), cardiac index (CI, in mL/min per 100 g BW), and RV/LV+S are given. Iloprost, tolafentrine, or both agents were applied as continuous intravenous infusion by osmotic minipumps from days 14 to 28 (MCT [28d]/Ilo [n=8], MCT [28d]/Ilo/Tola [n=8] or from days 28 to 42 (MCT [42d]/Ilo [n=8], MCT [28d]/Ilo/Tola [n=8] and MCT [28d]/Ilo/Tola [n=10]) or from days 28 to 42 (MCT [42d]/Ilo/Tola [n=7]). Control animals received sham injection of saline (n=9). Control; †P<0.05 vs MCT [28d]; ‡P<0.05 vs MCT [28d]/Ilo; #P<0.05 vs MCT [42d].

Paraffin Embedding and Microscopy

Fixation was performed by immersion of the lungs in a 3% paraformaldehyde solution. For paraffin embedding, the entire lungs were dissected in tissue blocks from all lobes. Sectioning at 10 µm was performed from all paraffin-embedded blocks. H&E and elastica staining was performed according to common histopathological procedures. Light microscopic slides were analyzed in a blind fashion without knowledge of treatment groups. In each rat, 30 to 40 intra-acinar arteries were categorized as muscular (ie, with a complete medial coat of muscle), partially muscular (ie, with only a crescent of muscle), or nonmuscular (ie, with no apparent muscle), as reported. The ratio of alveoli to pulmonary arteries was determined, as described previously.

Western Blot Assay

Frozen pulmonary arteries were homogenized in Lysis buffer containing 50 mmol/L Tris-HCl, pH 7.6, 10 mmol/L CaCl2, 150 mmol/L NaCl, 60 mmol/L NaN3, and 0.1% wt/vol Triton X-100 using a tissue homogenizer. Samples were centrifuged at 13 000 rpm for 30

![Figure 1. Influence of long-term treatment with intravenous iloprost (Ilo) and tolafentrine (Tola) on hemodynamics and right heart hypertrophy in MCT-induced pulmonary hypertension. Animals were investigated 14 days (MCT [14d]; n=8), 28 days (MCT [28d]; n=8), or 42 days (MCT [42d]; n=8) after MCT treatment. Right ventricular systolic pressure (RVPsys, in mm Hg), systemic arterial pressure (SAP, in mm Hg), cardiac index (CI, in mL/min per 100 g BW), and RV/LV+S are given. Iloprost, tolafentrine, or both agents were applied as continuous intravenous infusion by osmotic minipumps from days 14 to 28 (MCT [28d]/Ilo [n=8], MCT [28d]/Ilo/Tola [n=8], and MCT [28d]/Ilo/Tola [n=10]) or from days 28 to 42 (MCT [42d]/Ilo/Tola [n=7]). Control animals received sham injection of saline (n=9). Control; †P<0.05 vs MCT [28d]; ‡P<0.05 vs MCT [28d]/Ilo; #P<0.05 vs MCT [42d].]
minutes. The supernatants were measured for protein content using Dye Reagent Concentrate (Bio-Rad). Extracts containing equal amounts of protein (10 μg for MMP-2 and 30 μg for MMP-9) were denatured by boiling for 10 minutes in Laemmli’s buffer containing β-mercaptoethanol and separated on 7.5% SDS-polyacrylamide gels at 100 V. The separated proteins were blotted on polyvinylidene fluoride membrane with a semidry transfer unit at 100 mA for 2 hours. The blots were blocked and developed with 0.3 and 0.2 μg/mL of rabbit polyclonal IgG antibodies for specific rat MMP-2 and MMP-9 and a 1/7500 dilution of horse radish peroxidase–labeled goat anti-rabbit IgG (Abcam Ltd, Cambridge, UK). The bands were visualized using an enhanced chemiluminescence detection system (Amersham) and quantified by densitometry. The antibodies were raised against the catalytic domains of the proteins and thus detect both the pro and active forms of MMPs. Samples of pulmonary arteries were normalized to GAPDH.

