JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585

JPET #191585

Sphingosine-1-phosphate induced airway hyperreactivity in rodents is mediated by the $S1P_3$ receptor

Alexandre Trifilieff and John R. Fozard

Novartis Institutes for BioMedical Research, Respiratory Diseases Area, Novartis AG, CH-4002 Basel, Switzerland

Running tile: S1P-induced airway hyperreactivity

Author for correspondence:

Alexandre Trifilieff

WSJ-386.03.09, Novartis Pharma AG, Postfach, CH-4002 Basel, SWITZERLAND

Phone: +41 61 6969483

E-mail: alexandre.trifilieff@novartis.com

Text pages: 18

Tables: 3

Figures: 7

References: 27

Abstract: 239 words

Introduction: 407 words

Discussion: 1470 words

Abbreviations: S1P, sphingosine-1-phosphate; R_L, airway resistance.

Section: Inflammation, Immunopharmacology, and Asthma

ABSTRACT

There is a need to better understand the mechanism of airway hyperreactivity, a key feature of asthma. Evidence suggests that sphingosine-1-phosphate (S1P) could be a major player in this phenomenon. The purpose of this work was to define the S1P receptor responsible for this phenomenon. We have studied, in the rat, the effect of two S1P synthetic receptor ligands, FTY720 (which in its phosphorylated form is a potent agonist at each S1P receptor except $S1P_2$) and AUY954 (a selective $S1P_1$ agonist) on lung function in vivo. This was complemented by in vitro studies using isolated trachea from the rat, the $S1P_3$ receptor-deficient mouse and its wild type counterpart. Following oral administration, FTY720 induced a generalized airway hyperreactivity to a range of contractile stimuli. This was observed as early as one hour post dosing, lasted for at least 24 hours and was not subject to desensitization. In both the rat and wild type mouse isolated trachea, pre-incubation with the active phosphorylated metabolite of FTY720, induced hyperresponsiveness to 5-HT. This effect was not seen in the isolated tracheas from $S1P_3$ receptor-deficient mice. AUY954, did not mimic the effect of FTY720 either in vivo or in vitro. Our data are consistent with activation of the S1P pathway inducing a generalized airway hyperreactivity in rats and mice that is mediated by the $S1P_3$ receptor. $S1P_3$ receptor antagonists might prove useful as new therapeutic strategies aimed at blocking the airway hyperreactivity observed in asthma.

INTRODUCTION

Airway hyperreactivity to bronchoconstrictor stimuli is a key feature of asthma. It is defined as an exaggerated sensitivity and reactivity of the airways to a variety of bronchoconstrictor stimuli. Several cell types and/or mediators have been implicated in the genesis of this phenomenon. However, the cellular and molecular mechanisms that are involved in airway hyperreactivity remain unclear (Maddox and Schwartz, 2002). Although, the inflammatory component of asthma in most patients is well controlled with anti-inflammatory drugs, such as inhaled steroids, the airway hyperreactivity is refractory to such treatments (Brusasco et al., 1998; Lundgren et al., 1988). There is therefore a need to better understand the mechanisms leading to the airway hyperreactivity observed in asthma.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that is known to regulate diverse biological functions of many cell types, from proliferation and survival to migration and secretion. S1P exerts most of its actions by activation of five G protein-coupled receptors designated $S1P_{1-5}$ (Alexander et al., 2011). S1P has been demonstrated to be an important regulator of vascular and gastric smooth muscle contraction (Schuchardt et al., 2011; Zhou and Murthy, 2004). At the level of airway smooth muscle, S1P has been demonstrated to stimulate contraction of human airway isolated smooth muscle cells (Rosenfeldt et al., 2003) and guinea pig isolated trachea (Kume et al., 2007). In addition, it has been demonstrated that S1P causes an increased sensitivity to methacholine-induced contraction of guinea pig isolated trachea (Kume et al., 2007) and mouse isolated bronchus and whole lung (Roviezzo et al., 2007; Roviezzo et al., 2010). Although, these studies point strongly towards a role for S1P as an important mediator that induces bronchoconstriction and airway hyperreactivity in vitro or ex vivo, the same phenomenon has not yet been described

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

in vivo and none of the above studies has defined unequivocally the S1P receptor(s) responsible for the effect. In the present study, we have used two synthetic S1P receptor agonists (FTY720, which in its phosphorylated form is a potent agonist at each S1P receptor except $S1P_2$) and AUY954 (a selective $S1P_1$ receptor agonist) and $S1P_3$ receptor-deficient mice in an attempt to characterize the S1P receptor subtype responsible for the in vivo and in vitro airway hyperreactivity induced by activation of the S1P pathway. Taken together, our results suggest that, in rats and mice, activation of the S1P pathway induces airway hyperreactivity that is mediated by activation of the $S1P_3$ receptor.

METHODS

Animals

Male Brown Norway rats weighing 200-300 g, were supplied by Charles River (L'Abresle, France). Wild type (C57BL/6) or $S1P_3$ receptor-deficient mice (Ishii et al., 2001) were bred in house and used at the age of 11-15 weeks. Animals were kept at an ambient temperature of 22 ± 2 °C under a 12 hours normal phase light-dark cycle. Food and drinking water was freely available. Animals were acclimatized for a period of at least 7 days upon arrival in the laboratory before any experimental work began. All experiments were carried out with the approval of the Veterinary Authority of the City of Basel (Kantonales Veterinaeramt, Basel-Stadt).

Lung function measurements

Measurement of airway resistance and cardiovascular parameters was carried out in anaesthetized rats as described previously (Ellis et al., 2004). Briefly, naive or ovalbumin-sensitized rats were anaesthetized with pentothal (80 mg/kg), then given vecuronium bromide (12 mg/kg) to suppress natural breathing. Animals were ventilated via a tracheal cannula with a mixture of air and oxygen (1:1). Heparinised polyethylene catheters were inserted into the right carotid artery for recording blood pressure and heart rate and into the left jugular vein for bronchospasmogen administration. Body temperature was maintained at 37 °C by means of a heated table. Airway resistance was calculated with a digital electronic respiratory analyzer (LFR, Mumed Ltd., U.K.) for each respiratory cycle.

For the measurement of ovalbumin-induced bronchoconstriction, cumulative intravenous injections of ovalbumin (0.3, 1 and 3 mg/kg) were given to ovalbumin-sensitized animals (Ellis et al., 2004) with a 10 minutes interval between two doses.

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

The measurement of responses to other bronchoconstrictor stimuli was performed in non-sensitized rats. The spasmogens (5-HT, methacholine and adenosine) were injected intravenously with a 5 minutes period between doses, sufficient to allow the effects of the preceding dose to have returned to baseline. The time interval between each sequence of bronchoconstrictor agents was 15 minutes.

Drugs treatment for in vivo experiments

FTY720 was dissolved in 0.9 % saline and given either intravenously (bolus of 0.2 ml) or orally by gavage (2 ml/kg). AUY954, a selective $S1P_1$ receptor agonist (Pan et al., 2006), was dissolved in 10 % tween 80 + 40 % polyethylene glycol 200 in distilled water and given orally by gavage (2 ml/kg).

