Antiserotonergic Properties of Terguride in Blood Vessels, Platelets, and Valvular Interstitial Cells

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ABSTRACT
Serotonin (5-hydroxytryptamine; 5-HT) is involved in heart valve tissue fibrosis, pulmonary arterial fibrosis, and pulmonary hypertension. We aimed at characterizing the antiserotonergic properties of the ergot alkaloid derivative terguride [1,1-diethyl-3-(6-methyl-8-α-ergolinyl)urea] by using functional receptor assays and valvular interstitial cell culture. Terguride showed no vasoconstrictor effect in porcine coronary arteries (5-HT2A receptor bioassay) and no relaxant effect in porcine pulmonary arteries (5-HT2B receptor bioassay). Terguride behaved as a potent antagonist at 5-HT2A receptors (noncompetitive antagonist parameter pD2 9.43) and 5-HT2B receptors (apparent pA2 8.87). Metabolites of terguride (N'-monodeethylterguride and 6-norterguride) lacked agonism at both sites. N'-monodeethylterguride and 6-norterguride were surmountable antagonists at 5-HT2A receptors (pA2 7.62 and 7.85, respectively) and 5-HT2B receptors (pA2 7.90 and 7.11, respectively). Kinetic studies on the effects of terguride in pulmonary arteries showed that the rate to reach drug-receptor equilibrium for terguride was fast. Washout experiments showed that terguride easily disappeared from the receptor biophase. Pretreatment with terguride inhibited 5-HT-induced amplification of ADP-stimulated human platelet aggregation (IC50 16 nM). In porcine valvular interstitial cells, 5-HT-induced activation of extracellular signal-regulated kinase (ERK) 1/2, an initiator of cellular proliferation and activity, was blocked by terguride as shown by Western blotting. In these cells, the stimulatory effect of 5-HT on [3H]proline incorporation (index of extracellular matrix collagen) was blocked by terguride. Because of the inhibition of both 5-HT2A and 5-HT2B receptors, platelet aggregation, and cellular proliferation and activity (ERK1/2 phosphorylation and collagen production) terguride may have therapeutic potential in the treatment of fibrotic disorders.

Introduction
Terguride [1,1-diethyl-3-(6-methyl-8-α-ergolinyl)urea; Fig. 1] is an ergot alkaloid derivative that acts as a partial agonist at dopamine D2 receptors (Newman-Tancredi et al., 2002a). This may explain the beneficial effect of this drug in the treatment of hyperprolactinemia. However, ergot alkaloid derivatives (e.g., the antiparkinsonian drugs pergolide and cabergoline) may induce severe adverse effects such as valvular, retroperitoneal, or pleuropulmonary fibrosis (Antonini et al., 2009). Cardiopulmonary fibrotic side effects have also been observed with appetite-suppressant drugs such as flutamide and drugs of abuse, for example, 3,4-methylenedioxyamphetamine (also known as ecstasy) (Simonneau et al., 2003; Roth, 2007; Hutcheson et al., 2009). Cardiopulmonary fibrotic side effects have also been observed with appetite-suppressant drugs such as flutamide and drugs of abuse, for example, 3,4-methylenedioxyamphetamine (also known as ecstasy) (Simonneau et al., 1998; Setola et al., 2003; Roth, 2007; Hutcheson et al., 2011). Heart valve tissue fibrosis, pulmonary arterial fibrosis, and hypertension are associated with activation of 5-hydroxytryptamine 2B (5-HT2B) receptors and/or 5-HT2A receptors (Launay et al., 2002; Roth, 2007; Fabre et al., 2008; Connolly et al., 2009). In addition, platelet hyper-reactivity, local activation of platelets in the pulmonary circulation, and formation of microthrombi have been implicated in the pathogenesis of pulmonary hypertension (Chauvat et al., 1996). The response of platelets and smooth muscles to 5-HT is mediated predominantly by 5-HT2A receptors (Nishihira et al., 2006).

Signaling molecules downstream from 5-HT2A and 5-HT2B receptors include extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphorylation of ERK1/2 promotes processes of cell proliferation, cell growth, and differentiation (Knauer et al., 2009). The development of fibrotic changes in lungs and hearts by 5-HT has been associated with an enhanced production of extracellular matrix components such as collagen, fibronectin, and tenascin-C (Knauer et al., 2009). The development of fibrotic changes in lungs and hearts by 5-HT has been associated with an enhanced production of extracellular matrix components such as collagen, fibronectin, and tenascin-C.
of such metabolites in the interaction with 5-HT2A and ride and N(6)-demethylterguride (6-norterguride), were also main metabolites of terguride, such as ergolines (Jähnichen et al., 2005). In the present study, the antagonism was studied in the pig in comparison with other 5-HT2B receptor-mediated signaling might be promising in the treatment of fibrotic heart valvulopathies and pulmonary fibrosis.

