



Cardiovascular Pharmacology

C-122, a novel antagonist of serotonin receptor 5-HT_{2B}, prevents monocrotaline-induced pulmonary arterial hypertension in ratsDavid A. Zopf^a, Liomar A.A. das Neves^{b,*}, Kristen J. Nikula^c, Jinbao Huang^b, Peter B. Senese^b, Michael R. Gralinski^b^a Corridor Pharmaceuticals, Inc., Towson, MD, USA^b CorDynamics, Inc., Chicago, IL, USA^c Seventh Wave Laboratories, LLC, Chesterfield, MO, USA

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a chronic disease characterized by sustained elevation of pulmonary arterial pressure that leads to right ventricle failure and death. Pulmonary resistance arterioles in PAH undergo progressive narrowing and/or occlusion. Currently approved therapies for PAH are directed primarily at relief of symptoms by interfering with vasoconstrictive signals, but do not halt the microvascular cytoproliferative process. In this study we show that C-122 (2-amino-N-(2-{4-[3-(2-trifluoromethylphenothiazin-10-yl)-propyl]-piperazin-1-yl}-ethyl)-acetamide trihydrochloride, a novel antagonist of serotonin receptor 5-HT_{2B} (K_i = 5.2 nM, IC₅₀ = 6.9 nM), when administered to rats for three weeks in daily oral 10 mg/kg doses, prevents not only monocrotaline (MCT)-induced elevations in pressure in the pulmonary arterial circuit (19 ± 0.9 mm Hg vs. 28 ± 2 mm Hg in MCT-vehicle group, P < 0.05) and hypertrophy of the right ventricle (right ventricular wt./body wt. ratio 0.52 ± 0.02 vs. 0.64 ± 0.04 in MCT-vehicle group, P < 0.05), but also muscularization of pulmonary arterioles (23% vs. 56% fully muscularized in MCT-vehicle group, P < 0.05), and perivascular fibrosis in the lung. C-122 is orally absorbed in the rat, and partitions strongly into multiple tissues, including heart and lung. C-122 has significant off-target antagonist activity for histamine H-1 and several dopamine receptors, but shows no evidence of crossing the blood–brain barrier after a single 10 mg/kg oral dose in rats. We conclude that C-122 can prevent microvascular remodeling and associated elevated pressures in the rat MCT model for PAH, and offers promise as a new therapeutic entity to suppress vascular smooth muscle cell proliferation in PAH patients.

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1. Introduction

Pulmonary arterial hypertension (PAH) is a chronic disease characterized by sustained elevation of pulmonary arterial pressure that leads to right ventricle failure and death. Pulmonary resistance arterioles in PAH undergo progressive narrowing and/or occlusion due to intimal hyperplasia, medial hypertrophy, perivascular fibrosis, microthrombosis, inflammatory cell infiltration, and angioproliferative plexiform lesions (McLaughlin et al., 2009). Functional alterations in pathways that regulate smooth muscle tone include enhanced expression of phosphodiesterase 5 (PDE5) (Corbin et al., 2005; Wharton et al., 2005), upregulation of endothelin expression (Galiè et al., 2004; Giaid et al., 1993), and decreased production of prostaglandin I₂ (PGI₂) (Christman et al., 1992; Tuder et al., 1999). Current therapies for PAH include pharmacologic agents that 1) inhibit PDE5, 2)

antagonize endothelin, or 3) supplement the prostaglandin pathway with exogenous prostacyclins (Humbert et al., 2004). These treatments improve longevity and performance of activities of daily life for PAH patients (Macchia et al., 2010), but do not halt the ongoing cytoproliferative process that inexorably modifies pulmonary vascular architecture, and leads to lung transplant.

Evidence that serotonin (5-HT) plays a role in both the proliferative and functional components of PAH pathogenesis has been accumulating for decades (Esteve et al., 2007; Fanburg and Lee, 1997; MacLean and Dempsey, 2010). Ninety-five percent of total body 5-HT is produced outside the central nervous system, mainly in enterochromaffin cells in the gut (Sirek and Sirek, 1970). Platelets take up 5-HT in the blood (Jernej et al., 2000), and deliver 5-HT at sites of microvascular injury and coagulation (Yoshioka et al., 1993). The pressor response to 5-HT in the pulmonary circulation is reduced by selective blockade of the 5-HT_{2A} receptors (Breuer et al., 1985). 5-HT is a mitogen for a wide variety of cell types, including rat and human pulmonary endothelial cells, smooth muscle cells, and myofibroblasts, where the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT₇ receptors are expressed (Esteve et al., 2007; Königshoff et al., 2010; Pitt

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et al., 1994; Ullmer et al., 1995; Ullmer et al., 1996). Elevated serotonin levels in the carcinoid syndrome enhance the risk of developing thickened and dysfunctional heart valves (Simula et al., 2002), a negative outcome that is mirrored in patients who ingest 5-HT_{2B} agonists (Roth, 2007), many of which have been withdrawn from the market because of increased risk of both proliferative cardiac valvulopathies and PAH (Rothman et al., 2000). Blockade of serotonin receptors inhibits smooth muscle proliferation due to 5-HT (Dumitrascu et al., 2011; Launay et al., 2002; Lee et al., 1991), and selective deletion of the 5-HT_{2B} receptor prevents the development of PAH in the hypoxic mouse model (Launay et al., 2002). Recently, PRX-08066, a selective 5-HT_{2B} receptor antagonist (Porvasnik et al., 2010), and terguride, an antagonist of both 5-HT_{2A} and 5-HT_{2B} receptors (Dumitrascu et al., 2011), were shown to prevent the development of PAH in the rat monocrotaline (MCT) model.

The present study demonstrates that C-122, a novel 5-HT_{2B} and 5-HT₇ receptor antagonist, prevents vascular remodeling and hemodynamic changes associated with PAH in the rat MCT model.

2. Methods

2.1. Experimental design

Adult male Sprague–Dawley rats (287 ± 4 g) were obtained from Charles River Laboratories (Raleigh, NC). Animals housed individually in a temperature/humidity controlled room with 12-hour light/dark cycles had free access to water and food and were acclimated for one week prior to the study. All experimental protocols were approved by the University of Illinois at Chicago Care and Use Committee, and all experiments were conducted in accordance with the NIH guidelines for animal welfare.