Isolated tracheal preparation

Animals were killed by exposure to carbon dioxide. The trachea was removed and two (mouse) or four (rat) segments, containing 4 cartilaginous rings, were cut. Each segment was opened longitudinally, opposite to the smooth muscle band and set up for recording isotonic tension in 10 ml organ baths containing modified Krebs' solution (mM: NaCl, 118; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11) at 37 °C bubbled with 95 % O_2 / 5 % CO_2. Resting tension was maintained at 1 and 0.5 g, for rat and mouse segments, respectively. After a stabilization period of 1 hour, during which time the tissues were repeatedly washed, the experiment started as follows. For the rat segments, following addition of a supramaximal concentration of KCl (50 mM) and several washes, Compounds were added for 30 minutes before the start of a concentration response curve to 5-HT (10^{-7}

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

to 10^{-5} M). For the mouse segments, the same sequence was used without the initial addition of KCl. FTY720-P and AUY954 were dissolved in 100% dimethyl sulfoxide (final bath concentration 0.2%).

Data analysis

In vivo data are expressed as variation from the baseline measurements. As an index of airway sensitivity to the contractile agents, the provocative dose of the contractile agents required to increase lung resistance by 100% from baseline (PC_{100}) was calculated and data expresed as average and 95% confidence interval.

For isolated rat and mouse trachea, data are expressed as a percentage of the contraction obtained with 50 mM KCl or with 10 μM 5-HT during the first concentration-response curve, respectively. All other data are presented as means ± S.E.M. Statistical significance, for the in vivo experiments, was established with Student's *t*-test and the Hommel-Hochberg multiple comparison test using the 'Excel' V.8.0 software package. For the in vitro experiments, Student's *t*-test for paired data was used and the Hochberg correction was applied to adjust for multiple comparisons. Significance was assumed at the 5 % probability level.

Material

Unless stated otherwise, all reagents were obtained from Sigma-Aldrich (Buchs, Switzerland). FTY720, FTY720-P and AUY954 were synthesized by the Novartis Global Department of Chemistry (Basel, Switzerland). The S1P receptors selectivity profile for the compounds, as determined by GTPγS binding assay, are as follows: (in nM) FTY720-P ($S1P_1$, 0.3 ± 0.1; $S1P_2$, >10,000; $S1P_3$, 3.1 ± 0.4; $S1P_4$, 0.6 ± 0.2 and

$S1P_5$, 0.3 ± 0.2); AUY954 ($S1P_1$, 1.2 ± 0.6; $S1P_2$, > 10,000; $S1P_3$, 1210 ± 127; $S1P_4$, > 1000 and $S1P_5$, 340 ± 80) (Pan et al., 2006).

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Intravenous injection of FTY720 but not AUY954 induces a delayed and long lasting bronchoconstriction that is reversed by salbutamol

When given as a bolus intravenous injection to the rat, 10 mg/kg FTY720 induced an immediate and short lasting (less than 2 minutes) decrease in mean arterial blood pressure and heart rate. Although the hypotensive effect was transient, the bradychardia was long lasting and still present at 1 hour; the latest time point studied (Fig. 1) Approximately 10 minutes after the intravenous bolus injection of FTY720 airway resistance began to increase. The bronchoconstrictor response developed slowly in a dose-dependent manner, and reached a maximal effect at approximately 50 minutes (Figs. 1 and 2). The effect on the airway resistance, induced by 10 mg/kg FTY720, was almost completely reversed by an intravenous bolus injection of 10 µg/kg salbutamol (inhibition of 95 ± 2%, n = 4) (Fig. 1), consistent with the increase in airway resistance induced by FTY720 being due to a constrictor effect on the airway smooth muscle. In contrast, when 10 mg/kg AUY954 was given as a bolus intravenous injection, no bronchoconstriction was observed (Figs. 1 and 2). However, AUY954 induced a bradycardia and a hypotensive effect with a similar magnitude and kinetic aspect to FTY720 (Fig. 1).

Oral administration of FTY720 but not AUY954 induces a long lasting generalized airway hyperreactivity which does not show tachyphylaxis

When given orally FTY720 (0.1 to 10 mg/kg) did not induce bronchoconstriction but from the dose of 1 mg/kg a long lasting bradycardia was observed (Table 1). Three hours after oral dosing, FTY720 induced a dose-dependent increase in the airway responses to a range of spasmogens, including agonists that act directly on the smooth

muscle (5-HT, methacholine), an agent that activates mast cells (adenosine) or allergen challenge with ovalbumin (Fig. 3, Table 2). The increase in sensitivity was selective for the airways since the hypotensive response and effects on heart rate induced by all spasmogens tested were not altered by FTY720 (Fig. 3). In contrast to FTY720, three hours after oral treatment with 30 mg/kg of AUY954, no hyperreactivity to 5-HT, methacholine or adenosine was observed (Fig. 4), suggesting that the phenomenon observed with FTY720 was not mediated by the $S1P_1$ receptor. Treatment with AUY954 did not modify the hypotensive response or the effects on heart rate induced by all spasmogens (not shown).

In order to investigate the time course for the FTY720-induced increase in airway reactivity to spasmogens, animals were treated orally with 1 mg/kg FTY720 and airway reactivity measured at different time points thereafter. The increase in airway reactivity was present as early as one hour after dosing, reached its maximum at 3 hours and was still present 24 hours post dosing (Fig. 5). This airway hyperreactivity phenomenon was not subject to desensitization, since the effect was similar whether the animals were treated acutely with 1 mg/kg FTY720 or following 2-weeks chronic treatment with 1 mg/kg FTY720 and the measurements made 3 hours after the last dose (Fig. 6).

FTY720-P-induced hypersensitivity to the 5-HT-induced contraction in rat isolated trachea

Since the reversal of the bronchoconstriction observed following intravenous injection of FTY720 by salbutamol suggested a direct effect on smooth muscle, we used the rat isolated tracheal segment model to further study this effect. When applied to the organ bath, the active phosphorylated metabolite of FTY720 (FTY720-P) induced an

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

immediate concentration-dependent contraction (tension in % of the response to 50 mM KCl; 0.1 µM, 1.9 ± 0.89; 1 µM, 27.9 ± 5.8; 10 µM, 98.9 ± 16.1). In addition, it increased concentration-dependently the 5-HT-induced contraction when compared to control (Fig. 7, top left panel) but did not affected its potency with calculated pEC_{50} values ranged from 5.98 to 6.06 (Table 3). Under similar conditions, AUY954 (0.1-10 µM) had neither an intrinsic contractile effect, nor any influence on the contractile response to 5-HT (Fig. 7, top right panel; Table 3).