The main aim of the present study was to examine the pharmacological properties of terguride on a broader basis. 5-HT2A receptor antagonism by terguride was studied previously in the rat (Kren et al., 2004), and 5-HT2B receptor antagonism was studied in the pig in comparison with other ergolines (Jähnichen et al., 2005). In the present study, the main metabolites of terguride, such as N\(^{-}\)monodeethylterguride and N(6)-demethylterguride (6-norterguride), were also included in our analysis to assess the potential contribution of such metabolites in the interaction with 5-HT2A and 5-HT2B receptors. 5-HT2A receptor-mediated responses were examined in endothelium-denuded porcine coronary arteries by measurement of vasoconstriction (Görnemann et al., 2008; Kekewksa et al., 2011). 5-HT2B receptor-mediated responses were investigated in porcine pulmonary arteries by measurement of endothelium-dependent relaxation (Görneman et al., 2008; Kekewksa et al., 2011). Furthermore, studies on the kinetics of terguride were performed in porcine pulmonary arteries. In addition, we studied the ability of terguride to inhibit 5-HT-potentiated platelet aggregation. Molecular biological studies were performed to assess whether terguride inhibited the 5-HT-induced increase in ERK1/2 phosphorylation in porcine valvular interstitial cells (PVICs). Radioimetric measurement of \(^{3}H\)proline was applied to characterize the direct effect of terguride on collagen biosynthesis in PVICs.

Fig. 1. Chemical structure of terguride.

Materials and Methods

Tissue Preparation. Lungs and hearts from pigs were obtained from the Lehr- und Versuchsanstalt für Tierzucht und Tierhaltung (Teltow Ruhlsdorf, Germany) and placed in ice-cold oxygenated Krebs-Henseleit solution (KHS) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\) (1.6 mM for coronary arteries), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), and 10 mM D-glucose, pH 7.4. Small branches of pulmonary arteries were dissected from the lungs and coronary arteries (left anterior descending and left circumflex) from the hearts. The tissues were cleaned of fat and adhering tissue. The vessels were cut into rings (pulmonary arteries, 2–3 mm long and 2 mm i.d.; coronary arteries, 3–4 mm long and 2–3 mm i.d.). The intimal surface of coronary artery rings was gently rolled with a pair of tweezers to destroy the endothelium. Vascular rings were horizontally suspended between two L-shaped stainless-steel hooks (300-\(\mu\)m diameter). The tissues were mounted in water-jacketed 20-ml organ chambers and constantly exposed to oxygenated KHS (95.5\% \(O_2\)/5\% \(CO_2\), pH 7.4, 37°C). Preparations were connected to an isometric force transducer (FMI TIM-1020; FMI Föhr Medical Instruments, Seeheim-Jugenheim, Germany) attached to a TSE 4711 transducer coupler (TSE Systems, Bad Homburg, Germany) and a Siemens C 1016 compensograph (Siemens AG, Erlangen, Germany) for the continuous recording of changes in tension.

Porcine Coronary Artery (Functional 5-HT\(_{2A}\) Receptor Assay). Resting tension was adjusted to 20 mN at the beginning of the experiment. The tissues were stabilized for 60 min with replacement of the bathing medium after 30 min. During the following equilibration period (115 min) the vessels were stimulated twice with KCl (50 mM) for 30 min. The rings were rinsed with KHS for 5 min to wash out KCl. Resting tension was readjusted to 20 mN 15 min after the first contraction with KCl had returned to baseline. The absence of endothelium was verified by the failure of bradykinin (0.1 \(\mu\)M) to cause relaxation after the second contraction with KCl. Cumulative concentration-response curves to 5-HT or terguride were constructed until a maximal response was observed. Antagonists were added to the bathing medium 60 min before the construction of a concentration-response curve to 5-HT. Antagonist affinity (pA\(_2\)) for ketanserin (5-HT\(_{2A}\) receptor antagonist) versus 5-HT estimated by Schild plot analysis was 8.9 (Görnemann et al., 2008). Contractile effects were expressed as a percentage of the second KCl-induced contraction. All experiments were performed in the continuous presence of prazosin (0.1 \(\mu\)M), cocaine (6 \(\mu\)M), and indomethacin (5 \(\mu\)M) to block \(\alpha\)-adrenoceptors and inhibit neuronal uptake of 5-HT and vascular eicosanoid production by cyclooxygenase.

