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Terguride ameliorates monocrotaline induced pulmonary hypertension in rats

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Running Title

Terguride in pulmonary hypertension

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Abstract

BACKGROUND: Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by vasoconstriction and remodeling of the pulmonary vasculature. The serotonin (5-hydroxytryptamine [5-HT]) pathway has been shown to play a major role in the pathogenesis of PAH, but pharmacological modulation of this pathway for treatment of pulmonary arterial hypertension is to date at preclinical level. Terguride is a 5-HT receptor (5-HTR) antagonist which is well tolerated and clinically approved for ovulation disorders.

METHODS and RESULTS: Immunohistochemistry against $5\text{-HT}_{2A/B}$ receptors on human lungs revealed their localization to vascular smooth muscle layer and quantitative RT-PCR showed 5-HTR_{2B} up regulation in pulmonary artery smooth muscle cells (PASMC) isolated from PAH patients. Proliferation and migration of cultured primary human PASMC were dose-dependently blocked by terguride. Therapeutic 5-HT signaling inhibition was *first* demonstrated in isolated ventilated and perfused rat lungs and *second* by chronic terguride treatment of rats with monocrotaline-induced pulmonary hypertension in a preventive or curative approach.

CONCLUSION: Terguride inhibited proliferation of pulmonary artery smooth muscle cells and abolished 5-HT induced pulmonary vasoconstriction. Chronic terguride treatment prevented dose-dependently the development and progression of MCT-induced PAH in rats. Thus, terguride represents a valuable novel therapeutic approach in PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by an increase of pulmonary artery pressure resulting from endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells [1]. When untreated, the disease finally results in right ventricular (RV) failure and death. Several important signaling systems have been shown to be dysregulated in pulmonary hypertension (PH). In patients with idiopathic pulmonary arterial hypertension, a reduced excretion of prostaglandin (PG)I_2 and an enhanced excretion of thromboxane metabolites has been noted [2]. Moreover, enhanced expression of phosphodiesterase (PDE) 5, which hydrolyzes the NO-induced second messenger cGMP, was observed in pulmonary hypertension [3]. In addition, the vasoconstrictor endothelin is upregulated in PAH [4] and correlates with the degree of the disease [5]. Furthermore, the anorexic epidemic implied a role of serotonin (5-hydroxytryptamine [5-HT]) in the pathogenesis of PH [6]. Expression analysis of lung tissues from PAH patients undergoing lung transplantation, revealed an increased expression of 5-HT transporter (5-HTT) and an enhanced proliferative growth response of isolated pulmonary arterial smooth muscle cells (PASMC) to 5-HT [7]. While most of above mentioned pathways are currently addressed clinically for treatment of PAH, e.g. by infusion or inhalation of prostanoids [8, 9], oral application of PDE 5 inhibitors [10, 11] and endothelin antagonists [12], the 5-HT pathway is still studied only on a preclinical level. Experimentally it has been shown that inhibitors of 5-HTT, e.g. fluoxetine, reversed monocrotaline-induced pulmonary hypertension in rats [13] and 5-HTT overexpressing mice develop spontaneously pulmonary hypertension [14]. Furthermore, the 5-HT receptors 5-HTR_{1B}, 5-HTR_{2A}, 5-HTR_{2B} and 5-HTR_7 are expressed in smooth muscle and endothelial cells of the pulmonary vasculature [15] , and 5-HT levels are increased in plasma of pulmonary hypertensive patients [16, 17]. Strong evidence for the 5-HT_{2B} receptor as a therapeutic target in PAH has emerged. 5-HT_{2B} knockout animals are resistant to hypoxia-induced pulmonary hypertension [18] and administration of the specific 5-HT_{2B} receptor

antagonist RS-127445 prevented the increase in pulmonary arterial pressure in mice which were challenged to hypoxia. Here we address the question of whether terguride, a potent 5-$HTR_{2A/2B}$ antagonist, might not only cause acute vasodilation in the lung, but exert anti-remodeling effects upon long-term use in chronic experimental pulmonary hypertension. Terguride is approved for ovulation disorders due to hyperprolactinemia by acting as a partial dopamine D_2 receptor agonist in the pituitary (for review see [19]). In addition, it is a strong antagonist of the 5-HT_{2A} [20, 21] and 5-HT_{2B} receptor [22] and therefore seems well suited for treatment of PAH. In this study we investigated expression and localization of 5-$HT_{2A/B}$ receptors in human lungs from healthy versus PAH condition. Next, we addressed the question whether terguride could inhibit proliferation and/or migration of cultured human primary pulmonary artery smooth muscle cells. In isolated rat lung we examined the acute vasorelaxant efficacy of terguride on 5-HT induced vasoconstriction. Furthermore pulmonary arterial anti-remodeling effects and therapeutic efficacy of long-term terguride treatment in experimental pulmonary hypertension were studied. To this purpose, the model of monocrotaline-induced pulmonary hypertension was employed. Monocrotaline (MCT) is a toxin derived from plants of the *Crotalaria* species [23], which causes pulmonary arterial endothelial cell injury and subsequent pulmonary artery smooth muscle hypertrophy [24].

Material and Methods

Human samples and patient characteristics

Human lung tissue was obtained from ten donor patients and ten patients diagnosed as suffering of idiopathic pulmonary arterial hypertension (IPAH) undergoing lung transplantation (5 female, 5 male, mean age 36.2 ± 3.9, mean pulmonary arterial pressure (PAP) 66.0 ± 9.4 mm Hg). However, none of the 10 patients with IPAH had a BMPR2 mutation. After surgery, human lung tissue was directly preserved on ice for PASMC isolation or snap frozen in liquid nitrogen for mRNA isolation, or transferred into 4% buffered paraformaldehyde for histopathological investigation. Tissue donation was approved by the national ethical committee and national law. All patient enrolled in this study gave written informed consent.

Reverse Transcription and quantitative real-time PCR

Lung homogenates and freshly explanted PASMCs were subjected to gene expression analysis of the 5-HT_2 receptors (5-HTR_{2A} and 5-HTR_{2B}), 5-HT transporter (5-HTT) and dopamine receptors (DRD_1, DRD_2, DRD_3 and DRD_4). For this purpose total RNA extraction, cDNA synthesis, and quantitative (q)RT-PCR using the primers listed in Supplemental Table 1 were performed. Under identical cycling conditions, all primer sets worked with similar efficiencies to obtain simultaneous amplification in the same run, as described before [25]. Sequences were taken from GeneBank, all accession numbers are denoted. Hypoxanthine phosphoribosyltransferase (HPRT), an ubiquitously and equally expressed gene free of pseudogenes, was used as a reference gene in all qRT-PCR reactions. Relative transcript abundance is expressed as ΔCt value ($\Delta Ct = Ct^{reference} - Ct^{target}$), where higher ΔCt values indicate higher transcript abundances, and negative ΔCt values represent genes that are less expressed compared with the reference gene. Similarly, gene expression analysis of

proinflammatory cytokines such as interleukin 1 beta (IL1β), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα) and monocyte chemotactic protein-1 (MCP-1) was assessed in rat lung homogenates.

Western blotting

PASMCs were homogenized in lysis buffer containing 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$, 150 mM NaCl, 60 mM NaN_3 and 0.1% (w/v) Triton X-100 using a tissue homogenizer. Samples were centrifuged at 13000 rpm for 20 min at 4°C, and the supernatant protein content was measured using Dye Reagent Concentrate (Bio-Rad, Muenchen, Germany). Extracts containing equal amounts of protein were denatured and subjected to electrophoresis on a 10% SDS polyacrylamide gel and blotted on to polyvinylidene fluoride membrane with a semidry transfer unit (Biometra, Goettingen, Germany). The membrane was then incubated with anti-5-HTR_{2B} (Santa Cruz Biotechnology, Santa Cruz, CA) and then with the appropriate HRP-conjugated secondary antibody. Equal protein loading was confirmed by blotting membranes with an antibody against GAPDH. The bands were visualized using an enhanced ECL detection kit (Amersham Bioscience, Freiburg, Germany) and quantified by densitometry.

Migration assay of Human pulmonary artery smooth muscle cells (PASMC)

PASMC were explanted from pulmonary arteries as previously described [26]. They were cultured in Smooth Muscle Cell Growth Medium 2 (PromoCell) enriched with ComplementMix C-39267 at 37°C in a 5% CO_2, 95% O_2 atmosphere. At 70% confluence, PASMCs were treated with terguride at concentrations of 0, 0.01 μM or 1 μM for 24 hours. PASMC were then trypsinized and further incubated with terguride at concentrations of 0, 10^{-6}M or 10^{-8}M for 1 hour prior to assessment of their migration ability to PDGF (10ng/ml) using a Boyden chamber (Neuro Probe, Gaithersburg, MD) as described [27].