Effect of FTY720-P on 5-HT-induced contraction of isolated mouse trachea

Because of the lack of selective antagonists for the $S1P_3$ receptor, we used the $S1P_3$ receptor-deficient mouse to study the possible involvement of this receptor in the phenomenon observed. In contrast to the rat isolated trachea, FTY720-P had no intrinsic contractile effect in the mouse trachea isolated from wild type animals. However, as observed in the rat, FTY720-P, from a concentration of 1 µM, induced an increase in the contractile response to 5-HT in the isolated trachea from wild type mice (Fig. 7, middle left panel). When applied under the same conditions to tracheal segment isolated from $S1P_3$ receptor-deficient mice, FTY720-P did not induce hypersensitivity to 5-HT (Fig 7, bottom left panel). The 5-HT potency was not different when compared with segments from wild type and $S1P_3$ receptor-deficient mice and was also not affected by FTY720-P as indicated by the pEC_{50} values ranging from 5.91 to 6.04 and 5.71 to 6.07 for the wild type and $S1P_3$ receptor-deficient animals, respectively (Table 3). AUY954 (0.1-10 µM) when applied to tracheal segments isolated from rat (Fig. 7, top right panel), wild type (Fig. 7, middle right panel) or $S1P_3$ receptor-deficient (Fig. 7, bottom right panel) mice had neither an

intrinsic contractile effect, nor did it influence the contractile response to 5-HT (Table 3).

DISCUSSION

Airway hyperreactivity is a prominent feature of human asthma (Maddox and Schwartz, 2002) that is insensitive to currently available treatments (Brusasco et al., 1998; Lundgren et al., 1988). A significant body of data now indicates that S1P has pro-inflammatory effects and recent studies have demonstrated that this lipid could be involved not only in the inflammatory reaction seen in asthmatic patients (Nixon, 2009) but also could play a role in airway hyperreactivity.

The role of endogenously produced S1P has been assessed, in mouse models of asthma and in all cases, the airway hyperreactivity was abrogated (Lai et al., 2008; Nishiuma et al., 2008; Haberberger et al., 2009; Chiba et al., 2010b). These data suggest that the increased S1P levels seen in the airways of asthmatic patients following provocation with allergen (Ammit et al., 2001) could drive the airway hyperreactivity associated with this disease. There can be little doubt that the above studies establish a role for S1P pathways in airway hyperreactivity induced by allergen challenge in mice. Our data extend this phenomenon to the rat, and demonstrate for the first time, that airway hyperreactivity to a range of spasmogens can be induced in naive animals following oral administration of FTY720 a non-selective S1P receptor agonist. This phenomenon lasted for at least 24 hours and did not desensitize following chronic dosing for two weeks.

In the present study, using synthetic S1P receptor ligands, we have attempted to define the receptor responsible for the S1P-induced airway hyperreactivity in the rat. Although, it has been previously reported that a single subcutaneous injection of S1P was able to induce an increase in cholinergic reactivity of mouse bronchial tissue in vitro (Roviezzo et al., 2010), in the rat we could not see any effect of S1P itself either using a bolus intravenous injection or an infusion (not shown). This discrepancy is

hard to explain, but seems likely to have a pharmacokinetic explanation. Thus, it is known that levels of S1P in most tissues are low, probably due to the metabolic activity of S1P lyase (Schwab et al., 2005) whereas the concentration of S1P in plasma is in the micromolar range (Pappu et al., 2007). Taking these points into account, we do not find it surprising that intravenous administration of S1P has no effect in our model. Because we could not see any in vivo effect using S1P, we used FTY720, a stable synthetic analog of S1P that, once phosphorylated in vivo acts as an agonist at each of the S1P receptors except $S1P_2$ (Brinkmann et al., 2002). In contrast to S1P, it has been shown that following oral and intravenous administration, FTY720 distributes extensively to various tissues including lungs (Meno-Tetang et al., 2006). Using this agonist, we have shown that following intravenous injection, the compound induced a remarkable, slowly developing, bronchoconstriction. When given orally, the intrinsic bronchoconstrictor effect was not observed. However, a generalized airway hyperreactivity, to a range of contractile stimuli, was observed as early as one hour after oral dosing. The fact that FTY720 induces bronchoconstriction only when it is given intravenously is a puzzling observation. When dosed to the rat, FTY720 is extensively converted to its active metabolite FTY720-P and this has been demonstrated to take place in a wide range of tissues (Billich et al., 2003). We speculate that the lack of bronchoconstriction following oral dosing of FTY720 is related to its peak exposure. Indeed, in the rat, intravenous injection of FTY720 gives a high systemic exposure and a rapid diffusion into tissues including the lung. In contrast, when given orally, the absorption into tissues is slow and delayed with a very low systemic clearance (Meno-Tetang et al., 2006). Therefore the peak lung concentration following oral dosing of FTY720 might not be high enough to induce bronchoconstriction but its very long systemic half life together with its extensive

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

distribution into the lung might account for the airway hyperreactivity observed. Indeed, following oral dosing, it takes about 3 hours for FTY720 to fully distribute to the lung, which is the time needed to observed the maximal airway hyperreactivity (Fig. 5); whereas the lung diffusion is much faster following intravenous dosing (Meno-Tetang et al., 2006).

To obtain more information on the S1P receptor(s) responsible for the airway hyperreactivity, we used AUY954 a selective $S1P_1$ receptor agonist (Pan et al., 2006; Schuchardt et al., 2011). It was important to establish that the intervention with AUY954 was truly a consequence of a high degree of selectivity for the $S1P_1$ receptor at the dose level used. That this was the case is suggested by the fact that AUY954 did not mimic the effect of FTY720 in vivo in the present study, whereas the compound did block allograft rejection in a rat transplantation model at doses similar to FTY720 (Pan et al., 2006). Additionally, in the present experiments the compound did not mimic the effects of FTY720-P in the rat and mouse isolated trachea. These results suggest strongly that the $S1P_1$ receptor does not play a role in the induction of airway hyperreactivity. The fact that the airway hyperreactivity induced by FTY720, which has negligible affinity for the $S1P_2$ receptor, in vivo, was also seen on the isolated trachea in vitro and mimics the effect observed with S1P in the mouse isolated bronchi (Roviezzo et al., 2007) and in guinea pig isolated trachea (Kume et al., 2007), essentially rules out that the $S1P_2$ receptor plays a role in this phenomenon.

A previous study, in the mouse isolated bronchus, concluded that the $S1P_3$ receptor was not responsible for the potentiating effect of S1P on acetylcholine-induced contraction (Roviezzo et al., 2007). Nonetheless, this conclusion has to be challenged by the fact that it was based on the ineffectiveness of suramin and BML-241 to inhibit the S1P-driven increase in sensitivity to acetylcholine. However, suramin is a non

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

selective anionic polycyclic dye that blocks many receptor/ligand interactions and BML-241 has been shown not to be a $S1P_3$ antagonist but rather a non selective inhibitor of increases in intracellular calcium (Jongsma et al., 2006). In contrast, our data showing that the FTY720-P-induced hyperreactivity to 5-HT is abrogated in tracheal segments isolated from $S1P_3$ receptor-deficient mouse, provides powerful evidence that this phenomenon is mediated by the $S1P_3$ receptor in this species.

In contrast to previous reports describing an intrinsic contractile effect of S1P in human airway smooth cells (Rosenfeldt et al., 2003), in guinea pig isolated trachea (Kume et al., 2007) and an intrinsic contractile effect of FTY720-P in isolated rat trachea (this study), we could not demonstrate such an effect in the mouse isolated trachea with FTY720-P. Our observation is in line with the lack of effect of S1P reported in the isolated bronchus from naive mice (Roviezzo et al., 2007).

