Terguride ameliorates monocrotaline-induced pulmonary hypertension in rats

R. Dumitrascu*, C. Kulcke*, M. Königshoff*, F. Kouri*, X. Yang[#], N. Morrell[#], H.A. Ghofrani*, N. Weissmann*, R. Reiter[†], W. Seeger^{*,†}, F. Grimminger*, O. Eickelberg*, R.T. Schermuly^{*,†} and S.S. Pullamsetti^{*,†}

ABSTRACT: Pulmonary arterial hypertension (PAH) is a life-threatening disease characterised by vasoconstriction and remodelling of the pulmonary vasculature. The serotonin (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 PAH is, to date, at a pre-clinical level. Terguride is a 5-HT receptor (5-HTR) antagonist that is well tolerated and clinically approved for ovulation disorders.

Immunohistochemistry against 5-HTR_{2A/B} on human lungs revealed their localisation to the vascular smooth muscle layer and quantitative RT-PCR showed 5-HTR_{2B} upregulation 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 signalling inhibition was 1) demonstrated in isolated, ventilated and perfused rat lungs and 2) by chronic terguride treatment of rats with monocrotaline (MCT)-induced pulmonary hypertension in a preventive or curative approach.

Terguride inhibited proliferation of PASMCs 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.

KEYWORDS: Collagen, experimental therapeutics, inflammation, pulmonary hypertension, smooth muscle cells, vascular remodelling

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterised 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 signalling systems have been shown to be dysregulated in pulmonary hypertension (PH). In patients with idiopathic PAH (IPAH), a reduced excretion of prostaglandin I₂ and an enhanced excretion of thromboxane metabolites have been noted [2]. Moreover, enhanced expression of phosphodiesterase (PDE)-5, which hydrolyses the NO-induced second messenger cyclic guanosine monophosphate, was observed in PH [3]. In addition, the vasoconstrictor endothelin is upregulated in PAH [4] and correlates with the degree of the disease [5]. Furthermore, an epidemic of PH in patients using anorexic agents 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 these 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 pre-clinical level. Experimentally, it has been shown that inhibitors of 5-HTT, *e.g.* fluoxetine, reversed monocrotaline (MCT)-induced PH in rats [13] and 5-HTT-overexpressing mice spontaneously develop PH [14]. Furthermore, the 5-HT receptors (5-HTRs) 5-HTR_{1B}, 5-HTR_{2A}, 5-HTR_{2B} and 5-HTR₇ are expressed in smooth muscle and endothelial cells of the pulmonary vasculature [15], and 5-HT levels are increased in the plasma of PH patients

AFFILIATIONS

*University of Giessen Lung Center (UGLC), Giessen,

[†]Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany.

[#]Division of Respiratory Medicine, Addenbrooke's Hospital, Cambridge, UK.

[†]Ergonex Pharma GmbH, Appenzell, Switzerland.

CORRESPONDENCE

R.T. Schermuly

Max Planck Institute for Heart and Lung Research
Parkstrasse 1

Bad Nauheim 61231

Germany

E-mail: ralph.schermuly@mpi-bn.mpg.de

Received:

Aug 06 2010

Accepted after revision:

Aug 16 2010

First published online:

Oct 14 2010

This article has supplementary material available from www.erj.ersjournals.com

European Respiratory Journal

Print ISSN 0903-1936

Online ISSN 1399-3003

[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 PH [18] and administration of the specific 5-HT_{2B} antagonist RS-127445 prevented the increase in pulmonary arterial pressure (P_{pa}) in mice that were challenged to hypoxia. Here, we address the question of whether terguride, a potent 5-HT_{2A/2B} antagonist, might not only cause acute vasodilation in the lung, but also exert anti-remodelling effects upon long-term use in chronic experimental PH. Terguride is approved for ovulation disorders due to hyperprolactinaemia by acting as a partial dopamine receptor (DR)_{D2} agonist in the pituitary gland (for review, see [19]). In addition, it is a strong antagonist of 5-HT_{2A} [20, 21] and 5-HT_{2B} [22], and, therefore, seems well suited for treatment of PAH. In this study, we investigated expression and localisation of 5-HT_{2A/B} in human lungs from healthy *versus* PAH conditions. Next, we addressed the question of whether terguride could inhibit proliferation and/or migration of cultured human primary pulmonary artery smooth muscle cells (PASMCs). In isolated rat lung, we examined the acute vasorelaxant efficacy of terguride on 5-HT-induced vasoconstriction. Furthermore, pulmonary arterial anti-remodelling effects and therapeutic efficacy of long-term terguride treatment in experimental PH were studied. To this end, the model of MCT-induced PH was employed. 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 10 donor patients and 10 patients diagnosed as suffering from IPAH undergoing lung transplantation (five females and five males; mean $\pm$ SEM age 36.2 ± 3.9 yrs; P_{pa} 66.0 ± 9.4 mmHg). However, none of the 10 patients with IPAH had a bone morphogenetic protein receptor II mutation. After surgery, human lung tissue was immediately preserved on ice for PASMC isolation, snap-frozen in liquid nitrogen for mRNA isolation or transferred into buffered 4% paraformaldehyde for histopathological investigation. Tissue donation was approved by the German national ethical committee and German law. All patients enrolled in this study gave written informed consent.