Proliferation Assay of Human pulmonary artery smooth muscle cells (PA-SMC)

Freshly isolated human PASMCs were plated onto a 48 well plate. They were subjected to starvation for the 24 hours using smooth muscle cell medium containing 0.5% supplement. Subsequently cells were treated with terguride or vehicle for 24 hours. Then, [^{3}H]-Thymidine (Amersham, UK) was added to each well for 6 hours. After washing with PBS cells were lysed in 0.5M NaOH and [^{3}H]-Thymidine incorporation was quantified by scintillation technique as described [28].

RNA extraction, Reverse Transcription and Semi-Quantitative PCR analysis

Explanted PASMCs were cultured as above described. At 70% confluence they were treated with terguride at concentrations of 0, 0.01 μM or 1 μM for 24 hours. Then, mRNA was extracted from PASMC using the Qiagen kit according to manufacturer's instructions and reverse transcribed to cDNA using the Promega ImPro II reverse transcriptase. Semi-quantitative PCR analysis was performed for collagen type A_1 and A_2 and fibronectin. The band intensities were normalized to the loading control, heat shock protein 70 (HSP 70). Specific primers used for sequence detection were as follow: for collagen type A_1, 5'AATGGTGCTCCTGGTATTGC3' (forward) and 5'GGAAACCTCTCTCGCCTCTT3' (reverse); for collagen type A_2, 5'TTATTCCCAATTAAAAGTATGCAGATTATT3' (forward) and 5'GAAGATGAAAATGAGACTGGCAAA3' (reverse); for fibronectin, 5'CCGACCAGAAGTTTGGGTTCT3' (forward) and 5'CAATGCGGTACATGACCCCT3' (reverse); for HSP 70 5'TGTGTCTGCTTGGTAGGAATGGTGGTA3' (forward) and 5'TTACCCGTCCCCGATTTGAAGAAC3' (reverse). Relative mRNA levels were calculated by densitometry and normalized to HSP 70.

Animals

Adult male Sprague Dawley rats (body weight of 300 to 350 grams) were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were housed under controlled conditions with free access to rodent chow and tap water. All experiments were conducted according to the institutional guidelines that comply with national and international regulations.

Animal experimental protocol

Acute vasodilatory effects of terguride were investigated in isolated ventilated and perfused rat lungs. For the experiments, lungs were prepared from five groups of five rats each. 5-HT induced pulmonary vasoconstriction was assessed in presence of defined concentrations of terguride, ketanserin, SB 204741, ropirinole and vehicle.

For chronic treatment studies, seven groups with MCT-induced pulmonary hypertension were studied, three with "early" intervention or sham intervention, and four with "late" intervention or sham intervention. Terguride treatment groups comprised $Ter1_{0-28}$ (0.4 mg/kg, bid from day 0 to day 28), group $Ter2_{0-28}$ (1.2 mg/kg, bid from day 0 to day 28), group $Ter3_{14-28}$ (0.4 mg/kg bid from day 14 to day 28), group $Ter4_{14-28}$ (1.2 mg/kg bid from day 14 to day 28). The corresponding controls include vehicle treated groups MCT_{0-28}, MCT_{14-28} and MCT_{14} as well as a healthy control group for reference purposes. Doses of terguride were chosen according to preceding pilot experiments, addressing long-term tolerability of this agent.

MCT-induced PH and chronic treatment

Chronic progressive pulmonary hypertension was induced in rats as previously described [28, 29]. Briefly, rats received a single subcutaneous injection of 60 mg monocrotaline/kg body weight. Control animals were administered subcutaneously saline solution. Monocrotaline-

injected animals were randomized for placebo or chronic terguride therapy. Long term treatment was administered by intraperitoneal injection. Terguride was dissolved in ethanol, subsequently diluted with sodium acetate prior to pH adjustment to 7.4. Ethanol concentration in the injected solution was less than 5% (v/v). Terguride was administered at dose levels as described above in a volume of 0.25 ml/rat by twice daily intraperitoneal injection. Placebo groups received ethanol/sodium acetate solution at the same volume.

Hemodynamics, arterial oxygenation and cardiac output

In order to monitor hemodynamics, animals were anaesthetized by intraperitoneal injection with ketamine/xylazine as described [28]. Tracheotomy was performed and animals were artificially ventilated with 10 ml/kg body weight. Inspiratory oxygen fraction (FiO_2) was set at 0.5 and a positive endexpiratory pressure of 1.0 cm H_2O was used throughout. Systemic arterial pressure was monitored by cannulating carotid artery with a polyethylene cannula connected to a fluid filled transducer (Braun, Germany). Pulmonary pressure expressed as right ventricular systolic pressure was assessed by right heart catheterisation through the jugular vein. Animals were placed on a heating pad in order to maintain body temperature for the duration of the experiment. Arterial and venous blood samples were collected during hemodynamic measurement and analyzed by an automatic blood analyzer (ABL 500).

Isolated ventilated and perfused lung

The acute vasorelaxant effects of terguride were investigated in isolated ventilated and perfused rat lungs. Briefly, animals were deeply anaesthetized and lungs were removed from the thoracic chest. Lungs were ventilated with room air and perfused in a recirculating system as described previously [30]. A fluid filled catheter connected to a transducer was placed into pulmonary artery for pulmonary artery pressure assessment throughout the experiment. Defined terguride concentrations of 0, 1, 3 and 10 nM were applied in the recirculating buffer

ten minutes before 5-HT challenge and pulmonary pressure was recorded. Similarly, specific 5-HTR_{2A} and 5-HTR_{2B} inhibition was induced using ketanserin (concentration range 0-10 nM) and SB 204741 (concentration range 0-1 μM). Dopaminergic agonism was achieved using ropirinole in a concentration range from 0 to 1μM.

Tissue processing

The right lungs from monocrotaline injected rats were preserved and snap frozen in liquid nitrogen. Left lungs were perfused through the pulmonary artery and through tracheae with Zamboni fixative (formaldehyde 2%, picric acid 15% in 0.1M phosphate buffer) at a constant pressure of 22 and 11 cm H_2O, respectively. Lung lobes were immersed in Zamboni reagent. For paraffin embedding lung lobes were dissected in tissue blocks from all lobes. Sectioning at 3 μm thickness was performed from all paraffin-embedded blocks.

Right heart hypertrophy assessment

Hearts were removed and right ventricles were dissected from the left ventricles and septum. They were dried and weighted. Right ventricular hypertrophy was assessed by the ratio (RV/(LV+S)), i.e. the ratio of weight of right ventricular wall (RV) versus left ventricular wall plus septum (LV+S).

Histology and immunohistochemistry

To address the cellular localization of 5-HTR_{2A} and 5-HTR_{2B} in human and rat lung tissue, histological sections of lungs from donors and IPAH patients undergoing lung transplantation or lungs from MCT-treated or non-treated control rats were used. Human lung tissues were fixed for 24 hours with 4% buffered paraformaldehyde at 4°C and embedded in paraffin. Rat tissue was fixed as described above. 3 μm thick sections were immunohistochemically stained against 5-HTR_{2A} (polyclonal antibody, ImmunoStar, U.S., Art. Nr: 24288, dilution 1:100) and

5-HTR_{2B} (polyclonal antibody, Abcam plc, Cambridge, UK, Art. Nr: 13292, dilution 1:800). Representative histological photographs were acquired at a 200 fold magnification. For assessment of wall thickness of small peripheral pulmonary arteries, histological sections of rat lungs were used. For this purpose elastica staining was performed according to published histopathological procedures.

Muscularization degree of small pulmonary arteries was assessed by means of double immunostaining of the 3-µm sections with anti-α-smooth muscle actin antibody (dilution 1:900, clone 1A4, Sigma, Saint Louis , Mo) and anti-human von Willebrand factor antibody (dilution 1:900, Dako, Hamburg, Germany) as previously described [28]. Sections were counterstained with methyl-green and examined by light microscopy. All samples were analyzed in a blind fashion by two independent anatomopathologists.

Collagen deposition was estimated in rat lung sections after Sirius red and trichrome Masson staining. Quantification was performed in light microscopy by image analysis using an automated morphometric system (Qwin, Leica, Wetzlar, Germany). The automated analysis was set to differentiate positively stained from negatively stained areas of the image. In addition, sections were analysed under polarization microscopy. Collagen deposition is expressed as a percent of positive staining from total analysed area.

Morphological assessment of lung vasculature

Wall thickness of small pulmonary arteries was investigated on elastica-stained lung sections by light microscopy with the use of a computerized morphometric system (Qwin, Leica, Wetzlar, Germany).

