PRX-08066, a Novel 5-hydroxytryptamine Receptor 2B (5-HT_{2B}) Receptor Antagonist,
Reduces Monocrotaline-induced Pulmonary Arterial Hypertension and Right Ventricular
Hypertrophy in Rats

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5-HT_{2B} Receptor Antagonist Reduces PAH

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Abbreviations

5-HT	5-hydroxytryptamine
AE	alveolar edema
AMI	alveolar macrophage infiltration
DF	dexfenfluramine
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
FOV	field of view
iPAH	idiopathic pulmonary arterial hypertension
LVP	left ventricular pressure
LVSB	left ventricular septal bowing
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MCT	Monocrotaline
OLP	overall lung pathology
PA	pulmonary artery
PAH	pulmonary arterial hypertension
PAP	peak pulmonary artery pressures
PASMC	pulmonary arterial smooth muscle cell
PBS	phosphate buffered saline
PE	perivascular edema
RVP	right ventricular pressure
RVSP	right ventricular systolic pressure

Abstract

Pulmonary arterial hypertension (PAH) is a life threatening disease that results in right ventricular failure. PRX-08066 is a selective 5-hydroxytryptamine receptor 2B (5-HT_{2B}) receptor antagonist that causes selective vasodilation of pulmonary arteries. In the current study, the effects of PRX-08066 were assessed using the monocrotaline (MCT) -induced PAH rat model. Male rats received 40 mg/kg of MCT or PBS and were treated orally twice a day with vehicle, 50 mg/kg or 100 mg/kg of PRX-08066 for 5 weeks. Pulmonary and cardiac functions were evaluated by hemodynamics, heart weight, MRI, pulmonary artery (PA) morphology and histology. Cardiac MRI demonstrated that PRX-08066 (100 mg/kg) significantly ($P < 0.05$) improved right ventricular ejection fraction. PRX-08066 significantly reduced peak PA pressure at 50 mg/kg and 100 mg/kg ($P < 0.05$, $P < 0.01$, respectively) compared to MCT control animals. PRX-08066 therapy also significantly reduced RV/body weight and RV/LV+Septum ($P < 0.01$, $P < 0.001$, respectively) compared to MCT treated animals. Morphometric assessment of pulmonary arterioles revealed a significant reduction in medial wall thickening and lumen occlusion associated with both doses of PRX-08066 ($P < 0.01$). The 5-HT_{2B} receptor antagonist PRX-08066 significantly attenuated the elevation in PA pressure, RV hypertrophy, and maintained cardiac function. Pulmonary vascular remodeling was also diminished compared to MCT control rats. PRX-08066 prevents the severity of PAH in the MCT rat model.

Introduction

Pulmonary arterial hypertension (PAH) is an elevation in pulmonary vascular resistance due to vasoconstriction and pulmonary vascular remodeling resulting in increased pulmonary arterial pressure. Idiopathic pulmonary arterial hypertension (iPAH) has no known underlining cause. PAH can also develop as a consequence of coexisting disease, such as chronic obstructive pulmonary disease, hypoxia, portal hypertension or HIV infection (Archer and Rich, 2000; Li et al., 2006). PAH is usually progressive and invariably fatal. The median survival without treatment in adult patients with PAH was 2.8 years after diagnosis and in children the median survival was only 10 months (D'Alonzo et al., 1991). Recent advances in PAH therapies, specifically epoprostenol treated patients, have resulted in the median survival reaching more than 6 yrs in adults (Barst et al., 1994) and significant improvement in survival for children with reported rates at 1, 5, and 10 years being 94%, 81%, and 61%, respectively (Yung et al., 2004). Although survival rates have improved with new therapeutic modalities, the prognosis is still poor and development of more effective therapies is clearly needed.

Serotonin or 5-hydroxytryptamine (5-HT) was recognized for playing a role in the development of PAH as a relationship between PAH patients and diet pills such as aminorex, dexfenfluramine, and fenfluramine was observed (Kew, 1970; Kay et al., 1971; Kramer and Lane, 1998). 5-HT remodels the pulmonary vasculature associated with PAH by vasoconstriction, promotion of platelet aggregation and pulmonary arterial smooth muscle cell (PASMC) proliferation (Egermayer et al., 1999; MacLean et al., 2000; Dempsie and MacLean, 2008). Out of the 14 different 5-HT receptors, the 5-HT1B, 5-HT2A and 5-HT2B receptors show evidence in playing a role in the pathobiology of PAH (MacLean, 2007). 5-HT2B receptors (5-HT2BRs) are expressed in pulmonary endothelial and smooth muscle cells and