RT and quantitative real-time PCR

Lung homogenates and freshly explanted PASMCs were subjected to gene expression analysis of 5-HT_{2A} and 5-HT_{2B}, 5-HTT, and DRD₁, DRD₂, DRD₃ and DRD₄. For this purpose, total RNA extraction, cDNA synthesis and quantitative (q)RT-PCR using the primers listed in online supplementary 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 GenBank; all accession numbers are denoted. Hypoxanthine phosphoribosyltransferase, a 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 a Δ Ct value (Δ Ct = $C_t^{\text{reference}} - C_t^{\text{target}}$), where higher Δ Ct values indicate higher transcript abundances and negative Δ Ct values represent genes that are less expressed compared with the reference gene. Similarly, gene expression analysis of proinflammatory

cytokines, such as interleukin (IL)-1 β , IL-6, tumour necrosis factor (TNF)- α and monocyte chemotactic protein (MCP)-1, was assessed in rat lung homogenates.

Western blotting

PASMCs were homogenised in lysis buffer containing 50 mM Tris-HCl pH 7.6, 10 mM CaCl₂, 150 mM NaCl, 60 mM NaN₃ and 0.1% (w/v) Triton X-100 using a tissue homogeniser. Samples were centrifuged at $16,000 \times g$ (13,000 rpm) for 20 min at 4°C, and the supernatant protein content was measured using Dye Reagent Concentrate (Bio-Rad, Munich, Germany). Extracts containing equal amounts of protein were denatured and subjected to electrophoresis on a sodium dodecylsulfate–10% polyacrylamide gel and blotted on to polyvinylidene fluoride membrane with a semidry transfer unit (Biometra, Göttingen, Germany). The membrane was then incubated with anti-5-HT_{2B} (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with the appropriate horseradish peroxidase-conjugated secondary antibody. Equal protein loading was confirmed by blotting membranes with an antibody against GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The bands were visualised using an enhanced chemiluminescence detection kit (Amersham Bioscience, Freiburg, Germany) and quantified by densitometry.

Human PASMC migration assay

PASMCs were explanted from pulmonary arteries as described previously [26]. They were cultured in Smooth Muscle Cell Growth Medium 2 (PromoCell, Heidelberg, Germany) enriched with Complement Mix C-39267 (PromoCell) at 37°C in a 5% CO₂, 95% O₂ atmosphere. At 70% confluence, PASMCs were treated with terguride at concentrations of 0, 0.01 or 1 μ M for 24 h. PASMCs were then trypsinised and further incubated with terguride at concentrations of 0, 10⁻⁶ or 10⁻⁸ M for 1 h prior to assessment of their migration ability in response to 10 ng·mL⁻¹ platelet-derived growth factor (PDGF) using a Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) as described previously [27].

Human PASMC proliferation assay

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

RNA extraction, RT and semi-quantitative PCR analysis

Explanted PASMCs were cultured as described. At 70% confluence, they were treated with terguride at concentrations of 0, 0.01 or 1 μ M for 24 h. Then, mRNA was extracted from PASMCs using the QIAGEN RNeasy Mini Kit according to manufacturer's instructions and reverse transcribed to cDNA using the Promega ImPro II reverse transcriptase (Promega, Mannheim, Germany). Semi-quantitative PCR analysis was performed for collagen types A₁ and A₂, and fibronectin. The band intensities were normalised to the loading control, heat shock protein (HSP)70. Specific primers used for sequence detection were as follows. Collagen type A₁: 5'-AATGGTG

CTCCTGGTATTGC-3' (forward) and 5'GGAAACCTCTCTC GCCTCTT3' (reverse); collagen type A₂: 5'-TTATCCCAATT AAAAGTATGCAGATTATT-3' (forward) and 5'-GAAGATG AAAATGAGACTGGCAAA-3' (reverse); fibronectin: 5'-CCG ACCAGAAGTTTGGGTCT-3' (forward) and 5'-CAATGCG GTACATGACCCCT-3' (reverse); HSP70: 5'-TGTGTCTGCTT GGTAGGAATGGTGGTA-3' (forward) and 5'-TTACCCGTC CCCGATTTGAAGAAC-3' (reverse).

Animals

Adult male Sprague Dawley rats (body weight 300–350 g) 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 the presence of defined concentrations of terguride, ketanserin, SB204741, ropirinole and vehicle.

For chronic treatment studies, seven groups with MCT-induced PH were studied: three with “early” intervention or sham intervention, and four with “late” intervention or sham intervention. Terguride treatment groups comprised group Ter_{1–28} (0.4 mg·kg⁻¹ *b.i.d.* from day 0 to day 28), group Ter_{20–28} (1.2 mg·kg⁻¹ *b.i.d.* from day 0 to day 28), group Ter_{3–14–28} (0.4 mg·kg⁻¹ *b.i.d.* from day 14 to day 28) and group Ter_{4–14–28} (1.2 mg·kg⁻¹ *b.i.d.* from day 14 to day 28). The corresponding controls include vehicle-treated groups MCT_{0–28}, MCT_{14–28} and MCT₁₄, 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 PH was induced in rats as previously described [28, 29]. Briefly, rats received a single subcutaneous injection of 60 mg·kg⁻¹ MCT, while control animals were administered subcutaneous saline solution. MCT-injected animals were randomised for placebo or chronic terguride therapy. Long-term treatment was administered by intraperitoneal injection. Terguride was dissolved in ethanol and subsequently diluted with sodium acetate prior to pH adjustment to 7.4. Ethanol concentration in the injected solution was <5% (v/v). Terguride was administered at dose levels as described above in a volume of 0.25 mL·rat⁻¹ *b.i.d.* by intraperitoneal injection. Placebo groups received ethanol/sodium acetate solution at the same volume.