The degree of muscularization of small pulmonary arteries was assessed as previously described [29]. Briefly, 80 to 100 intra-acinar lung vessels accompanying either alveolar ducts or alveoli were analyzed at a 400-fold magnification. Vessels were categorized as non-, partially- or fully-muscularized according to a smooth muscle content of <5%, 5 to 75% or

>75%, respectively. The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number counted in the whole experimental group. Both, muscularization degree and wall thickness were analyzed in a blinded fashion.

Data analysis

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

Results

Pulmonary expression of serotonin receptors (5-HTR) and serotonin transporter (5-HTT) in pulmonary hypertension

We investigated by immunostaining the expression and localization of serotonin receptors 5-HTR_{2A} and 5-HTR_{2B} in lung tissue from healthy donors and IPAH patients undergoing lung transplantation. Positive 5-HTR_{2A} immunostaining was observed in the smooth muscle cells layer, while 5-HTR_{2B} stained pulmonary vascular endothelium and vascular smooth muscle layer (Figure 1 Aa-p). Similar pattern was observed in rat lung tissues obtained from monocrotaline-treated and control animals (Figure 3a-p). Gene expression analysis revealed that 5-HTR_{2A}, 5-HTR_{2B} and 5-HT transporter (5-HTT) were expressed in lung homogenates and confirmed expression of theses genes in isolated PASMCs (Figure 1 B, C). No significant difference in gene expression was noted in lung homogenates from IPAH patients when compared with donor patients. With respect to the expression of dopamine receptor isoforms DRD_1, DRD_2, DRD_3 and DRD_4 in lung homogenate no significant differences in expression between lung tissue from donors and IPAH patients were observed (Supplemental Figure 1).

In contrast and despite no significant difference in lung homogenates of donors and IPAH patients were observed, 5-HTR_{2B} expression in PASMCs from IPAH patients was up-regulated at both mRNA and protein expression (Figure 1 C-E). 5-HTT was expressed to comparable extents in PASMCs from donors and IPAH patients.

Effects of terguride on collagen synthesis, cell migration and proliferation of pulmonary arterial smooth muscle cells (PASMC)

To study the effects of terguride on serum induced PASMC proliferation, serum starved PASMCs were stimulated with 5% FCS in the presence or absence of terguride. Stimulation of cultured PASMCs with 5% FCS induced proliferation (Figure 2 A). In the presence of 0.1 μM Terguride inhibited the FCS stimulated [^{3}H]-Thymidine incorporation in PASMCs to 66.3

± 3.5% vs. serum stimulated control (Figure 2 A). Subsequently, the involvement of distinct 5-HT receptor isoforms in the observed proliferative effects in the presence of 5% FCS was studied by ketanserin and SB 204741, which selectively inhibit 5-HTR_{2A} and 5-HTR_{2B} receptors, respectively. As shown in Figure 3 A, in the presence of 1 μM ketanserin and SB 204741 proliferation of PASMCs was inhibited to 51.4 ± 7.3% and 47.4 ± 7.1%, respectively. When the same experiment was performed in PASMCs derived from IPAH lungs, inhibition of FCS stimulated proliferation was observed to a comparable extent in the presence of terguride, ketanserin and SB 204741 when compared with PASMCs from donor lungs (Figure 2 B).

Furthermore, when PASMCs were assessed for cell migration activity towards PDGF (10 ng/ml) in a Boyden chamber, preincubation with 1 μM terguride significantly inhibited PASMCs migration (Figure 2 C). In addition, expression of collagen A_1, collagen A_2 and fibronectin in 5% FCS stimulated PASMCs were assessed by semi-quantitative PCR analysis. In the presence of 1 μM terguride a significant down-regulation of collagen A_2 mRNA was observed while collagen A_1 and fibronectin mRNA expression levels were not significantly changed (Figure 2 D).

Effects of Terguride on constricted lung vasculature in isolated rat lungs.

5-HT at a concentration of 1 μM induced reproducibly a pulmonary vasoconstriction with a 20.17 ± 1.51% increase in the vascular pressure when compared to lungs perfused in the absence of 5-HT. This rise in pressure was inhibited by terguride in concentration-dependent manner with 34.7 ± 9.1%, 69.8 ± 12.8% and 89.9 ± 4.2% in the presence of 1, 3, and 10 nM terguride, respectively (Figure 4). Addition of the specific 5-HTR_{2A} inhibitor ketanserin to the perfusate markedly inhibited 5-HT induced vasoconstriction. In contrast, SB 204741, a specific 5-HTR_{2B} inhibitor, did not ameliorate vasoconstriction by 5-HT. Similarly, the

presence of ropinirol, an agonist on DRD_2 and DRD_3 receptors, did not change vascular pressure regardless of the presence or absence of 5-HT in the perfusate (Figure 4).

Effects of Terguride treatment from day 0 to day 28 on pulmonary pressure, right heart hypertrophy and gas exchange in rats with monocrotaline-induced pulmonary hypertension.

Rats injected with monocrotaline developed progressive pulmonary hypertension within 28 days. This is demonstrated by the sustained significant increase in right ventricular systolic pressure (RVSP) to 66.1 ± 5.5 mmHg at day 28 versus 26.1 ± 1.5 mmHg for control animals ($p<0.05$). Elevated pulmonary pressure was accompanied by right heart hypertrophy measured as ratio of the right ventricle per left ventricle plus septum weight ratio (RV/(LV+S)). This increased significantly 28 days after monocrotaline injection to 0.71 ± 0.03 versus 0.30 ± 0.01 in control animals ($p<0.05$). Daily treatment of monocrotaline-injected animals from day 0 to day 28 with terguride attenuated the above mentioned pathophysiological changes. Treatment of animals with 0.4 mg terguride/kg body weight/bid significantly reduced pulmonary pressure (47.8 ± 6.3 versus 66.1 ±5.5 for vehicle treated animals, $p<0.05$) (Figure 5 A) and RV/LV+S ratio (0.28 ± 0.01 versus 0.71 ± 0.03 of vehicle treated animals, $p<0.05$) (Figure 5 B). Treatment with 1.2 mg/kg body weight terguride led to almost complete abolishment of changes in pulmonary pressure (36.4 ± 1.7 versus 66.1 ± 5.5, $p<0.05$) induced by MCT. Likewise, changes in RV/LV+S were completely abolished by this treatment (0.26 ± 0.01 versus 0.71 ± 0.03, $p<0.05$) (Figure 5 A and B). In addition, terguride treatment at these dose levels improved arterial oxygenation which was impaired in MCT-injected rats after 28 days (318 ± 56 mmHg in MCT versus 430 ± 55 mmHg and 436 ± 21 mmHg in terguride treated animals with 0.4 and 1.2 mg/kg, respectively) (Figure 5 C). Moreover, treatment with 1.2 mg/kg body weight terguride led to increased survival. However, terguride treatment didn't show any significant effects on systemic arterial pressure

(SAP), systemic vascular resistance index (SVRI) and bodyweight in MCT-injected rats after 28 days (Supplemental table 2).

With respect to the pulmonary vasculature, we found in MCT-injected rat lungs after 28 days a significant increase in medial wall thickness of vessels between 25-50 μm in diameter (18.6 ± 0.4% in control versus 28.7 ± 0.3% in MCT, $p<0.05$) and vessels between 51-100 μm in diameter (17.0 ± 0.4% versus 23.0 ± 0.5%, $p<0.05$) (Figure 5 D). In addition, monocrotaline injection led after 28 days to significant muscularization of small pulmonary vessels – assessed as percentage of fully muscularized vessels (0.2 ± 0.1% for control versus 68.2 ± 7.2% for vehicle treated animals) (Figure 5 E). Medial wall thickness as well as vascular muscularization was prevented by chronic treatment with terguride at two different doses. Medial wall thickness in animals treated with terguride reached values similar to those of saline injected animals (19.8 ± 0.3% and 20.3 ± 0.3% for terguride treated animals with 0.4 and 1.2 mg/kg body weight and vessels with diameter between 20 to 50 μm, respectively) (Figure 5 D). Similarly, number of fully muscularized vessels was significantly reduced in animals chronically treated with terguride (12.5 ± 15 and 19.9 ± 4.0 for terguride treated animals with 0.4 and 1.2 mg/kg body weight, respectively) when compare to non-treated MCT rats (Figure 5 E).

Effects of Terguride treatment from day 14 to day 28 on pulmonary pressure, right heart hypertrophy and gas exchange in rats with monocrotaline-induced pulmonary hypertension.

Terguride treatment from days 14 to 28 reduced significantly pulmonary pressure in a dose dependant manner (Figure 6 A) when compared to 4 weeks MCT and vehicle treated rats (53.8 ± 4.6 mmHg for terguride 0.4 mg/kg body weight and 47.3 ± 5.7 mmHg for terguride 1.2 mg/kg body weight, versus 66.1 ± 5.5 mmHg for vehicle treated animals). Pulmonary pressure was in both groups still higher than that in animals two weeks after MCT injection.

