

ORIGINAL ARTICLE

A prostaglandin D₂ receptor antagonist modifies experimental asthma in sheep

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Summary

Background Prostaglandin (PG) D₂ is the major cyclooxygenase metabolite released by mast cells upon allergen stimulation, and elicits responses through either the prostanoid DP1 receptor and/or the chemoattractant receptor homologous molecule expressed on T-helper type 2 (Th2) cells (CRTH2/DP2). Experimental evidence suggests that stimulation of one or both these receptors contributes to asthma pathophysiology.

Objective The aim of this study was to test the hypothesis that the prostanoid DP1 receptor contributes to asthma pathophysiology by determining the efficacy of an orally active antagonist for this receptor, S-5751, on allergen-induced bronchoconstriction, airway hyperresponsiveness (AHR) and cellular inflammation in the sheep model of asthma.

Methods PGD₂-induced cyclic adenosine monophosphate (cAMP) production in platelet-rich plasma was used to establish the *in vitro* efficacy of S-5751. *In vivo*, sheep naturally allergic to *Ascaris suum* were challenged with an aerosolized antigen with and without S-5751 treatment (given 4 days before and for 6 days after the challenge).

Results S-5751 inhibited PGD₂-induced cAMP production in platelet-rich plasma with an IC₅₀ value of 0.12 µM. S-5751 at 30 mg/kg, but not at 3 mg/kg, reduced the early bronchoconstriction and inhibited the late bronchoconstriction. AHR and inflammatory cell infiltration in bronchoalveolar lavage fluid at days 1 and 7 were also inhibited with the 30 mg/kg dose. The responses observed with S-5751 at 30 mg/kg were comparable with those with montelukast treatment (0.15 mg/kg, twice a day, intravenous); however, S-5751 did not block inhaled leukotrieneD₄-induced bronchoconstriction.

Conclusion Prostanoid DP1 receptor inhibition may represent an alternative target for asthma therapy.

Keywords asthma, PGD₂, prostanoid DP1 receptor, S-5751, sheep

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Introduction

Prostaglandin (PG) D₂ is the major cyclooxygenase metabolite produced by mast cells responding to various allergic stimuli [1]. Allergen challenge in patients with bronchial asthma [2] induces a rapid elevation of PGD₂ in bronchial lavage fluid and PGD₂ itself, causes airway narrowing [3], eosinophil infiltration [4], increased blood flow and other biological effects that contribute to allergic disease. The actions of PGD₂ are mediated through two different receptors: the prostanoid DP1 receptor and chemoattractant receptor homologous molecule expressed on T-helper type 2 (Th2) cells (CRTH2/DP2) [5].

Recent studies suggest that prostanoid DP1 stimulation can have either pro- or anti-inflammatory effects, while CRTH2 stimulation is mostly associated with inflammatory processes [6]. In prostanoid DP1 receptor-deficient mice, ovalbumin (OVA)-induced airway hyperresponsiveness (AHR), accumulation of lymphocytes and eosinophils in airway lavage fluid, and production of Th2 cytokines were reported to be reduced [7] when compared with wild-type littermates. However, these results are in contrast to those of Hammad et al. [8, 9], who showed in mice that the prostanoid DP1 agonist BW245C, when administered at the time of antigen challenge, reduced allergic inflammation and airway responsiveness and that this protective

effect was lost in prostanoid DP1 knock-out mice. These results were generally consistent with earlier studies by Spik et al. [10], who showed in OVA-sensitized and challenged mice that activation of CRTH2 with the agonist DK-PGD₂ given during each antigen challenge caused an increase in tissue and bronchoalveolar lavage (BAL) eosinophilia and in AHR, but that when BW245C was used there was a slight reduction in tissue eosinophilia and AHR was blocked. While the latter two studies support a role for CRTH2-mediated inflammation, the study by Matsuoka et al. [7] suggests that stimulation of prostanoid DP1 is linked to inflammation and AHR. While we cannot fully explain the differences between the study by Matsuoka et al. [7], and the studies of Hammad et al. [8, 9] and Spik et al. [10], our previous studies [11, 12] support the study by Matsuoka et al. [7] because they show that treatment with the selective prostanoid DP1 antagonist, S-5751, does not exacerbate, but improves allergic airway function.

S-5751 is an orally available antagonist of the prostanoid DP1 receptor and functionally antagonizes cyclic adenosine monophosphate (cAMP) production by human platelets with an IC₅₀ value of 0.9 nM [11, 12]. Our previous studies demonstrated that S-5751 significantly inhibits early and late nasal responses and eosinophil infiltration in an allergic rhinitis model, plasma exudation in an allergic conjunctivitis model and eosinophil infiltration into the lung in allergic asthma in guinea-pigs [11, 12]. Thus, our previous findings support the hypothesis that the prostanoid DP1 receptor contributes to the pathophysiological events in allergic diseases, and therefore, that S-5751 is a reasonable molecule to further assess the potential role of the prostanoid DP1 receptor in modulating these events.