Haemodynamics, arterial oxygenation and cardiac output

In order to monitor haemodynamics, animals were anaesthetised by intraperitoneal injection with ketamine/xylazine as described previously [28]. Tracheotomy was performed and animals were artificially ventilated at 10 mL·kg⁻¹. Inspiratory oxygen fraction (F_IO₂) was set at 0.5 and a positive end-expiratory pressure of 1.0 cmH₂O was used throughout. Systemic arterial pressure was monitored by cannulating the

carotid artery with a polyethylene cannula connected to a fluid-filled transducer (Braun, Kronberg, Germany). Pulmonary pressure expressed as RV 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 haemodynamic measurement and analysed using an automatic blood analyser (ABL 500; Radiometer Medical ApS, Brønshøj, Denmark).

Isolated, ventilated and perfused lung

The acute vasorelaxant effects of terguride were investigated in isolated, ventilated and perfused rat lungs. Briefly, animals were deeply anaesthetised and lungs were removed from the thoracic cavity. 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 the pulmonary artery for P_{pa} assessment throughout the experiment. Defined terguride concentrations of 0, 1, 3 and 10 nM were applied in the recirculating buffer 10 min before 5-HT challenge and pulmonary pressure was recorded. Similarly, specific 5-HTR_{2A} and 5-HTR_{2B} inhibition was induced using ketanserin (0–10 nM) and SB204741 (0–1 µM). Dopaminergic agonism was achieved using ropirinole in a concentration range from 0 to 1 µM.

Tissue processing

The right lungs from MCT-injected rats were preserved and snap-frozen in liquid nitrogen. Left lungs were perfused through the pulmonary artery and tracheae with Zamboni fixative (2% formaldehyde, 15% picric acid in 0.1 M phosphate buffer) at a constant pressure of 22 and 11 cmH₂O, 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 weighed. RV hypertrophy was assessed by the ratio RV/(LV+S), *i.e.* the ratio of weight of RV wall *versus* left ventricular wall plus septum (LV+S).

Histology and immunohistochemistry

In order to address the cellular localisation 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 untreated control rats were used. Human lung tissues were fixed for 24 h with buffered 4% paraformaldehyde at 4°C and embedded in paraffin. Rat tissue was fixed as described. 3-µm thick sections were immunohistochemically stained against 5-HTR_{2A} (polyclonal antibody 24288; ImmunoStar, Hudson, WI, USA; dilution 1:100) and 5-HTR_{2B} (polyclonal antibody 13292; Abcam plc, Cambridge, UK; dilution 1:800). Representative histological photographs were acquired at a 200× 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.

The degree of muscularisation of small pulmonary arteries was assessed by means of double immunostaining of the 3- μ m sections with anti-smooth muscle α -actin antibody (clone 1A4; Sigma, St Louis, MO, USA; dilution 1:900) and anti-human von Willebrand factor antibody (Dako, Hamburg, Germany; dilution 1:900) as described previously [28]. Sections were counterstained with methyl green and examined by light microscopy. All samples were analysed 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 by light microscopy image analysis using an automated morphometric system (Qwin; Leica, Wetzlar, Germany). The automated analysis was set to differentiate positively stained areas from negatively stained areas of the image. In addition, sections were analysed under polarisation microscopy. Collagen deposition data are presented as % 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 computerised morphometric system (Qwin, Leica, Wetzlar, Germany).

The degree of muscularisation of small pulmonary arteries was assessed as previously described [29]. Briefly, 80–100 intra-acinar lung vessels accompanying either alveolar ducts or alveoli were analysed at a 400 $\times$ magnification. Vessels were categorised as nonmuscularised, partially muscularised or fully muscularised according to a smooth muscle content of <5, 5–75 or >75%, respectively. The percentage of pulmonary vessels in each muscularisation category was determined by dividing the number of vessels in that category by the total number counted in the whole experimental group. Both, muscularisation degree and wall thickness were analysed in a blinded fashion.

Data analysis

Data are presented as mean $\pm$ SEM. Differences between groups were assessed by ANOVA and Student Newman–Keuls *post hoc* test for multiple comparisons, with a *p*-value <0.05 regarded to be significant.

RESULTS

Pulmonary expression of 5-HTR and 5-HTT in PH

We investigated by immunostaining the expression and localisation 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 cell layer, while anti-5-HTR_{2B} stained pulmonary vascular endothelium and vascular smooth muscle layer (fig. 1a–p). A similar pattern was observed in rat lung tissues obtained from MCT-treated and control animals (fig. 2). Gene expression analysis revealed that 5-HTR_{2A}, 5-HTR_{2B} and 5-HTT were expressed in lung homogenates and confirmed expression of these genes in isolated PSMCs (fig. 1q and r). 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 DRD₁, DRD₂, DRD₃ and DRD₄ in lung homogenate, no significant differences in expression between lung tissue from donors and IPAH patients were observed (online supplementary fig. 1).