Porcine Pulmonary Artery (Functional 5-HT\(_{2B}\) Receptor Assay). Resting tension was adjusted to 20 mN at the beginning of the experiment. During an initial stabilization period of 60 min, the bathing medium was replaced once after 30 min. The tissue rings were then stimulated at intervals of 45 min once with KCl (30 mM) and three times with 9.11-dideoxy-11a,9\(\beta\)-epoxy methano prostaglandin \(\text{F}_2\alpha\) (U46619) (0.01 \(\mu\)M) until the contractile response had become constant. After each of the stimulations, the rings were rinsed with KHS for 5 min to wash out KCl or U46619. Fifteen minutes after the KCl and the first U46619 stimulation had returned to baseline, resting tension was readjusted to 20 mN. The presence of endothelium was verified by the ability of bradykinin (0.01 \(\mu\)M) to cause relaxation after the second contraction with U46619. The relaxant response to 5-HT (or terguride) was studied after the third U46619-induced contraction had stabilized. Antagonist affinity (pA\(_2\)) for N-(1-methyl-1H-5-indolyl)-N(3-methyl-5-isothiazolyl) urea (SB204741) (5-HT\(_{2B}\) receptor antagonist) versus 5-HT estimated by Schild plot analysis was 6.8 (Glusa and Pertz, 2000). In agonist experiments with terguride, a noncumulative concentration-response curve to the agonist (1–1000 nM) was established by adding only one concentration of agonist to each tissue. In experiments where the antagonist properties of terguride were studied, a cumulative concentration-response curve to 5-HT was constructed on each tissue 60 min after the addition of terguride. In additional experiments, terguride (10 nM) was added to the bathing fluid for 5, 10, 20, 30, and 60 min after the third U46619-induced contraction had plateaued. Thereafter, a cumulative concentration-response curve to 5-HT was constructed. In further experiments, terguride (10 nM) was administered for 30 min to the bathing fluid followed by washout periods of 0, 15, 30, 45, and 60 min before establishing the third U46619 stimulation followed by the generation of a cumulative concentration-response curve to 5-HT. When the maximal relaxant response to 5-HT or the test agonist had been attained, relaxation was accomplished by the addition of bradykinin (0.01 \(\mu\)M). Relaxant effects were expressed as a percentage of the relaxation induced by the agonist plus bradykinin. All experiments were performed in the continuous presence of ketanserin (0.1 \(\mu\)M) to block 5-HT\(_{2A}\) receptors.
Human Platelet Aggregation Assay. Venous blood was obtained from healthy volunteers and anticoagulated with 3.8% sodium citrate (9 volumes blood + 1 volume citrate). Platelet-rich plasma (PRP) was prepared by centrifugation at 150g at room temperature for 12 min. To obtain platelet-poor plasma (PPP), the erythrocyte-rich sediment was centrifuged at 2500g for 10 min. For aggregation studies, the PRP was adjusted to a platelet count of 2.5 to 3 x 10^9/ml with autologous PPP as described previously (Glusza and Markwardt, 1984). Platelet aggregation was measured turbidometrically at 37°C by using a four-channel aggregometer, APACT 4 (Labor GmbH, Ahrensburg, Germany). The aggregation was monitored over a period of 10 min. PRP (170 µl) was preincubated at 37°C for 10 min. Thereafter, 20 µl of physiological saline or inhibitor solution were added, and 3 min later the aggregation was induced by the addition of 5-HT (1 µM) and ADP (0.5–1 µM). The concentration of ADP used was that causing to 20% of maximal aggregation, and 5-HT increased the extent of this aggregation approximately three times. In the case of aggregation induced by the combination of 5-HT and ADP, the maximal aggregation induced by ADP alone was subtracted from the aggregation response after the addition of 5-HT and ADP to assess the effects of 5-HT on the aggregation response specifically. The aggregation was measured automatically by using the APACT software PAAS. The PRP was adjusted to 0% transmission, and the PPP was adjusted to 100% transmission.

