SHORT COMMUNICATION

Effects of 5-Hydroxytryptamine Class 2 Receptor Antagonists on Bronchoconstriction and Pulmonary Remodeling Processes

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Serotonin [5-hydroxytryptamine (5-HT)] is associated with several chronic pulmonary diseases, recognizing 5-HT₂ receptor antagonists as potential inhibitors of tissue remodeling. However, the effects of 5-HT₂ receptors, especially 5-HT_{2B} receptors on airway function and remodeling, are unclear. We investigated the role of 5-HT_{2B} receptors on airway smooth muscle contractility and remodeling processes. Murine precision-cut lung slices were pretreated with 5-HT_{2B} receptor antagonists (EXT5, EXT9, RS 127445, and PRX 08066), as well as ketanserin (5-HT_{2A/2C} receptor antagonist) (1, 10 μ mol/L), before addition of cumulative concentrations of 5-HT to induce bronchoconstriction. Remodeling effects after treatment with 10 μ mol/L 5-HT and 5-HT₂ receptor antagonists were further studied in distal lung tissue by examining release of profibrotic transforming growth factor (TGF)- β 1 and proliferation of human bronchial smooth muscle cells (HBSMCs). 5-HT-induced bronchoconstriction was significantly reduced by EXT5, EXT9, and ketanserin, but not by RS 127445 or PRX 08066. The 5-HT_{2B} receptor antagonists significantly reduced TGF- β 1 release. 5-HT, in combination with TGF- β 1, increased proliferation of HBSMCs, a process reduced by EXT5 and EXT9. Our results indicate that EXT5 and EXT9 may relieve bronchoconstriction in murine airways and serve as an add-on effect in attenuating pulmonary remodeling by improving airway function. The antiproliferative effect on HBSMCs and the inhibition of TGF- β 1 release further support a role of 5-HT_{2B} receptors in pathologic remodeling processes. (*Am J Pathol* 2018; 188: 1113–1119; <https://doi.org/10.1016/j.ajpath.2018.01.006>)

Structural alterations of airways are common features of several respiratory disorders, such as idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma, with increased airway narrowing and/or bronchial hyper-responsiveness as a result.^{1–3} The smooth muscle cell layer in the airways is subjected to structural alterations, presenting hypertrophy and hyperplasia of airway smooth muscle cells.^{4,5} These cells have large plasticity, demonstrating either a contractile phenotype, affecting airways and vessels, or a secretory phenotype, influencing inflammatory responses, cell recruitment, and remodeling.⁴ Serotonin [5-hydroxytryptamine (5-HT)] and the class 2 receptors (5-HT₂) have been associated with several pulmonary functions, such as airway narrowing and vascular remodeling.⁶ 5-HT induces bronchoconstriction in airways of

guinea pig, rat, and mice,^{7–9} and elevated levels of 5-HT are detected in patients with asthma.¹⁰ In a recent study, we reported antifibrotic effects of the 5-HT_{2B} receptor

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antagonists, EXT5 and EXT9, in modeled pulmonary fibrosis *in vivo*.¹¹ The receptor antagonists attenuated fibrotic remodeling with reduced tissue deposition and number of extracellular matrix (ECM)-producing cells in pulmonary parenchymal tissue¹¹; however, effects on airway function were not evaluated.

We speculated that 5-HT_{2B} receptors may regulate both functions and remodeling processes in the airways. To examine this hypothesis, 5-HT-induced bronchoconstriction in murine precision-cut lung slices (PCLSs) was studied using preventive treatment with selective 5-HT₂ receptor antagonists. PCLSs offer an *ex vivo* model to study several physiological functions in naïve pulmonary tissue.^{12–14} Mechanical forces exerted during bronchoconstriction may trigger the release of the profibrotic growth factor, transforming growth factor (TGF)- β 1, known to enhance airway remodeling.¹⁵ To examine the effect of 5-HT_{2B} receptor antagonism on remodeling processes in murine lung tissue, the release of TGF- β 1 was measured after treatment with 5-HT and 5-HT_{2B} receptor antagonists. Studies were also performed in human bronchial smooth muscle cells (HBSMCs), assessing cell proliferation with TGF- β 1 and 5-HT stimuli, in combination with 5-HT₂ receptor antagonist treatment.