In contrast, and despite the fact that no significant difference in lung homogenates of donors and IPAH patients were observed, 5-HTR_{2B} expression in PSMCs from IPAH patients was upregulated, as assessed by both mRNA and protein expression (fig. 1q–t). 5-HTT was expressed to comparable extents in PSMCs from donors and IPAH patients.

Effects of terguride on collagen synthesis, cell migration and proliferation PSMCs

To study the effects of terguride on serum-induced PASM proliferation, serum-starved PSMCs were stimulated with 5% fetal calf serum (FCS) in the presence or absence of terguride. Stimulation of cultured PSMCs with 5% FCS induced proliferation (fig. 3a). 0.1 μ M terguride inhibited FCS-stimulated [³H]thymidine incorporation in PSMCs to $66.3 \pm 3.5\%$ versus the serum-stimulated control (fig. 3a). Subsequently, the involvement of distinct 5-HTR isoforms in the observed proliferative effects in the presence of 5% FCS was studied using ketanserin and SB204741, which selectively inhibit 5-HTR_{2A} and 5-HTR_{2B} receptors, respectively. As shown in figure 3a, in the presence of 1 μ M ketanserin and SB204741, proliferation of PSMCs was inhibited to 51.4 ± 7.3 and $47.4 \pm 7.1\%$, respectively. When the same experiment was performed in PSMCs derived from IPAH lungs, inhibition of FCS-stimulated proliferation was observed to a comparable extent in the presence of terguride, ketanserin and SB204741 when compared with PSMCs from donor lungs (fig. 3b).

Furthermore, when PSMCs were assessed for cell migration activity towards PDGF (10 ng·mL⁻¹) in a Boyden chamber, pre-incubation with 1 μ M terguride significantly inhibited PASM migration (fig. 3c). In addition, expression of collagen A₁, collagen A₂ and fibronectin in 5% FCS-stimulated PSMCs was assessed by semi-quantitative PCR analysis. In the presence of 1 μ M terguride, a significant downregulation of collagen A₂ mRNA was observed, while collagen A₁ and fibronectin mRNA expression levels were not significantly changed (fig. 3d).

Effects of terguride on constricted lung vasculature in isolated rat lungs

1 μ M 5-HT induced reproducibly a pulmonary vasoconstriction with a $20.17 \pm 1.51\%$ increase in the vascular pressure when compared with lungs perfused in the absence of 5-HT. This increase in pressure was inhibited by terguride in a concentration-dependent manner by 34.7 ± 9.1 , 69.8 ± 12.8 and $89.9 \pm 4.2\%$ in the presence of 1, 3 and 10 nM terguride, respectively (fig. 4). Addition of the specific 5-HTR_{2A} inhibitor ketanserin to the perfusate markedly inhibited 5-HT-induced vasoconstriction. In contrast, SB204741, a specific 5-HTR_{2B} inhibitor, did not ameliorate vasoconstriction by 5-HT. Similarly, the presence of ropinirol, an agonist on DRD₂ and DRD₃, did not change vascular pressure regardless of the presence or absence of 5-HT in the perfusate (fig. 4).

FIGURE 1. Figure legend presented on following page.

FIGURE 1. Pulmonary vascular expression and localisation of 5-hydroxytryptamine (5-HT) receptors (5-HTR)_{2A} and 5-HTR_{2B} in lung tissues from donor and idiopathic pulmonary arterial hypertension (IPAH) patients. a–p) 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 the disease condition, while immunostaining against 5-HTR_{2B} was associated with pulmonary vascular smooth muscle cells and pulmonary vascular endothelial cells. n=4; scale bars=50 µm. The mRNA levels of 5-HTR_{2A}, 5-HTR_{2B} and 5-HT transporter (5-HTT) were assessed in q) lung homogenates and r) explanted pulmonary arterial smooth muscle cells (PASMCs) from donor and IPAH patients. Results are representative for 10 donor and 10 IPAH patients. Hypoxanthine phosphoribosyltransferase was used as reference gene. s) Protein expression and t) quantification of 5-HTR_{2B} in explanted PASMCs from donor and IPAH patients. Results are representative for four donor and four IPAH patients. Data are presented as mean±SEM. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a housekeeping gene. H&E: haematoxylin and eosin; vWF: von Willebrand factor. *: p<0.05 versus donor; #: p=0.062.

Effects of terguride treatment from day 0 to day 28 on pulmonary pressure, right heart hypertrophy and gas exchange in rats with MCT-induced PH

Rats injected with MCT developed progressive PH within 28 days. This is demonstrated by the sustained, significant

increase in RV systolic pressure 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 RV/(LV+S). This increased significantly 28 days after MCT injection to 0.71 ± 0.03 versus 0.30 ± 0.01 in

FIGURE 2. Pulmonary vascular localisation of 5-hydroxytryptamine (5-HT) receptors (5-HTR)_{2A} and 5-HTR_{2B} in rat lungs. a–h) Immunostaining of histological sections from control and monocrotaline (MCT)-injected rats revealed vascular smooth muscle localisation for 5-HTR_{2A} with strong positive immunoreactivity in MCT-injected rats. i–p) Positive immunostaining for 5-HTR_{2B} was noted in the endothelium and smooth muscle layer of the pulmonary vasculature in control and MCT-injected animals. n=4; scale bars=50 µm.