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stimulate calcium release in human endothelial cells from the pulmonary artery (Esteve et al., 2007). In the chronic hypoxic mouse model of pulmonary hypertension (PH), researchers demonstrated that 5-HT₂BRs are involved in the development of PH by mediating chronic hypoxic responses in wild type mice compared to the complete absence of PH and vascular remodeling in 5-HT₂BR^{-/-} mice (Launay et al., 2002). Both aminorex and fenfluramine cause an elevation of 5-HT levels by increasing the release of 5-HT from platelets and inhibiting the metabolisms or the reuptake of 5-HT (MacLean, 1999; Fitzgerald et al., 2000; Belohlavkova et al., 2001). While dexfenfluramine (DF) binds weakly to the 5-HT₂A, 5-HT₂B and 5-HT₂C receptors, its main metabolite, N-deethylated DF (norDF) is a potent agonist of the 5-HT₂BR (Hong et al., 2004). These results suggest that 5-HT₂BR may play a critical role in the development of PAH pathogenesis.

PRX-08066 (5-((4-(6-chlorothieno[2,3-d]pyrimidin-4-ylamino)piperidin-1-yl)methyl)-2-fluorobenzonitrile monofumarate) (Orbach et al., 2006) is a highly potent (K_i ~ 3.4 nM) and selective 5-HT₂BR antagonist that causes selective vasodilation of pulmonary arteries (Orbach, 2006). In a series of in vitro studies designed to test the effect of PRX-08066 on the 5-HT-induced vascular muscularization, PRX-08066 inhibited the 5-HT-induced mitogen-activated protein kinase (MAPK) activation (IC₅₀~12nM) and markedly reduced thymidine incorporation (IC₅₀~3nM) in Chinese Hamster Ovary (CHO) cells expressing the human 5-HT₂BR. This suggests that PRX-08066 can potentially inhibit the pathologic 5-HT induced vascular muscularization associated with PAH. PRX-08066 significantly reduced the hypoxia-dependent increase in right ventricular systolic pressure (RVSP) in both rats and mice without affecting the systemic mean arterial pressure (MAP) in the animals (Orbach, 2006). PRX-08066 has the

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potential to provide direct and selective vasodilation of the pulmonary vasculature without affecting systemic blood pressure.

We selected the monocrotaline (MCT) induced rat model of pulmonary hypertension (PH) since it is well documented that 5-HT plays an important role in the pathogenesis of MCT-induced PH (Kanai et al., 1993; Miyata et al., 2000). The MCT-induced PH in rats is a commonly used animal model of inflammatory PH that produces endothelial injury and changes to the pulmonary vasculature similar to human forms of PAH. In this study, we investigated the effect of PRX-08066 to see if this 5-HT₂BR antagonist could prevent or reduce the severity of MCT-induced PH.

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Methods

Formulation of PRX-08066

A suspension of PRX-08066 at 20mg/mL for oral gavage was developed. The compound was weighed and suspended in water and the pH was adjusted to between 6.5 and 7.5 by slow addition of 1N NaOH (sodium hydroxide). Methylcellulose in water (1% w/v) and 10x phosphate buffered saline (PBS) was added to achieve a final concentration of 20 mg/mL PRX-08066 in 0.5% methylcellulose (w/v) and 1x PBS. The suspension was wrapped in aluminum foil and stored at room temperature. The suspension was kept homogeneous by continuous stirring prior to dosing.

MCT induced PAH in Rats and Experimental Design

Male Sprague Dawley®TM rats (Harlan, Indianapolis, IN) weighing between 200 and 225 g were housed in conventional conditions in the Animal Care Services Facility at the University of Florida (UF). All experiments were approved by the Institutional Animal Care and Use Committee guidelines at UF. Before entering the study, rat hearts were prescreened for any abnormalities by echocardiography using a Phillips Sonos® 5500 echo machine (Hewlett Packard, Andover, MA) with a 12-MHz ultrasound probe.

The rats were randomly assigned to the following four experimental groups: 1) PBS control+vehicle (n=10); 2) MCT+vehicle (n=10); 3) MCT+PRX-08066, 50 mg/kg (n=8); and 4) MCT+PRX-08066, 100 mg/kg (n=13). Compound PRX-08066 or the vehicle (VH), 0.5% methylcellulose (w/v) and 1x PBS, was administered twice daily by oral gavage for five weeks. PAH was induced on day 0 approximately two to four hours after the first gavage treatment by a single dose of MCT, 40 mg/kg, subcutaneously (Sigma-Aldrich, St. Louis, MO). Rats were weighed twice a week and dosages were adjusted appropriately. At the end of the four-week

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treatment regimen each rat was assessed for PAH using cardiac magnetic resonance imaging. At the end of five weeks, invasive hemodynamic measurements were assessed, blood samples were taken, and the rats were sacrificed.