Culture of Porcine Valvular Interstitial Cells. Porcine hearts were transported in sterile KHS containing 100 U/ml penicillin and 0.1 mg/ml streptomycin. Aortic and mitral valve cusps were excised and then placed with a low serum concentration of 0.5%. After 48 h the cells were incubated in DMEM/F12 supplemented with 10 µM phenelzine (a nonselective monoamine oxidase inhibitor) and 0.6 mM ascorbic acid. 5-HT (in the absence and presence of terguride) and terguride alone were added. Terguride was added 30 min before 5-HT. Cells were then incubated for 48 h in the presence of 1 µCi/ml [3H]proline (PerkinElmer, Rodgau-Jügesheim, Germany). Cells were washed twice with ice-cold PBS before precipitation with ice-cold 10% trichloroacetic acid for 1 h at 4°C. The precipitates were solubilized in 0.3 N NaOH/0.1% SDS solution at 37°C under gentle agitation, mixed with scintillation cocktail, and measured in a β-scintillation counter. Experiments were performed in triplicate or quadruplicate. Results are presented as fold-changes compared with untreated control cells.

Materials. U46619 was a gift from Upjohn (Kalamazoo, MI). ADP disodium salt was from Serva (Heidelberg, Germany). Bradykynin triacetate, indomethacin, phenelzine sulfate, and prazosin hydrochloride were from Sigma-Aldrich. Ascorbic acid and caffeine hydrochloride were from Merck (Darmstadt, Germany). 5-Hydroxytryptamine creatinine sulfate was from Agros Organics (Geel, Belgium). Terguride, N-monoacetylterguride, and N(6)-demethylterguride were from Alfarona sro (Cernosice, Czech Republic). Cells were grown in DMEM/F12 supplemented with 10% dialyzed HyClone fetal calf serum (Thermo Fisher Scientific, Waltham, MA) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). At first confluence, the cells were cryopreserved and used for experiments at passages 1 to 3.

Western Blot Analysis of Extracellular Signal-Regulated Kinases. PVICs were grown to 80 to 90% confluence and made quiescent by incubation in DMEM/F12 containing 0.2% fetal calf serum for 48 h. 5-HT or terguride (when tested as an agonist) were added for 5 min. Terguride (when tested as an antagonist) was added 30 min before the treatment with 5-HT. After two rinses with PBS, cells were scraped on ice and lysed with radioimmunoprecipitation assay buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with protease inhibitors (2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 mM EDTA, and 10 mM sodium fluoride). The cell lysates were centrifuged, mixed with loading buffer, heated to 95°C for 5 min, and stored at -20°C before further processing. Total protein concentrations were determined after precipitation and staining with Amido Black solution. Protein samples (10 µg) were separated on 10% resolving gel for SDS-polyacrylamide gel electrophoresis (35 mA, 12°C) and then transferred onto polyvinylidene fluoride membranes (Westsan S; pore size 0.2 µm) by semidyed electroblotting (1.5 mA/m², 2 h, 4°C). Membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with a primary antibody against the phosphorylated form of ERK1/2 (1:2000; Cell Signaling Technology, Danvers, MA) and β-actin as loading control (1:10,000; Abcam plc, Cambridge, UK) for 1 h overnight at 4°C. After washing with PBS/Tween 20 (0.1%), bands were detected by probing with a horseradish peroxidase-conjugated secondary antibody (1:10,000; Rockland Immunonchemicals, Gilbertsville, PA). Bands were visualized by chemiluminescence with LumiGLO Reagent (Cell Signaling Technology) on Kodak Biomax films (Eastman Kodak, Rochester, NY). Quantification of bands was obtained by digital image analysis using National Institutes of Health Image software (http://rsb.info.nih.gov/nih-image/).

Measurement of [3H]Proline Incorporation. Collagen synthesis activity was assessed by measuring the incorporation of [3H]proline as follows: 3 x 10^5 cells were seeded in 24-well plates and grown overnight in DMEM/F12 medium supplemented with 10% dialyzed fetal calf serum and penicillin/streptomycin. The medium was replaced with a low serum concentration of 0.5%. After 48 h the cells were incubated in DMEM/F12 supplemented with 10 µM phenelzine (a nonselective monoamine oxidase inhibitor) and 0.6 mM ascorbic acid. 5-HT (in the absence and presence of terguride) and terguride alone were added. Terguride was added 30 min before 5-HT. Cells were then incubated for 48 h in the presence of 1 µCi/ml [3H]proline (PerkinElmer, Rodgau-Jügesheim, Germany). Cells were washed twice with ice-cold PBS before precipitation with ice-cold 10% trichloroacetic acid for 1 h at 4°C. The precipitates were solubilized in 0.3 N NaOH/0.1% SDS solution at 37°C under gentle agitation, mixed with scintillation cocktail, and measured in a β-scintillation counter. Experiments were performed in triplicate or quadruplicate. Results are presented as fold-changes compared with untreated control cells.