Rats were randomly assigned to one of five experimental groups (n = 10 per group). Rats in groups 1 and 2 served as healthy controls; the remaining rats were injected subcutaneously on Day 0 with 60 mg/kg body weight monocrotaline, the toxic alkaloid of *Crotalaria spectabilis*, (dissolved in DMSO at a concentration of 60 mg/ml, Sigma Aldrich, St. Louis, MO). On days 1–21, rats were dosed via oral gavage (2 ml/kg) with vehicle (PBS), or C-122 (2-amino-N-(2-{4-[3-(2-trifluoromethyl-phenothiazin-10-yl)-propyl]-piperazin-1-yl}-ethyl)-acetamide trihydrochloride (molecular weight free base = 493.6 Da); Corridor Pharmaceuticals, Inc., Towson, MD) at 1 mg/kg or 10 mg/kg. Rats were weighed daily, and the dosages of C-122 were adjusted appropriately.

2.2. Hemodynamic measurements

On day 21, the animals were anesthetized by intra-muscular injection of ketamine/xylazine (80/10 mg/kg) and placed on a heating pad to maintain body temperature at 37 °C. A Millar catheter 1.4 French (Millar Instruments, Houston, TX) was inserted into the femoral artery to measure arterial blood pressure. Additionally, the pulmonary artery and right ventricular (RV) pressures were measured as described previously (Stinger et al., 1981). Briefly, a 3.5 French umbilical vessel catheter (Utah Medical Products LTD, Midvale, Utah), angled to 90° over the distal 1 cm and curved slightly at the tip, was introduced into the right external jugular vein, with the angle directed interiorly, the catheter was inserted proximally, which placed the catheter in the right atrium. The catheter was rotated 90° counterclockwise and inserted further, which placed the catheter in the right ventricle, and then advanced approximately 1.5 cm, into the pulmonary artery. Placement at each stage was confirmed by monitoring the respective pressure contours. Hemodynamic values were automatically calculated by the physiological data acquisition system NOTOCORD-Hem Software 4.1 (NOTOCORD Inc., Croissy sur Seine, France).

2.3. Right ventricular hypertrophy measurements

At the end of the study, rats were euthanized by pentobarbital overdose and hearts were isolated, flushed with saline and dissected to separate the right ventricle from the left ventricle + septum (LV + S). Dissected samples were weighed and the ratio of the RV weight to body weight [RV/BW] for each heart was calculated to obtain an index of RV hypertrophy.

2.4. Evaluation of histopathology

After the lungs were harvested, they were instilled with 10% neutral buffered formalin and immersed in the same fixative. The left and right caudal lung lobes were trimmed to produce six transverse samples per rat and these samples were routinely processed and embedded in paraffin blocks. Sections (approximately 5 µm thick) were stained with Verhoeff's elastin/eosin stain and examined by light microscopy. Histopathological findings were classified as: 1— alveolar inflammation and septal remodeling, 2— perivascular inflammation and edema, 3— perivascular fibrosis, and 4— arteriolar medial hypertrophy. The findings were graded by a pathologist without knowledge of treatment group assignment as 0 (not present), 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked). The distribution of each finding, if present was classified as multifocal or diffuse. The degree of muscularization of small peripheral pulmonary arteries was assessed by examination of sections immunohistochemically reacted with an anti-alpha-smooth muscle actin antibody (rabbit polyclonal ab5694 diluted 1:100, Abcam, Cambridge, MA). These sections were stained with Verhoeff's elastin stain and examined by light microscopy with the aid of an eyepiece micrometer. Eighty intra-acinar pulmonary arterioles with diameter of 10 to 50 µm were categorized as non-muscularized (exhibit elastin but no apparent smooth muscle), partially-muscularized (incomplete medial layer of smooth muscle), or fully-muscularized (concentric medial layer of smooth muscle) (Schermuly et al., 2004). The percentage of pulmonary vessels in each muscularization category was determined for each rat.

2.5. Pharmacokinetics and biodistribution

2.5.1. Sample collection

Male Sprague Dawley rats weighing 301 ± 12 g were surgically fitted with a jugular cannula and allowed to acclimate to laboratory conditions for at least 10 days. Animals were housed one per cage and supplied with a commercial rodent diet ad libitum prior to study initiation. Food was withheld for a minimum of 12 h prior to dosing, and returned 4 h post-dose. Water was supplied ad libitum. Nine rats were dosed via oral gavage (10 ml/kg) with C-122 dissolved in phosphate buffered saline, pH 7.4 for 14 consecutive days. Blood samples were collected after 0.5, 1, 2, 4, 8 and 24 h (3 rats per time point) via the jugular vein cannula on days 1 and 14 and placed into chilled tubes containing K₃EDTA as an anticoagulant. Samples were centrifuged at 16,000 × g, 4 °C for 5 min. Plasma was aspirated, placed on dry ice, and stored at –80 °C. To monitor biodistribution, tissue samples including heart, lung, brain, bone marrow, spleen, kidney, liver, thymus, large intestine and small intestine were collected from a parallel set of 3 animals per time point sacrificed 4 h, 1 day, 3 days, or 7 days after a single oral dose of 10 mg/kg C-122. Plasma was derived as described above, and tissues were dissected, rinsed with saline to remove residual blood or intestinal contents, and weighed. To each tissue sample a volume of 20:80 methanol:water (vol/vol) sufficient to make 4 ml/1 g tissue was added and the mixtures were homogenized on ice with a Virsonic 100 ultrasonic homogenizer and stored at –80 °C.

2.5.2. Sample analysis

Plasma and tissue homogenates were extracted manually by acetonitrile precipitation as follows: to a 50 µl sample of plasma or tissue

homogenate or blank matrix, 50 μ l of 50:50 (vol:vol) acetonitrile:water was added, followed by 100 μ l acetonitrile containing 0.1% formic acid plus 100 ng/ml ritonavir (internal standard). After vortex mixing, the tube was centrifuged at 16,000 \times g for 10 min at room temperature. Supernatant was analyzed by HPLC using a Phenomenex Synergi Polar RP C₁₈, 50 \times 2.0 mm id, 4 μ m column eluted at 300 μ l/min at ambient temperature with 0.2% aqueous formic acid (A) plus 0.2% formic acid in acetonitrile (B) in a gradient: 95% A to 100% B. The column eluate was analyzed using a PE Sciex API4000 mass spectrometer. Calibration standards were prepared in a blank plasma or tissue matrix for each plasma or tissue homogenate type. An eight-point calibration curve was prepared for each matrix spiked with C-122 at concentrations ranging from 1 to 1000 ng/ml. Accuracy and precision (coefficient of variation (CV)) of the method for samples spiked with C-122 at 50 ng/ml was determined to be 102% (CV 7.8%) for plasma and 97.6% (CV 4.3%) for brain homogenates. The lower limit of quantification for the method for the plasma matrix was 0.5 ng/ml.