Materials and Methods

Receptor Antagonists

The following 5-HT₂ receptor antagonists were evaluated: 5-HT_{2A/2C} receptor antagonist ketanserin (Tocris, Bristol, UK)¹⁶ and 5-HT_{2B} receptor antagonists RS 127445 (Tocris),¹⁶ PRX 08066 (eNovation Chemicals LLC, Bridgewater, NJ),¹⁷ and EXT5 and EXT9 (AnaMar AB, Lund, Sweden).¹¹ Compounds were dissolved in dimethyl sulfoxide. All receptor antagonists were evaluated at 1 and 10 μ mol/L at $\leq 0.1\%$ dimethyl sulfoxide, to avoid cellular toxicity and unspecific receptor binding.

Preparation of PCLSs

C57BL/6 mice (female, aged >10 weeks) (Scanbur Research A/S, Karlslunde, Denmark) were sacrificed with i.p. injection of pentobarbital (0.3 mL of 60 mg/mL). The aorta was cut for exsanguination. With an open abdomen and intact thorax, lungs were filled with prewarmed low melting agarose solution (0.75% to 1.5%) through a fixed positioned tracheal needle inserted into the trachea. After 10 minutes on ice, heart and lungs were removed and put in minimal essential medium on ice to allow the agarose to solidify. Cores were manually cut along airways and cut into 200 to 250 μ m PCLSs with a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL). Slices were incubated at 37°C, 5% CO₂ overnight, with repeated medium change during the first 4 hours. Animal experiments were approved by the local ethics committee (Malmö/Lund, Sweden, M103-14).

Pharmacologic Interventions in PCLSs

PCLSs in medium were positioned in 24-well cell culture plates using nylon threads anchored on platinum weights. PCLSs were pretreated with 1 and 10 μ mol/L of ketanserin, RS 127445, PRX 08066, EXT5, or EXT9 for 20 minutes, before the addition of cumulative concentrations of 5-HT hydrochloride (0.01 to 100 μ mol/L) (Sigma-Aldrich, St. Louis, MO).¹² In addition, cumulative concentrations of a stable derivate of 5-HT, α -methyl-5-HT maleate (Tocris) (0.01 to 100 μ mol/L), were examined for bronchoconstriction. Images of airways were taken (Nikon Eclipse TS100 microscope and Digital sight DS-U2 camera; Nikon, Amsterdam, the Netherlands) every 7 seconds, with 5-minute intervals per cumulative dose of 5-HT. Area of airway was analyzed with NIS-Element AR 3.2 (Nikon).

TGF- β 1 Release from Distal Lung Tissue

In a culture plate, manually cut pieces of distal murine lung tissue were pretreated for 30 minutes with 10 μ mol/L 5-HT_{2B} receptor antagonist EXT5, EXT9, RS 127445, or PRX 08066 before treatment with 10 μ mol/L 5-HT every 2 hours (for a total of 6 hours) at 37°C, 5% CO₂. After 24 hours $\pm$ 2 hours after first addition of 5-HT, cell medium was collected and analyzed for TGF- β 1 using the ELISA Quantikine TGF- β 1 kit (MB100B; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Lung tissue treated with medium containing 0.1% dimethyl sulfoxide served as untreated control. TGF- β 1 content was normalized to total wet tissue weight per sample.

Immunocytochemistry of 5-HT₂ Receptors

Primary HBSMCs (CC-2576; Lonza, Basel, Switzerland) were cultured in SmGM-2BulletKit medium (Lonza) in 5% fetal bovine serum at 37°C, 5% CO₂. Cells were seeded approximately 20,000 cells/well in 4-well glass chamber slides (154526; Thermo Scientific, Waltham, MA) and incubated overnight at 37°C, 5% CO₂. Cells were fixed in 4% formaldehyde solution for 15 minutes, followed by 10-minute rehydration in tris-buffered saline solution. Cells were incubated 90 minutes with primary antibody, 5-HT_{2A} (1 μ g/mL; sc-166775; Santa Cruz Biotechnology, Dallas, TX), 5-HT_{2B} (1.7 μ g/mL; OAAF04758; Aviva System Biology, San Diego, CA), or 5-HT_{2C} (2.3 μ g/mL; PA5-27164; Thermo Scientific) receptor, followed by washing steps in tris-buffered saline solution. Cells were incubated 60 minutes with Cy5-conjugated secondary antibody (1:200) goat anti-rabbit (A21246), goat anti-mouse (A21235), and DAPI (150 nmol/L) for nuclei staining, followed by washing in tris-buffered saline solution and mounting. ImageJ software version 1.51n (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) was used for image visualization.