**Gelatin Zymography**

Pulmonary arteries were homogenized at 4°C in buffer containing 1% Triton X-100, 150 mmol/L NaCl, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 10 μmol/L EDTA, and 20 mmol/L Tris-HCl, pH 7.5, with 20 mg/mL CaCl₂. The gel was kept in fixative solution containing 40% methanol and 7% acetic acid for 1 hour. After this, the gel was washed twice with buffer volume. Activity of MMPs was analyzed by SDS-PAGE zymography, in which the enzymes hydrolyze gelatin substrate present in the gel and form a clear band. The samples, under nonreducing conditions, were electrophoresed through a 10% polyacrylamide gel copolymerized with gelatin (1 mg/mL) at 4°C. Shortly after electrophoresis, the gel was incubated for 1 hour at 25°C in 2.5% Triton X-100 solution. The gel was then washed twice with water, each for 20 minutes, before incubating it overnight at 37°C in 0.05 mol/L Tris-HCl buffer, pH 8.0, containing 5 mmol/L CaCl₂. The gel was kept in fixative solution containing 40% methanol and 7% acetic acid for 1 hour. After electrophoresis, the gel was incubated for 1 hour at 25°C in 2.5% Triton X-100 solution. The gel was then washed twice with water, each for 20 minutes, before incubating it overnight at 37°C in 0.05 mol/L Tris-HCl buffer, pH 8.0, containing 5 mmol/L CaCl₂. The gel was kept in fixative solution containing 40% methanol and 7% acetic acid for 1 hour. After this, the gel was stained with 0.25% Coomassie brilliant blue R250 dye for at least 1 hour and then destained in a solution of 10% methanol and 7% acetic acid. Gels were visualized with a Biodoc analyzer (Whatman Biometra), in which activity of MMP-2 and MMP-9 was shown as a clear white band against the blue background.

**Experimental Protocols**

In the first set of experiments, the acute hemodynamic effects of iloprost and tolafentrine were investigated in rats that had developed MCT-induced pulmonary hypertension. MCT-treated rats (28 days) were prepared as described, and either iloprost or tolafentrine or vehicle was applied as short-term infusion (60 minutes). Hemodynamics were monitored at the end of the infusion period. The following doses were applied: 1.6, 8, 16, and 80 ng/kg per minute iloprost and 0.62, 3.2, 6.2, and 20 μg/kg per minute tolafentrine.

For chronic intervention studies, rats were randomized to receive either saline, iloprost, tolafentrine, a combination of iloprost and tolafentrine, or vehicle by osmotic minipumps. The following six groups were studied, four with early intervention or sham intervention and two with late intervention or sham intervention: group ILO₂₅₅ (8.3 ng/kg per minute from day 14 to day 28), group Tola/ILO₂₅₅ (625 ng/kg per minute), group Tola/ILO₁₄₂₈ (625/8.3 ng/kg per minute), group Tola/ILO₂₅₅ (625/8.3 ng/kg per minute from day 28 to day 42), group saline/ILO₂₅₅ (5 μL/h), and group saline/ILO₂₅₅ (5 μL/h). The doses of iloprost and tolafentrine were chosen according to preceding pilot experiments, addressing long-term tolerability of the agents under investigation.

**Data Analysis**

All data are given as mean±SEM. Differences between groups were assessed by ANOVA and Student’s Newman-Keuls post hoc test for multiple comparisons, with P<0.05 regarded as significant.