Results

Effects of Terguride and Metabolites in Porcine Coronary Arteries (5-HT3A Receptor Assay). 5-HT (3–300 nM) induced a concentration-dependent contraction in porcine coronary arteries. In contrast, terguride (1 nM-1 µM)
failed to elicit a contraction in porcine coronary artery but antagonized 5-HT-induced contractions (Fig. 2). Terguride (1 nM) did not cause a rightward shift of the concentration-response curve to 5-HT but induced a powerful depression of the maximal 5-HT response; $E_{\text{max}}$ was reduced from $34 \pm 4$ to $9 \pm 1\%$ ($P < 0.05$; Fig. 2). Thus terguride behaved as a noncompetitive antagonist of the contractile response to 5-HT (noncompetitive antagonist parameter $pD_2$ 9.43 ± 0.08; $n = 6$). Metabolites of terguride (N'-monodeethylterguride and 6-norterguride) were also devoid of agonist activity in porcine coronary artery. 5-HT curves in the presence of the metabolites were shifted to the right in a parallel manner; thus these drugs behaved as surmountable antagonists of the 5-HT response (Fig. 3). Antagonist potencies for 6-norterguride (apparent $pA_2$ 7.30 ± 0.02; $n = 4$) and N'-monodeethylterguride (pA2 7.82 ± 0.06; $n = 5$) were lower than the antagonist activity of the parent drug terguride.

Effects of Terguride and Metabolites in Porcine Pulmonary Arteries (5-HT2B Receptor Assay). 5-HT (0.1–300 nM) induced a concentration- and endothelium-dependent relaxation of U46619 (10 nM)-precontracted porcine pulmonary arteries. Terguride (1 nM-1 µM) failed to induce a relaxation in these vessels but antagonized the relaxant 5-HT response. 5-HT curves in the presence of terguride (10 nM) were shifted to the right in a nonparallel manner with a depression of the maximal response (apparent pA2 8.87 ± 0.06; reduction of $E_{\text{max}}$ from 74 ± 3 to 38 ± 4%; $P < 0.05$; Fig. 4). Metabolites of terguride (N'-monodeethylterguride and 6-norterguride) were also devoid of agonist activity. Both metabolites antagonized the relaxant response to 5-HT at a higher concentration (0.5 µM) than that of terguride. 5-HT curves in the presence of the metabolites were shifted to the right in a parallel manner. The apparent pA2 for N'-monodeethylterguride was 7.30 ± 0.02 ($n = 4$) and that for 6-norterguride was 7.11 ± 0.08 ($n = 4$; Fig. 5); thus these drugs behaved as surmountable antagonists of the 5-HT response.

Kinetic Studies on Terguride in Porcine Pulmonary Arteries. These experiments were performed to estimate the rate for terguride to reach drug-receptor equilibrium and the rate to disappear from the receptor biophase. When terguride (10 nM) was added to the bathing fluid, 5, 10, 20, 30, and 60 min before the construction of the concentration-response curve to 5-HT, there was no difference between the pEC50 values and the $E_{\text{max}}$ values of the 5-HT curves after 5-, 10-, 20-, 30-, and 60-min incubation of terguride. Figure 6 shows the effects after 5- and 60-min incubation of terguride (10 nM). Thus terguride showed fast-onset kinetics. In another set of experiments, terguride (10 nM) was administered for 30 min to the bathing fluid followed by washout periods of 0,
15, 30, 45, and 60 min before the addition of U46619. After reaching the plateau of the U46619-induced contraction, cumulative concentration-response curves to 5-HT were nearly identical under the different washout conditions compared with the respective 5-HT control curve (no differences in pEC\textsubscript{50} and \(E_{\text{max}}\) values). Figure 7 shows the effects after 0 and 15 min of washout. Thus terguride showed fast-offset kinetics.

**Inhibition of 5-HT Amplified Platelet Aggregation by Terguride.** A standard turbidometric aggregometry assay was used to assess the ability of terguride to inhibit 5-HT-mediated amplification of ADP-induced aggregation of human platelets. At a concentration of 1 \(\mu\)M, ADP per se aggregated human platelets by approximately 20%. 5-HT (1 \(\mu\)M) alone induced a very low transient or no aggregation (data not shown). In contrast, the combination of 5-HT (1 \(\mu\)M) with a submaximal concentration of ADP (0.5–1 \(\mu\)M) caused a synergistic aggregation response of 66% (Fig. 8). The 5-HT-induced amplification of ADP aggregation was concentration-dependently blocked when platelets were preincubated with terguride or ketanserin (5-HT\(_2A\) receptor antagonist). The concentration-response curves for the inhibitory potency of terguride and ketanserin are shown in Fig. 8. Terguride inhibited platelet aggregation with an IC\textsubscript{50} of 16 (5–57) nM that was more than 10-fold lower than that of ketanserin [IC\textsubscript{50} 240 (169–339) nM].