The sheep model of experimental asthma is frequently used to determine the potential of early-stage clinical candidates to modify airway functional changes induced by an allergen challenge [13–19]. One advantage of this model is that the animals used are naturally sensitive to the inhaled antigen, *Ascaris sum* [13], which removes concerns about sensitization protocols. Another advantage is that antigen challenge results in reproducible early and late bronchoconstrictor responses, AHR and increased numbers of inflammatory cells in BAL fluids [13]. Furthermore, the antigen-induced airway responses are not influenced by vehicle treatment, inactive drugs or active drugs given at inadequate doses [15–18]. Previous studies in this model have documented significant increases in PGD₂ levels in BAL fluid obtained immediately after allergen challenge, similar to those reported in challenge studies in human subjects [14]. In the context of the present discussion, then, the sheep model would be appropriate for the study of the potential efficacy of a prostanoid DP1 receptor antagonist against antigen-induced airway functional changes. The results of such a

study would also seem timely, because as pointed out by Pettipher [6], there is a paucity of data regarding airway functional changes after an antigen challenge in the presence of DP1 antagonists.

Therefore, in the present study, we determined whether S-5751 could modify antigen-induced responses in the sheep model of experimental asthma and compared the effects of this compound with the responses obtained after treating the sheep with the cysteinyl-leukotriene (cys-LT1) antagonist, montelukast, a drug that was previously shown to be effective in the sheep model before its clinical availability [20]. The results show that S-5751 inhibited PGD₂-induced cAMP production in a concentration-dependent fashion in sheep platelet-rich plasma, and that *in vivo* S-5751 provided protection against antigen-induced responses similar to that seen with montelukast. S-5751, however, showed no activity against inhaled leukotriene (LT)D₄, suggesting that its effects did not result from direct cys-LT1 blockade. These findings suggest that prostanoid DP1 receptor antagonism may be potentially beneficial in the treatment of allergic airway diseases.

Materials and methods

Reagents

S-5751, (Z)-7-[(1R,2R,3S,5S)-2-(5-hydroxybenzo[b]thiophen-3-ylcarbonylamino)-10-norpinan-3-yl]hept-5-enoic acid (purity: 98.3%), was synthesized at Shionogi & Co. Ltd (Osaka, Japan). Montelukast sodium was extracted at Shionogi & Co. Ltd from the commercially available Singulair (Alfresa Pharma Co., Osaka, Japan; purity: 97.7%). PGD₂, 3-isobutyl-1-methylxanthine (IBMX) and triton X-100 were obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

The Mount Sinai Medical Center Animal Research Committee approved all the procedures used in this protocol. This committee is responsible for assuring the humane care and use of experimental animals. All animals (female, weighing between 32 and 46 kg, average 40 kg) used had previously demonstrated both early and late bronchoconstriction and AHR to carbachol aerosol following an inhalation challenge with *A. suum* antigen [13, 21]. As reported previously, these airway responses are reproducible and animals that demonstrate these characteristics do not spontaneously convert to single responders [13, 21].

Selectivity assay

Human prostanoid DP1, TP, IP, FP, EP1, EP2, EP3 or EP4 receptor cDNA inserted into the expression vector was transfected into HEK293 cells or Jurkat T cells. Stable

transfectants were selected in the presence of G418 (0.5 mg/mL; Nacalai Tesque Inc., Kyoto, Japan). To assess the binding of S-5751 to each receptor, [^3H]-PGD₂ (Perkin Elmer Life and Analytical Sciences Inc., Waltham, MA, USA), [^3H](+)-S-145 (TP antagonist, Shionogi & Co. Ltd.), [^3H]-iloprost (GE Healthcare UK Limited, London, UK), [^3H]-PGF_{2 α} (Perkin Elmer Life and Analytical Science) and [^3H]-PGE₂ (Perkin Elmer Life and Analytical Science) were used as ligands. Briefly, the membranes of transfectants were incubated with a tritium-labelled ligand in the presence of S-5751 for 60 min at room temperature, and the incubation was terminated by rapid vacuum filtration using a glass fibre filter (GF/C; Whatman, Maidstone, UK). The membranes were washed several times, and then the radioactivity of tritium retained on the filters was measured using a liquid scintillation counter (PerkinElmer Life Science, Norwalk, CT, USA). To assess the efficacy of S-5751 on CRTH2, a receptor-binding assay and calcium mobilization induced by PGD₂ was used with CRTH2-transfected cells as reported previously [22]. Binding of S-5751 to the LTD₄ receptor was assessed with [^3H]LTD₄ and guinea pig lung in MDS Pharma Services (Minneapolis, MN, USA).