2.6. Receptor binding

Specific binding of C-122 with a panel of drug receptors was determined by measuring displacement of bound radiolabeled agonist or antagonist at a series of C-122 concentrations. In functional assays, EC₅₀ values (concentration producing half-maximal specific agonist response) and IC₅₀ values (concentration causing half-maximal inhibition of a control specific agonist response) were determined by non-linear regression analysis of the C-122 concentration-response curves. The receptor panel included the following: serotonin receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₃, 5-HT_{4e}, 5-HT_{5A}, 5-HT₆, and 5-HT₇; histamine receptors H-1, H-2, H-3, and H-4; norepinephrine receptors Adr α ₁, Adr α ₂, Adr β non-selective, Adr β ₁, Adr β ₂, Adr β ₃, calcitonin Calc, and CGRP₁; chemokine receptors CCR₁, CCR_{2B}, CCR₄, CCR₅, CX3CR₁, CXCR_{1/2}, CXCR₂, and CSCR₄; endothelin receptors ETA and ETB; insulin receptor; receptors for interleukins IL1, IL2, and IL6; leptin receptor; leukotriene receptors LTB₄, CysLT₁, and CysLT₂; N-formyl peptide receptors FPR₁ and FPRL₁; opiate receptor (nonselective); benzodiazepine receptor PBR, phorbol ester receptor PE; prostenoid receptors, CRTH₂, DP, EP₂, EP₄, FP, and TP; sigma receptor (nonselective); tachykinin receptors, NK₁ and NK₂; thyroid hormone receptors, ThyRH and ThyR Rel Hor; transporters, AdenosineT, CholineT, DopamT GABAT, GlyT, MonoamineT, NET, and 5-HTT; receptor for tumor necrosis factor, TNF; urotensin receptor, Urot II; vanilloid receptor, Van; vasoactive peptide receptor VIP₁; vasopressin receptors V_{1A}, V_{1B}, and V₂; acetylcholine choline receptors, M₁, M₂, M₃, M₄, and M₅; dopamine receptors D₁, D_{2L}, D_{2S}, D₃, D_{4,2}, D_{4,4}, and D_{4,7}. All receptor binding assays were performed at Cerep (Le Bois L'Evescault, France) using well-established procedures (<http://www.cerep.fr/cerep/users/pages/catalog/assay/catalog.asp?domaine=1&classestest=33>).

2.7. Transport by P-glycoprotein

Partitioning of C-122 across MDR-MDCK cell monolayer's in vitro to assess transport by P-glycoprotein (P-gp) was measured as described by Wang, et al. (2005). The apparent permeability coefficient, *Papp*, was calculated after addition of 5 μ M C-122 separately to the apical and basolateral surfaces of cell monolayers as $(dQr/dt)/A \times C_o$, where *dQr/dt* is the cumulative amount in the receiver compartment versus time; *A* the area of the cell monolayer; *C_o* the initial concentration of the dosing solution.

2.8. Drug binding to plasma protein

Equilibrium dialysis studies were carried out in human plasma (Bioreclamation, Hicksville, NY), Sprague–Dawley rat plasma (Bioreclamation, Hicksville, NY), Beagle dog plasma (Lampire Biological

Laboratories, Pipersville, PA). Amika MicroEquilibrium Dialyzers and Ultra-Thin Membranes (molecular weight cutoff 5000 Da) were purchased from Harvard Bioscience (Holliston, Massachusetts). Aliquots of C-122 in DMSO were dosed into 2 ml of plasma at 10 μ M for C-122 and control compounds atropine and warfarin. PBS (500 μ l) and 500 μ l of plasma were loaded into opposite sides of dialysis chambers which were incubated at 37 °C overnight on a rocker. After ~22 h of incubation, aliquots (50 μ l for donor, 250 μ l for receiver) were removed from the chambers and placed into a 96-well plate. Plasma (50 μ l) was added to the wells containing the receiver samples, and 250 μ l of PBS was added to the wells containing the donor samples. Two volumes of acetonitrile were added to each well, and the plate was mixed and then centrifuged at 3000 rpm for 10 min. Aliquots of the supernatant were removed, diluted 1:1 into distilled water, and analyzed by LC/MS/MS as described above in Section 2.5.2. A seven point standard curve was prepared in 5:1 PBS:plasma, with concentrations ranging from 5.0 μ M to 5.0 nM for the test compounds. The standards were then diluted with two volumes of acetonitrile, mixed, and centrifuged at 900 \times g for 10 min. The supernatant was removed, diluted 1:1 into distilled water, and analyzed by LC/MS/MS. Recovery and protein binding values were calculated as follows: % Bound = [(Conc. in

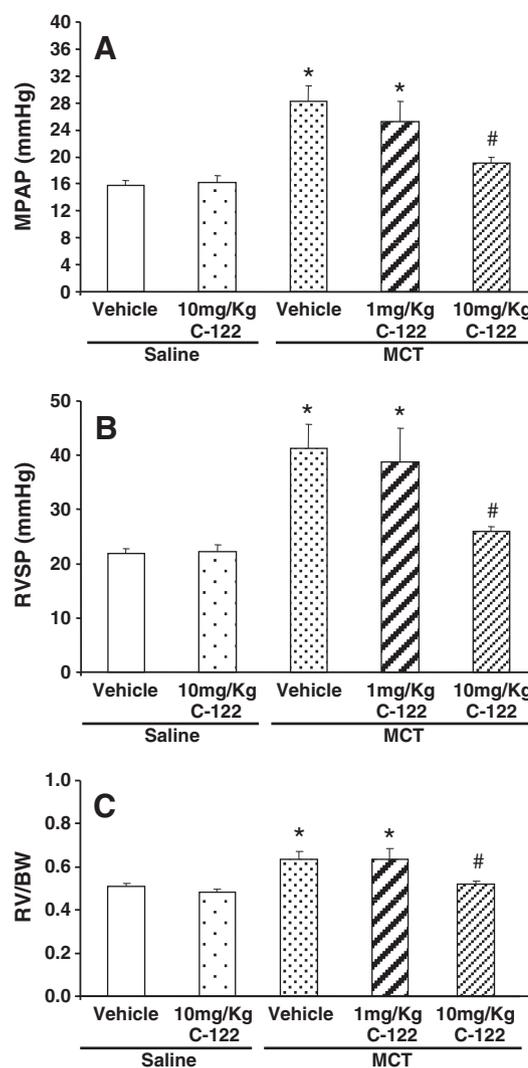


Fig. 1. Effect of C-122 on hemodynamics in monocrotaline (MCT) induced pulmonary hypertension in rats. Right ventricular systolic pressure (RVSP), mean pulmonary arterial pressure (PAP), and RV to body weight (RV/BW) ratio in saline-injected control and MCT-injected (MCT) rats receiving vehicle and 10 mg/kg or 1 mg/kg C-122 for 21 days. Data are presented as mean \pm S.E.M. (n = 10). * = *p* < 0.05 vs. saline-injected/vehicle; # = *p* < 0.05 vs. MCT/vehicle.