FIGURE 3. Terguride inhibits pulmonary arterial smooth muscle cell (PASC) a, b) proliferation, c) migration and d) collagen gene expression. Serum stimulation (5% fetal calf serum (FCS)) induces PASC proliferation compared with serum-starved cells. Specific 5-hydroxytryptamine receptor (5-HT)_{2A} inhibition by 1 μM ketanserin or 5-HT_{2B} inhibition by 1 μM SB204741 reduces proliferation of a) donor- or b) idiopathic pulmonary arterial hypertension (IPAH)-derived PASCs induced by FCS. In addition, 5-HT_{2A} and 5-HT_{2B} blockade by terguride in cultured PASCs reduces a) donor- or b) IPAH-derived PASCs proliferation. Results are representative for six donor and six IPAH patients. c) Terguride reduces platelet-derived growth factor (PDGF)-BB (10 ng·mL⁻¹)-induced PASC migration. d) In the presence of terguride, collagen A₂ mRNA level was down-regulated with no subsequent changes in collagen A₁ and fibronectin in cultured PASCs. Heat shock protein (HSP)70 was used as a loading control. Data are presented as mean $\pm$ SEM. #: $p < 0.05$ versus 5% FCS alone; *: $p < 0.05$ versus PDGF-BB.

control animals ($p < 0.05$). Daily treatment of MCT-injected animals with terguride from day 0 to day 28 attenuated these pathophysiological changes. Treatment of animals with 0.4 mg·kg⁻¹ terguride *b.i.d.* significantly reduced pulmonary pressure (47.8 ± 6.3 versus 66.1 ± 5.5 mmHg for vehicle-treated animals; $p < 0.05$; fig. 5a) and RV/LV+S (0.28 ± 0.01 versus 0.71 ± 0.03 for vehicle-treated animals; $p < 0.05$; fig. 5b). Treatment with 1.2 mg·kg⁻¹ terguride led to almost complete abolition of the changes in pulmonary pressure (36.4 ± 1.7 versus 66.1 ± 5.5 mmHg; $p < 0.05$) induced by MCT. Likewise, changes in RV/LV+S were completely abolished by this treatment (0.26 ± 0.01 versus 0.71 ± 0.03 ; $p < 0.05$; fig. 5a and b). In addition, terguride treatment at these doses improved arterial oxygenation, which was impaired in MCT-injected rats after 28 days (318 ± 56 versus 430 ± 55 mmHg and 436 ± 21 mmHg in 0.4 and 1.2 mg·kg⁻¹ terguride-treated

animals, respectively; fig. 5c). Moreover, treatment with 1.2 mg·kg⁻¹ terguride led to increased survival. However, terguride treatment did not show any significant effects on systemic arterial pressure (SAP), systemic vascular resistance index (SVRI) and bodyweight in MCT-injected rats after 28 days (online supplementary table 2).

With respect to the pulmonary vasculature, in MCT-injected rat lungs after 28 days we found a significant increase the proportion of in medial wall thickness of vessels 25–50 μm in diameter ($18.6 \pm 0.4\%$ in control versus $28.7 \pm 0.3\%$ in MCT-injected rats; $p < 0.05$) and 51–100 μm in diameter ($17.0 \pm 0.4\%$ versus $23.0 \pm 0.5\%$; $p < 0.05$) (fig. 5d). In addition, after 28 days, MCT injection led to significant muscularisation of small pulmonary vessels, assessed as percentage of fully muscularised vessels ($0.2 \pm 0.1\%$ for control versus $68.2 \pm 7.2\%$ for

FIGURE 4. Terguride antagonises 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-hydroxytryptamine receptor (5-HT_{2A}) was antagonised by ketanserin, but not in case of 5-HT_{2B} inhibitor SB204741 or the dopaminergic agonist ropirineol. $n=5$. Data are presented as mean $\pm$ SEM. ΔP_{pa} : change in pulmonary arterial pressure. *: $p < 0.05$ versus control.

vehicle-treated animals) (fig. 5e). Medial wall thickness and vascular muscularisation 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 ($19.8 \pm 0.3\%$ and $20.3 \pm 0.3\%$ for animals with treated with 0.4 and 1.2 mg·kg⁻¹ terguride, respectively, and vessels with diameter 20–50 μ m) (fig. 5d). Similarly, the number of fully muscularised vessels was significantly reduced in animals treated chronically with terguride (12.5 ± 15 and 19.9 ± 4.0 for animals with treated with 0.4 and 1.2 mg·kg⁻¹ terguride, respectively) when compared with nontreated, MCT-injected rats (fig. 5e).