**Results**

**MCT-Treated (14-, 28-, and 42-Day) Rats**

A single injection of MCT resulted in significant increase in right ventricular systolic pressure on day 28 (62.9±3.4 mm Hg, n=8), with additional increase on day 42 (70.5±7.4 mm Hg, n=8) compared with saline-injected control animals (25.5±0.0 mm Hg, n=9) (Figure 1). This was paralleled by right ventricular hypertrophy, with the RV/LV+S ratio increasing from 0.29 to 0.52±0.03 (28 days) and 0.71±0.07 (42 days), respectively. Compared with control animals (37.0±4.1 mL/min per 100 g BW), cardiac index was significantly decreased on day 28 (28.9±2.4 mL/min per 100 g BW) and day 42 (29.5±1.8 mL/min per 100 g BW). No changes in systemic arterial pressure occurred. When analyzed after 14 days of MCT treatment, the same tendency was noted, but changes in hemodynamics and RV/LV ratio did not differ significantly from control lungs. Both arterial PO₂ and central venous oxygen saturation (SvO₂) decreased on MCT treatment (Figure 2). The MCT-treated groups had significantly lower BW than timematched control animals, and 100% (8 of 8), 80% (8 of 10), and 60% (8 of 13) animals survived the 14-, 28-, and 42-day MCT treatment. The hemodynamic changes were accompanied by significant media hypertrophia of pulmonary vessels, as depicted in Figure 3 (42 days after injection of MCT). Treatment with MCT was associated with a significant increase in distal pulmonary artery muscle (Figure 4A) and a reduction in the number of peripheral pulmonary arteries (Figure 4B).
protein expression of gelatinases MMP-2 and MMP-9 in pulmonary artery was detected (Figure 5A) compared with control animals. As shown in Figure 5A, there was a time-dependent increase in MMP-2 and MMP-9 expression in pulmonary arteries of rats after 2, 4, and 6 weeks of treatment with MCT. Activity was monitored by zymography and showed highly elevated gelatinolytic capacities, with MMP-2 as major fraction (Figure 6). MMP-2 activity from MCT-treated rats was seen as triple bands, with molecular weight values of 66, 62, and 59 kDa, with 62 kDa being the predominant form, as previously described.14,15

**Immediate Vasodilatory Effect of Iloprost and Tolafentrine in Rats With MCT-Induced Pulmonary Hypertension**

Both intravenous iloprost and intravenous tolafentrine reduced right ventricular systolic pressure in a dose-dependent manner (Figure 7). Pulmonary vasodilatation was accompanied by a decrease in systemic arterial pressure and an increase in cardiac index.

**Long-Term Treatment With Iloprost or Tolafentrine**

**Treatment From Days 14 to 28**

All treatment groups had significantly lower RVSP values than MCT-treated animals with saline infusion (Figure 1). Intravenous iloprost decreased RVSP to 42.4±3.1 mm Hg and tolafentrine to 37.6±3.0 mm Hg compared with the MCT 28-day group. The most effective approach was the combination of iloprost and tolafentrine, which resulted in complete normalization of RVSP (25±1 mm Hg) with even increased cardiac index. This was also true for right heart hypertrophy, which decreased in response to both iloprost and tolafentrine treatment (ILO14-28 0.37±0.03 [n=8]; TOLA14-28 0.38±0.04 [n=9]) and was fully normalized in response to combined treatment (Tola/ILO 14-28 0.29±0.01 [n=10]). Cardiac index increased significantly in the tolafentrine-treated groups, even surpassing normal values (TOLA14-28 42.2±4.2 mL/min per 100 g BW; Tola/ILO 14-28 42.0±4.1 mL/min per 100 g BW). Systemic arterial pressure did not change significantly. The decrease in arterial PO2 in response to MCT treatment was fully normalized in the iloprost/tolafentrine-treated animals (Figure 2). A total of 80%, 90%, and 100% of animals survived the 28-day period in the iloprost, tolafentrine, and combined-therapy groups. Protein content of MMP-2 and MMP-9, as well as gelatinolytic activities, decreased in the treatment groups, most impressively in the Tola/ILO 14-28 group, in which normal values were reached for the MMP-2
and MMP-9 expression (Figure 5B). As depicted in Figure 4A, a significant decrease in the presence of muscularized peripheral pulmonary arteries was achieved by all treatment protocols. This was accompanied by a normalization of the ratio of alveoli to pulmonary artery, indicating an attenuation of the MCT-induced loss of vessels (Figure 4B).