Quantitation of PRX-08066 in Blood Plasma

Blood samples were collected in potassium EDTA tubes 2 hours post-morning-dose on day 4, 7, 14, 21 and centrifuged at 5500 g for 10 minutes. Plasma samples were mixed with 3 volumes of acetonitrile containing reserpine (Sigma-Aldrich, St Louis, MO) as an internal standard. Precipitate and supernatant were separated by centrifugation and the supernatant was analyzed by liquid chromatography/mass spectrometry using the Agilent 1100 HPLC system, (Agilent Technologies Inc, Santa Clara, CA), equipped with Waters Symmetry C18 column, 2.1 x 30 mm (Waters Corporation, Milford, MA); solvent A 0.1% formic acid in water and B 0.1% formic acid in acetonitrile; 1.5 mL/min gradient elution from 90% to 50% of A in 2 min to 5% in 0.5 min to 90% in 0.3 min; total run time 3.5 min; LC/MSD Trap XCT Ultra (Thermo Fisher Scientific Inc, Waltham, MA) in electrospray positive ion mode using transitions m/z 403.2 $\rightarrow$ 217.1 for PRX-08066 and 609.7 $\rightarrow$ 448.0 for reserpine).

PRX-08066 concentration was determined by interpolation with a standard curve obtained from $1/x^2$ -weighted linear regression of peak-to-internal standard ratios of calibration standards to their respective nominal concentration. Standards were prepared by spiking PRX-08066 in blank plasma using the protocol described above for unknown samples.

Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) of the rat cardiac cycle was performed using a 4.7 Tesla Bruker Avance spectrometer (Bruker Biospin, Billerica, MA). The rats were anesthetized using a mixture of 1.5-2% isoflurane and oxygen (O₂). Monitoring of respiration and

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electrocardiogram (ECG) for use during data acquisition was conducted using a Small Animal Instrument monitoring and gating system (S/A Instruments, Inc, Stony Brook, NY). Rats were placed in the prone position on a quadrature surface receive/quadrature volume transmit coil and inserted feet first into the magnet. Following the acquisition of an ungated survey sequence, two chamber and four chamber cine sequence were acquired using a Fast Low Angle Shot (FLASH) sequence (TR ~ 200 msec, TE = 2.5 msec, matrix = 256 x 128, FOV = 70 mm x 30 mm, thickness = 1 mm, 3 slices) in order to prescribe short axis images. After identification of the most apical and basal segments of the ventricles using the two-chamber and four-chamber images, a short axis image stack was collected with a FLASH sequence to (TR ~ 200 msec, TE = 2.7 msec, matrix = 256 x 192, FOV = 40 mm x 30 mm, thickness = 1 mm, approximately 12 slices). The rat's ECG was used to trigger each phase encode segment at the peak of the R-wave. Repetition time was dependent on the heart rate of the animal and R-R interval. Approximately 14 to 20 frames were used to capture the entire cardiac cycle. Right ventricle (RV) and left ventricle (LV) volumetric and RV wall thicknesses (in short axis mid-ventricle images) measurements were performed using CAAS MRV FARM software (Pie Medical Imaging, Netherlands).

Invasive Hemodynamic Analysis

To obtain peak pulmonary artery pressures (PAP), the rats were anesthetized with isoflurane at 4-5 % in 100% O₂ then intubated intratracheally with a 14 gauge angiocatheter and placed on an SAR-830 small animal ventilator (CWE, Inc, Ardmore, PA). The isoflurane was decreased to a flow rate of 2.0% with an O₂ flow rate of 0.3 L/min maintained at 1.7 mL tidal volume and respiratory rate of 65 breath/min. The rat's chest was opened to expose the heart. A 1.4F Millar MIKRO-TIP® catheter transducer (Millar Instruments Inc., Houston, TX) was

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inserted directly into the left ventricle to record left ventricular pressure (LVP). After the LVP was recorded, the Millar catheter was removed, inserted into the right ventricle and advanced into the PA to record right ventricular pressure (RVP) and PAP. The hemodynamic tracings were evaluated using Power Lab equipment and software (Power lab v5.2.2, ADI instruments, Castle Hill, Australia).

RV Hypertrophy and Histology Samples

The rats were perfused with PBS through the left ventricle over a 5 minute period using a 24g angiocatheter after blood samples were collected. The hearts were removed and weighed. The great arteries and atria were removed and the ventricles were dissected apart. The free right ventricle wall and the left ventricle with septum were weighed. The left lung and liver were excised and divided into 2mm thick sections. The tissues were fixed in 10% neutral buffered formalin for 8 hours, transferred to 70% ethyl alcohol and embedded in paraffin. Four micrometer tissue sections were cut and stained with hematoxylin-eosin (H&E) stain. An extra lung slide was cut and stained by the Verhoeff-van Gieson method to highlight the elastic lamina of the pulmonary arteries.