We did not directly investigate the mechanism of the FTY720-induced airway hyperreactivity in vivo. However, many different cell types that are responsive to S1P could explain the in vivo airway hyperreactivity (Ryan and Spiegel, 2008). We believe that the data of the present study, strongly suggest that this phenomenon is driven by a direct effect on the bronchial smooth muscle that has been shown to express the $S1P_3$ receptor (Roviezzo et al., 2007; Chiba et al., 2010a). In support of this, the response observed in vivo could be abrogated by salbutamol, a recognized bronchodilator. Furthermore, in vivo airway hyperreactivity of similar magnitude was observed for a mechanistically diverse range of broncho spasmogens including agonists that act directly at the levels of smooth muscle (i.e. 5-HT, methacholine), an agent that activate mast cells (i.e. adenosine) or more complex challenges such as ovalbumin. In addition, the in vivo phenomenon could be reproduced in the mouse and rat isolated trachea. All these observations, point toward a crucial role for the

smooth muscle in the airway hyperreactivity induced by activation of the S1P pathway. Although we did not attempt to delineate the intracellular pathway(s) involved in the airway hyperreactivity, we feel that the downstream mechanism of action for S1P induced contraction and/or airway hyperreactivity in various airway smooth muscle preparations, including human (Rosenfeldt et al., 2003) and guinea pig (Kume et al., 2007) has been published numerous time and shown to involve activation of Rho kinase.

In conclusion, evidence is presented in this paper that activation of the S1P pathway, in rats and mice, induces a generalized increase in airway reactivity in vivo and in vitro that is mediated by the $S1P_3$ receptor. It bears emphasis that the long duration of action of the airway hyperreactivity induced by FTY720 and the fact that it does not desensitize are features which are characteristic of human asthma. It follows, therefore, that selective antagonists at the $S1P_3$ receptor could help to elucidate the mechanism of this phenomenon and possibly facilitate the development of new therapeutic strategies aimed at suppressing the airway hyperreactivity observed in conditions such as asthma.

ACKNOWLEDGMENTS

The authors would like to thank Francois Baur and Emilie Renard for their technical expertise and Lazzaro Mazzoni for many helpful discussions.

AUTHORSHIP CONTRIBUTIONS.

Participated in research design: Trifilieff and Fozard.

Performed data analysis: Trifilieff and Fozard.

Wrote the manuscript: Trifilieff and Fozard.

REFERENCES

Alexander SP, Mathie A and Peters JA (2011) Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* **164 Suppl 1**:S1-324.

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S and Panettieri RA, Jr. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* **15**:1212-1214.

Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N and Baumruker T (2003) Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem* **278**:47408-47415.

Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M and Lynch KR (2002) The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* **277**:21453-21457.

Brusasco V, Crimi E and Pellegrino R (1998) Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* **53**:992-998.

Chiba Y, Suzuki K, Uechi M, Kurihara E, Goto K, Sakai H and Misawa M (2010a) Downregulation of sphingosine-1-phosphate receptors in bronchial smooth muscle of mouse experimental asthma. *Pharmacol Res* **62**:357-363.

Chiba Y, Takeuchi H, Sakai H and Misawa M (2010b) SKI-II, an inhibitor of sphingosine kinase, ameliorates antigen-induced bronchial smooth muscle hyperresponsiveness, but not airway inflammation, in mice. *J Pharmacol Sci* **114**:304-310.

Ellis KM, Cannet C, Mazzoni L and Fozard JR (2004) Airway hyperresponsiveness to bradykinin induced by allergen challenge in actively sensitised Brown Norway rats. *Naunyn Schmiedebergs Arch Pharmacol* **369**:166-178.

Haberberger RV, Tabeling C, Runciman S, Gutbier B, Konig P, Andratsch M, Schutte H, Suttorp N, Gibbins I and Witzenrath M (2009) Role of sphingosine kinase 1 in allergen-induced pulmonary vascular remodeling and hyperresponsiveness. *J Allergy Clin Immunol* **124**:933-941.

Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJA, Kingsbury MA, Zhang G, Brown JH and Chun J (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LPB3/EDG-3. *J Biol Chem* **276**:33697-33704.

Jongsma M, Hendriks-Balk MC, MICHEL MC, Peters SL and Alewijnse AE (2006) BML-241 fails to display selective antagonism at the sphingosine-1-phosphate receptor, S1P(3). *Br J Pharmacol* **149**:277-282.

Kume H, Takeda N, Oguma T, Ito S, Kondo M, Ito Y and Shimokata K (2007) Sphingosine 1-Phosphate Causes Airway Hyper-Reactivity by Rho-Mediated Myosin Phosphatase Inactivation. *J Pharmacol Exp Ther* **320**:766-773.

Lai WQ, Goh HH, Bao Z, Wong WSF, Melendez AJ and Leung BP (2008) The Role of Sphingosine Kinase in a Murine Model of Allergic Asthma. *J Immunol* **180**:4323-4329.

Lundgren R, Soderberg M, Horstedt P and Stenling R (1988) Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J* **1**:883-889.

Maddox L and Schwartz DA (2002) The pathophysiology of asthma. *Annu Rev Med* **53**:477-498.

Meno-Tetang GM, Li H, Mis S, Pyszczynski N, Heining P, Lowe P and Jusko WJ (2006) Physiologically based pharmacokinetic modeling of FTY720 (2-amino-2[2-(-4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) in rats after oral and intravenous doses. *Drug Metab Dispos* **34**:1480-1487.

Nishiuma T, Nishimura Y, Okada T, Kuramoto E, Kotani Y, Jahangeer S and Nakamura S (2008) Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol* **294**:L1085-L1093.

Nixon GF (2009) Sphingolipids in inflammation: pathological implications and potential therapeutic targets. *Br J Pharmacol* **158**:982-993.

Pan S, Mi Y, Pally C, Beerli C, Chen A, Guerini D, Hinterding K, Nuesslein-Hildesheim B, Tuntland T, Lefebvre S, Liu Y, Gao W, Chu A, Brinkmann V, Bruns C, Streiff M, Cannet C, Cooke N and Gray N (2006) A monoselective sphingosine-1-phosphate receptor-1 agonist prevents allograft rejection in a stringent rat heart transplantation model. *Chem Biol* **13**:1227-1234.

Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG and Coughlin SR (2007) Promotion of Lymphocyte Egress into Blood and Lymph by Distinct Sources of Sphingosine-1-Phosphate. *Science* **316**:295-298.

Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA, Jr. and Spiegel S (2003) Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J* **17**:1789-1799.

Roviezzo F, D'Agostino B, Brancaleone V, De GL, Bucci M, De DG, Orlotti D, D'Aiuto E, De PR, Rossi F, Sorrentino R and Cirino G (2010) Systemic administration of sphingosine-1-phosphate increases bronchial hyperresponsiveness in the mouse. *Am J Respir Cell Mol Biol* **42**:572-577.