**Treatment From Days 28 to 42**

The combined infusion of tolafentrine and iloprost during this late period decreased RVSP significantly to 50.6±2.3 mm Hg (control, 70 mm Hg; P<0.05; n=7) and increased cardiac index from 29.5±2.8 to 46.9±8.2 mL/min per 100 g BW (P<0.05). In addition, the RV/LV+S ratio was significantly reduced by this treatment. As shown in Figure 3, there was a marked reduction of vascular remodeling in MCT rats undergoing late iloprost/tolafentrine treatment, both in comparison with MCT (28-day) control animals (onset of treatment) and with MCT (42-day) control animals (end of experiments). Compared with MCT-treated rats, the number of fully muscularized distal pulmonary arteries was markedly reduced in iloprost/tolafentrine-treated animals (Figure 4A). Moreover, the ratio of alveoli to pulmonary artery decreased, suggesting the presence of new vessel development (Figure 4B). This was accompanied by a decrease in MMP-2 and MMP-9 protein content and gelatinolytic activity for MMP-2 (Figures 5C and 6). No changes in arterial blood gases occurred (Figure 2). Seventy percent of the animals survived the 42-day period in the combined-treatment group.

**Discussion**

In this study, we demonstrated that the prostacyclin analogue iloprost, the phosphodiesterase 3/4 inhibitor tolafentrine, and a combination of both agents attenuated and reversed development of pulmonary hypertension and right heart hypertrophy in MCT-treated rats. Two time schedules of intervention were used in these experiments. When administered from days 14 to 28 after MCT application, efficacy of both single agents was noted and, most impressively, combined administration of iloprost and tolafentrine fully normalized the hemodynamic changes and completely prevented right heart hypertrophy. Moreover, this combined regimen significantly reversed the extent of pulmonary circulatory abnormalities when administered after full establishment of pulmonary
hypertension, from days 28 to 42, in MCT rats that were already severely ill.

MCT has repeatedly been used for induction of chronic pulmonary hypertension in rats, a species suitable for testing acute hemodynamic and gas exchange effects of vasodilators and chronic antiremodeling effects of antiinflammatory and antiproliferative agents. As anticipated, well-reproducible induction of severe, progressive pulmonary hypertension was also noted in the present investigation, indicated by a dramatic increase in RVSP, a subsequent decrease in cardiac index and \( \text{SvO}_2 \) values, and impressive right heart hypertrophy. Moreover, a decrease in arterial oxygenation was noted, and the animals experienced substantial weight loss because of the severity of the disease. The critical loss of cross-sectional area of the precapillary resistance vessels is reflected by histological and morphometric demonstration of marked media hypertrophy in these vessels.

When assessing acute hemodynamic effects of iloprost and tolafentrine in animals with established MCT-induced pulmonary hypertension, both substances displayed characteristic features of pulmonary vasodilators. Because of the intravenous mode of administration, the RVSP reduction was accompanied by a corresponding decrease in systemic arterial pressure, whereas cardiac index increased, indicating reduction of pulmonary and systemic resistances. Interestingly, maximum reduction of peripheral vascular resistance index (estimated by decrease in RVSP and increase in cardiac index) caused by tolafentrine (~35%) slightly surpassed that elicited by iloprost (~20%). This finding supports recent experimental and clinical findings that this dual-selective PDE3/4 inhibitor is a potent vasodilator agent in lung circulation under conditions of pulmonary hypertension.