Figure 1 Effect of 5-HT₂ receptor antagonists on 5-HT-induced bronchoconstriction. **A:** To induce bronchoconstriction, precision-cut lung slices (PCLSs) from C57BL/6 mice were treated with cumulative concentrations (0.01 to 100 $\mu\text{mol/L}$) of 5-HT or α -methyl-5-HT. **B–F:** PCLSs received preventative treatments (20 minutes) with 5-HT_{2A} receptor antagonist ketanserin (**B**) or with 5-HT_{2B} receptor antagonists RS 127445, PRX 08066, EXT5, or EXT9 (**C–F**, respectively), before addition of cumulative concentrations of 5-HT (5-minute intervals). Bronchoconstriction was monitored every 7 seconds, and changes in total airway area were measured and presented as percentage airway area, of initial area (set to 100%). Significant differences are shown in comparison to 5-HT treatment without receptor antagonist. Data are expressed as means $\pm$ SEM (**A–F**). $n = 4$ to 5 (**A**); $n = 4$ (**B**); $n = 3$ to 4 (**C**); $n = 3$ (**D**); $n = 5$ (**E**); $n = 5$ to 6 (**F**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus 5-HT treatment without receptor antagonist.

Proliferation of HBSMCs

HBSMCs were seeded 20,000 cells per well, in a 96-well cell culture plate, and incubated overnight at 5% CO₂, 37°C. Cells were serum starved in 1% fetal bovine serum 1 day before pretreatment with 1 and 10 $\mu\text{mol/L}$ of EXT5, EXT9, RS 127445, PRX 08066, or ketanserin. After 1 hour, cells were treated with 10 ng/mL TGF- β 1 (R&D Systems) in combination with 1 $\mu\text{mol/L}$ 5-HT and the receptor antagonists. Bromodeoxyuridine was added after 24 hours. After an additional 24 hours, cell proliferation was measured with a photospectrometer and quantified by relating the amount of bromodeoxyuridine incorporation over time, according to the manufacturer's instructions (ab126556; Abcam, Cambridge, UK).

Statistical Analysis

Changes in airway area were presented as sigmoidal dose-response curve (variable slope), with values represented as means $\pm$ SEM and analyzed with two-way analysis of variance with Dunnett's multiple comparisons test. Significant differences in pEC₅₀ of 5-HT were evaluated with Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. *Ex vivo* and *in vitro* data were presented as means $\pm$ SD, using one-sample *t*-test for statistical evaluation, as recommended when the numbers in groups are small, because nonparametric tests based on ranks with low *n* numbers seldom demonstrate any significant differences.¹⁸ For correction of multiple comparisons, one-way analysis of variance using two-stage linear setup

procedure of Benjamini, Krieger, and Yekutieli was applied. $P < 0.05$ was considered statistically significant. Statistical software program GraphPad Prism 7 (GraphPad Software, La Jolla, CA) was used for statistical calculations.