Chronic terguride treatment from day 14 to day 28 reduced significantly right heart hypertrophy (0.38 ± 0.02 for terguride dose of 0.4 mg/kg body weight and 0.39 ± 0.03 for terguride dose of 1.2 mg/kg body weight) (Figure 6 B). The above mentioned changes were accompanied by improvement in alveolar gas exchange assessed as pO2/FiO2 (390 ± 73 for terguride at a dose 0.4 mg/kg body weight and 521 ± 30 for terguride at a dose 1.2 mg/kg body weight, versus 318 ± 56 for vehicle treated animals) (Figure 6 C), but with no significant effects on systemic arterial pressure (SAP), systemic vascular resistance index (SVRI) and bodyweight (Supplemental table 2).

Medial wall thickness of small pulmonary arteries with a diameter between 25 to 50 µm was significantly higher 14 days (26 ± 0.4) and 28 days (28.7 ± 0.3) after MCT injection (Figure 6 D) as compared to healthy controls. Treatment with 0.4 or 1.2 mg terguride /kg body weight bid reduced significantly medial wall thickness (22.8 ± 0.4 and 22.1 ± 2.0). Consistent with these findings, vascular muscularization indicated a reduction in fully muscularized vessels in the lung upon chronic terguride treatment (39.7 ± 3.6% for terguride 0.4 mg/kg and 29.6 ± 3.5% for terguride 1.2 mg/kg) (Figure 6 E).

Effects of Terguride treatment on collagen deposition and inflammatory cytokines/ chemokines in rats with monocrotaline-induced pulmonary hypertension.

Masson trichrome and Sirius Red staining showed striking collagen deposition in pulmonary arteries of the lung of 4 weeks MCT rats compared to vehicle treated rats (Figure 7 Aa-l). Total collagen fibers (yellow-red and green stained) were increased in MCT-injected rats as shown by polarization microscopy of Sirius red–stained lung sections. Interestingly, chronic terguride treatment from day 1 to day 28 reduced significantly pulmonary vascular total collagen (Figure 7 Aa-l). Additionally, quantitative analysis indicated a reduction in total collagen content in MCT-challenged rats upon chronic terguride treatment (46.1% for terguride 0.4 mg/kg and 47.3% for terguride 1.2 mg/kg) (Figure 7 B).

Following exposure of rats to MCT resulted in 8, 16, 4 and 4 fold increase in expression of IL1β, IL6, TNFα and MCP-1, respectively, over control animals, after 28 days. Expression of these inflammatory cytokines and MCP-1 as detected by RT-PCR, was strongly reduced or normalized in lung tissue of rats treated with terguride (Figure 8).

Discussion

Clinical observations in patients have provided evidence for the presence of increased systemic 5-HT concentration in idiopathic PAH [16, 17, 31, 32]. Furthermore, weight-loss drugs such as Fen-Phen, Aminorex, fenfluramine, and phentermine, which either *per se* or through their respective metabolites exert 5-HT agonistic properties and interact with the 5-HT transport system have emphasised a pacemaker role of the 5-HT pathway in this drug-induced PAH epidemic [33, 34]. Effects of 5-HT in the lung are mediated through the 5-HT transporter and distinct 5-HT receptor isoforms. Working hypotheses for a contribution of 5-HTT, 5-HTR_{1B}, 5-HTR_{2A} and 5-HTR_{2B} to the pathophysiology of PAH have been proposed. Animal studies have generated findings in support for a role of single transporter or receptors as potential targets for therapeutic intervention although conflicting data in support or rebuttal of the involvement of these targets can be found in literature. We studied the expression profile of 5-HTR_{2A}, 5-HTR_{2B} and 5-HTT in human lung tissue and primary pulmonary arterial smooth muscle cells of healthy donors and IPAH patients. In our study differences in expression of 5-HTT in human lung tissue as well or in human PASMCs between IPAH patients and donors were not detectable, while 5-HTR_{2B} expression was found to be up-regulated in PASMCs. In contrast, Marcos *et al*. [7] and by Eddahibi *et al*. [35, 36] report a strong up-regulation of 5-HTT expression, but no changes in 5-HTR_{2A} and 5-HTR_{2B} expression in PASMCs of PPH patients as compared to controls. On the other hand our findings seem to be in agreement with the work by Launay *et al.*, which reports a 4.3 fold increase in binding sites of 5-HTR_{2B} in biopsies of human pulmonary arteries from PH patients as compared to non-PH patients, while 5-HTR_{2A} expression remained unchanged.

The involvement of 5-HTR_{2A} and 5-HTR_{2B} signaling in the pathogenesis of PAH has been studied previously. In the MCT model of pulmonary hypertension inhibition of 5-HTR_{2A}

signaling by specific inhibitors like DV-7029 or sarpogrelate with an approximately 100 fold selectivity over the 5-HTR_{2B} receptor [37] resulted in a marked suppression of rise of pulmonary arterial pressure, medial wall thickening and right heart hypertrophy, when treatment was started immediately after MCT treatment [38-40]. It was not detectable, when treatment was delayed for 3 weeks after MCT administration [38]. A marked improvement of pulmonary vascular endothelial activation/injury, suppression of P-selectin expression, reduction of the accumulation of mononuclear cell, macrophages and mast cells in the lung as well as an upregulation of eNOS in lung tissue have been demonstrated. Mechanisms related to inhibition of acute inflammation, counteracting hyper-responsiveness of pulmonary arteries to 5-HT as well as a decrease in proliferation have been implicated in the action of 5-HTR_{2A} antagonists. Although acute administration of ketanserin failed to demonstrate significant dilatory effects on pulmonary hemodynamics [41], suppressive effect of sarpogrelate on respiratory failure and right ventricular failure with pulmonary hypertension in patients with systemic sclerosis during long term treatment were observed [42].

A key role of 5-HTR_{2B} activation in vascular remodelling processes and the development and progression of pulmonary hypertension has been suggested by Launay *et al* [18]. Briefly, absence of vascular remodeling during chronic hypoxia has been demonstrated in a 5-$HTR_{2B}^{-/-}$ mouse model as well as by pharmacological inhibition with the 5-HTR_{2B} antagonists RS 127445 [43] and PRX-08066 [44] in animal models of chronic hypoxia or MCT-induced pulmonary hypertension, respectively [18, 44, 45]. A possible clinical relevance of these findings is supported by 2 lines of evidence: Firstly, a mutation causing premature truncation of the 5-HTR_{2B} receptor was described in a PAH patient associated with fenfluramine use [46]. Although originally considered a loss of function mutation, subsequent analysis indicated that this mutation was associated with a complete loss of inositol 1,4,5-trisphosphate and a partial loss of nitric-oxide synthase stimulation, but also with a strong gain of efficacy in cell proliferation [47]. Secondly, acute administration of PRX-08066 resulted in a reduction in systolic pulmonary

blood pressure during exercise-induced hypoxia in humans without effect on systemic blood pressure [48]. 5-HTR_2 antagonism might be even more promising as a target for therapeutic intervention in pulmonary hypertension, since 5-HTR_{2A} and 5-HTR_{2B} signaling is not restricted to the lung, but has also been implicated in the development of heart hypertrophy and heart failure [49, 50]. Combined inhibition of excessive 5-HTR_{2A} and 5-HTR_{2B} activation in lung and heart in PAH provides a strong rationale for a clinical evaluation of such agents in the treatment of PAH. Terguride is a partial dopamine agonist at DRD_2 and DRD_3 with potent anti-serotoninergic effects [51]. Although the dopamine receptors DRD_1, DRD_2, DRD_3 and DRD_4 are expressed in lung tissue, the expression levels do not differ between donors and PAH patients. In bioassays, an insurmountable antagonism on 5-HTR_{2A} and 5-HTR_{2B} has been demonstrated [20, 22, 52]. Terguride has been approved for treatment of ovulation disorders due to hyperprolactinemia and hyperprolactinemic pituitary adenoma and shown to have a well-established safety profile.