Evaluation of Histopathology

Histological evaluation was completed on 41 lung H&E slides. Tissues were scored by a pathologist blinded to the experimental conditions. The following parameters were evaluated in the lung tissue: alveolar edema, alveolar macrophage infiltrate, type II pneumocyte hyperplasia, general arterial thickening, thickening of tunica intima, thickening of tunica media, and perivascular edema. Each parameter was evaluated and scored from 0-5, with 0 being no discernable lesions and 5 indicating the most severe lesions. Final scores for the overall lung pathology were calculated by averaging all parameters for each individual treatment group. The

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experimental groups were revealed after the histological scoring was completed and individual animals were placed in the appropriate groups for statistical analysis.

Morphometric Analysis of Pulmonary Arteries

Lung slides stained with Verhoeff-van Gieson were used to measure arterioles ranging from 25-100 μm for muscularization of medial wall thickness at a magnification of 400x. Fifteen arteriole images per lung section were taken using a Zeiss Axioskop microscope, AxioCam camera, and AxioVision software (Carl Zeiss MicroImaging, Inc, Thornwood, NY). MetaMorph Offline imaging software (MetaMorph®Imaging System, Universal Imaging Corporation, Downingtown, PA) was used to calculate the area of the lumen and the area of total vessel. A ratio of lumen area to total vessel area multiplied by 100 (L/TV%) was then calculated as a relative measurement of arteriole wall thickness and lumen carrying capacity (Ohar et al., 1998; Zhou et al., 2006).

Statistical Analysis

All data are expressed as means $\pm$ SEM unless otherwise indicated. Differences between treatment groups were analyzed using one-way ANOVA and a post-hoc Tukey multiple comparison test. Comparison between variables measured was completed using a Pearson correlation test. A *P*-value < 0.05 was considered statistically significant.

Results

Stability of PRX-08066 Plasma Concentrations

PRX-08066 plasma levels were measured 2h post-dose ($t_{\max}$) every week throughout the study. Plasma levels were stable throughout the study (Figure 1) and drug concentration increased approximately two-fold with a two-fold increase in dose as expected from a linear pharmacokinetic behavior. Indeed, average of all plasma levels were 2780 ± 180 (N = 48 measurements) and 1250 ± 110 ng/mL of plasma (N = 32) at 100 and 50 mg/kg dose respectively. Stable plasma levels of PRX-08066 at 2h post-dose confirmed that there was no accumulation of drug and no increase in elimination over the course of the study. During week 4 of dosing, plasma levels were determined at 6-7h post-dose (trough) and found to be 835 ± 209 and 328 ± 60 ng/mL at 100 and 50 mg/kg dose respectively.

Correction of Cardiac Function Determined by MRI

Diastolic and systolic images are illustrated in Figure 2 for all four experimental groups. PRX-08066 treated groups (Figure 2A, 2B, 2E, and 2F) demonstrated less right ventricular hypertrophy and septal flattening than the MCT control group (Figure 2C and 2G). In MCT rats that were treated with 100 mg/kg of PRX-08066, the RV mass was significantly decreased when compared to the MCT + VH group as seen in Figure 2I ($293 \text{ mg} \pm 16.69$ and $458 \text{ mg} \pm 38.10$, respectively; $P < 0.001$) and approached the normal RV mass value of the PBS + VH control ($265 \text{ mg} \pm 10.29$). The 50 mg/kg dose of PRX-08066 showed less severe RV hypertrophy but was not significant ($342.4 \text{ mg} \pm 46.69$) when analyzed with the MCT control. The RV ejection fraction (Figure 2J) of rats treated with 50 mg/kg (55.41 ± 6.12) and 100 mg/kg of PRX-08066 was greater than that of the MCT+VH rats, but only the MCT+100 mg/kg group reached statistical significance at 57.44 ± 2.6 versus 41.46 ± 4.39 ($P < 0.05$). However treatment of

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PRX-08066 after 4 weeks increased RV ejection fractions closer to normal cardiac values demonstrated by the PBS+VH control rats (66.00 ± 1.71).