Results

Reduced Bronchoconstriction with 5-HT₂ Receptor Antagonists

Airway contraction was studied in murine PCLSs to evaluate the role of 5-HT₂ receptors in 5-HT-induced bronchoconstriction. 5-HT demonstrated distinct bronchocontractile effects with increasing cumulative concentrations (0.01 to 100 $\mu\text{mol/L}$). At 100 $\mu\text{mol/L}$ of 5-HT, the initial airway area (100%) was reduced to $48\% \pm 10.4\%$ (Figure 1A). Treatment with the more stable tryptamine derivative of 5-HT, α -methyl-5-HT (0.01 to 100 $\mu\text{mol/L}$) showed similar effects on bronchoconstriction, with a reduction to $46\% \pm 13.8\%$ at 100 $\mu\text{mol/L}$ (Figure 1A). The 5-HT_{2A/2C} receptor antagonist, ketanserin, at both 1 and 10 $\mu\text{mol/L}$, elicited potent inhibitory effects on 5-HT-induced bronchoconstriction ($P = 0.0001$) (Figure 1B). The 5-HT_{2B} receptor antagonists' RS 127445 and PRX 08066 showed no significant effects at either 1 or 10 $\mu\text{mol/L}$ (Figure 1, C and D). Both EXT5 and EXT9 significantly inhibited 5-HT-induced bronchoconstriction, at 1 $\mu\text{mol/L}$ of EXT5 ($P = 0.0015$) and 10 $\mu\text{mol/L}$ of EXT9 ($P = 0.0214$) at 100 $\mu\text{mol/L}$ of 5-HT (Figure 1, E and F). Characterization of the sigmoidal dose-response curves statistically confirmed the attenuating bronchodilatory effects of 1 $\mu\text{mol/L}$ EXT5 and 10 $\mu\text{mol/L}$ EXT9, as seen with

Table 1 Characterization of the Sigmoidal Dose-Response Curves

Treatment	n	E _{max}	pEC ₅₀	Hill slope
5-HT (control)	4	54.4 ± 14.9	5.8 ± 0.5	−0.71
α-Methyl 5-HT	5	52.1 ± 8.2	6.7 ± 0.5	−0.91
5-HT (control)	5	53.5 ± 6.8	7.2 ± 1.0	−0.62
EXT5, 1 μmol/L	4	22.1 ± 11.5	5.7 ± 0.9*	−0.63
EXT5, 10 μmol/L	4	44.4 ± 12.3	6.8 ± 1.2	−0.54
5-HT (control)	7	59.2 ± 9.3	6.0 ± 0.3	−0.8
EXT9, 1 μmol/L	5	64.3 ± 6.4	5.3 ± 0.2	−1.1
EXT9, 10 μmol/L	5	41.7 ± 15.4	4.9 ± 0.4*	−1.7
5-HT (control)	3	50.3 ± 6.9	5.7 ± 0.3	−0.95
PRX 08066, 1 μmol/L	3	37.0 ± 8.6	5.7 ± 0.4	−1.1
PRX 08066, 10 μmol/L	3	70.9 ± 6.2	5.7 ± 0.2	−1
5-HT (control)	6	51.1 ± 8.8	6.4 ± 0.5	−0.6
RS 127445, 1 μmol/L	4	76.9 ± 85.5	5.5 ± 1.8	−0.3
RS 127445, 10 μmol/L	3	32.8 ± 8.4	7.2 ± 1.5	−1.1
5-HT (control)	4	55.6 ± 5.0	6.3 ± 0.2	−1
Ketanserin, 1 μmol/L	4	12.8 ± 8.5	NA	NA
Ketanserin, 10 μmol/L	4	4.2 ± 1.3	NA	NA

Murine precision cut lung slices were pretreated (20 minutes) with 5-HT₂ receptor antagonists (1 and 10 μmol/L) before addition of cumulative concentrations of 5-HT (0.01 to 100 μmol/L) to induce bronchoconstriction. From the sigmoidal dose-response (variable slope) curves, E_{max} and pEC₅₀ were calculated and expressed as means ± SEM, including hill slope and n value.

**P* < 0.05. Significant difference of pEC₅₀ from 5-HT control.
E_{max}, maximal possible effect; pEC₅₀, negative logarithm of EC50; NA, not applicable.

significant differences in pEC₅₀ of 5-HT, in comparison to control (Table 1).