**Effect of Terguride on PVIC ERK1/2 Phosphorylation.** 5-HT (1 \(\mu\)M) increased pERK1/2 in PVIC cultures as shown by Western blotting. Terguride (1 and 10 \(\mu\)M) per se had no stimulatory effect on pERK1/2 but significantly inhibited the effect of 1 \(\mu\)M 5-HT (Fig. 9).

**Effect of Terguride on Collagen Synthesis in PVICs.** In contrast to 5-HT (1 \(\mu\)M), terguride (1 \(\mu\)M) did not cause

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**Fig. 5.** Antagonism of 5-HT-induced relaxation by \(N''\)-monodeethylterguride (A) and 6-norterguride (B) in U46619 precontracted porcine pulmonary arteries. Relaxations are expressed as a percentage of the total relaxation elicited by bradykinin (10 nM). Points are mean values ± S.E.M. from four animals.

**Fig. 6.** Antagonism of 5-HT-induced relaxation by terguride in U46619 precontracted porcine pulmonary arteries. Terguride was added to the bathing fluid when the plateau of the U46619 contraction had been attained. After 5 or 60 min a concentration-response curve to 5-HT was constructed. Relaxations are expressed as a percentage of the total relaxation elicited by bradykinin (10 nM). Points are mean values ± S.E.M. from five animals.

**Fig. 7.** Antagonism of 5-HT-induced relaxation by terguride in porcine pulmonary arteries. Terguride (10 nM) was added to the bathing fluid followed by no washout or washout for 15 min. Tissues were then contracted with U46619 (10 nM) followed by relaxation to 5-HT. Relaxations are expressed as a percentage of the total relaxation elicited by bradykinin (10 nM). Points are mean values ± S.E.M. from four animals.
Serotonin 5-HT₂ₐ and/or 5-HT₂ₕ receptor activation is involved in fibrotic disorders such as valvular heart disease, idiopathic pulmonary fibrosis, skin fibrosis, liver fibrosis, and nephropathies (Grewal et al., 1999; Ruddell et al., 2006; Roth, 2007; Königshoff et al., 2010; Dees et al., 2011). In addition, 5-HT₂ₕ receptor expression was found to be up-regulated in pulmonary artery smooth muscle cells isolated from patients with idiopathic pulmonary hypertension (Launay et al., 2002; Dumitrascu et al., 2011). Thus, drugs that inhibit 5-HT₂ₐ₂ₕ receptors may be beneficial in the treatment of various fibrotic disorders.

In the present study, we assessed the potential antagonist effects of terguride at 5-HT₂ₐ and 5-HT₂ₕ receptors in cardiopulmonary tissue. We used tissue from pigs because pigs have anatomical, physiological, histological, and biochemical characteristics similar to those of humans. Terguride is an N(1)-unsubstituted ergoline compound, and N(1)-unsubstituted ergolines have been shown to display nearly identical characteristics similar to those of humans. Terguride is an N(1)-unsubstituted ergoline compound, and N(1)-unsubstituted ergolines have been shown to display nearly identical affinities for pig and human 5-HT₂ₐ receptors (Nelson et al., 1993). In addition, pig and human 5-HT₂ₕ receptors show 95% sequence homology (Ullmer et al., 1995).

In our studies, terguride lacked agonist activity at porcine 5-HT₂ₕ receptors but behaved as a potent antagonist of 5-HT. This is in line with our previous studies in porcine pulmonary arteries (Jähnichen et al., 2005) and with observations at human recombinant 5-HT₂ₕ receptors expressed in Chinese hamster ovary cells (Newman-Tancredi et al., 2002b). Obviously, 5-HT₂ₕ receptor agonism seems not to be a class effect of the ergolines (Jähnichen et al., 2005). The ability of terguride to behave as a silent antagonist at porcine 5-HT₂ₐ receptors in our studies is consistent with previous studies using rat vascular tissues (Kren et al., 2004). However, our data on the antagonist effect of terguride at 5-HT₂ₐ receptors in porcine coronary arteries are in contrast to the partial agonist effect of this drug at recombinant 5-HT₂ₐ receptors (Newman-Tancredi et al., 2002b). This discrepancy indicates that functional responses using transfected cells and those using native tissues may be different. Because transfected cells express a larger number of receptors such cells may not