Effects of terguride treatment from day 14 to day 28 on pulmonary pressure, right heart hypertrophy and gas exchange in rats with MCT-induced PH

Terguride treatment from days 14 to 28 significantly reduced pulmonary pressure in a dose-dependant manner (fig. 6a) when compared with 4 weeks MCT- and vehicle-treated rats (53.8 ± 4.6 mmHg for 0.4 mg·kg⁻¹ and 47.3 ± 5.7 mmHg for 1.2 mg·kg⁻¹ terguride-treated versus 66.1 ± 5.5 mmHg for vehicle-treated animals). Pulmonary pressure was still higher in both groups than that in control animals 2 weeks after MCT injection. Chronic terguride treatment from day 14 to day 28 significantly reduced right heart hypertrophy (RV/(LV+S) 0.38 ± 0.02 for 0.4 mg·kg⁻¹ and 0.39 ± 0.03 for 1.2 mg·kg⁻¹ terguride-treated rats; fig. 6b). These changes were accompanied by an improvement in alveolar gas exchange assessed as the oxygen tension/ F_{I,O_2} ratio (390 ± 73 for 0.4 mg·kg⁻¹ and 521 ± 30 for 1.2 mg·kg⁻¹ terguride-treated versus 318 ± 56 for vehicle-treated animals; fig. 6c), but with no significant effects on SAP, SVRI or body weight (online supplementary table 2).

The proportion of small pulmonary arteries with a diameter of 25–50 μ m was significantly higher 14 days ($26 \pm 0.4\%$) and

28 days ($28.7 \pm 0.3\%$) after MCT injection (fig. 6d), as compared with healthy controls. Treatment with 0.4 or 1.2 mg·kg⁻¹ terguride *b.i.d.* significantly reduced medial wall thickness (22.8 ± 0.4 and $22.1 \pm 2.0\%$). Consistent with these findings, vascular muscularisation indicated a reduction in fully muscularised vessels in the lung upon chronic terguride treatment ($39.7 \pm 3.6\%$ for 0.4 mg·kg⁻¹ and $29.6 \pm 3.5\%$ for 1.2 mg·kg⁻¹ terguride; fig. 6e).

Effects of terguride treatment on collagen deposition and inflammatory cytokines/chemokines in rats with MCT-induced PH

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

Exposure of rats to MCT resulted in eight-, 16-, four- and four-fold increases in expression of IL-1 β , IL6, TNF- α 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 normalised in lung tissue of rats treated with terguride (fig. 8).

DISCUSSION

Clinical observations in-patients have provided evidence for the presence of increased systemic 5-HT concentration in IPAH [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 effects 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 5-HTT and distinct 5-HT₂ isoforms. Working hypotheses for a contribution of 5-HTT, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} to the pathophysiology of PAH have been proposed. Animal studies have generated findings in support for a role of a 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-HT_{2A}, 5-HT_{2B} and 5-HTT in human lung tissue and primary PSMCs of healthy donors and IPAH patients. In our study, differences in expression of 5-HTT in human lung tissue or in human PSMCs were not detectable between IPAH patients and donors, while 5-HT_{2B} expression was found to be up-regulated in PSMCs. In contrast, MARCOS *et al.* [7] and by EDDAHIBI and co-workers [35, 36] report a strong upregulation of 5-HTT expression, but no changes in 5-HT_{2A} or 5-HT_{2B} expression in PSMCs of primary PH patients, as compared with controls. However, our findings seem to be in agreement with the work by LAUNAY *et al.* [18], who report a 4.3-fold increase in binding sites of 5-HT_{2B} in biopsies of human pulmonary arteries from PH patients as

FIGURE 5. Tergide (Ter) prevents development of pulmonary hypertension in rats after monocrotaline (MCT)-injection. Chronic treatment with tergide *b.i.d.* at a dose of 0.4 or 1.2 mg·kg⁻¹ from day 0 to day 28 after MCT-injection reduced almost completely pathophysiological changes in a) pulmonary pressure, b) right heart hypertrophy, c) improved arterial oxygenation, d) prevented medial wall thickening and e) muscularisation of small pulmonary arteries. Tergide treatment groups comprised group Ter₁₀₋₂₈ (0.4 mg·kg⁻¹ *b.i.d.* from day 0 to day 28) and group Ter₂₀₋₂₈ (1.2 mg·kg⁻¹ *b.i.d.* from day 0–28). MCT₀₋₂₈ represents the corresponding vehicle-treated control. n=8. Data are presented as mean±SEM. RVSP: right ventricular systolic pressure; RV: weight right ventricular wall; LV: weight of left ventricular wall; s: weight of septum; P_{O_2} : oxygen tension; F_{i,O_2} : inspiratory oxygen fraction; N: nonmuscularised; P: partially muscularised; M: muscularised. *: p<0.05 versus control; #: p<0.05 versus MCT₀₋₂₈.

compared with non-PH patients, while 5-HTR_{2A} expression remained unchanged.

The involvement of 5-HTR_{2A} and 5-HTR_{2B} signalling in the pathogenesis of PAH has been studied previously. In the MCT model of PH, inhibition of 5-HTR_{2A} signalling by specific inhibitors, such as DV-7029 or sarpogrelate, with a ~100-fold selectivity over the 5-HTR_{2B} receptor [37] resulted in a marked suppression of the increase in P_{pa} , medial wall thickening and right heart hypertrophy when treatment was started immediately after MCT treatment [38–40]. This 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, and an upregulation of endothelial nitric oxide synthase in lung tissue have been demonstrated. Mechanisms related to inhibition of acute inflammation, counteracting hyperresponsiveness of pulmonary arteries to 5-HT, and 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 haemodynamics [41], a suppressive effect of sarpogrelate on respiratory