Release of TGF-β1 after Treatment with 5-HT and 5-HT₂ Receptor Antagonists

Mechanical tensions may contribute to progression of fibrosis, by triggering an increased release of active TGF-β1 after contraction of myofibroblast and smooth muscle cells.^{19,20} Levels of TGF-β1 were studied in medium extracted from murine distal lung tissue treated with 10 μmol/L 5-HT and 10 μmol/L 5-HT_{2B} receptor antagonists. Mouse lung tissue treated with 5-HT showed tendencies (*P* = 0.0881) in stimulating the release of TGF-β1, with six of eight mice showing elevated levels of TGF-β1 (within 95% CI). Treatment with 10 μmol/L 5-HT_{2B} receptor antagonist RS 127445, EXT9, and EXT5 inhibited the release of TGF-β1 to levels of 67.5% (*P* = 0.0084), 72.7% (*P* = 0.0221), and 64.2% (*P* = 0.0092), respectively, in comparison to 5-HT control (100%) (Figure 2). Indications of reduced levels of TGF-β1 were also seen in initial evaluation with 10 μmol/L PRX 08066 (data not shown).

Expression of 5-HT₂ Receptors and Effect of 5-HT₂ Receptor Antagonists on Human Bronchial Smooth Muscle Cell Proliferation

The accumulation of smooth muscle cells causes hyperplastic changes of the airway wall, affecting airway responsiveness.

Airway smooth muscle cells respond to and secrete proinflammatory agents, chemokines, and growth factors, such as TGF-β1,^{21,22} that may contribute to airway remodeling. HBSMCs expressed the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, shown with antibody labeling (Figure 3, A–E). By using a combinatory treatment with the profibrotic stimuli TGF-β1 in combination with 5-HT, HBSMCs showed an increased cell proliferation (51% ± 17%; *P* = 0.002) after 48 hours, compared with unstimulated control (medium with 0.1% dimethyl sulfoxide) (Figure 3F). Initial evaluation of effects after induction with 5-HT alone did not generate a distinct increased proliferation of HBSMCs (data not shown), an event commonly seen with 5-HT. It has been described as a helper agonist and establishes its effect when combined with other agents, as shown in other published studies.^{11,23} Compounds EXT5 and EXT9 significantly reduced proliferation at 10 μmol/L [75% ± 12% (*P* = 0.0091) and 75% ± 20% (*P* = 0.0519), respectively] in comparison to 5-HT in combination with TGF-β1 (100%). Comparable effects were also seen in the initial evaluation with 10 μmol/L PRX 08066, resulting in reduced proliferation (data not shown). RS 127445 at 1 μmol/L caused a slight, but significant, increase in proliferation; however, at 10 μmol/L, no significant effect was detected. Ketanserin (1 and 10 μmol/L) showed no significant effect on HBSMC proliferation (Figure 3F).

Figure 2 The release of transforming growth factor (TGF)-β1 in 5-HT-treated murine lung tissue. Murine pieces of distal lung tissue were treated with medium alone (untreated, dimethyl sulfoxide 0.1%) or with 10 μmol/L 5-HT in combination with or without 30-minute pretreatment with 10 μmol/L EXT5, EXT9, or RS 127445. Lung tissues were repeatedly exposed to 5-HT, three times for a duration of 6 hours, and medium was collected after 24 hours and measured for TGF-β1. Concentrations were normalized to wet tissue weight. Results are represented as individual values (within 95% CI, categorized in symbol shapes for each individual animal), normalized to 5-HT control (100%). Significant difference is shown from tissue cultured with 5-HT. Untreated tissue served as control. Data are expressed as means ± SD. *n* = 5 to 9 animals. **P* < 0.05, ***P* < 0.01.

Figure 3 Expression of 5-HT₂ receptors on human bronchial smooth muscle cells (HBSMCs) and the effect of 5-HT₂ receptor antagonists on HBSMC proliferation. **A–C:** Cultured HBSMCs were labeled with anti-5-HT_{2A} (**A**), anti-5-HT_{2B} (**B**), and anti-5-HT_{2C} (**C**) receptor antibodies, with DAPI for cell nuclear staining (blue). **D** and **E:** Negative control for secondary antibodies shows no positive staining. **F:** The relative amount of proliferating HBSMCs was measured with bromodeoxyuridine (BrdU) incorporation. Cells were treated for 48 hours with 10 ng/mL TGF-β1 and 1 μmol/L 5-HT with or without pretreatment (1 hour) with 5-HT₂ receptor antagonists (EXT5, EXT9, RS 127445, or ketanserin) (1 and 10 μmol/L). **F:** BrdU incorporation, shown in percentage to TGF-β1 and 5-HT stimuli, is presented as individual values, with a minimum of four conducted experiments. Significant difference from cells treated with TGF-β1 in combination with 5-HT. Untreated cells served as control. Data are expressed as means ± SD (**F**). $n = 4$ to 5 (**F**). $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar = 30 μm (**A–E**).