![Fig. 8. Inhibition of 5-HT/ADP-induced aggregation of human platelets by terguride and ketanserin, respectively. Points are mean values ± S.E.M. for three to eight separate experiments. Inset, aggregation of human platelets induced by ADP (0.5–1 µM) alone and amplification by combination of ADP and 5-HT (1 µM). Data are mean values ± S.E.M. for 40 experiments.](image)

![Discussion](image)

![Fig. 9. Western blots of porcine aortic and mitral valvular interstitial cells demonstrating the effect of terguride (Terg; 1 and 10 µM), 5-HT (1 µM), and terguride (1 and 10 µM) plus 5-HT (1 µM) on ERK1/2 phosphorylation. A, representative blot. The levels of β-actin served as the loading control. B, pERK1/2 levels from four different experiments. The mean value of the controls (−) was set at 1 (control value), and all data were expressed as percentages relative to control value. *, P < 0.05.](image)

![Fig. 10. Porcine aortic and mitral valvular interstitial cell culture showing the increase in collagen biosynthesis by tritiated proline incorporation at ASPET Journals on April 9, 2017 jpet.aspetjournals.org Downloaded from](image)
accurately reflect the physiological conditions that occur in native tissues (Sanders-Bush and Canton, 1995).

It has been reported that the active metabolites of anorectic drugs (e.g., fenfluramine) or antidepressive drugs (e.g., methysergide) may contribute to fibrotic changes in cardiovascular tissues (Launay et al., 2002; Roth, 2007). The metabolites norfenfluramine and methylergonovine had higher agonist potencies and efficacies at 5-HT2A and 5-HT2B receptors than their parent drugs, fenfluramine and methysergide (Rothman et al., 2000). An active metabolite may also act as an agonist while the parent drug behaves as a silent antagonist. For example, meta-chloro-phenylpiperazine, the active metabolite of the antidepressive drug trazodone, exerts agonist activity at 5-HT2A and 5-HT2C receptors, whereas trazodone itself is an antagonist at these 5-HT receptor subtypes (Stahl, 2009). Therefore, it was of interest to find out whether metabolites of terguride such as N-demethylethylterguride and 6-norterguride may stimulate 5-HT2A and 5-HT2B receptors. Neither N-demethylethylterguride nor 6-norterguride activated both receptors in our study. We were surprised to find that the metabolites of terguride surmountably antagonized 5-HT2A and 5-HT2B receptor-mediated 5-HT responses albeit with lower affinity than terguride. The surmountable antagonism of the metabolites is in contrast to the effect of the parent drug terguride, which behaved as a noncompetitive 5-HT2A receptor antagonist and an insurmountable 5-HT2B receptor antagonist. Thus structurally closely related compounds such as terguride and its metabolites may show different modes of antagonism at 5-HT2A and 5-HT2B receptors.

Ergot derivatives such as metergoline and methysergide antagonize 5-HT-induced contractions in blood vessels by blocking 5-HT2A receptors; however, both drugs show slow-onset and -offset kinetics at these receptors (Bond et al., 1989; Pertz and Eich, 1992). Maximal inhibitory effects of terguride (1 nM) at 5-HT2A receptors of porcine coronary artery were reached only over a longer time period (i.e., after 60 min of equilibration). It should be emphasized, however, that the slow kinetics of 5-HT2A receptor antagonists may not be restricted to ergot derivatives but are a general phenomenon, especially when low concentrations of antagonist are used. For example, equilibrium was not reached in porcine coronary artery; when the contractile response to 5-HT was antagonized by a low concentration (3 nM) of ketanserin (pA2 8.9) or spiperone (pA2 9.3), both are competitive 5-HT2A receptor antagonists (H. H. Pertz and S. Jähnichen, unpublished data). We suggest that the rate for terguride to reach equilibrium depends on the receptor involved in the pharmacological response and the concentration used. Terguride reveals differential binding kinetics at endothelial 5-HT2B and smooth muscle 5-HT2A receptors. Our experiments clearly demonstrate that this drug exhibits fast-onset and -offset kinetics at 5-HT2B Receptors.