In this study we investigated terguride as a prototypical drug for translational research on therapeutic intervention of 5-HTR_2 signaling in PAH and demonstrate therapeutic efficacy of this compound in experimental pulmonary hypertension induced by monocrotaline (MCT) in rats. Subcutaneous injection of the plant alkaloid MCT in rats induces severe progressive pulmonary hypertension similar to human IPAH. It is characterized by vascular structural changes such as medial wall thickening, *de novo* muscularization of normally non-muscularized small pulmonary arteries and vascular fibrosis. Monocrotaline injected rats were administered dose levels of 0.4 and 1.2 mg/kg terguride. Based on pharmacokinetic data and AUC estimates (unpublished data) this corresponds to mean plasma concentrations of 11.8 and 35.2 nM terguride in rats. Marked species differences in the binding of ergots and tryptamines to 5-HTR_{2A} and 5-HTR_{2B} exist [53, 54]. In particular, N1-unsubstituted ergots, which includes also terguride, have higher affinity for the human 5-HTR_2 when compared to the

receptor in rats. Binding of terguride and 5-HT to recombinant expressed rat and human 5-HTR_{2B} receptors has been compared by Fielden et al. 2009 and emphasizes a 10 fold difference in the ratio of binding constants for 5-HTR_{2B} of terguride vs. 5-HT [55]. This corroborate the view that dose requirements for inhibition of human 5-HTR_{2A} and 5-HTR_{2B} by terguride is overestimated by studies in rats and that clinically relevant plasma concentrations in patients are achieved in the dose range of 1-3 mg/d.

We demonstrate that terguride a) improved hemodynamics, b) reduced right heart hypertrophy, c) restored arterial oxygenation and d) prevented and reversed pulmonary vascular structural changes induced by MCT in rats. Daily terguride treatment of MCT-injected rats has protected against PH development. It prevented in a dose-dependant manner elevation of right ventricular systolic pressure and completely prevented right heart hypertrophy. These effects were accompanied by significant reduction in the number of fully muscularized small pulmonary arteries, a significantly reduced medial wall thickness index, a decrease in vascular fibrosis and a marked down-regulation of inflammatory cytokines. More impressively, chronic daily treatment with terguride from day 14 to day 28 post MCT injection in two different doses exhibited potent therapeutic effect of this drug comparative to those when treatment was approached in a preventive manner. This experimental setup provides evidence that terguride has antiremodeling potency.

We have provided evidence that several mechanisms contribute to effects of terguride. To begin with, in isolated and perfused rat lungs 5-HT acutely induced pulmonary vasoconstriction, which was concentration-dependently inhibited by terguride. In the presence of the selective 5-HTR_{2A} antagonist ketanserin 5-HT-induced vasoconstriction was concentration dependently reversed. It remained unaffected in the presence of 1 μM SB 204741, a selective 5-HTR_{2B} antagonist [56]. This argues that vasoconstriction by 5-HT is not mediated by the 5-HTR_{2B} receptor, but involves 5-HTR_{2A} receptor signaling. In view of reports on increased plasma concentrations of 5-HT in IPAH patients, which may contribute

to the vasoconstriction, this may be of therapeutic relevance. A potential contribution of dopamine agonistic effects of Terguride to the vasodilatory effects might be considered as a decrease in pulmonary vascular resistance in response to dopamine or to the selective DRD_1 agonists SK&F 38393 and fenoldopam [57-59] has been reported. Terguride is a partial dopamine agonist with a high affinity to DRD_2 and DRD_3 [21] and binds with a 70 fold lesser affinity to DRD_1 *in vitro* [60]. The absence of a dopaminergic component in the vasodilatory activity of Tergruide is corroborated by the fact, that the non-selective dopamine DRD_2 and DRD_3 receptor agonist ropinirol at pharmacologically relevant concentrations did not affect basal vascular tone or 5-HT-induced vasoconstriction.

Secondly, on a cellular level inhibition by terguride on proliferation of primary PASMCs in response to 5% FCS as a source of 5-HT as well as of peptidic growth factors was demonstrated. Using specific inhibitors of the 5-HTR_{2A} and 5-HTR_{2B}, it was shown, that the 5-HT dependent proliferation response in donor derived PASMCs involved signaling *via* 5-HTR_{2A} and-5-HTR_{2B} receptors. This is in contrast to work by Marcos et al. 2003 and Eddahibi et al. 2001, which reports a lack of inhibition in 5-HT stimulated proliferation of human PASMCs derived from donors and patients with PAH in the presence of 5-HTR_{2A} and 5-HTR_{2B} antagonists, but anti-proliferative activity of the 5-HT reuptake inhibitor fluoxetine [61, 62] . However, our findings confirm the suppression of the mitogenic response of cultured PASMCs to 5-HT by ketanserin or other 5-HT_2 inhibitors, which has been reported by Pitt et al. 1994 and Lee et al. 1991 [63, 64]. In PASMCs derived from PAH lungs, inhibition of cell proliferation by Terguride, ketanserin and SB 204741 was comparable to extent of inhibition observed in PASMCs from donor lungs. This finding may limit the conclusion of a prominent role of 5-HTR_2 signaling in proliferative responses of PASMCs in PAH. However, intraindividual differences in responsiveness to and the extent of proliferative stimulation by 5-HT of PASMCs among PAH patients and possibly the applied experimental conditions in vitro may have resulted in a low sensitivity of PASMCs to 5-HT and may have affected

proliferative responses to 5-HT. This might provide a possible explanation for the apparent discrepancy of findings between the histology from the animal model and cell cultures. The up-regulation in expression of 5-HTR_{2B} in pulmonary hypertension as shown in Figure 1 and studies from Launay *et al* where 5-HT-dependent proliferation of cells in vascular beds from mice exposed to hypoxia is increased when compared to vascular beds from normoxic mice, but normalized in the presence of the 5-HTR_{2B} inhibitor RS-127445 or in tissue derived from 5-$HT_{2B}^{-/-}$ mice [18] supports the view that 5-HTR_{2B} signaling in PASMCs from lung tissue of PAH patients is differentially increased as compared to 5-HTR_{2A} activation. Furthermore, terguride also inhibited migration of PASMCs, and inhibited expression of collagen A_2 in PASMCs in cell culture. Thus, a number of pathomechanisms such as hypercontraction and hyperplasia of PASMCs, which are implicated in the muscularization of small pulmonary vessels and remodelling processes in PAH, are inhibited by terguride. Finally, terguride also markedly down-regulates the increased expression of IL-1β, IL-6, TNFα and MCP-1 in MCT-treated rats. However, it should be kept in mind that although the MCT model of pulmonary hypertension highlights some components of pulmonary hypertension pathogenesis, such as exaggerated pulmonary vascular inflammation, striking differences with human PAH exist. The development of PAH in humans usually takes years and although the role of inflammatory processes is not clinically predominant in IPAH, it may play a role in PAH associated with connective tissue diseases. Nevertheless, increased levels of pro-inflammatory cytokines have been reported in PAH patients [51, 65-67] and the presence of perivascular inflammatory cell infiltrates in plexiform lesions of lungs from PAH patients highlight the clinical importance of inflammatory processes in PAH .

5-HT receptor antagonism in experimental pulmonary hypertension has been addressed by few other research groups with the use of highly selective inhibitors or knockout animals, focusing on inhibition of solely 5-HTR_{2A} or 5-HTR_{2B} receptors, respectively, as a molecular target.

The present study represents a translational approach combining both experimental and clinical findings. It provides evidence that combined inhibition of 5-HTR_{2A} and 5-HTR_{2B}, even when administered as late intervention (i.e. starting 14 day after MCT treatment) exerted marked therapeutic effects. Our data propose for treatment of pulmonary arterial hypertension a drug which is clinically approved and well tolerated. Due to its vasorelaxant, anti-proliferative, anti-fibrotic and anti-inflammatory properties terguride represents a new therapeutical approach in the treatment of PH in accordance with the modern clinical therapeutical concepts.

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Figure legends:

Figure 1. Pulmonary vascular expression and localization of serotonin receptors 5-HTR_{2A} and 5-HTR_{2B} in lung tissues from donor and IPAH patients. Immunostaining of histological sections from donors and IPAH patients revealed positive immunoreactivity of pulmonary smooth muscle cells for 5-HTR_{2A} with strong immunoreactivity in disease condition (Aa-h). immunostaining against while 5-HTR_{2B} was associated with pulmonary vascular smooth muscle cells and pulmonary vascular endothelial cells (i-p). n=4, Scale bar 50 μm. mRNA expression of 5-HT_2 receptors and the 5-HT transporter in IPAH. The mRNA levels of 5-HTR_{2A}, 5-HTR_{2B} and 5-HTT were assessed in lung homogenates (B) and explanted PASMC (C) from donor and IPAH patients. Results are representative for 10 donor and 10 IPAH patients. Hypoxantine phosporibosyltransferase (HPRT) was used as reference gene. *$p<0.05$ versus donor. Protein expression (D) and quantification (E) of 5-HTR_{2B} receptor in explanted PASMCs from donor and IPAH patients. Results are representative for 4 donor and 4 IPAH patients. GAPDH was used as housekeeping gene.