The positive effects of PRX-08066 treatment on RV remodeling resulted in preserved LV diastolic, systolic and stroke volume (Figure 3). The LV diastolic volume (Figure 3A) for the 50 mg/kg, $461 \mu\text{L} \pm 45.87$, and 100 mg/kg, $473.8 \mu\text{L} \pm 20.77$, groups of PRX-08066 was significantly maintained close to normal values over the MCT+VH group ($287.8 \mu\text{L} \pm 41.33$; $P < 0.01$, $P < 0.001$, respectively). The MCT+100 mg/kg group also maintained a significantly higher LV systolic volume (Figure 3B) compared to the MCT+VH control ($164.4 \mu\text{L} \pm 13.84$ versus $112.8 \mu\text{L} \pm 17.30$, $P < 0.05$). The LV stroke volume increased significantly with treatment by PRX-08066 (Figure 3C). The LV stroke volume of the PBS+VH and the MCT+100 mg/kg PRX-08066 rats ($347.40 \mu\text{L} \pm 11.47$ and $309.40 \mu\text{L} \pm 8.67$, respectively) was significantly greater than the MCT+VH rats, $175.1 \mu\text{L} \pm 25.71$, $P < 0.001$. Rats receiving the MCT+50 mg/kg PRX-08066 dose, $295 \mu\text{L} \pm 33.70$, also showed beneficial effects when compared to the MCT-vehicle rats ($P < 0.01$).

Changes in Hemodynamic Variables after Treatment of PRX-08066

The hemodynamic variables after 5 weeks of treatment with PRX-08066 were greatly improved (Figure 4). Compared to the MCT control group ($63.44 \text{ mmHg} \pm 7.78$), peak PAP in the MCT+50 mg/kg and MCT+100 mg/kg groups (Figure 4A) were significantly decreased ($43.07 \text{ mmHg} \pm 5.10$, $P < 0.05$; and $40.75 \text{ mmHg} \pm 3.47$, $P < 0.01$; respectively). The peak PAP in the MCT+VH was notably elevated by a two-fold increase compared to the PBS+VH group ($26.95 \text{ mmHg} \pm 1.24$, $P < 0.001$). No significant difference was observed in the peak LV pressure (Figure 4B). The peak RV:LV ratio shown in Figure 4C was significantly lower in the MCT+100 mg PRX-08066 rats (0.43 ± 0.04) when compared to the MCT+VH rats, (0.75 ± 0.10 ,

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$P < 0.01$) indicating PRX-08066 having a favorable effect in the prevention and modulation of pathological changes initiated by the MCT injection.

Effects of PRX-08066 on Body Weight and RV Hypertrophy

Comparison and statistical analysis of body weight and ventricular remodeling from all four experimental groups can be seen in Table 1. Rats in the MCT+50 mg/kg treatment group gained significantly more weight than the rats receiving MCT+VH ($P < 0.01$). The weight gain observed for rats receiving MCT+100 mg/kg of PRX-08066 was not significant when compared to the MCT+VH control group.

Rats treated with 50 mg/kg and 100 mg/kg of PRX-08066 demonstrated a marked reduction in RV hypertrophy illustrated by the RV necropsy mass to body weight ratio (Table 1). PRX-08066 attenuated RV hypertrophy after 5 weeks of treatment by 32-34% after MCT induction. The MCT+VH control rats developed a 2 fold increase of RV hypertrophy over the PBS+VH control rats. The RV/LV+S ratio was also significantly decreased in the PRX-08066 treated groups compared to the MCT+VH group.

Relationship between MRI derived RV performance and invasive outcome measures

The positive correlation seen between the calculated MRI RV end-diastolic myocardial mass and the RV mass obtained at necropsy ($P < 0.0001$, $r = 0.82$) is shown in Figure 5A. This signifies the robust non-invasive tool MRI can be used to follow stages of PAH. RV hypertrophy, MRI RV derived mass, as a function of peak PAP ($P < 0.0001$, $r = 0.78$) depicts the RV overload that occurs as the pulmonary arterial vasculature thickens (Figure 5B). A negative correlation is observed between the RV/LV+S mass ratio and the MRI RV ejection fractions ($P < 0.0001$, $r = -0.84$) illustrating how PRX-08066 treated rats have increased ejection fractions compared to the MCT+VH animals (Figure 5C).

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Histological Changes with PRX-08066 Treatment on Development of MCT-induced PH

Treatment with 50 mg/kg and 100 mg/kg PRX-08066 reduced the following pathological features associated with MCT-induced PH: alveolar macrophage infiltration (AMI), alveolar edema (AE), type II pneumocyte hyperplasia (Type II HP) and perivascular edema (PE). In both the 50 and 100 mg/kg PRX-08066 treated groups, the severity of lesions was significantly decreased compared to the MCT+VH rats (Figure 6A). There was a significant reduction in AMI ($P<0.05$) and AE ($P<0.01$) in rats receiving MCT+100 mg/kg of PRX-08066 compared to the MCT+VH rats (Figure 6B). The MCT+50 mg/kg PRX-08066 group significantly reduced AE ($P<0.05$) only compared to the MCT+VH control. These results show a dose dependent reduction of lung lesion characteristics with increasing doses of PRX-08066. The overall lung pathology (OLP) is also markedly decreased with both treatment groups, but significant only in the MCT+100 mg/kg group versus the MCT+VH control ($P<0.05$). While the lung lesions in both drug groups were not completely prevented or cured, they were significantly diminished in both groups.