Cyclic adenosine monophosphate assay with platelet-rich plasma

A total volume of 20–30 mL blood was collected from allergic sheep, which had not been exposed to an allergen challenge for at least 2 weeks, in a tube containing 1/9-V of 3.2% buffered sodium citrate (BD Vacutainer Systems, Franklin Lakes, NJ, USA). The samples were centrifuged at 180 g for 10 min at room temperature. The upper plasma layer (platelet-rich plasma) was collected. The platelet-rich plasma prepared was pre-treated with 2.5 mM IBMX and then stimulated with several concentrations of PGD₂ for 2 min for the concentration response study. For the evaluation of antagonistic activity of S-5751, platelet-rich plasma was incubated with S-5751 (0.01–3 μM) for 10 min and then stimulated with 0.3 μM PGD₂ for 2 min at 37 °C. The reaction was stopped with 0.1N HCl and cells were lysed with 1% Triton X-100. The concentration of cAMP was quantified using the cAMP enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ, USA).

Measurement of airway mechanics

These methods have been published extensively [16–19]. Lung resistance (R_L) was measured using the oesophageal balloon technique. Analysis of 5–10 breaths was used for the determination of R_L in cm H₂O/L/S.

Aerosol delivery systems

Aerosols of *A. suum* extract (diluted 20:1 with phosphate-buffered saline; 82,000 PNU/mL) carbachol aero-

sols for determination of airway responsiveness and LTD₄ were generated using a disposable medical nebulizer (Raindrop[®], Puritan Bennett Corp., Carlsbad, CA, USA)-dosimeter-controlled system previously described by us [16–19]. Aerosols were delivered at a tidal volume of 500 mL and a rate of 20 breaths/min.

Concentration response curves to inhaled carbachol

Airway responsiveness was determined from cumulative concentration response curves to inhaled carbachol (10 breaths of increasing concentrations of carbachol solution (0.25, 0.5, 1.0, 2.0 and 4.0% w/v) as described previously [16–19]. The cumulative carbachol concentration [in breath units (BU)] that increased R_L by 400% over the post-PBS value (PC_{400}) was calculated by interpolation from the concentration response curve. One BU was defined as one breath of a 1% w/v carbachol aerosol solution [16–19].

Bronchoalveolar lavage

As previously described by us [16–19], BAL was performed by infusion and aspiration of 3×30 mL aliquots of phosphate-buffered saline pH 7.4 without Ca and Mg (PBS) at 39 °C, using 30 mL syringes. For each time-point, the lavages from three different subsegmental bronchi were pooled. Return volume was measured and the total number of cells was counted in a haemocytometer from a sample of unconcentrated lavage fluid by phase-contrast microscopy. The effluent was centrifuged and the cell pellet was resuspended in PBS at a concentration of 10⁶ cells/mL. The total viable cells were estimated by trypan blue exclusion. The cell suspension was spun in a cytospin and stained with Diff-Quik (Baxter, McGraw Park, IL, USA) for a differential cell count.

Experimental protocol

A schematic of the dosing regimen and experimental protocol is shown in Fig. 1. Ten sheep underwent a screening antigen challenge with *A. suum* and were divided into two groups ($n = 5$, each) (Fig. 1). In assigning the animals to a group, we focused on eliminating any statistical difference in the late response and the AHR, as these are key readouts in assessing molecules that may have anti-inflammatory properties. Table 1 shows that there was no statistically significant difference between the animals assigned to Groups A and B in terms of the antigen-induced functional responses. Group A was used as a vehicle (0.5% methyl cellulose) and then for 30 mg/kg of S-5751 in 0.5% methyl cellulose, p.o. Group B for 3 mg/kg of S-5751 in 0.5% methyl cellulose, p.o. and then for montelukast [0.15 mg/kg in normal saline, twice a day intravenously (i.v.)]. The doses of S-5751 used in this

study were chosen based on our preliminary work, and the dose and route of administration chosen for montelukast were based on previous studies demonstrating efficacy [15, 23]. At least 3 weeks after the screening antigen challenge to designate the groups, baseline concentration response curves to aerosol carbachol and baseline BAL fluids were obtained 1–4 days before the start of the first treatments. The sheep in Group A were treated with vehicle and those in Group B with 3 mg/kg S-5751 (Fig. 1). Treatments were once a day from day 4 through day 6. To ensure drug delivery, the S-5751 was administered via a nasogastric tube. On the challenge day (day 0) baseline values of R_L were obtained and then the sheep received

vehicle or S-5751 orally 2 h before antigen challenge. Measurements of R_L were repeated 1.5 h after treatment and then the sheep were challenged with *A. suum* antigen. Measurements of R_L were obtained immediately after challenge, hourly from 1 to 6 h after challenge and on the half hour from 6.5 to 7.5 h after challenge. Measurements of R_L were obtained 24 h after challenge, followed by determination of PC_{400} , after which the post-challenge BAL was performed. PC_{400} and BAL were also measured 7 days after the challenge. At least 3 weeks after the antigen challenge described above, the same protocol was repeated, except that the sheep in Group A were treated with 30 mg/kg, p.o. of S-5751, whereas those in Group B were treated with i.v. montelukast sodium. On the antigen challenge day, S-5751 was given orally 2 h before and montelukast sodium intravenously 30 min before and 4 h after antigen challenge.