FIGURE 6. Terguride (Ter) reverses monocrotaline (MCT)-induced pulmonary hypertension in rats. Chronic treatment with tergruride at a dose of 0.4 or 1.2 mg·kg⁻¹ was initiated by day 14 and continued up to day 28 after MCT injection. 5-hydroxytryptamine receptor (5-HTR)_{2A/2B} inhibition by tergruride reversed MCT-induced pathophysiological changes, such as a) pulmonary pressure, b) right heart hypertrophy and c) arterial oxygenation, d) reversed medial wall thickening and e) muscularisation of small pulmonary arteries. Terguride treatment groups comprised group Ter₃₋₁₄₋₂₈ (0.4 mg·kg⁻¹ *b.i.d.* from day 14 to day 28) and group Ter₄₋₁₄₋₂₈ (1.2 mg·kg⁻¹ *b.i.d.* from day 14 to day 28). MCT₁₄ and MCT₁₄₋₂₈ represent the corresponding vehicle-treated controls. n=8. Data are presented as mean ± SEM. RVSP: right ventricular systolic pressure; RV: weight right ventricular wall; LV: weight of left ventricular wall; s: weight of septum; PO₂: oxygen tension; F_iO₂: inspiratory oxygen fraction; N: nonmuscularised; P: partially muscularised; M: muscularised. *: p < 0.05 versus control; #: p < 0.05 versus MCT 28 days.

failure and right ventricular failure with PH 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 PH has been suggested by LAUNAY *et al.* [18]. Briefly, absence of vascular remodelling during chronic hypoxia has been demonstrated in a 5-HTR_{2B}^{-/-} mouse model and by pharmacological inhibition with the 5-HTR_{2B} antagonists RS127445 [43] and PRX-08066 [44] in animal models of chronic hypoxia

or MCT-induced PH, respectively [18, 44, 45]. A possible clinical relevance of these findings is supported by two lines of evidence. First, a mutation causing premature truncation of 5-HTR_{2B} was described in a patient with PAH 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, 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

FIGURE 7. Terguride (Ter) reverses monocrotaline (MCT)-induced collagen deposition. Chronic daily treatment with terguride at a dose of 0.4 or 1.2 mg·kg⁻¹ from day 0 to day 28 after MCT-injection reversed collagen deposition as detected by a-h) Sirius red staining and i-l) Masson trichrome staining. e-h) Polarisation light revealed a clear accumulation of total collagen fibres (yellow, red and green). m) Quantitative image analysis data. Terguride treatment groups comprised group Ter1₀₋₂₈ (0.4 mg·kg⁻¹ *b.i.d.* from day 0 to day 28) and group Ter2₀₋₂₈ (1.2 mg·kg⁻¹ *b.i.d.* from day 0-28). MCT₀₋₂₈ represents the corresponding vehicle-treated control. Data are presented as mean ± SEM. n=4. Scale bars=50 μm. *: p<0.05 versus control; #: p<0.05 versus MCT₀₋₂₈.

exercise-induced hypoxia in humans without effect on systemic blood pressure [48]. 5-HTR₂ antagonism might be even more promising as a target for therapeutic intervention in PH, since 5-HTR_{2A} and 5-HTR_{2B} signalling are not restricted to the lung, but have 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, acting on DRD₂ and DRD₃, with potent antiserotonergic effects [51]. Although the dopamine receptors DRD₁, DRD₂, DRD₃ and DRD₄ are expressed in lung tissue, their 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 hyperprolactinaemia and hyperprolactinaemic 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 targeting 5-HTR₂ signalling in PAH, and demonstrated therapeutic efficacy of this compound in experimental PH induced by MCT in rats. Subcutaneous injection of the plant alkaloid MCT in rats induces severe progressive PH similar to human IPAH. It is characterised by vascular structural changes, such as medial wall thickening, *de novo* muscularisation of normally

FIGURE 8. Terguride (Ter) reduces monocrotaline (MCT)-induced lung pro-inflammatory cytokine and chemokine gene expression. Chronic treatment with terguride at a dose of 0.4 or 1.2 mg·kg⁻¹ from day 0 to day 28 and from day 14 to day 28 after MCT-injection decreased mRNA levels of interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 in lung homogenates. Hypoxanthine phosphoribosyltransferase was used as reference gene. MCT₀₋₂₈ represents the corresponding vehicle-treated control. n=4. *: p<0.05 versus MCT₀₋₂₈.

nonmuscularised small pulmonary arteries and vascular fibrosis. MCT-injected rats were administered 0.4 or 1.2 mg·kg⁻¹ terguride. Based on pharmacokinetic data and area under the curve estimates (data not shown), 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 include terguride, have higher affinity for human 5-HTR₂ when compared with the receptor in rats. Binding of terguride and 5-HT to recombinant rat and human 5-HTR_{2B} have been compared by FIELDEN *et al.* [55], who emphasise a 10-fold difference in the ratio of binding constants for 5-HTR_{2B} of terguride *versus* 5-HT. This corroborates the view that dose requirements for inhibition of human 5-HTR_{2A} and 5-HTR_{2B} by terguride are overestimated by studies in rats and that clinically relevant plasma concentrations in patients are achieved in the dose range of 1–3 mg·day⁻¹.