Reduction of pulmonary arterial remodeling with PRX-08066

Medial wall thickness in the pulmonary arteries was markedly decreased in both treatment groups of PRX-08066 compared to the MCT+VH group as seen in the representative photomicrographs in Figure 7A. The reduction of arterial thickness was observed not only in the tunica media, but also in the intima and adventitia regions. The decrease in overall arterial hypertrophy was significant between groups ($P<0.002$) as determined by ANOVA (Figure 7B). The arteriole wall thickness was increased which resulted in the lumen carrying capacity (L/TV %), shown in Figure 7C, being markedly decreased in the MCT+VH group when compared to the PBS+VH group (22.06 ± 1.92 and 54.09 ± 2.32 , respectively; $P < 0.001$). Twice daily dosing

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of both 50 and 100 mg/kg of PRX-08066 attenuated an increase in pulmonary artery carrying capacity from that of the MCT-vehicle rats (36.72 ± 4.28 and 37.2 ± 2.88 , respectively; $P < 0.01$).

Discussion

This study showed PRX-08066 attenuates the pathological effects of MCT-induced PAH in rats. MCT severely injures the pulmonary vasculature ultimately resulting in increased vascular resistance and PAP leading to RV pressure overload and RV hypertrophy similar to PAH seen in humans (Hessel et al., 2006). We demonstrated that the 5-HT_{2B}R antagonist PRX-08066 prevented severe PAH and preserved cardiac function similar to control rats. Increased ejection fractions and decreased RV hypertrophy were observed by both MRI and pathologic analysis. Rats given PRX-08066 had improved lung pathology and reduced medial wall thickness consistent with prevention of severe MCT-induced PAH. We did not see a complete prevention PAH but treatment with PRX-08066, especially in rats receiving 100 mg/kg, resulted in a significant decrease in PAP and attenuated PAH development. The present results also did not prove a dose response between the 50 and 100 mg/kg doses of PRX-08066 which was confirmed using the post-hoc Tukey multiple comparison analysis. However, both doses were effective in the majority of outcome measures, with a more optimal effect achieved at the 100 mg/kg dose. The lower p value in the 50 mg/kg treatment group was probably due to lower animal numbers (N=8) compared to the 100 mg/kg group (N=13) which resulted in reduced statistical power. In addition the study was not designed as a dose response study. The pathologic changes induced by MCT were not completely prevented, but reduced in both dose groups.

This study also demonstrated tolerance of orally administered PRX-08066 twice a day without notable gastrointestinal side effects. Plasma levels of PRX-08066 in both dose groups showed linear pharmacokinetic behavior and good bioavailability through the oral route. PRX-08066 also attenuated the hemodynamic and pathologic characteristics of PAH. As part of the

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study protocol, rat weight changes were closely monitored. The 50 mg/kg PRX-08066 dose group maintained close to normal rat weights compared to control. Although the 100 mg/kg dose group gained less weight than the lower dose group, they thrived compared to the MCT group. Despite the lower body weights of the 100mg PRX-08066 group, the treatment resulted in a milder disease state with conserved cardiac function.

The chronic hypoxic mouse and MCT-induced rat models of PAH are used to evaluate the development, pathogenesis and treatment of PAH. Prior studies have investigated the role of 5-HT₂BRs in the development of lung vascular remodeling and PAH using the chronic hypoxic mouse model. Similar to our results the 5-HT₂BR antagonist, RS-127445, also demonstrated attenuation of PAH and prevented an increase in RV systolic pressure, RV/LV+S, and medial wall thickness in the chronic hypoxic mouse model (Launay et al., 2002; Callebert et al., 2006). These results differ from other findings that showed RS-127445 did not attenuate the development of MCT-induced PAH (Guignabert et al., 2005). That study used a dose of 2mg/kg/d in the rat MCT model and the lack of response may have resulted from the dose being too low.