Donor – Conc. in Receiver)/(Conc. in Donor)] \times 100%; Recovery = [(Conc. in Donor + Conc. in Receiver)/(Dose Conc.)] \times 100%.

2.9. Data analysis

All data are expressed as mean \pm S.E.M. or otherwise noted. The different experimental groups were analyzed by one-way ANOVA and Newman–Keuls post hoc test for multiple comparisons. Significance was fixed at $P < 0.05$.

3. Results

3.1. C-122 administration normalizes hemodynamics and right ventricular hypertrophy in MCT-induced PAH

Twenty-one days after exposure to MCT (60 mg/kg, i.p.) or saline, rats underwent catheterization to assess hemodynamics. Mean pulmonary arterial pressure (MPAP) increased 78% in MCT rats dosed orally with PBS (MCT/vehicle) compared with saline-injected controls dosed orally with PBS: MPAP = 28.2 ± 2.3 mm Hg vs. 15.8 ± 0.7 mm Hg, respectively ($P < 0.05$, Fig. 1A). Similarly, MCT treatment increased right ventricular systolic pressure (RVSP) by 88% (41.3 ± 4.5 mm Hg vs. 21.9 ± 0.8 mm Hg $P < 0.05$, Fig. 1B) and RV/BW ratio by 25% (0.64 ± 0.04 vs. 0.51 ± 0.01 mm Hg in saline-injected/vehicle control animals, $P < 0.05$, Fig. 1C). Daily oral treatment of MCT rats with C-122 at 10 mg/kg for 21 days reduced MCT-induced elevations of MPAP, RVSP, and RV/BW by 75%, 78% and 81%, respectively ($P < 0.05$, Fig. 1A–C). At a dose of 1 mg/kg, C-122 did not attenuate the effects of MCT on MPAP, RVSP and RV/BW (Fig. 1A–C). Saline-injected rats exposed to 10 mg/kg C-122 exhibited no changes in MPAP, RVSP or RV/BW compared with vehicle controls (Fig. 1A–C). Mean arterial pressure (MAP) and heart rate (HR) were unmodified compared with controls in all C-122-treated groups (Fig. 2). Daily

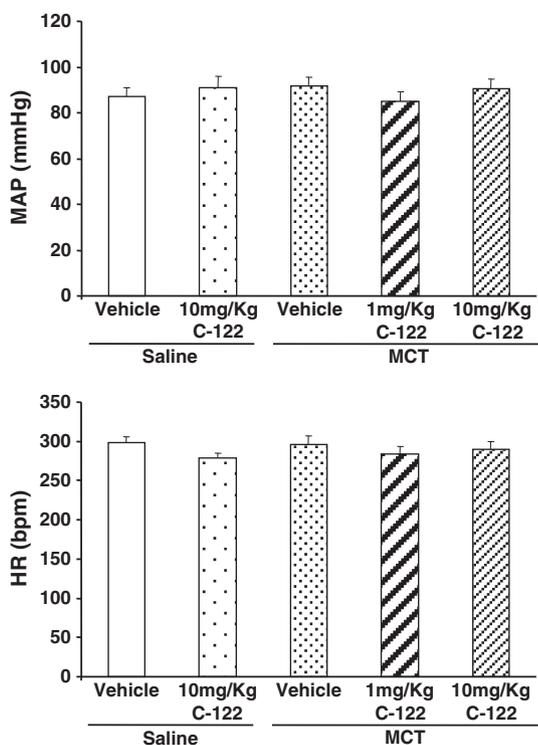


Fig. 2. Effect of C-122 on hemodynamics in monocrotaline (MCT) induced pulmonary hypertension in rats. Mean arterial pressure (MAP) and heart rate in saline-injected control and MCT-injected (MCT) rats receiving vehicle and 10 mg/kg or 1 mg/kg C-122 for 21 days. Data are presented as mean \pm S.E.M. ($n = 10$).

clinical evaluation showed no evidence of physical or behavioral drug-related toxicity.

3.2. C-122 administration prevents pulmonary vascular remodeling in MCT-induced PAH

3.2.1. Histopathology

Microscopic evaluation of lungs from MCT/vehicle rats revealed alveolar inflammation and septal remodeling, perivascular inflammation and edema, perivascular fibrosis, and arteriolar medial hypertrophy (Fig. 3) as indicated by greater incidences and severity scores for all parameters evaluated as compared with saline-injected/vehicle controls (Table 1). MCT rats treated with 10 mg/kg C-122 had a marked decrease in the incidences and severities of perivascular fibrosis and arteriolar medial hypertrophy compared to MCT/vehicle rats (Table 1 and Fig. 3). The severities of alveolar inflammation and septal remodeling, and perivascular inflammation and edema were also clearly diminished in the MCT rats treated with 10 mg/kg C-122 as compared to the MCT/vehicle group (Table 1). Low dose treatment with C-122 had no meaningful affect on the incidences and severity scores of MCT-induced histopathological changes (Table 1). The histopathological findings in saline-injected controls treated with 10 mg/kg C-122 were similar to saline-injected controls dosed orally with vehicle, except for the presence of occasional, focal aggregates of vacuolated alveolar macrophages (Fig. 3F).