Concentration response curves to inhaled leukotriene D_4

To ensure that S-5751 was not acting as a cys-LT1 antagonist, *in vivo*, we determined whether the 30 mg/kg dosing regimen of S-5751 had any effect on LTD $_4$ -induced bronchoconstriction. Three sheep were challenged with 10 breaths of increasing concentrations (50, 100 and 150 μ g/mL) of LTD $_4$ once without treatment and then after treating the animals with 30 mg/kg S-5751 for 4 days and on the fifth day –2 h before LTD $_4$ challenge (to mimic the dosing for the antigen challenge studies). R_L was measured within 5 min after each concentration of LTD $_4$ to construct a concentration response curve.

Statistical analysis

An analysis of variance (ANOVA) was used to test for the overall effects of the area under the curve for the early (AUC_{0-4h}), the late response ($AUC_{4-7.5h}$) and the post/pre-challenge PC_{400} ratios, among the vehicle control and drug treatment groups. If a significant difference was detected, then Dunnett's test was used for pairwise

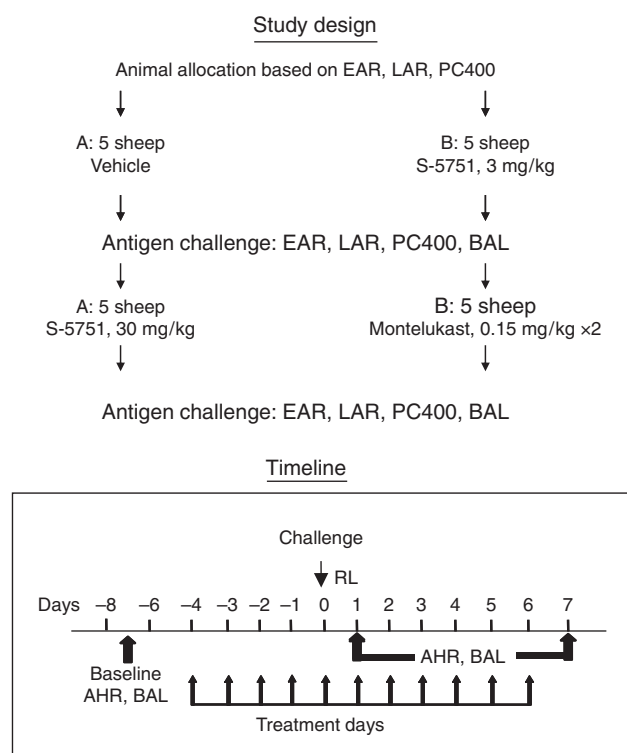


Fig. 1. Schematic of experimental design and protocol.

Table 1. Antigen-induced early (0–4 h) and late (4–7.5 h) bronchoconstriction and airway hyperresponsiveness values used to formulate Groups for the Pharmacological Study

	AUC		Post/pre-challenge PC_{400} ratio	
	R_L (%) 0–4 h (early asthmatic response)	R_L (%) 4–7.5 h (late asthmatic response)	Day 1	Day 7
Screening antigen trial				
Group A mean $\pm$ SE	610 $\pm$ 26%	478 $\pm$ 11%	0.5 $\pm$ 0.0	0.5 $\pm$ 0.0
Group B mean $\pm$ SE	494 $\pm$ 64%	484 $\pm$ 14%	0.5 $\pm$ 0.0	0.6 $\pm$ 0.0
<i>P</i> -value (unpaired Student's <i>T</i> -test)	0.153	0.751	0.694	0.213

Early and late bronchoconstriction are expressed as AUC.

Airway responsiveness is expressed as a ratio of the post/pre-challenge value of PC_{400} . A ratio of 1 indicates that there was no change in the airway responsiveness, whereas a ratio < 1 indicates the development of airway hyperresponsiveness. Values are mean $\pm$ SEM for five sheep.

AUC, area under the curve.

comparisons, which is appropriate as all results were compared with the vehicle (control group) response. ANOVA and Dunnett's test were also used to determine differences in cAMP production with each concentration of PGD₂, with and without S-5751-treatment. Significance was accepted when $P < 0.05$. All values in the text, figures and tables are expressed as mean $\pm$ SEM.

Results

In vitro efficacy of S-5751

The selectivity of S-5751 binding to various prostanoid receptors was estimated with cells transfected with human prostanoid receptors. As shown in Table 2, binding of S-5751 to the prostanoid DP1 receptor (K_i value of 2.4 nM) was more potent than those to receptors for TP (about

130-fold), IP (>2000-fold), FP (>1000-fold), EP1 (>3000-fold), EP2 (70-fold), EP3 (>1000-fold) and EP4 (about 40-fold). Importantly, there was no observed binding or functional antagonism to human CRTH2 (Table 2). Moreover, additional studies showed that S-5751 had an $IC_{50} > 10 \mu\text{M}$ for the binding of [³H]-LTD₄ in guinea-pig lung.