We demonstrate that terguride 1) improved haemodynamics, 2) reduced right heart hypertrophy, 3) restored arterial oxygenation and 4) prevented and reversed pulmonary vascular structural changes induced by MCT in rats. Daily terguride treatment of MCT-injected rats protected against PH development. It prevented, in a dose-dependent manner, elevation of RV systolic pressure and completely prevented right heart hypertrophy. These effects were accompanied by significant reduction in the number of fully muscularised small pulmonary arteries, a significantly reduced medial wall thickness index, a decrease in vascular fibrosis and a marked downregulation of inflammatory cytokines. More impressively, chronic daily treatment with terguride from day 14 to day 28 after MCT injection at two different doses exhibited a potent therapeutic effect of this drug comparable to those seen when treatment is approached in a preventive manner. This experimental setup provides evidence that terguride has antiremodelling 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 SB204741, a selective 5-HTR_{2B} antagonist [56]. This argues that vasoconstriction by 5-HT is not mediated by 5-HTR_{2B}, but involves 5-HTR_{2A} signalling. 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 DRD₁ agonists SKF38393 and fenoldopam [57–59] has been reported. Terguride is a partial dopamine agonist with a high affinity for DRD₂ and DRD₃ [21] and binds with 70-fold less affinity to DRD₁ *in vitro* [60]. The absence of a dopaminergic component in the vasodilatory activity of terguride is corroborated by the fact that the nonselective dopamine DRD₂ and DRD₃ agonist ropinirol did not affect basal vascular tone or 5-HT-induced vasoconstriction at pharmacologically relevant concentrations.

Thirdly, at a cellular level, inhibition by terguride on proliferation of primary PSMCs 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 PSMCs involved signalling *via* 5-HTR_{2A} and 5-HTR_{2B}. This is in contrast to work by MARCOS *et al.* [61] and EDDAHIBI *et al.* [62], which reports a lack of inhibition of 5-HT-stimulated proliferation of human PSMCs derived from donors and patients with PAH in the presence of 5-HTR_{2A} and 5-HTR_{2B} antagonists, but antiproliferative activity of the 5-HT reuptake inhibitor fluoxetine. However, our findings confirm the suppression of the mitogenic response of cultured PSMCs to 5-HT by ketanserin or other 5-HTR₂ inhibitors, which has been reported by PITT *et al.* [63] and LEE *et al.* [64]. In PSMCs derived from PAH lungs, inhibition of cell proliferation by terguride, ketanserin and SB204741 was comparable to extent of inhibition observed in PSMCs from donor lungs. This finding may limit the conclusion of a prominent role of 5-HTR₂ signalling in proliferative responses of PSMCs in PAH. However, intra-individual differences in responsiveness to and the extent of proliferative stimulation by 5-HT of PSMCs among PAH patients and, possibly, the applied experimental conditions *in vitro* may have resulted in a low sensitivity of PSMCs 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 upregulation in expression of 5-HTR_{2B} in PH as shown in figure 1 and studies from LAUNAY *et al.* [18], 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 normalised in the presence of the 5-HTR_{2B} inhibitor RS-127445 or in tissue derived from 5-HT_{2B}^{-/-} mice, supports the view that 5-HTR_{2B} signalling in PSMCs from lung tissue of PAH patients is differentially

increased compared with 5-HT_{2A} activation. Furthermore, terguride also inhibited migration of PSMCs and inhibited expression of collagen A₂ in PSMCs in cell culture. Thus, a number of pathogenic mechanisms, such as hypercontraction and hyperplasia of PSMCs, which are implicated in the muscularisation of small pulmonary vessels and remodelling processes in PAH, are inhibited by terguride. Finally, terguride also markedly downregulates the increased expression of IL-1 β , IL-6, TNF- α and MCP-1 in MCT-treated rats. However, it should be kept in mind that, although the MCT model of PH highlights some components of PH 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_{2A} antagonism in experimental PH has been addressed with the use of highly selective inhibitors or knockout animals by few other research groups, who focused solely on inhibition of 5-HT_{2A} or 5-HT_{2B} 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-HT_{2A} and 5-HT_{2B}, even when administered as late intervention (*i.e.* starting 14 days after MCT treatment), exerted marked therapeutic effects. Our data lead us to propose terguride, which is clinically approved and well tolerated, as a novel treatment of PAH. Due to its vasorelaxant, antiproliferative, antifibrotic and anti-inflammatory properties, terguride represents a new therapeutic approach in the treatment of PH, in accordance with modern clinical therapeutic concepts.

SUPPORT STATEMENT

This study was funded by an unrestricted research grant from Ergonex, Deutsche Forschungsgemeinschaft grant SFB547 (Project C6) and the European Commission under the Sixth Framework Program (contract number LSHM-CT-2005-018725, PULMOTENSION).

STATEMENT OF INTEREST

Statements of interest for H.A. Ghofrani and R. Reiter, and for the study itself can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

ACKNOWLEDGEMENTS

We thank W. Klepetko (Dept of Cardiothoracic Surgery, University of Vienna, Vienna, Austria) for kindly providing the human lung tissue used in this study. We also thank A. Voigt and E. Bieniek for their excellent technical assistance with physiology and histology used in this study, and R. Morty for proofreading the manuscript (all University of Giessen Lung Center, Giessen, Germany).

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