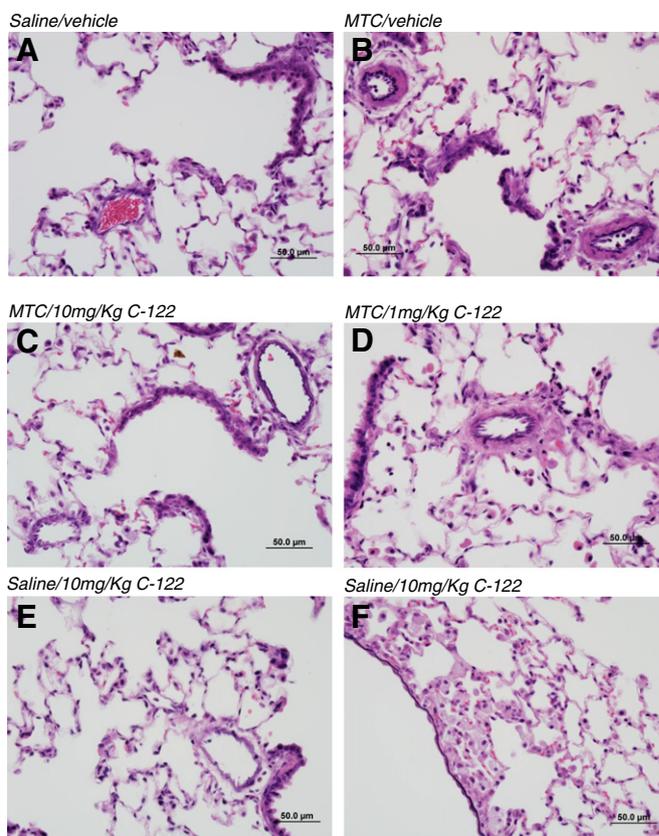


Fig. 3. Panels A–E illustrate centriacinar pulmonary arterioles in Verhoeff's elastin/eosin-stained sections of rat lung tissue. (A) saline-injected/vehicle control lung. (B) MTC/vehicle lung: prominent arteriolar medial hypertrophy and fibrils of collagen in the periarteriolar space (perivascular fibrosis). (C) MTC/10 mg/kg C-122 lung: the arteriolar walls are clearly less thick than those in the MTC/vehicle lung, and do not exhibit perivascular fibrosis. Note the similarity in size and wall thickness of the arteriole in the lower left of this image to that in A. (D) MTC/1 mg/kg C-122: this arteriole exhibits mild medial hypertrophy and perivascular fibrosis. (E) saline-injected/10 mg/kg C-122: the arteriole is similar to those observed in saline-injected/vehicle lungs. (F) Saline-injected/10 mg/kg C-122: vacuolated macrophages in alveolar spaces of the peripheral lung.

Table 1

Pulmonary histopathology incidence table from saline-injected control and MCT-injected (MCT) rats receiving vehicle, 10 mg/kg or 1 mg/kg C-122 for 21 days.

Pulmonary lesion	Dose group (n = 10)				
	Saline + vehicle	Saline + 10 mg/kg C-122	MCT + vehicle	MCT + 1 mg/kg C-122	MCT + 10 mg/kg C-122
<i>Alveolar inflammation/septal remodeling</i>					
Severity score (0–4) ^a	0	2	1	–	–
	1	8	9	2	3
	2	–	–	5	4
	3	–	–	2	1
	4	–	–	1	2
<i>Perivascular inflammation/edema</i>					
Severity score (0–4) ^a	0	3	4	–	–
	1	6	5	4	4
	2	1	1	6	5
	3	–	–	–	1
	4	–	–	–	–
<i>Perivascular fibrosis</i>					
Severity score (0–4) ^a	0	10	10	1	2
	1	–	–	8	5
	2	–	–	1	3
	3	–	–	–	–
	4	–	–	–	–
<i>Arteriolar medial hypertrophy</i>					
Severity score (0–4) ^a	0	7	10	–	–
	1	3	–	–	1
	2	–	–	3	6
	3	–	–	7	3
	4	–	–	–	–

^a Severity of lesions was scored as follows: 0 = finding not present; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked.

3.2.2. Analysis of pulmonary arteriolar muscularization

Categorization of 10 to 50 μ m diameter pulmonary arterioles as fully, partially, or non-muscularized revealed a 3-fold increase in completely muscularized arterioles and 8.1-fold and 1.5-fold decreases in non-muscularized and partially muscularized arterioles, respectively, in MCT-vehicle rat lungs at day 21 compared with saline-injected/vehicle controls (Fig. 4A). In contrast, MCT rats treated with high-dose C-122 exhibited no significant differences in the degree of muscularization of pulmonary arterioles compared with saline-injected/vehicle controls (Fig. 4A).

Low-dose treatment with C-122 resulted in non-significant trends toward fewer completely muscularized arterioles and more frequent partially- and non-muscularized arterioles compared to MCT-vehicle rat lungs. Saline-injected controls treated with 10 mg/kg C-122 were not different from saline-injected/vehicle controls with respect to degree of muscularization of arterioles (Fig. 4A).

There were clear differences in the morphology of each category of arteriole across the treatment groups. The fully muscularized arterioles in lungs from the MTC/vehicle group exhibited hypertrophy of the smooth muscle wall and remodeling with alteration of the elastin layers, whereas arterioles from the MTC/10 mg/kg C-122 group were similar to those from the saline-injected/vehicle group (Fig. 4B, top row). The partially muscularized arterioles from MTC/vehicle lungs generally had thicker muscular walls compared to similarly sized arterioles from saline-injected/vehicle and MTC/10 mg/kg C-122 lungs (Fig. 4B, middle row). In the MTC/vehicle lungs, 10–20 μ m diameter arterioles were frequently fully or partially muscularized whereas arterioles of this size were non-muscularized in saline-injected/vehicle and MTC/10 mg/kg C-122 lungs (Fig. 4B, bottom row).

3.3. Pharmacokinetics and biodistribution of C-122 in rats

3.3.1. Pharmacokinetics

Plasma concentration-time profiles for C-122 following single oral doses of 2 mg/kg or 10 mg/kg in male rats, and following the 14th consecutive daily dose at 10 mg/kg, are shown in Fig. 5. Calculated

pharmacokinetic parameters are presented in Table 2. After dosing on day 1 or day 14, the mean concentration of C-122 reached a broad peak between 0.5 and 4 h, and declined thereafter to a value near the lower level of quantification for the drug assay (0.5 ng/ml) at 24 h. Increasing the dose on day 1 from 2 to 10 mg/kg resulted in a slightly less than dose-proportional (3.98-fold) increase in plasma exposure (AUC_{0-24h}) but a greater than dose proportional (9-fold) increase in C_{max} . In rats receiving C-122 daily via oral gavage, the ratio of exposures on day 14 versus day 1 [$(AUC_{0-24h}$ day 14)/(AUC_{0-24h} day 1)] was 1.19, indicating only minor accumulation of C-122 in plasma after repeat dosing. The apparent volume of distribution (V_z/F) for C-122 was much greater than total body water in the rat (1920 vs. 0.7 l/kg), suggesting that drug may be sequestered into a tissue compartment.