To examine the *in vitro* efficacy of S-5751 on sheep prostanoid DP1 receptors, an assay of functional cAMP production was performed with sheep platelets. PGD₂ induced significant cAMP production in sheep platelet-rich plasma in a concentration-dependent manner (Fig. 2, left). Figure 2 (right) shows that S-5751 inhibited the cAMP production in platelet-rich plasma stimulated with $0.3 \mu\text{M}$ PGD₂ with an IC_{50} value of $0.12 \mu\text{M}$.

Efficacy on airway responses

The effects of S-5751 at 3 and 30 mg/kg, p.o. and montelukast at $0.15 \text{ mg/kg} \times 2$, i.v. on the time course of the antigen-induced bronchoconstriction and AHR are shown in Figs 3 and 4, with statistical comparisons presented in Table 3. We have grouped the data for S-5751 in Fig. 3 for comparative purposes. The responses of animals receiving S-5751 at 3 mg/kg showed no protective effect against the antigen-induced early or late bronchoconstriction (Fig. 3, top; Table 3). The antigen-induced responses of the animals given the 3 mg/kg dose (Group B) were virtually superimposable with the responses of the animals that received vehicle Group A). At the dose of 30 mg/kg, however, S-5751 significantly reduced the early response and blocked the late response (Fig. 3, top; Table 3). Figure 3 (bottom) shows the effects of S-5751 on AHR. At 3 mg/kg, S-5751 had no effect on the AHR 24 h after antigen challenge, but the continued treatment with this dose improved the resolution of the

Table 2. *In vitro* efficacy of S-5751 on prostanoid DP1 receptor and other prostanoid receptors

Target	Species	Preparation	Assay	Value
Receptor binding				K_i (nM)
DP1	Human	Transfectant	Binding	2.4
CRTH2/DP2	Human	Transfectant	Binding	> 5000
TP	Human	Transfectant	Binding	320
IP	Human	Transfectant	Binding	> 6800
FP	Human	Transfectant	Binding	4700
EP1	Human	Transfectant	Binding	9300
EP2	Human	Transfectant	Binding	170
EP3	Human	Transfectant	Binding	2600
EP4	Human	Transfectant	Binding	90
Functional antagonism to CRTH2				IC_{50} (nM)
CRTH2/DP2	Human	Transfectant	Calcium	> 10 000

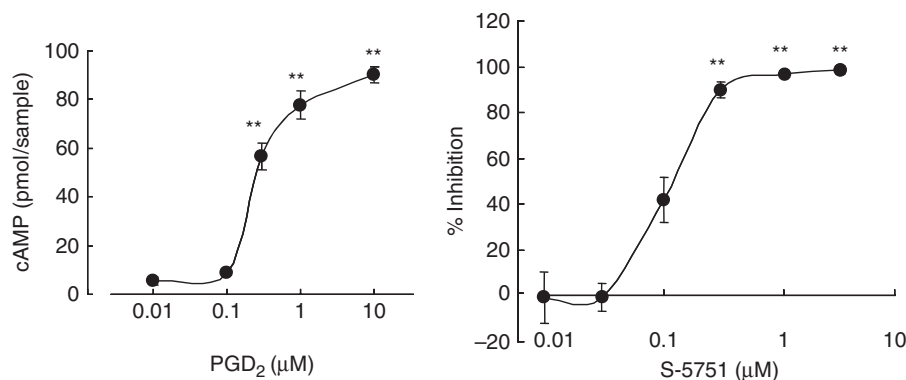


Fig. 2. Concentration response of prostaglandin (PG)D₂ on cyclic adenosine monophosphate (cAMP) production (left) and the effects of S-5751 on PGD₂-induced cAMP production (right) in sheep platelet-rich plasma. Platelet-rich plasma was stimulated by various concentrations (0.01–10 μM) of PGD₂ for 2 min at 37 °C (a). S-5751 (final concentration 0.01–3 μM) was treated for 10 min before stimulation with a suboptimal concentration of PGD₂ (0.3 μM) (b). The levels of cAMP were determined by enzyme immunoassay. Values are mean $\pm$ SEM. of three to four independent experiments. ** $P < 0.01$ vs. vehicle control.