Pulmonary hypertension is related to pulmonary vascular constriction and vascular thrombosis (Chaouat et al., 1996). The present in vitro studies suggest that terguride is a potent inhibitor of the 5-HT2A receptor-mediated platelet aggregation. 5-HT2A receptor antagonists have previously been shown to prevent femoral artery occlusion in an animal model (Nishihira et al., 2006) and coronary artery stenosis in patients (Klein et al., 1990). The antiplatelet effects of terguride may be of particular relevance for a therapeutic use in pulmonary hypertension. Thrombotic arteriopathy, which is highly prevalent in pulmonary hypertension, is an important pathophysiological feature of this disease. Regardless of the etiology, thrombotic vascular lesions have been found as an important feature of pulmonary vascular pathology that also includes vascular stenosis and remodeling (Chauvat et al., 1996). Current evidence suggests that primary and/or secondary abnormalities of platelet aggregation and disturbances of the blood coagulation and the fibrinolytic system may contribute to a prothrombotic state (Swaisgood et al., 2000). Hence, the inhibitory effect of terguride on 5-HT-amplified platelet aggregation may support the therapeutic benefit of this drug in pulmonary arterial fibrosis and hypertension.

Valvular heart disease is linked to ERK1/2 activity (Xu et al., 2002). Recent research by our group showed that 5-HT and cabergoline, another ergot alkaloid derivative, increased ERK1/2 phosphorylation in porcine aortic and mitral VICs (Keckeswka et al., 2011). The present study demonstrates that terguride did not stimulate the formation of pERK1/2 but inhibited pERK1/2 activation induced by 5-HT. The stimulatory effect of 5-HT on pERK1/2 in human, canine, and porcine VICs is susceptible to blockage by selective 5-HT2A receptor antagonists (Connolly et al., 2009; Keckeswka et al., 2011). This argues for a role of the 5-HT2A receptor in the inhibitory effect of terguride on pERK1/2 activation by 5-HT. Furthermore, in our study using porcine VICs, the 5-HT2A and 5-HT2B receptor antagonist terguride inhibited the 5-HT-induced incorporation of [3H]proline (index of extracellular matrix collagen). Our results are in line with the reduction of the collagen content in lung and skin by terguride in the mouse model of bleomycin-induced lung fibrosis (Königshoff et al., 2010) and skin fibrosis (Dees et al., 2011). It is noteworthy that bleomycin-induced lung fibrosis in mice was reduced by both ketanserin (5-HT2A receptor antagonist) and 6-chloro-5-methyl-N-quinolin-4-yl-2,3-dihydroindole-1-carboxamide (SB215505) (5-HT2B receptor antagonist) as demonstrated by reduced collagen content and procollagen 1 and procollagen 3 mRNA expression (Fábre et al., 2008). In addition, terguride reversed collagen disposition in a rat model of monocrotaline-induced hypertension in rats (Dumitrascu et al., 2011). Thus the combined inhibition of 5-HT2A and 5-HT2B receptors by terguride may offer a promising approach in the treatment of fibrotic lung disorders (Königshoff et al., 2010; Dumitrascu et al., 2011). A general consensus has emerged that 5-HT2B receptor agonism is the most likely cause of fibrotic valvular heart disease (Roth, 2007; Hutcheson et al., 2011). It has been demonstrated that 5-HT and pergolide-induced valvulopathy in the rat can be prevented by terguride and cyproheptadine, respectively (Hauso et al., 2007; Droogmans et al., 2009). Cyproheptadine is an antagonist with 3-fold higher affinity for 5-HT2A receptors over 5-HT2B receptors (Bonhaus et al., 1997). Based on the observations of the present study, the 5-HT2A receptor blocking properties of terguride may be responsible for the decrease of the 5-HT-induced collagen production in PVICs. Accordingly, the increase in collagen induced by cabergoline, a partial 5-HT2A and full 5-HT2B receptor agonist, was inhibited by (R)-(+)α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (MDL100907) (5-HT2A receptor antagonist) but not by SB204741 (5-HT2B receptor antagonist; Keckeswka et al., 2011). Admittedly, our cell cul-
tured processes have focused only on early remodeling mechanisms of 5-HT-induced heart valve disease. This may not reflect the in vivo situation because 5-HT-related heart valve disease is a chronic process. Moreover, it should be emphasized that the molecular mechanism by which 5-HT induces fibrotic disorders obviously depends on a complex network of different pathways.

In conclusion, the present study suggests that the combined antagonism at 5-HTTA and 5-HTTB receptors, inhibition of platelet aggregation, and inhibition of ERK1/2 phosphorylation and collagen production by terguride could provide a therapeutic rationale for the use of this drug in the treatment of fibrotic diseases such as fibrotic lung disorders and valvular heart disease.

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Authorship Contributions

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