3.3.2. Tissue biodistribution

Analysis of C-122 levels in various tissues 4 h after a 10 mg/kg oral dose revealed tissue drug levels (ng/g) in lung, heart, kidney, liver, large and small intestine, bone marrow and spleen 20 to 15,000 times higher than in plasma (2 ng/ml) (Fig. 6); C-122 was not detected in the brain. Three days after dosing, C-122 was not detected in plasma, brain, bone marrow or kidney, but was detected at decreased levels in the other tissues examined. Seven days post-dose C-122 was undetectable in plasma and tissues.

3.3.3. Plasma protein binding

C-122 was shown by equilibrium dialysis to be 96.5%, 99.6%, and 96.9% protein-bound in plasma from rat, dog, and human, respectively.

3.4. P-glycoprotein transport of C-122

To determine whether C-122 is a substrate for P-gp, bidirectional efflux of C-122 across monolayers of MDR-MDCK cells was determined. At a dose concentration of 5 μ M, the apparent permeability coefficient, P_{app} (A–B) was 0.23×10^6 cm/s, and P_{app} (B–A) was 28.6×10^6 cm/s. According to this well-characterized model, drugs

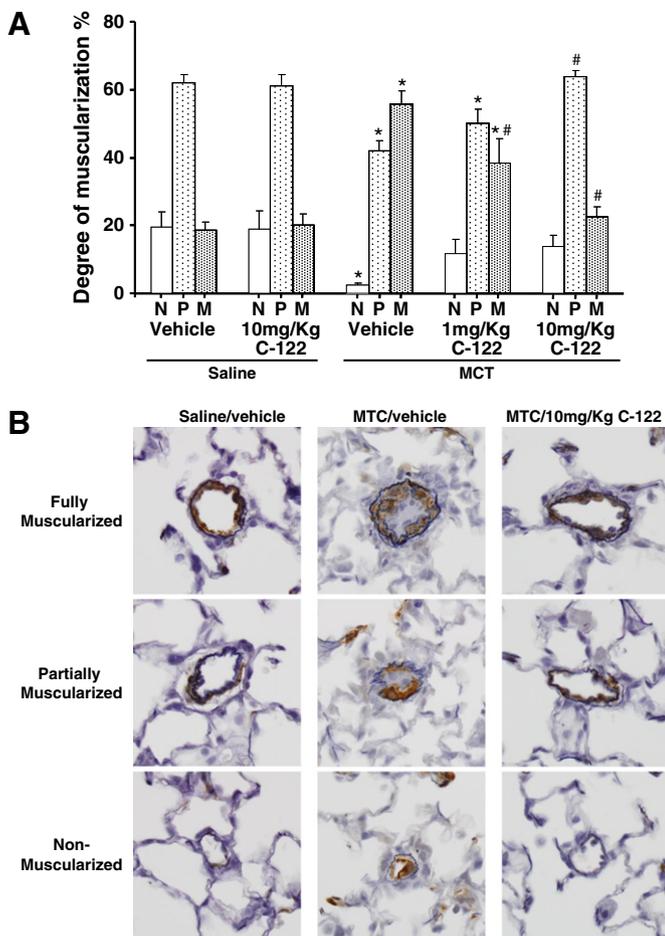


Fig. 4. A— Effect of C-122 on vascular remodeling in monocrotaline (MCT)-induced pulmonary hypertension. Mean percentage of non- (N), partially (P) and fully (M) muscularized vessels (10 to 50 μ m in diameter), is given for saline-injected control and MCT-injected (MTC) rats receiving vehicle and 10 mg/Kg or 1 mg/Kg C-122. Data are presented as mean \pm S.E.M. ($n = 10$). * = $p < 0.05$ vs saline-injected/vehicle; # = $p < 0.05$ vs MCT/vehicle. B— Peripheral arterioles in rat lungs immunostained for α -smooth muscle actin and counterstained with Verhoeff's elastin. The images in each row are shown at the same magnification and illustrate arterioles of similar size across treatment groups. Top row: fully muscularized arterioles. Note the thickened smooth muscle wall and remodeling resulting in duplication and altered organization of elastin layers in the MTC/vehicle lung. Middle row: partially muscularized arterioles. Note the thicker wall of the arteriole in the MTC/vehicle lung. Bottom row: the left (saline-injected/vehicle) and right (MTC/10 mg/kg C-122) lungs illustrate non-muscularized, small caliber peripheral arterioles. The middle image (MTC/vehicle) shows a fully muscularized arteriole of very small caliber ($\sim 20 \mu$ m). Fully muscularized peripheral arterioles of this size were a distinctive finding in MTC/vehicle lungs that distinguished them from saline-injected/vehicle and MTC/10 mg/kg C-122 lungs.

with Papp (A–B) $< 1 \times 10^6$ cm/s and efflux ratio [Papp(B–A)/Papp(A–B)] > 100 are highly unlikely to penetrate the blood brain barrier (Wang et al., 2005).

3.5. C-122 receptor binding

In a screen that included at least one representative from all seven classes of serotonin receptors, C-122 exhibited selective, high affinity binding to receptors 5-HT_{2B} ($K_i = 5.2$ nM) and 5-HT₇ ($K_i = 4.4$ nM) (Table 3). Weaker binding to serotonin receptor 5-HT_{2A} ($K_i = 61$ nM) also was detected, but binding was very weak ($K_i > 100$ nM) to receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2C}, and undetectable ($K_i > 1 \mu$ M) to receptors 5-HT₄, 5-HT_{5A}, 5-HT₆ and the serotonin transporter (5-HTT). Non-serotonin receptors for which C-122 showed significant affinity included the histamine receptor H-1 ($K_i = 3.6$ nM) and dopamine receptors D₁, D_{2L}, D_{2S}, D₃, and D_{4A} ($K_i = 0.085$ – 8.0 nM) (Table 3). No significant binding was detected to endothelin or prostenoid receptors, nor to a

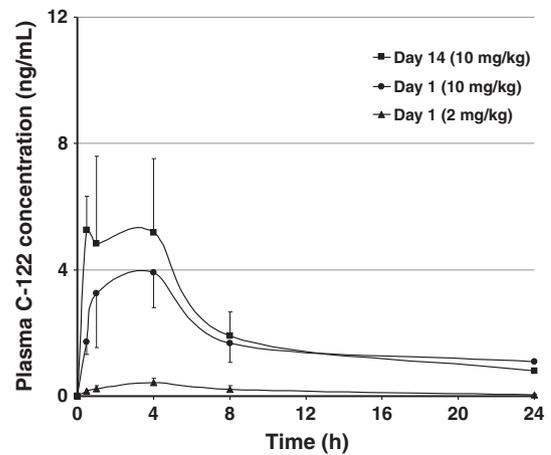


Fig. 5. Concentration-time profile of C-122 in plasma of male rats following a single oral dose of C-122 at 2 mg/kg (triangles) or 10 mg/kg (circles), or following the 14th consecutive daily oral dose (squares). Each point represents a mean concentration ($n = 3$); error bars illustrate standard deviations.