Figure 1

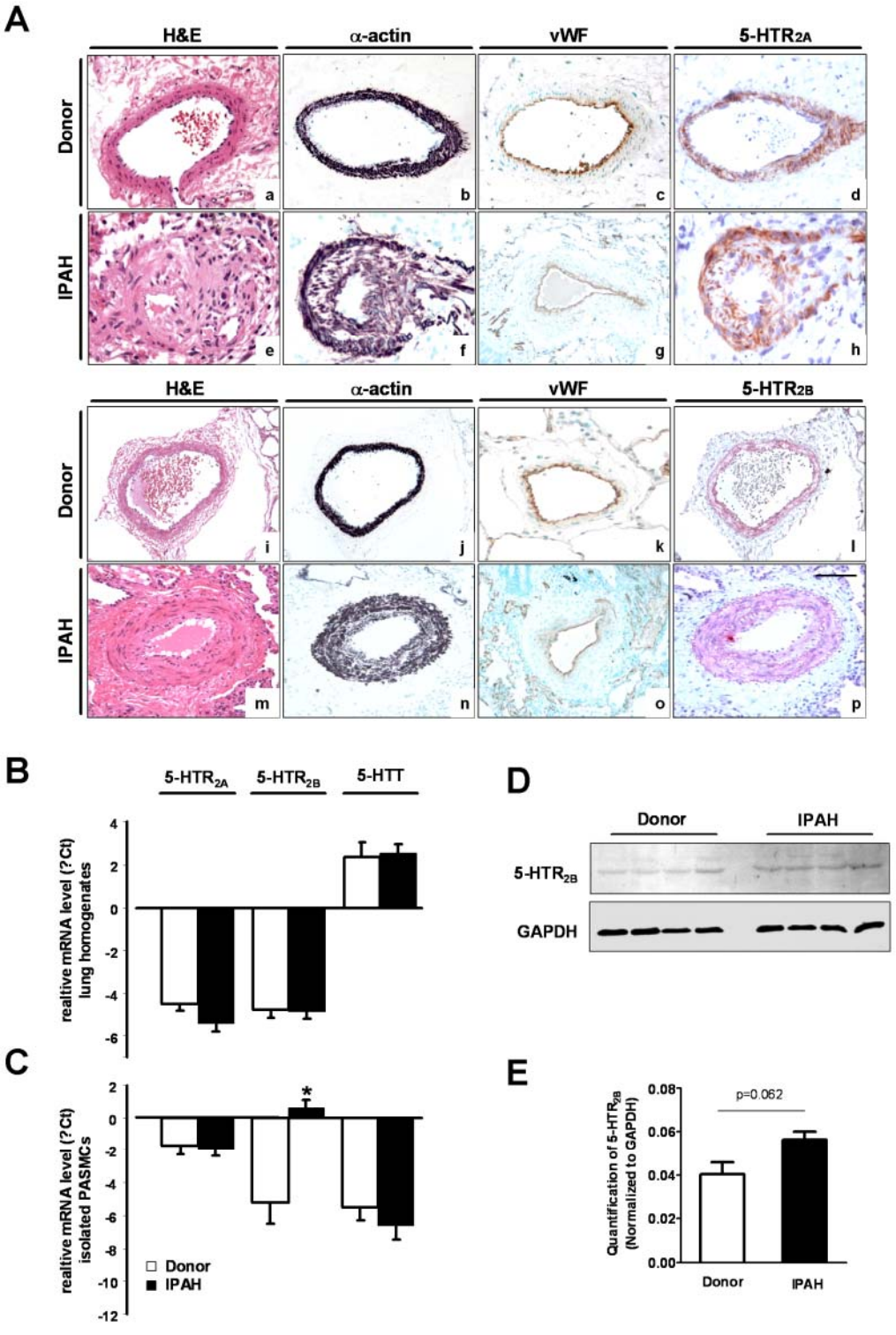


Figure 2. Terguride inhibits PASMCs proliferation, migration and collagen gene expression. Serum stimulation (5% FCS) induces PASMC proliferation compared to serum starved cells.

Specific 5-HTR_{2A} inhibition by 1 μM ketanserin or 5-HTR_{2B} inhibition by 1μM SB 204741 reduces proliferation of donor or IPAH derived PASMCs induced by FCS. In addition, 5-HTR_{2A} and 5-HTR_{2B} blockade in cultured PASMCs by terguride reduces donor or IPAH derived PASMCs proliferation (A, B). Results are representative for 6 donor and 6 IPAH patients. †, $p<0.05$ versus 5% FCS.

Terguride reduces PDGF-BB (10ng/ml) induced PASMCs migration (B). *, $p<0.05$ versus PDGF-BB. Collagen A_2 mRNA level was down-regulated with no subsequent changes in Collagen A_1 and Fibronectin in cultured PASMCs in presence of terguride. Heat shock protein 70 (HSP 70) was used as a loading control (C).

Figure 2

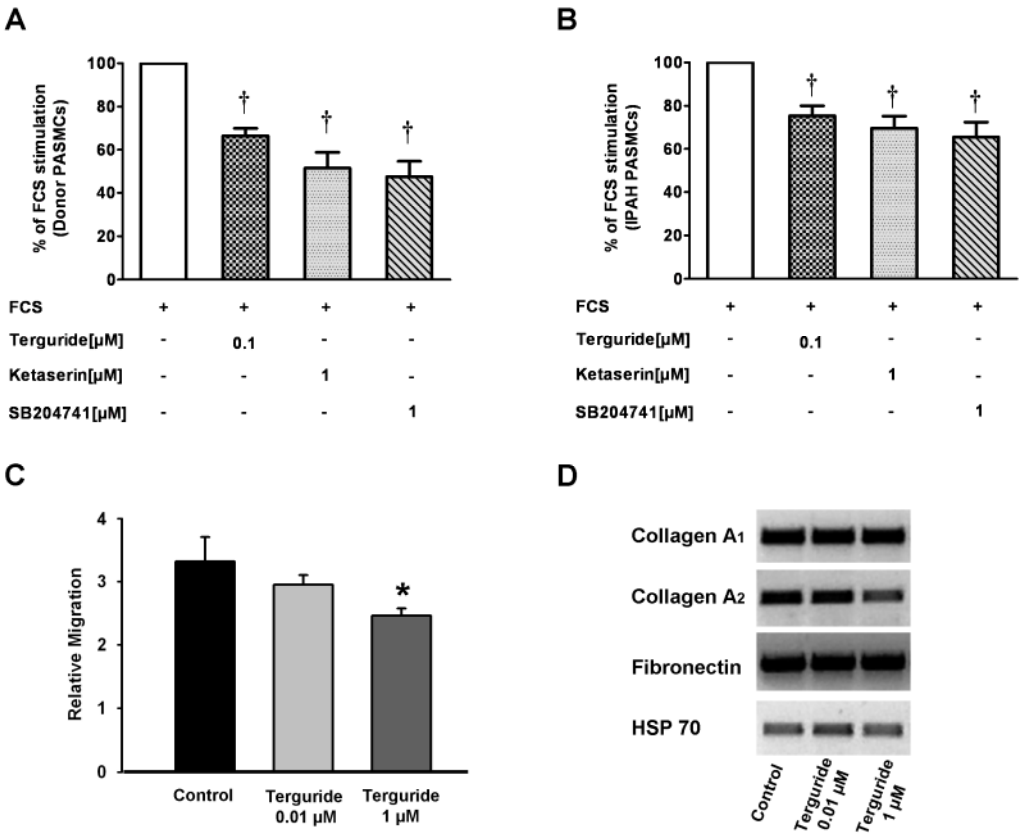


Figure 3. Pulmonary vascular localization of serotonin receptors 5-HTR_{2A} and 5-HTR_{2B} in rat lungs. Immunostaining of histological sections from control and monocrotaline-injected rats revealed vascular smooth muscle localisation for 5-HTR_{2A} with strong positive

immunoreactivity in monocrotaline-injected rats (a-h). Positive immunostaining for 5-HTR_{2B} was noted endothelium and smooth muscle layer of the pulmonary vasculature in control and monocrotaline-injected animals (i-p). n=4, Scale bar 50 μm.