Many PAH studies have targeted the main three pathways: prostacyclin, nitric oxide and the endothelin pathways, for treatment using the MCT animal model. Our data demonstrates that PRX-08066 ameliorates the pulmonary vascular pathology similarly to bosentan, sildenafil, beraprost, iloprost and combinations of these drugs. PRX-08066 decreased PAP by 32% in the 50mg/kg group and 36% in the 100 mg/kg group. A study using bosentan and sildenafil individually resulted in a 21% decrease, but the combination of the two drugs resulted in a 42% reduction in pressure (Clozel et al., 2006). Our study also showed less RV hypertrophy in the 50 and 100 mg/kg PRX-08066 group with a 34% and 36% decrease which is comparable to the

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combination treatment of bosentan and sildenafil that resulted in a 37% reduction (Clozel et al., 2006). In a comparative study evaluating NS-304, a diphenylpyrazine derivative, with beraprost, the RV systolic pressure was decreased 25% and 17% respectively and the reduction of RV hypertrophy was 24% for NS-304 and 12% for beraprost (Kuwano et al., 2008). Our results with PRX-08066 is also similar to the effect that inhaled and intravenous iloprost has on improving RV pressures (29% and 48%, respectively) and RV hypertrophy (35% and 40%, respectively) (Schermulý et al., 2004; Schermuly et al., 2005). Although PRX-08066 was not directly compared to bosentan, sildenafil, iloprost or the other agents, our data revealed comparable or enhanced improvement as a monotherapy in the PAH pathology. Additional investigations looking at combinational therapies are important for improving long-term patient treatment and are already being reported (Schermulý et al., 2004; Kuwano et al., 2008).

Experimental studies evaluating therapies for PAH report results on improved RV function, PAP and decreased pulmonary vasculature remodeling with little to no information on changes to LV function. The right and left ventricles are coupled and with RV hypertension or volume overload there are changes in LV filling that impede cardiac output. As pulmonary vascular resistance (PVR) increases, it limits RV stroke volume. This decrease in volume output limits the volume available for LV filling (Marcus et al., 2001). As the LV filling decreases, there is less systemic cardiac output and this was observed in the MCT-induced PAH rats who became systemically hypotensive. Contrary to previous reports with the 5HT_{2A} receptor blocker Ketanserin which caused systemic hypotension (Frishman et al., 1995; Frishman and Grewall, 2000), our experiment demonstrated no significant change in the LV pressure between the treatment groups and the PBS controls suggesting less peripheral vasodilatation with more selective vasodilatation of the pulmonary vasculature.

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Cardiac MRI is a new non-invasive tool being used for the diagnosis, evaluation and management of cardiac changes associated with PAH and the effects of therapeutic intervention on RV function and indices (van Wolferen et al., 2007; Chin et al., 2008). Changes in stroke volume, right and left ventricular volumes and RV mass can be measured in response to therapeutic treatments. We have chosen this tool over echocardiography due to better consistency, lower operator variability, and more importantly, the fact that MRI can give functional data and reliable images of the RV morphology (Nagendran and Michelakis, 2007; Benza et al., 2008). The characteristic crescent-shaped RV was illustrated in the MR cardiac images from rats receiving PRX-08066 compared to MCT control rats. Both PRX-08066 treatment groups had a smaller RV mass and improved function with increased ejection fractions. MR images also showed that the PRX-08066 treatment regimen had no left ventricular septal bowing (LVSB) which indicates less severe increase in PVR. The structural changes demonstrated by MR evaluation correlated well with the decreased PAP seen in the PRX-08066 treatment groups. RV and LV hemodynamics are affected by geometric shape and wall thickness of the RV. The severity of pressure overload in the RV was decreased in the PRX-08066 treatment groups compared to the MCT control group which developed a progressive afterload imposed by the PVR and resulted in LVSB and impaired LV filling.

Our results demonstrated that PRX-08066 attenuates the effect of MCT on the pulmonary vascular tree by decreasing the severity of PAH with decreased RV hypertrophy and PA pressure, reductions in histological changes and pulmonary arterial thickness, and improved RV function with increased ejection fractions. The 5-HT_{2B}R antagonist can be targeted as an additional pathway towards the treatment of PAH. Further studies designed to demonstrate therapeutic rather than preventative effect need to be done to prove efficacy. This compound

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could prove effective as a single agent or in combination with other clinically available medications used in treatment of PAH.

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Footnotes

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Legends for Figures

Figure 1. Plasma levels of PRX-08066 in both dose groups shows linear pharmacokinetic behavior (squares 100 mg/kg; diamonds 50 mg/kg; dashed line represents mean value throughout study; data are presented as mean $\pm$ SEM).