broad panel of potential off-target receptors at concentrations up to 1 μ M (see receptor listing in Section 2.7). In functional assays, C-122 was a potent antagonist for serotonin receptors 5-HT_{2B} ($IC_{50} = 6.9$ nM), a somewhat weaker antagonist of 5-HT₇ ($IC_{50} = 33$ nM) and 5-HT_{2A} (120 nM), and had very weak activity against 5-HT_{2C}, 5-HT₄, and 5-HT₆ receptors ($IC_{50} > 700$ nM) (Table 3). C-122 was a potent antagonist of dopamine receptors D₁, D_{2L}, D_{2S}, and D₃ (IC_{50} values 0.5–19 nM), and a weaker antagonist of dopamine receptors D_{4.2}, D_{4.4}, and D_{4.7} (IC_{50} values 49–230) and histamine receptor H-1 ($IC_{50} = 95$ nM). For all receptors where antagonist activities were measured for C-122, agonist activity also was measured and EC_{50} was found to be > 3000 nM.

4. Discussion

The present study demonstrates that C-122, a novel 5-HT receptor antagonist, provides protection against the development of MCT-induced pulmonary arterial hypertension in the rat. A single injection of MCT led to fibromuscular hypertrophy and hyperplasia in the walls of pulmonary resistance arterioles accompanied by elevated pulmonary arterial and right ventricular pressures as well as right ventricular hypertrophy. Daily oral treatment with C-122 for 21 days following MCT challenge largely prevented histopathologic changes and completely abolished increases in MPAP, RV systolic pressure and RV hypertrophy. C-122 treatment of saline-injected controls was without effect on normal vascular morphology or hemodynamics.

Prevention of PAH was observed in rats dosed orally with C-122 at 10 mg/Kg, but not 1 mg/kg. In pharmacokinetic experiments, mean plasma levels of C-122 reached $C_{max} = 4$ – 6 ng/ml during an interval of 1–4 h after a single 10 mg/kg oral dose, then gradually declined to < 1 ng/ml at 24 h. Because C-122 in rat plasma was 96.5% protein-

Table 2

C-122 Pharmacokinetic parameters following repeated daily oral administration 10 mg/kg C-122 to male rats ($n = 3$ rats/time point).

Study day	Dose (mg/kg)	T_{max} (h) ^a	C_{max} (ng/ml)	AUC_{0-24h} (ng * h/ml)	Vz/F (L/kg)
1	2	4	0.43	11.5	NA
1	10	4	3.9	45.8	1920
14	10	0.5	5.3	54.6	NA
1	Ratio 10 mg/kg:2 mg/kg	NA	9	3.98	NA
Ratio day14:day 1	10	NA	NA	1.19	NA

^a T_{max} – time of maximum concentration; C_{max} – maximum concentration; AUC_{0-24h} – area under the curve from 0 to 24 h; Vz/F – volume of distribution; NA – not applicable.

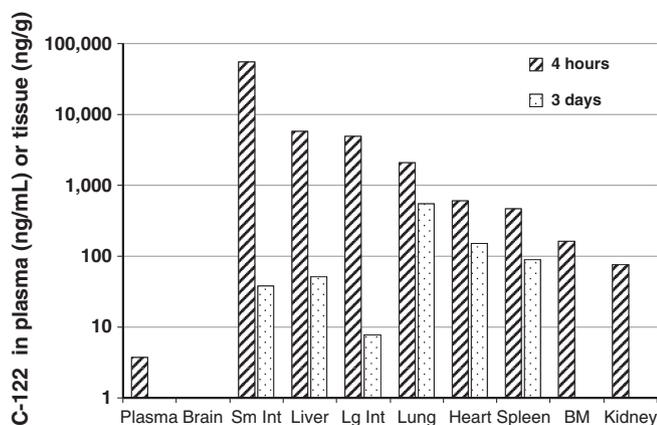


Fig. 6. Concentrations of C-122 in plasma (ng/ml) or tissues (ng/g) of male rats ($n=3$) sacrificed 4 h (solid bars) or 3 days (open bars) after a single oral dose of C-122. Abbreviations are as follows: Sm Int, small intestine; Lg Int, large intestine; and BM, bone marrow.

bound, the concentration of free drug at C_{max} was on the order of 0.1 nM. After the 14th consecutive daily dose at 10 mg/kg, C_{max} and AUC_{0-24h} were only slightly higher than after the initial dose, indicating little if any accumulation of C-122 in plasma upon repeat dosing. Although the present study was not designed to define dose–response, there is an apparent correlation between absence of efficacy at a 1 mg/kg dose in MCT rats and 9-fold lower C_{max} plus nearly 4-fold lower AUC_{0-24h} after a 2 mg/kg dose as compared with a 10 mg/kg dose in pharmacokinetic experiments. Biodistribution studies showed that C-122 partitions into many tissues, including heart and lung. After a single 10 mg/kg oral dose, the levels of C-122 in heart and lung exceed 500 ng/g for at least 4 days, and steady state levels during a daily dosing regimen may potentially be even higher. Because the tissue compartment into which C-122 rapidly exchanges is currently

Table 3
Binding constants of C-122 for various receptors.

Agonist	Receptor	(K_i) (nM)	Antagonist IC_{50} (nM) ^a
Serotonin	5-HT _{1A, 1B, 1D}	>100	ND
	5-HT _{2A}	61	120
	5-HT _{2B}	5.2	6.9
	5-HT _{2C}	>100	794
	5-HT ₃	>500	ND
	5-HT _{4e}	965	1400
	5-HT _{5A}	>500	ND
	5-HT ₆	92	1400
	5-HT ₇	4.4	33
	5-HTT	>1000	ND
Dopamine	D ₁	1.6	16
	D _{2L}	0.61	0.5
	D _{2S}	0.42	4.4
	D ₃	0.085	19
	D _{4,2}	40	73
	D _{4,4}	8.0	230
	D _{4,7}	121	49
Histamine	H-1	3.6	95
	H-2	>500	ND
	H-3	>1000	ND
	H-4	>1000	ND
Endothelin	ETA	>1000	ND
	ETB	>1000	ND
Prostanoid	CRTH ₂	>1000	ND
	DP	>1000	ND
	EP ₂	>1000	ND
	EP ₄	>1000	ND
	FP	>1000	ND
	TP	>1000	ND

^a Where antagonist functional activities are indicated, agonist activities also were measured and found to be >3000 nM.

unknown, it is difficult to estimate precisely the effective concentration of drug to which membrane bound receptors in lung vessel walls and interstitium are exposed.