Figure 3

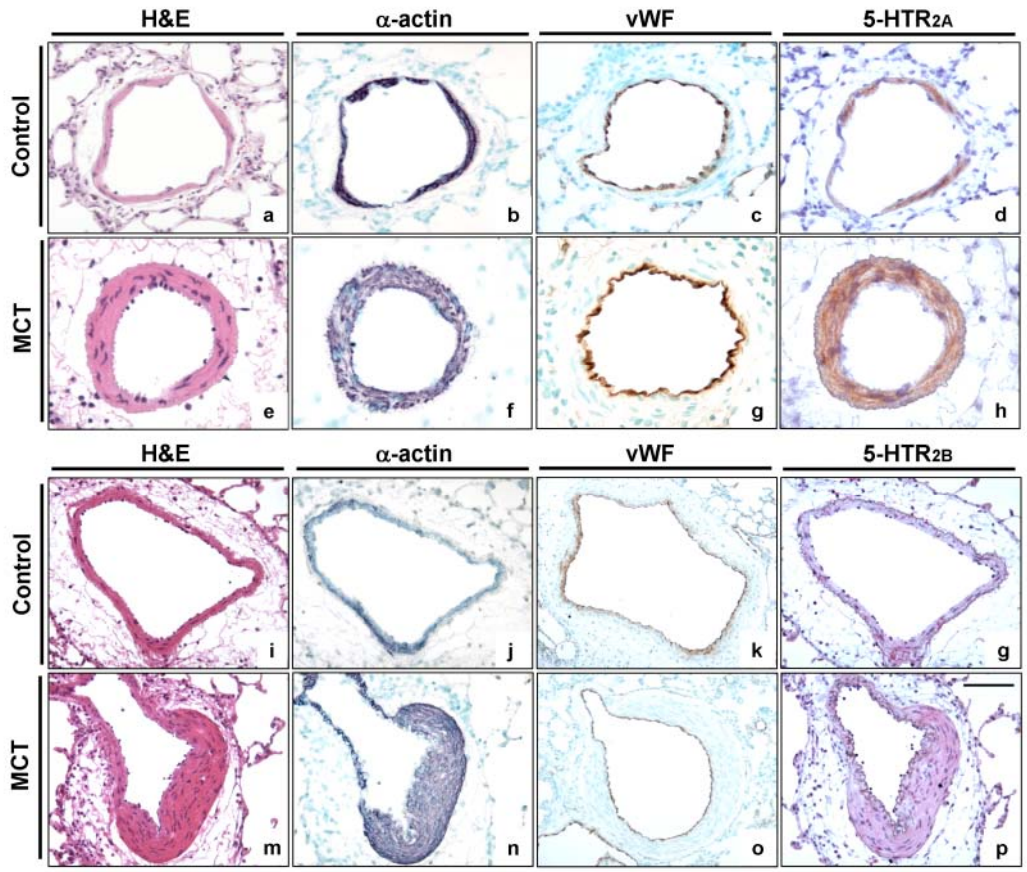


Figure 4. Terguride antagonizes serotonin-induced pulmonary vasoconstriction. Isolated ventilated and perfused rat lungs undergo vasoconstriction and pulmonary pressure elevation in response to serotonin. Presence of terguride diminished acute effects of serotonin on pulmonary pressure. Similar effects were observed when 5-HTR_{2A} were antagonised by ketanserin, but not in case of 5-HTR_{2B} inhibitor SB 204741 or dopaminergic agonist ropirinole.n =5, *, $p<0.05$ versus control.

Figure 4

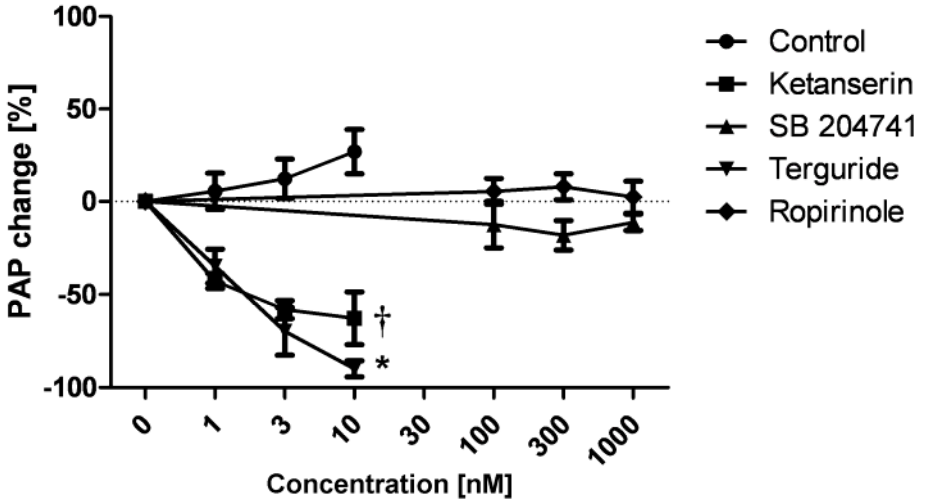


Figure 5. Terguride prevents development of pulmonary hypertension in rats after monocrotaline (MCT)-injection. Chronic treatment with terguride twice a day at a dose of 0.4 and 1.2 mg/kg from day 0 to day 28 after MCT-injection reduced almost completely pathophysiological changes in pulmonary pressure (A), right heart hypertrophy (B), improved arterial oxygenation (C), prevented media wall thickening (D) and muscularization (E) of small pulmonary arteries. n=8, *p<0.05 versus control, †p<0.05 versus MCT 28 days.

Figure 5

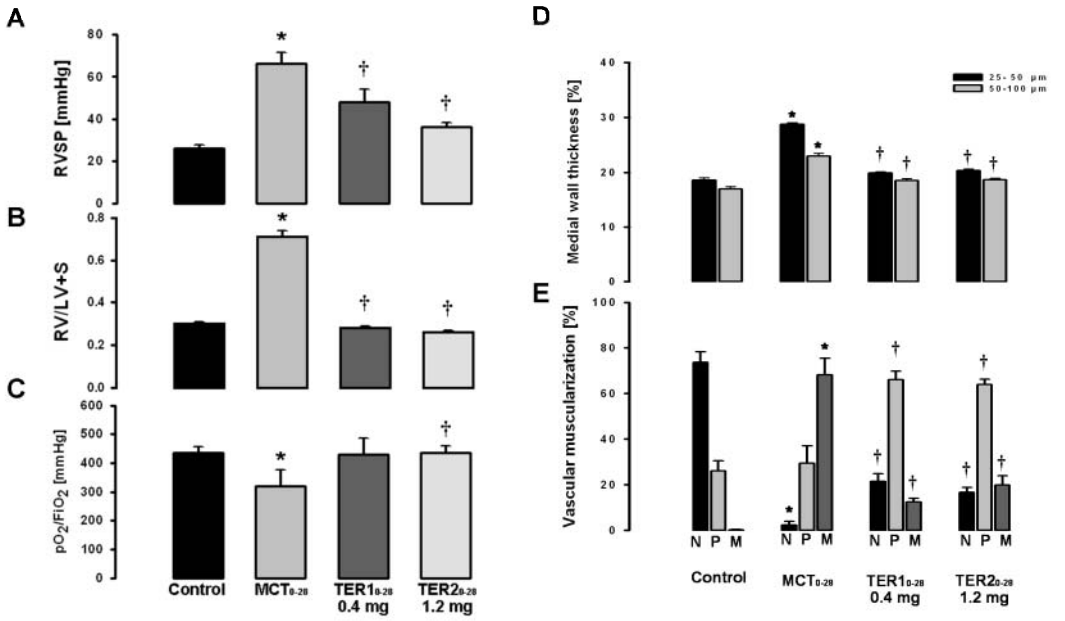


Figure 6. Terguride reverses monocrotaline (MCT)-induced pulmonary hypertension in rats. Chronic treatment with terguride at a dose of 0.4 and 1.2 mg/kg was initiated by day 14 and continued up to day 28 post MCT-injection. 5-$HTR_{2A/2B}$ inhibition by terguride reversed MCT-induced pathophysiological changes such as pulmonary pressure (A), right heart hypertrophy (B), arterial oxygenation (C) and reversed media wall thickening (D) and muscularization (E) of small pulmonary arteries. n=8, *p<0.05 versus control, †p<0.05 versus MCT 28 days.