Roviezzo F, Di LA, Bucci M, Brancaleone V, Vellecco V, De NM, Orlotti D, De PR, Rossi F, D'Agostino B and Cirino G (2007) Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. *Am J Respir Cell Mol Biol* **36**:757-762.

Ryan JJ and Spiegel S (2008) The role of sphingosine-1-phosphate and its receptors in asthma. *Drug News Perspect* **21**:89-96.

Schuchardt M, Tolle M, Prufer J and van der GM (2011) Pharmacological relevance and potential of sphingosine 1-phosphate in the vascular system. *Br J Pharmacol* **163**:1140-1162.

Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y and Cyster JG (2005) Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* **309**:1735-1739.

Zhou H and Murthy KS (2004) Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. *Am J Physiol Cell Physiol* **286**:C1130-C1138.

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

Figures Legends

Fig. 1. Representative trace for the effect of intravenous FTY720 (left panels) or AUY954 (right panels) on lung function and cardiovascular parameters in the Brown Norway rat. Anaesthetized rats were given intravenously 10 mg/kg of FTY720 or 10 mg/kg of AUY954 at time 0 and airway resistance and cardiovascular parameter measured for the following 60 minutes. For the animals given FTY720, the rats were injected with 10 μg/kg of intravenous salbutamol 60 minutes after dosing.

Fig. 2. Intravenously administered FTY720 but not AUY954 induces a delayed and sustained increase in airway resistance (R_L) in the Brown Norway rat in vivo. Anaesthetized rats were treated intravenously with vehicle, increasing doses of FTY720 or 10 mg/kg AUY954 and the airway resistance was monitored over time. Results are shown as change from baseline values and expressed as means ± S.E.M. of 3-6 animals per group. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

Fig. 3. Orally administered FTY720 induces airway hyperreactivity to a range of spasmogens in the Brown Norway rat in vivo. Increasing doses of FTY720 or its vehicle were administered orally to conscious rats. Three hours later, animals were anaesthetized and the effect of sequentially intravenously injected 5-HT, methacholine and adenosine on airway resistance (top panel) blood pressure (middle panel) and heart rate (bottom panel) were monitored. In a separate experiment, ovalbumin-sensitized rats were challenge with increasing dose of ovalbumin injected intravenously. Results are shown as change from baseline values and are expressed as

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

means ± S.E.M. of the number of animals indicated in parentheses. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

Fig. 4. Orally administered AUY954 does not induce airway hyperreactivity in the Brown Norway rat. Conscious rats were treated orally with vehicle or 30 mg/kg AUY954. Three hours after airway resistance (R_L) to spasmogens was recorded. Results are shown as change from baseline values and are expressed as means ± S.E.M.. of the number of animals indicated in parentheses.

Fig. 5. Time course for oral FTY720-induced airway hyperreactivity in the Brown Norway rat in vivo. Conscious rats were treated orally with 1 mg/kg of FTY720 and airway resistance (R_L) to spasmogens measured at different time points thereafter. Results are shown as change from baseline values and are expressed as means ± S.E.M. from 6 animals per group. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

Fig. 6. The effect of oral FTY720 is not subject to desensitization following chronic treatment in the Brown Norway rat. Conscious rats were treated orally with 1 mg/kg of FTY720 for 15 days (chronic). As a control, a group of animals was treated with vehicle for 15 days (vehicle) or with vehicle for 14 days followed by oral FTY720 1 mg/kg on day 15 (acute). Airway resistance (R_L) to intravenously injected spasmogens was measured 3 hours after the last treatment. Results are shown as change from baseline values and are expressed as means ± S.E.M. of the number of animals indicated in parentheses. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

Fig. 7. FTY720-P-induced hypersensitivity to 5-HT in the rat and mouse isolated trachea. Vehicle or increasing concentrations of FTY720-P or AUY954 were incubated for 30 minutes before the start of the 5-HT cumulative concentration response. For the rat, the 5-HT concentration-response curves in the presence of FTY720 were expressed as increase over the FTY720 effect. Results are expressed as means ± S.E.M. of the number of segments isolated from different animals indicated in Table 3.

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Baseline values for heart rate, blood pressure and airway resistance, 3 hours after oral administration of AUY954 or FTY720.

	Blood pressure (mm Hg)	Heart rate (beat/min)	Airway resistance ($cmH_2O/l/s$)
Vehicle (6)	136 ± 4	384 ± 16	128 ± 7
AUY954 30 mg/kg (4)	122 ± 8	354 ± 23	126 ± 5
FTY720 0.1 mg/kg (4)	132 ± 6	342 ± 11	132 ± 4
FTY720 1 mg/kg (4)	130 ± 7	298 ± 5*	132 ± 6
FTY720 10 mg/kg (5)	135 ± 5	201 ± 6*	154 ± 23

Data are expressed as mean ± S.E.M. of the number of animals indicated in parentheses. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. PC_{100} values for the various contractile agents following oral administration of FTY720.

	PC_{100}			
	Vehicle	FTY720 (0.1 mg/kg)	FTY720 (1 mg/kg)	FTY720 (10 mg/kg)
5-HT (μg/kg)	10.3 (6.8-26.5)	8.8 (5.2-42.1)	2.2* (1.9-2.6)	0.4* (0.1-1.6)
Methacholine (μg/kg)	27.1 (14.6-35.1)	21.1 (18.5-28.9)	11.5* (8.5-18.6)	5.5* (3.8-7.1)
Ovalbumin (mg/kg)	1.1 (0.5-1.8)	0.12* (0.05-0.31)	Not done	Not done

Data are expressed as mean and 95% confidence interval of the number of animals indicated in Figure 3. * $p < 0.05$ indicates significant difference by comparison with vehicle-treated animals.

JPET Fast Forward. Published on May 8, 2012 as DOI: 10.1124/jpet.112.191585
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. 5-HT pEC_{50} values in the rat and mouse isolated trachea preparations.

	pEC_{50}		
	Rat	Wild type mouse	S1P3 receptor-deficient mouse
Vehicle	5.98 ± 0.13 (9)	6.01 ± 0.02 (4)	5.94 ± 0.03 (4)
FTY720 0.1 μM	6.02 ± 0.21 (3)	6.04 ± 0.05 (4)	5.98 ± 0.07 (4)
FTY720 1 μM	6.01 ± 0.15 (7)	5.91 ± 0.07 (3)	5.71 ± 0.06 (4)
FTY720 10 μM	6.06 ± 0.09 (11)	6.04 ± 0.02 (5)	6.07 ± 0.05 (4)
AUY954 0.1 μM	6.01 ± 0.08 (3)	6.03 ± 0.04 (4)	6.01 ± 0.01 (3)
AUY954 1 μM	6.03 ± 0.12 (4)	6.09 ± 0.06 (3)	5.97 ± 0.07 (3)
AUY954 10 μM	6.03 ± 0.05 (4)	6.15 ± 0.05 (4)	5.86 ± 0.06 (4)

Data are expressed as mean ± S.E.M. of the number of animals indicated in parentheses.