In vitro screens for binding to serotonin and non-serotonin receptors showed that C-122 can displace high affinity labeled ligands from 5-HT_{2A}, 5-HT_{2B}, 5-HT₇, histamine H-1, and multiple dopamine receptors with K_i values in the low nanomolar range. C-122 showed a weak or absent interaction with a broad panel of drug receptors, including leukotriene, prostanoid, or endothelin receptors. Functional assays revealed that C-122 antagonizes the 5-HT_{2B} receptor with $IC_{50}=6.9$ nM and is a 4.7-fold and 17.4-fold less potent antagonist for 5-HT₇ and 5-HT_{2A} receptors, respectively. Potential off-target activities of C-122 include potent antagonism of D_{2L}, and D_{2S} ($IC_{50}=0.5$ nM and 4.4 nM, respectively) and weaker antagonism of the histamine H-1 receptor ($IC_{50}=95$ nM) and dopamine receptors D₁, 44.2, D_{4,4}, and D_{4,7} (IC_{50} values ranged from 16 nM to 230 nM). The absence of detectable C-122 in brain agrees with in vitro transcellular efflux studies that show the drug to be a good substrate for the P-gp drug transporter and suggest that at a 10 mg/kg oral dose of C-122 effective for prevention of PAH, there is minimal risk for central nervous system effects such as drowsiness or pseudo-parkinsonian symptoms associated with antagonism of H-1 and dopamine receptors, respectively.

MCT-induced PAH in the rat has been used extensively to demonstrate drug effectiveness in prevention and treatment of PAH. In this animal model, acute toxic injury provokes a tissue reaction that recapitulates in 2–3 weeks, an indolent human disease with a natural history of progression over several years. A prominent feature of both the rat MCT model and of human PAH is adherence of platelets to pulmonary endothelium with local release of serotonin and other mediators (Herve et al., 2001; Kanai et al., 1993). Details of ultrastructural changes in pulmonary architecture after a single subcutaneous injection of monocrotaline were described in the rat by Valdivia et al., 1967. The toxin is cleared from plasma within 24 h of dosing (Hayashi, 1966), but progressive interstitial alveolar edema, alteration of endothelial and interstitial cells, modification of basement membranes, increased numbers of mast cells, and formation of platelet thrombi occur during the ensuing 2–3 weeks (Lalich et al., 1977). As early as 6 h after dosing and persisting for 2 weeks, there is massive sequestration of platelets into the MCT-injured lung (White and Roth, 1988) accompanied by decreased capacity of the lung to clear 5-HT from plasma (Hilliker et al., 1982) and increased levels of circulating 5-HT (Hilliker et al., 1982; Kato et al., 1997). Higher levels of 5-HT in arterial vs. venous blood (Kato et al., 1997) in the MCT rat may reflect ongoing release of 5-HT from platelet microthrombi in the lung. Dynamics of 5-HT transport and release may be different in the human disease, however, as demonstrated by recent measurements of 5-HT in plasma and platelets from 13 patients suffering from PAH (Ulrich et al., 2011). Instead of elevated 5-HT in plasma of PAH patients relative to normal controls described by Herve et al. (1995), Ulrich et al. (2011) found no elevation of 5-HT in arterial or venous plasma from PAH patients, but surprisingly, found that platelets from both arterial and venous blood of PAH patients had significantly lower than normal 5-HT content. This effect was more pronounced in arterial blood platelets, suggesting that a subset of platelets enriched for 5-HT may be selectively depleted and their released 5-HT taken up from plasma during passage through the pulmonary capillary bed. Release of 5-HT from activated platelets in thromboemboli can lead to local plasma concentrations of 5-HT as high as 200 ng/ml (1.1 μ M) (Benedict et al., 1986) a value that far exceeds the K_i of 5-HT for the rat serotonin 5-HT_{2A} (5.75 nM) or 5-HT_{2B} (10.2 nM) receptors (Boess and Martin, 1994).

5-HT has been reported to exert effects in lung and in platelets via both the 5-HT transporter (5-HTT) and distinct receptors subtypes. Some investigators report up-regulation of 5-HTT expression, but little or no change from basal expression of 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and

5-HT₇ receptors in primary human lung tissue or pulmonary artery smooth muscle cell (PASMC) explants from idiopathic PAH patients compared with normal donors (Eddahibi et al., 2002; Marcos et al., 2004). In contrast, others found unchanged expression of 5-HTT, but increased expression of 5-HT_{2B} receptors in PASMCs from idiopathic PAH patients (Launay et al., 2002). Launay et al. (2002) proposed that 5-HT_{2B} plays a central role in vascular smooth muscle proliferation leading to PAH based upon preventive effects of selective 5-HT_{2B} receptor antagonists RS 127445 and PRX-08066 in MCT or hypoxia animal models, and abrogation of chronic hypoxia-induced PAH hypoxia in 5-HT_{2B}^(-/-) receptor knockout mice. In support of this proposal, Dumitrascu et al. (2011) demonstrated that daily treatment with terguride, an antagonist of 5HT_{2A} and 5-HT_{2B} receptors, prevented MCT-induced elevation in RVSP, RV hypertrophy, proliferative and inflammatory histopathologic changes in pulmonary arterioles, and increases in inflammatory cytokines. Furthermore, Porvasnik et al. (2010) showed that the specific 5HT_{2B} receptor antagonist, PRX08066 diminished vascular remodeling and attenuated the elevation of pulmonary arterial pressure and right ventricular hypertrophy in the MCT rat model.

In conclusion, the present study clearly demonstrates that C-122 administered daily for three weeks at a dose that shows no apparent toxicity effectively prevents the onset of increased muscularization of pulmonary resistance arterioles and associated elevated pressures in the rat MCT model for PAH. C-122 is a potent antagonist of the 5-HT_{2B} receptor in vitro and is orally absorbed, but its plasma C_{max} in the rat (0.1 nM free drug) is, paradoxically, lower than the observed IC₅₀ for the 5-HT_{2B} receptor in vitro (6.9 nM), suggesting that drug activity may rely upon relatively high tissue levels observed in several organs, including heart (>600 ng/g) and lung (>2000 ng/g). Although further study will be required to define its detailed mechanism of action, C-122 offers promise as a new therapeutic entity to address the critical need to suppress vascular smooth muscle cell proliferation in PAH patients.

Disclosures

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