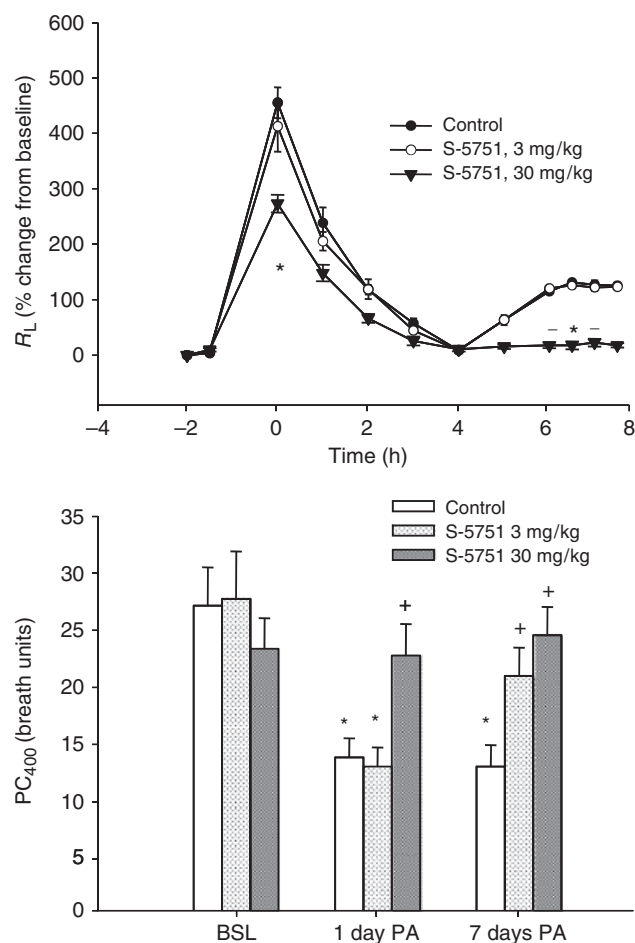


Fig. 3. Effect of 3 and 30 mg/kg of S-5751 on the time-course of antigen-induced changes in lung resistance (R_L) (top), and airway responsiveness to inhaled carbachol expressed as PC_{400} (bottom). Values are mean $\pm$ SEM of five sheep. Top: * P < 0.05 and vs. vehicle control. Bottom: * P < 0.05 vs. baseline; + P < 0.05 vs. vehicle control. Statistical comparisons are presented in Table 2.

AHR as indicated by the return of the PC_{400} to pre-challenge values by day 7 (Fig. 3, bottom; Table 3). S-5751 at 30 mg/kg completely blocked the post-antigen-induced AHR on days 1 and 7 (Fig. 3 bottom; Table 3).

The effects on airway responses in animals treated with montelukast are shown in Fig. 4. In the presence of montelukast, inhibition of the airway responses was similar to the inhibition seen with the 30 mg/kg dose of S-5751 (Table 3) so that no distinction between the responses in the presence of S-5751 and montelukast could be discerned.

Efficacy on cell infiltration in bronchoalveolar lavage fluid

Figure 5 shows the effect of S-5751 and montelukast on antigen-induced cell infiltration recovered in BAL fluid. In the vehicle-treated group, increases in total cells, macrophages and neutrophils were evident 1 day after

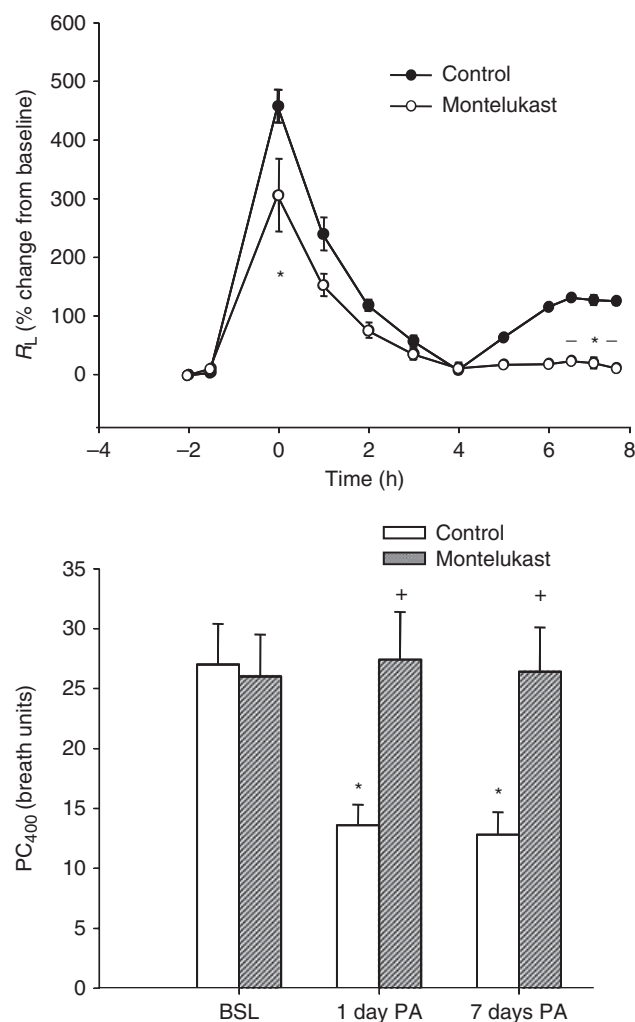


Fig. 4. Effect of montelukast on the time-course of antigen-induced changes in lung resistance (R_L) (top), and airway responsiveness to inhaled carbachol (bottom). Values are mean $\pm$ SEM of five sheep. Top: * P < 0.05 and vs. vehicle control. Bottom: * P < 0.05 vs. baseline; + P < 0.05 vs. vehicle control. Statistical comparisons are presented in Table 2.