Figure 2. MRI assessment of RV mass and ejection fraction after 4 weeks of PRX-08066 treatment. Representative systolic and diastolic heart images from MCT+50 mg/kg, MCT+100 mg/kg, MCT+VH and PBS+VH treatment groups (A-H). I, RV mass calculated from MRI images for all four experimental groups. RV mass was lower in the 100 mg/kg dose of PRX-08066 (* P <0.001) while the 50 mg/kg treatment group was not significantly decreased. J, MRI RV ejection fraction in PRX-08066 treated rats. Rats treated with the higher dose of PRX-08066 showed the most improvement (* P < 0.001; ** P < 0.05). Data are shown as the mean $\pm$ S.E.M.

Figure 3. LV diastolic, systolic and stroke volumes were measured from MR images. A, The LV diastolic volume was increased in rats that received treatment from PRX-08066, (* P <0.01, ** P <0.001) compared to the MCT+VH group. B, Rats treated with 50 and 100 mg/kg of PRX-08066 also showed marked improvement in LV systolic volume, (* P <0.01; ** P <0.05). The beneficial effects of 50 and 100 mg/kg of PRX-08066 was significantly demonstrated by an increase in LV stroke volume compared to the MCT+VH group, (* P < 0.01; ** P < 0.001). Data are shown as the mean $\pm$ S.E.M.

Figure 4. The effects of PRX-08066 on hemodynamic function in the rat MCT model. A, There was a significant decrease in peak PAP in both PRX-08066 dosing groups when compared to the

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rats that received MCT+VH, (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). B, There was no difference seen in the peak LV pressure in any of the experimental groups. C, The 100 mg/kg dose of PRX-08066 demonstrated the greatest improvement for peak RV:LV ratio, (* $P < 0.01$; ** $P < 0.001$). Data are shown as the mean $\pm$ S.E.M.

Figure 5. Interaction of MRI cardiac function with hemodynamics and RV hypertrophy. A and B, Positive linear correlations were observed between the RV mass at necropsy, peak PAP and the MRI derived end-diastolic (ED) RV mass. C, A negative correlation was observed between MRI calculated EF and the weight ratio RV/LV+S.

Figure 6. Pathological evaluation of H&E lung tissue after treatment of PRX-08066. A, The 50 and 100 mg/kg doses of PRX-08066 both showed improvement in alveolar macrophage infiltration (AMI) and edema (AE), type II pneumocyte hyperplasia (Type II PH) and perivascular edema (PE) when evaluated against the MCT+VH control; H+E, bar = 200 μ m. B, The MCT+100 mg/kg group had significant reductions seen in the AMI (* $P < 0.05$), AE (** $P < 0.01$), and overall lung pathology (OLP; * $P < 0.05$) while the MCT+50 mg/kg group showed marked improvement in AE (* $P < 0.05$). PBS+VH vs MCT+VH (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data are shown as the mean $\pm$ S.E.M.

Figure 7. Pulmonary artery remodeling after 5 weeks of PXR-08066 treatment. A, Sections of lung tissue with the tunica intima, media and adventitia shown by Verhoeff-van Gieson staining for elastic lamina; bar = 25 μ m. B, The overall pathology of the pulmonary arteries was better in the PRX-08066 dose groups with the arterial wall thickness being reduced (ANOVA; $P < 0.002$).

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C, The ratio of lumen area to total vessel area (L/TV%) with treatment of PRX-08066 significantly prevented medial wall thickening and luminal occlusion when compared to the MCT+VH group (*P < 0.01; **P < 0.001). Data are shown as the mean $\pm$ S.E.M.

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Tables

TABLE 1

Body weight, RV and LV remodeling in rats treated with vehicle or PRX-08066 (50 or 100 mg/kg) on day 35 after administration of PBS or MCT.

	PBS + VH	MCT + VH	MCT + 50mg PRX-08066	MCT + 100mg PRX-08066
Body Weight (kg)	0.336 ± 0.005*	0.269 ± 0.009	0.320 ± 0.007‡	0.294 ± 0.01†
RV / BW (g/kg)	0.61 ± 0.01*	1.31 ± 0.08	0.90 ± 0.10‡	0.87 ± 0.07*†
LV + S / BW (g/kg)	2.32 ± 0.07	2.16 ± 0.05	2.24 ± 0.04	2.20 ± 0.02
RV / LV+S (g)	0.264 ± 0.01*	0.61 ± 0.04	0.40 ± 0.05‡	0.39 ± 0.03*†

Values are means ± SEM; PBS, phosphate buffered saline; VH, vehicle; MCT, monocrotaline; RV, right ventricle; BW, body weight; LV, left ventricle; S, septum. * $P < 0.001$ vs MCT-VH group; † $P < 0.05$ vs PBS-VH group; ‡ $P < 0.01$ vs MCT-VH group.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B

C

Figure 6

Figure 7

A

B

C