Figure 6

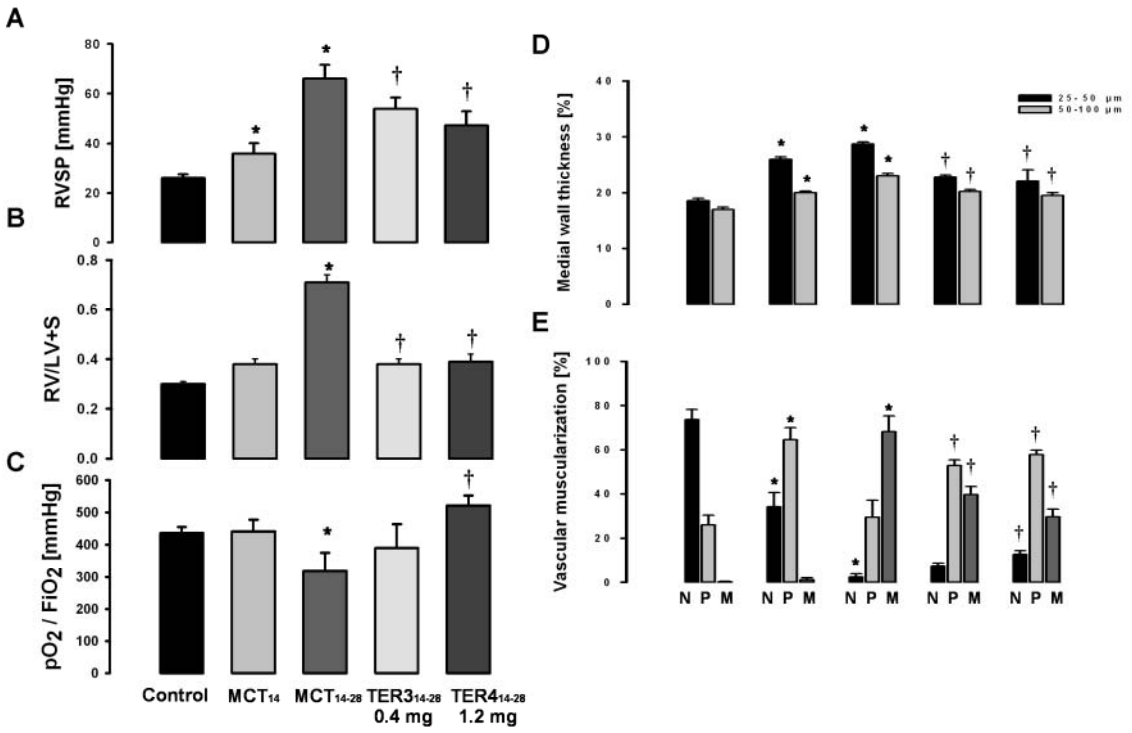


Figure 7. Terguride reverses monocrotaline (MCT)-induced collagen deposition. Chronic daily treatment with terguride at a dose of 0.4 and 1.2 mg/kg from day 0 to day 28 after MCT-injection reversed collagen deposition (A a-l, B) as detected by Sirius red staining (A a-h) and mason- trichrome staining (A i-l). Polarization light revealed a clear accumulation of total collagen fibers (yellow, red and green) (A e-h). The data of quantitative image analysis are given (B). n=4, *, $p<0.05$ versus control, †, $p<0.05$ versus MCT 28 days.

Figure 7

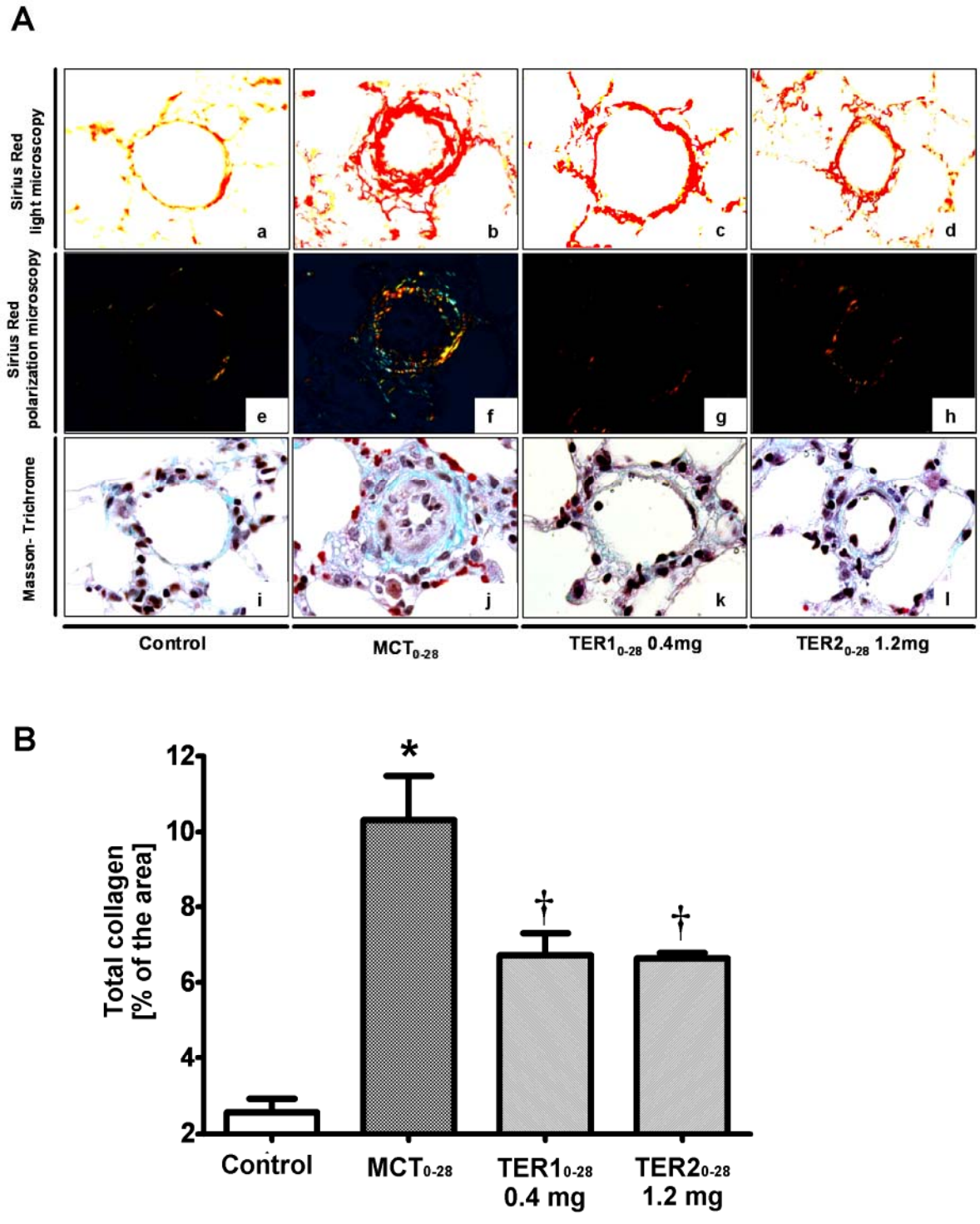
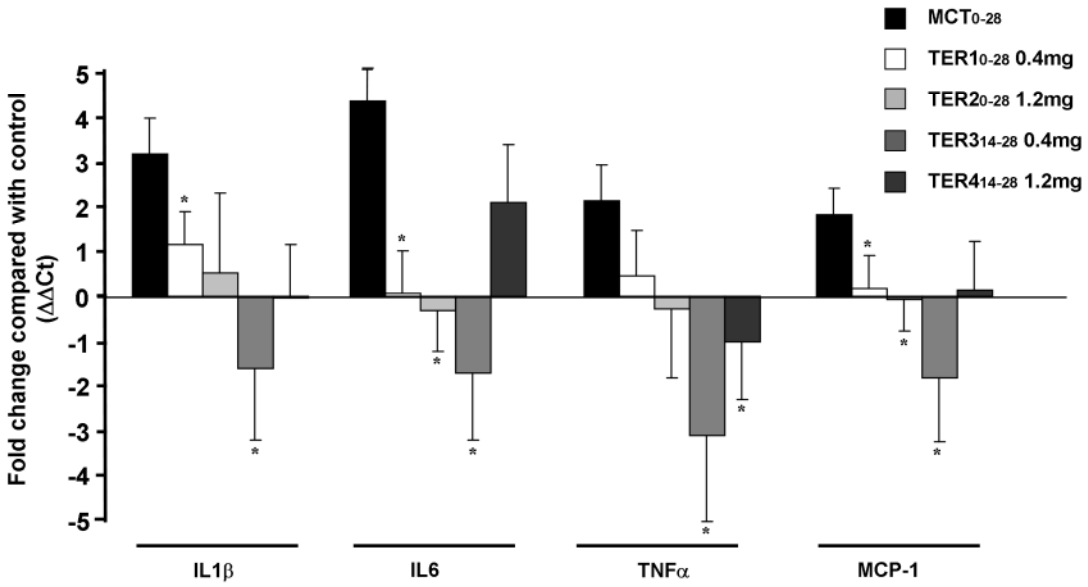


Figure 8. Terguride reduces monocrotaline (MCT)-induced lung pro-inflammatory cytokine and chemokine gene expression. Chronic treatment with terguride at a dose of 0.4 and 1.2

mg/kg from day 0 to day 28 and from day 14 to day 28 after MCT-injection decreased mRNA levels of interleukin 1 beta (IL1β), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα) and monocyte chemotactic protein-1 (MCP-1) in lung homogenates. Hypoxantine phosporibosyltransferase (HPRT) was used as reference gene. n=4, *, $p<0.05$ versus MCT 28 days.

Figure 8



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