challenge and these increases were maintained for 7 days after challenge. Treatment with 3 mg/kg S-5751 did not significantly affect these variables 1 day after challenge but 7 days later, reductions in total cells, macrophages and neutrophils were observed. At 30 mg/kg, S-5751 significantly reduced the total cell influx on day 1 and inhibited total cells, macrophages and neutrophils on day 7. Montelukast treatment resulted in inhibitory effects similar to those seen with the high dose of S-5751. Eosinophils were also evaluated at these times. In the vehicle trial, eosinophils recovered in BAL were $2.6 \pm 1.9 \times 10^3$ cells/mL at baseline. This number increased to $8.2 \pm 3.5 \times 10^3$ cells/mL 1 day after the challenge and $1.0 \pm 0.7 \times 10^3$ cells/mL 7 days after the challenge. These changes in cell numbers did not reach statistical significance and no significant changes were seen in the presence of either S-5751 or montelukast.

Table 3. Effect of S-5751 and montelukast on antigen-induced early (0–4 h) and late (4–7.5 h) bronchoconstriction and airway hyperresponsiveness

	AUC _{0–4 h} (Early)			AUC _{0–4 h} (Late)			AHR		
	R _L (%)	(%) inhibition (vs. vehicle)	P (vs. vehicle)	R _L (%)	(%) inhibition (vs. vehicle)	P (vs. vehicle)	Post/pre-challenge	P (vs. vehicle)	P (vs. vehicle)
Vehicle	652±48	–	–	318±9	–	–	0.50±0.03	–	–
S-5751 3 mg/kg	584±51	10	NS	314±14	1	NS	0.48±0.02	NS	<0.05
S-5751 30 mg/kg	385±33	41	<0.05	61±12	81	<0.05	0.96±0.02	<0.05	<0.05
Montelukast	423±62	35	<0.05	65±12	80	<0.05	1.00±0.05	<0.05	<0.05

Early and late bronchoconstriction are expressed as AUC.

Airway responsiveness is expressed as a ratio of the post/pre-challenge value of PC₄₀₀. A ratio of 1 indicates that there was no change in the airway responsiveness, whereas a ratio below 1 indicates the development of airway hyperresponsiveness. Values are mean±SEM, for five sheep.

AUC, area under the curve.

Efficacy on leukotrieneD₄-induced bronchoconstriction

Because the responses to S-5751 (30 mg/kg) and montelukast were similar *in vivo*, we tested the possibility that S-5751 could act as a cys-LT1 antagonist. Figure 6 shows that the response to inhaled LTD₄ with S-5751 treatment was not different from the response seen when the animals were challenged with LTD₄ alone.

Discussion

Our results demonstrate the functional efficacy of the prostanoid DP1 receptor antagonist, S-5751, in alleviating allergen-induced airway responses in the sheep model of asthma. Our findings are consistent with previous work in prostanoid DP1 receptor knockout mice where Matsuoka et al. [7] demonstrated that OVA-challenged mice lacking the prostanoid DP1 receptor showed reduced AHR and inflammatory cell infiltration, when compared with wild-type animals. Collectively, these studies support the concept that prostanoid DP1 receptor antagonism can modulate pathophysiological events associated with allergen challenge.

Our current findings, as well as our previous results [11, 12] and those of Matsuoka et al. [7] are, however, in contrast to studies reported by Hammad et al. [8, 9] and Spik et al. [10], which suggest that PGD₂ stimulation of the prostanoid DP1 receptor can abrogate allergic inflammatory responses. The reasons for the differences among the aforementioned studies are unclear but could include technical aspects, species differences, or, as reported for the vasculature, where the response to PGD₂ (pulmonary vasoconstriction or vasodilation) may depend on its concentration and/or maturation of the vascular system [24]. Such explanations in the context of the current findings are beyond the scope of the data presented and are speculative. What can be concluded, however, is that in the present study, S-5751, which, based on the data in Table 2, does not bind to CRTH2, inhibited allergen-induced airway responses.

Before initiating the *in vivo* studies, we first confirmed *in vitro* that S-5751 blocked prostanoid DP1 receptor function in the sheep. S-5751 blocked PGD₂-stimulated cAMP production in sheep platelet-rich plasma with an IC₅₀ value of 0.12 µM, indicating that S-5751 is active against the sheep prostanoid DP1 receptor. While these data do not allow us to claim with absolute certainty that the *in vivo* effects of S-5751 are directed specifically against the sheep prostanoid DP1 receptor, these data, combined with data from our previous publications regarding the pharmacology of S-5751 [11, 12], the highly selective binding data presented in Table 2, the pharmacology of montelukast [15] and the present results indicating that S-5751 neither directly binds to CRTH2 receptors, cys-LT1 receptors, nor inhibits LTD₄-induced