Extending these observations on acute vasodilatory effects, long-term infusion of both iloprost and tolafentrine was performed in MCT-treated rats. In contrast to previous investigations, which investigated the influence of an endothelin antagonist, prostaglandin E\(_1\), or a PDE 5 inhibitor by coapplication with MCT, we started therapeutic interventions when the development of pulmonary hypertension had already commenced, from weeks 2 to 4, by implantation of osmotic minipumps to deliver agents intravenously. Under these conditions, both iloprost and tolafentrine markedly attenuated the degree of pulmonary hypertension evolving in response to pyrrolizidine. This was true for systolic pulmonary artery pressure, consecutive decline of cardiac index and \( \text{SvO}_2 \) values, and right heart hypertrophy. The profile of changes induced by iloprost and tolafentrine was largely superimposable. However, the PDE3/4 inhibitor caused a more prominent increase in cardiac index, with data surpassing even normal values. This observation is well in line with the previous finding that tolafentrine markedly increases CO on short-term infusion in anesthetized rabbits with U46619-induced pulmonary hypertension.

The most impressive effect was caused by combination of iloprost and tolafentrine. When administered from days 14 to 28, this intervention fully normalized pulmonary artery pressure and completely prevented right heart hypertrophy and pulmonary vascular remodeling. For iloprost, such reasoning is well in line with its antithrombotic effects and with in vitro findings of an antiproliferative effect on pulmonary

![Figure 6. MMP-2 activity in pulmonary arteries. Homogenates of pulmonary arteries were separated on a polyacrylamide gel copolymerized with gelatin and examined for gelatinolytic activity. Equal loading was checked by measuring protein levels before the gel was loaded. Shown is MMP-2 activity in the staging groups (A) and early (B) and rescue (C) treatment groups. Activity assays are representative of n=4 gels for each group, showing identical results. *P<0.05 vs control; ***P<0.001 vs control; ††P<0.01 vs MCT [28d] or MCT [42d].](image-url)
Tolafentrine administration was also demonstrated when these two agents were used as rescue therapy after full establishment of MCT-induced pulmonary hypertension (days 28 to 42). Late-onset iloprost/tolafentrine treatment apparently reversed many of the MCT-induced changes. At the end of the observation period (42 days), systolic pulmonary artery pressure was not only lower than that in the 42-day control group but also ranged below that in the 28-day control group, with the onset of late therapeutic intervention. This observation again supports the view that the combined administration of a prostanoid and a corresponding PDE3/4 inhibitor does possess strong antiproliferative effects in the pulmonary circulation. The reduction in the number of peripheral arteries in MCT-treated animals reflects endothelial injury and occlusion of peripheral vessels, and the significant increase in vascularization by iloprost and tolafentrine may result from vascular endothelial growth factor, which is known to be induced by cAMP-increasing agents. The role of cAMP is additionally supported by measurements of the expression of the MMP-2 and MMP-9, which are 4- to 5-fold increased in pulmonary arteries of MCT-treated rats, indicating vascular remodeling processes. Both MMPs are markedly reduced by the different treatment regimens. Most impressive is the decrease of MMP-2 activity in the rescue therapy group from week 4 to 6. Both MMP-2 and MMP-9 are regulated by intracellular cAMP levels, and it has recently been shown that prostaglandin E2 and dibutyryl-cAMP suppress membrane type 1 matrix MMP, an activator of MMP-2. Another possibility that may underlie reduced activity of MMP-2 is an upregulation of tissue inhibitors of MMPs, which are also regulated by cAMP.

In conclusion, intravenous administration of iloprost and the dual-selective PDE3/4 inhibitor tolafentrine caused dose-dependent pulmonary vasodilation in MCT-induced pulmonary hypertension in rats. When using these agents for chronic infusion, development of vascular remodeling and right heart hypertrophy in response to MCT was attenuated, with preservation of gas exchange. Full suppression of any MCT-induced changes was noted on combined administration of iloprost and tolafentrine. Most impressively, this combined regimen was even effective to reverse the MCT-elicited pulmonary hypertension when used for late therapeutic intervention after full establishment of the pulmonary vascular abnormalities. We conclude that combined administration of iloprost and a dual-selective PDE3/4 inhibitor may offer a new therapeutic concept for antiremodeling therapy in severe pulmonary hypertension.

Acknowledgments

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