Figure 1

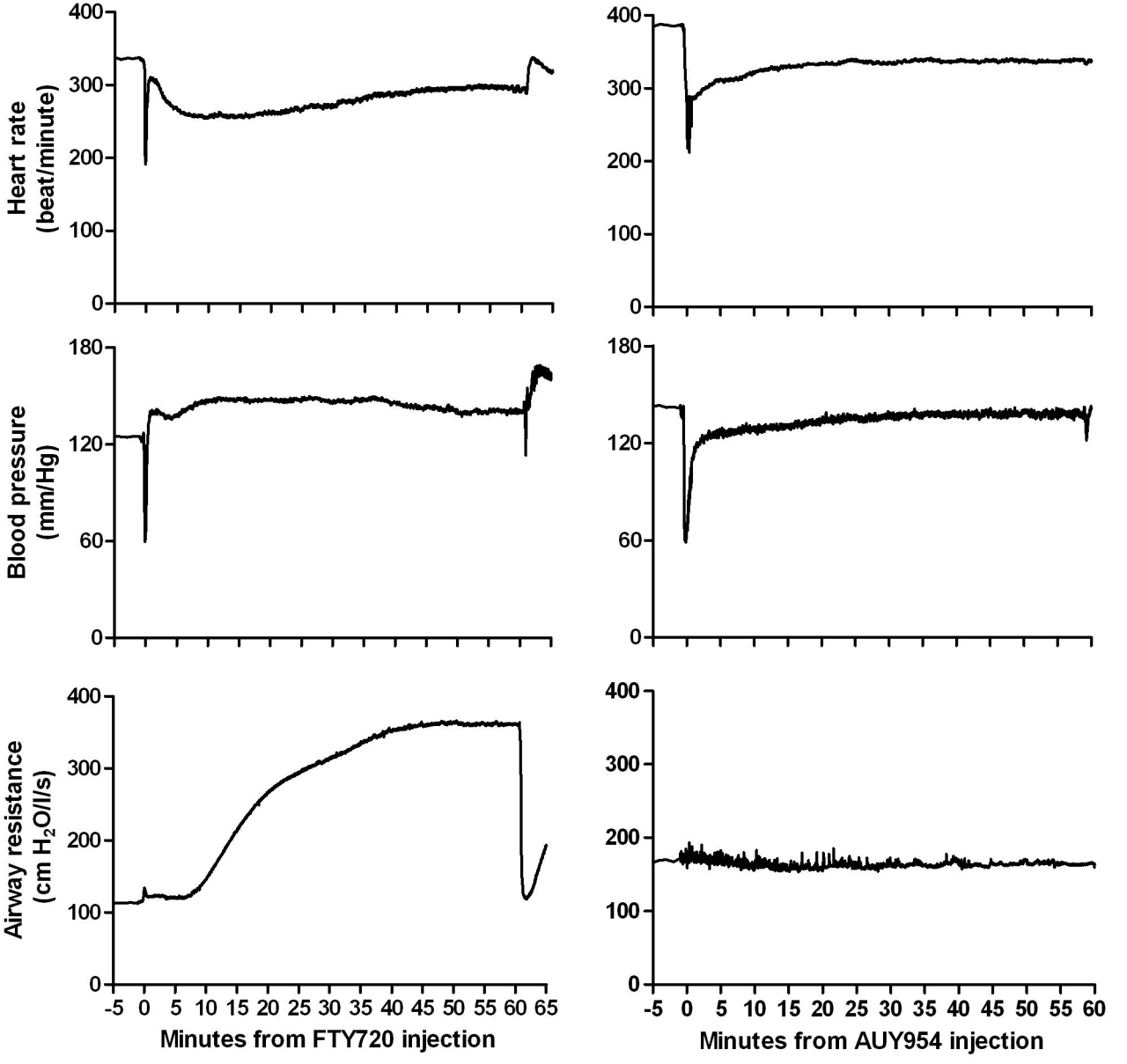

Heart rate (beat/minute)
Blood pressure (mm/Hg)
Airway resistance (cm H2O/l/s)
Minutes from FTY720 injection
Minutes from AUY954 injection

Figure 2

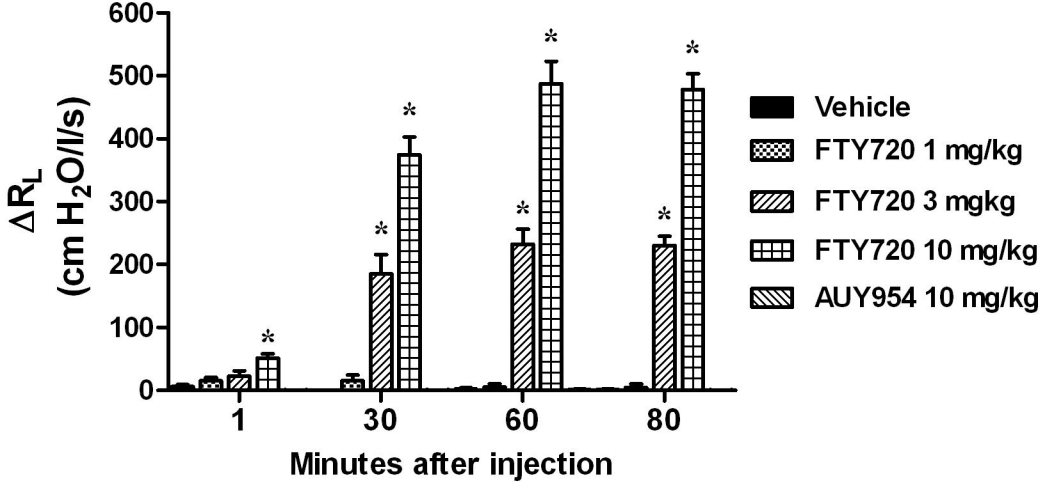

600
500
400
300
200
100
0
ΔRL
(cm H2O/l/s)
*
1
30
60
80
Minutes after injection
Vehicle
FTY720 1 mg/kg
FTY720 3 mgkg
FTY720 10 mg/kg
AUY954 10 mg/kg