Discussion

This study demonstrated that 5-HT–induced murine bronchoconstriction, TGF-β1 release, and proliferation of

smooth muscle cells can be inhibited by the 5-HT_{2B} receptor antagonists, EXT5 and EXT9. These results thereby identify the involvement of these compounds in regulating airway function and processes associated with tissue remodeling.¹¹

In early respiratory responses to noxious agents, there are a massive influx of inflammatory cells and release of inflammatory mediators, causing bronchial smooth muscle contractions and enhanced mucus secretion. With continuous inflammation and epithelial cell necrosis, airways undergo chronic pathologic remodeling and fibrosis, with increased airway smooth muscle cell layer and deposition of matrix proteins, resulting in airflow obstruction.²⁴ Small airway remodeling is present in patients with idiopathic pulmonary fibrosis,²⁵ as well as an increased pulmonary expression of the 5-HT_{2A} and 5-HT_{2B} receptors.²⁶ 5-HT activates several subtypes of receptors, generating a variety of pulmonary effects. Situated in close proximity to the airway smooth muscle layer are the mast cells, which release potent mediators, such as histamine and 5-HT, on cell degranulation, a process suggested to be ongoing in patients with symptomatic asthma.²⁷ In patients with idiopathic pulmonary fibrosis, mast cell density is increased, especially in areas of inflammation and fibrosis, correlating to the degree of tissue remodeling.^{28,29} An increased uptake of 5-HT with tianeptine (an antidepressant drug) reduced 5-HT in plasma and attenuated asthmatic symptoms.³⁰ However, the impact of 5-HT_{2B} receptor activation on pulmonary disorders remains incomplete and warrants further investigation. This study further characterizes effects of 5-HT on pulmonary functions, identifying a regulative role of the 5-HT₂ receptors on airway function and proliferation associated with remodeling. The tested orally available compounds, EXT5 and EXT9, with moderate diversity in functionality and binding to the 5-HT₂ receptors,¹¹ inhibited 5-HT–induced murine bronchoconstriction. In contrast to EXT5 and EXT9, the other two 5-HT_{2B} receptor antagonists PRX 08066 and RS 127445 showed no significant effect on 5-HT–induced bronchoconstriction. The effects presented between EXT5 and EXT9 may hypothetically be the result of these compounds' functionality profiles toward the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors,¹¹ favoring combinatory antagonistic effects of the 5-HT₂ receptors. Further studies addressing selectivity and receptor binding kinetics will help to fully understand the initial receptor characterization of EXT5 and EXT9, which are compounds in the early stage of evaluation.

The diversity in the compound effects between EXT5 and EXT9 was also previously observed in regulating pulmonary matrix production and levels of the proinflammatory cytokines, such as IL-1β, with a significant reduction in serum after EXT9 treatment in bleomycin-treated animals.¹¹ IL-1β has been shown to be elevated in bronchoalveolar lavage fluid in patients with symptomatic asthma.³¹ Interestingly, IL-1β potentiated 5-HT–induced bronchoconstriction in murine airways, a response hindered by the 5-HT_{2A/2C} receptor antagonist ketanserin.³² Speculatively, the immune modulating properties of EXT9 on IL-1β propose additional beneficial effects in attenuating airway hyperresponsiveness

in vivo.¹¹ Our obtained results further support an important role of the 5-HT_{2A} receptor in controlling airway contractility in mice, as well as recognizing EXT5 and EXT9 as regulators of airway function. The primary mechanism in regulating acute effects on murine bronchodilation *ex vivo* seems to be mediated via the 5-HT_{2A} receptor, as seen with lack of effect after treatment with highly selective 5-HT_{2B} receptor antagonists RS 127445 and PRX 08066.^{16,17} Conversely, in an induced allergic asthma study, airway hyperresponsiveness was blocked with a 5-HT_{2A/2C} receptor agonist *in vivo*.³³ However, the 5-HT_{2A/2C} receptor antagonist ketanserin has also proved to act as a bronchodilator in humans with asthma,³⁴ findings that highlight the complexity of serotonergic signaling in airways.