Figure 3

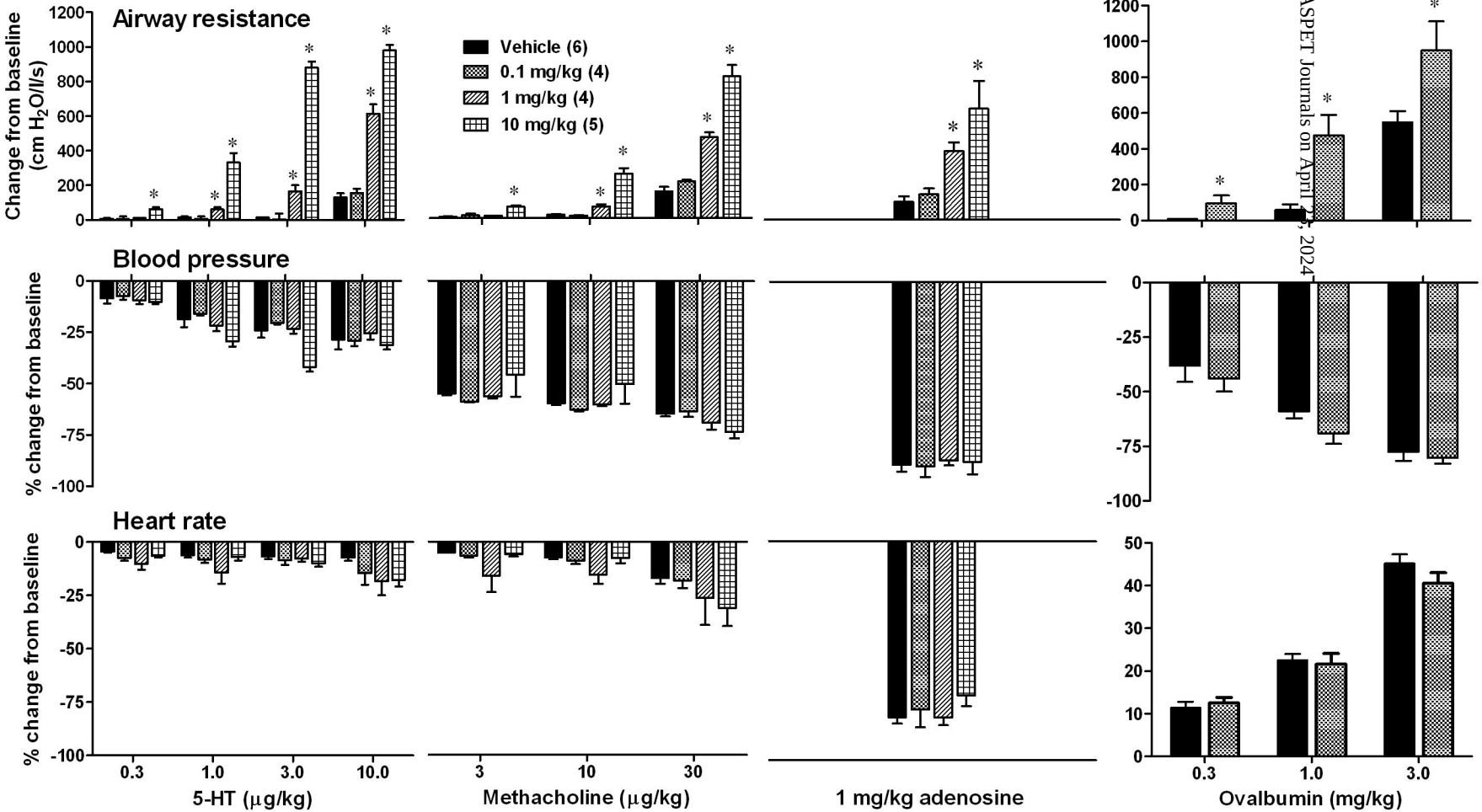


Figure 4

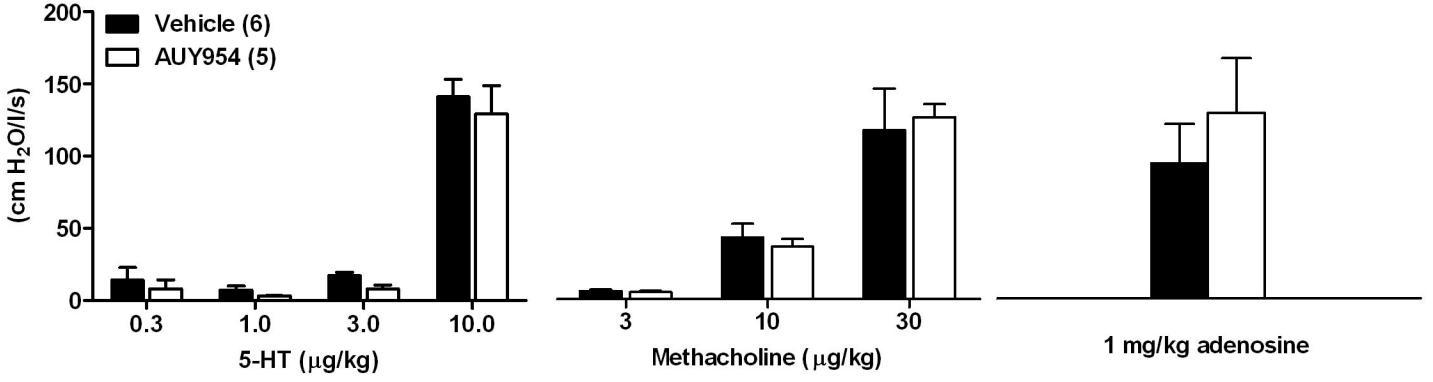

Vehicle (6)
AUY954 (5)
ΔRL
(cm H2O/l/s)
200
150
100
50
0
0.3
1.0
3.0
10.0
5-HT (μg/kg)
3
10
30
Methacholine (μg/kg)
1 mg/kg adenosine

Figure 5

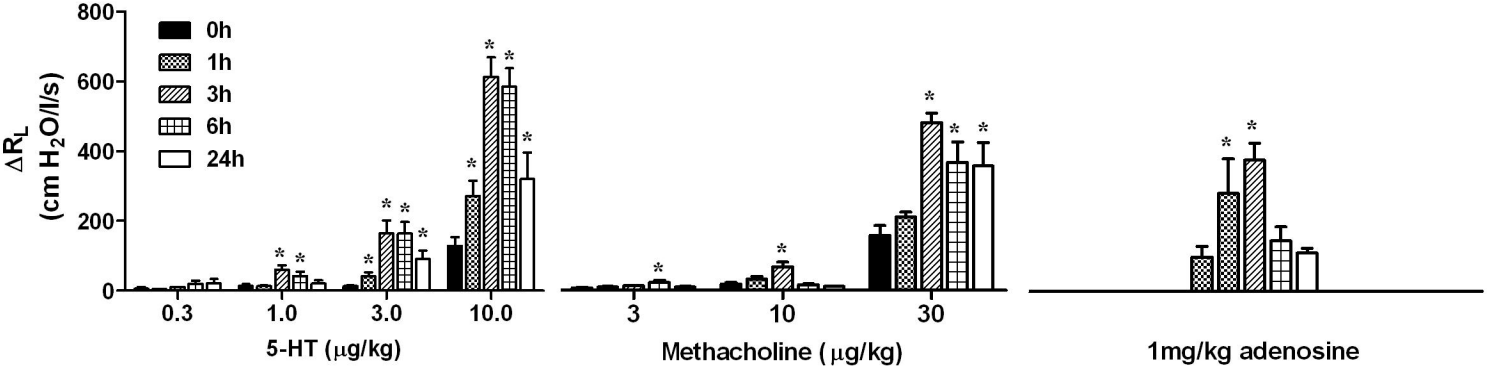

800
600
400
200
0
ΔR_L
(cm H2O/l/s)
0h
1h
3h
6h
24h
0.3
1.0
3.0
10.0
5-HT (μg/kg)
3
10
30
Methacholine (μg/kg)
1mg/kg adenosine

Figure 6

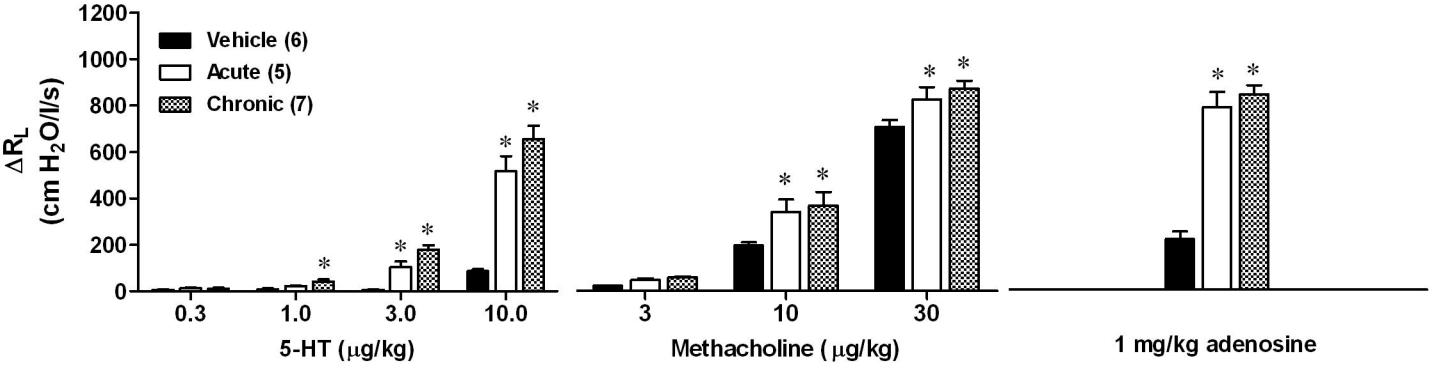
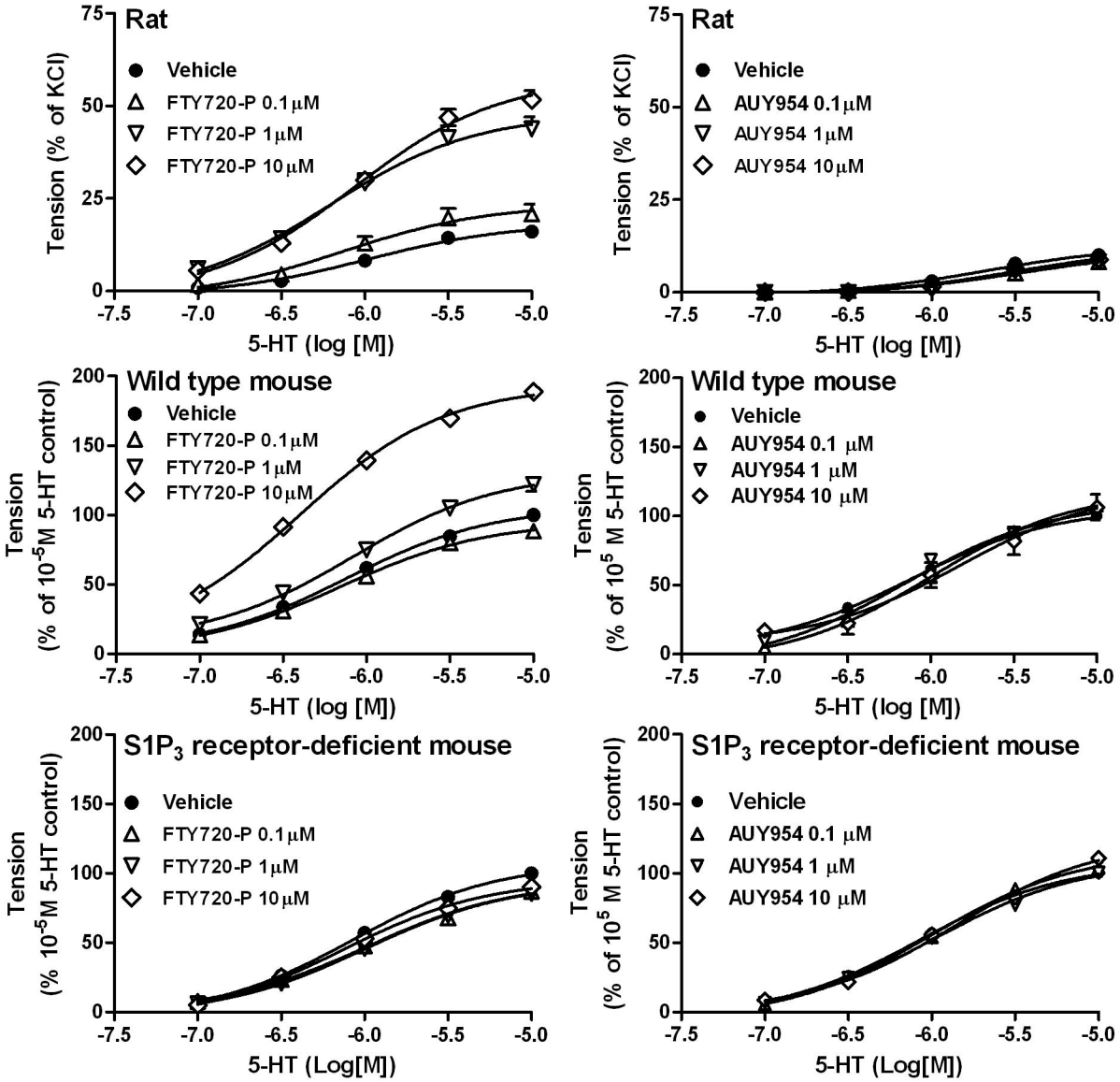

1200
1000
800
600
400
200
0
ΔR_L
(cm H2O/l/s)
Vehicle (6)
Acute (5)
Chronic (7)
*
0.3
1.0
3.0
10.0
5-HT (μg/kg)
3
10
30
Methacholine (μg/kg)
1 mg/kg adenosine

Figure 7


Rat
Vehicle
FTY720-P 0.1μM
FTY720-P 1μM
FTY720-P 10μM
Tension (% of KCl)
5-HT (log [M])
Rat
Vehicle
AUY954 0.1μM
AUY954 1μM
AUY954 10μM
Tension (% of KCl)
5-HT (log [M])
Wild type mouse
Vehicle
FTY720-P 0.1μM
FTY720-P 1μM
FTY720-P 10μM
Tension (% of 10-5M 5-HT control)
5-HT (log [M])
Wild type mouse
Vehicle
AUY954 0.1 μM
AUY954 1 μM
AUY954 10 μM
Tension (% of 10^5 M 5-HT control)
5-HT (log [M])
S1P3 receptor-deficient mouse
Vehicle
FTY720-P 0.1μM
FTY720-P 1μM
FTY720-P 10μM
Tension (% 10-5M 5-HT control)
5-HT (Log[M])
S1P3 receptor-deficient mouse
Vehicle
AUY954 0.1 μM
AUY954 1 μM
AUY954 10 μM
Tension (% of 10^5 M 5-HT control)
5-HT (Log[M])