5-HT has previously been shown to increase levels of TGF- β 1 and phosphorylated Smad3,³⁵ which have been linked to 5-HT_{2B} receptor activation.³⁶ Treatments with selective 5-HT_{2B} receptor antagonists have frequently shown to attenuate pathologic remodeling in both skin and pulmonary fibrosis.^{11,35,37} Our results further indicate a profibrotic role of the 5-HT_{2B} receptors, which, on receptor antagonism, inhibit the release of TGF- β 1 in murine lung tissue, as shown with the EXT compounds and two reference compounds. Little is known of the 5-HT₂ receptor turnover and half-life, as well as receptor internalization processes, resulting in difficulties in studying 5-HT-mediated effects, as seen when studying TGF- β 1 release after treatment with 5-HT. The mechanical tension elicited by cellular contractile fibers, such as α -smooth muscle actin, may result in the release of ECM-stored TGF- β 1, through interactions of integrins and the TGF- β 1-binding protein latency-associated protein.¹⁹ The mechanical properties of the tissue influence the release of TGF- β 1, a physical process that may connect to the composition of contractile fibers in cellular compartments. Treatment with 5-HT_{2B} receptor antagonist EXT5 or EXT9 in human lung fibroblasts reduced intracellular levels of α -smooth muscle actin, which, in part, may explain 5-HT_{2B} receptor regulation of TGF- β 1 release.¹¹ The decreased release of TGF- β 1 after treatment with 5-HT_{2B} receptor antagonist may thereby be explained by a reduction of intracellular levels of α -smooth muscle actin, as seen in human lung fibroblasts.¹¹ However, exposure to 5-HT may additionally increase the numbers of TGF- β 1-producing cells, through increased proliferation of smooth muscle cells and fibroblasts¹¹ or an increased endogenous production of TGF- β 1.³⁵

In the present study, translational studies in human primary cell culture system were applied, demonstrating that the combinatory stimuli of TGF- β 1 with 5-HT stimulated cell proliferation in HBSMCs, which was inhibited by the 5-HT_{2B} receptor antagonists EXT5, EXT9, and PRX 08066. RS 127445 did not display any statistical reducing effect on proliferation, although other studies support an anti-growth effect of RS 127445, with reducing thymidine incorporation and TGF- β levels in pulmonary arteries⁶ and in human lung fibroblasts (A.L., K.R.-T., A.-K.L.-C., C.W., G.W.-T., unpublished data), indicating RS 127445 as a compound with antiproliferative capacities.

The bronchodilatory and antiproliferative characteristics of EXT5 and EXT9 may serve as beneficial add-on effects to these compounds' previously reported inhibitory effect on ECM production.¹¹ The composition of the ECM determines the secretory phenotype and proliferative capacity of airway smooth muscle cells,^{38,39} highlighting the importance of cellular responses to an altered ECM. The ECM is important to support a contractile smooth muscle cell phenotype, with high expression of α -smooth muscle actin and low proliferation capacity.⁴⁰ In this study we have examined proliferative primary HBSMCs, a culture most likely composed of the secretory phenotype based on culture conditions and the proliferative status. The reduced proliferation elicited by the 5-HT_{2B} receptor antagonists may be linked to phenotypic switching, as seen in other cell types,⁴¹ thus promoting a contractile and less proliferative phenotype *in vitro*.

In summary, this study emphasized the important role of 5-HT and 5-HT₂ receptors on murine airway function, proliferation of human bronchial smooth muscle cells, and the release of TGF- β 1. The attenuating effects on airway remodeling are proposed to be mediated through 5-HT_{2B} receptor antagonism, whereas the acute bronchodilatory effects might involve a combination of 5-HT₂ receptors, warranting further investigations to fully understand effects mediated by the 5-HT₂ receptor antagonists.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.01.006>.

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