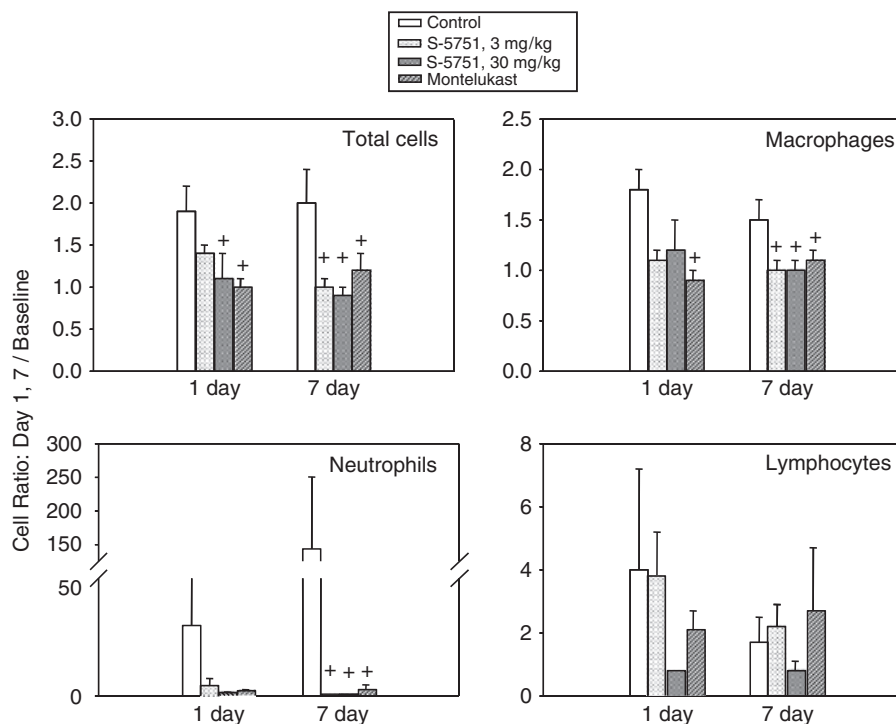


Fig. 5. Effect of S-5751 and montelukast on antigen-induced infiltration of inflammatory cells in sheep. Data are expressed as a ratio of post-challenge values on day 1s and 7 (after antigen challenge)/baseline. Values for total cells, macrophages, neutrophils and lymphocytes are shown. Values are mean $\pm$ SEM of five sheep. + P < 0.05 vs. vehicle.

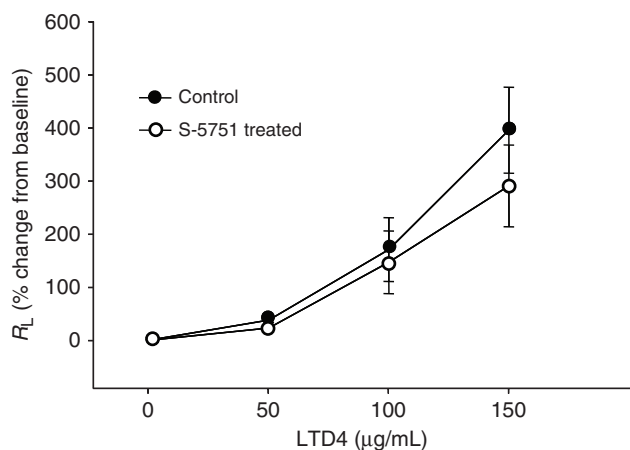


Fig. 6. Concentration-response curves to inhaled leukotriene (LT)₄ with and without treatment with S-5751. Animals were treated with 30 mg/mL S-5751 for 4 days and then on the fifth day 2 h before LTD₄ challenge. S-5751 had no effect on LTD₄-induced bronchoconstriction. Values are $\pm$ SEM for three sheep.

bronchoconstriction, make a compelling argument that the actions of S-5751 demonstrated in this study are based on its antagonism of the sheep prostanoid DP1 receptor.

S-5751 *in vivo* showed a concentration-dependent response with no inhibition at 3 mg/kg but significant inhibitory effects on airway responses at 30 mg/kg. These findings are in accordance with the plasma concentrations

of S-5751 at the two study doses. At the time of antigen challenge, plasma levels for the 3 and 30 mg/kg trials were approximately fivefold less (0.02 μ M) and threefold higher (0.36 μ M), respectively, than the IC₅₀ value needed for inhibition of PGD₂-induced cAMP production in platelet-rich plasma: 0.12 μ M, Fig. 2). These data suggest that plasma concentrations $\geq$ the *in vitro* IC₅₀ are necessary to achieve significant inhibition of early and late bronchoconstriction and 24-h AHR in sheep. Interestingly, 3 mg/kg of S-5751 improved AHR and cell infiltration into BAL fluids at 7 days post-challenge. These data suggest, indirectly, that there may be a low-level stimulation of prostanoid DP1 receptors for at least 7 days after antigen challenge, which can be blocked by consecutive day treatments with low doses of S-5751 over this time period.

Based on the data, it appears that the overall contribution of the prostanoid DP1 receptor is more important for the late bronchoconstriction, AHR and cellular responses, than for the early bronchoconstriction. The partial inhibition of the early response by S-5751 is consistent with a previous report in the sheep model using another prostanoid DP1 receptor antagonist [25]. These findings are consistent with the fact that PGD₂ itself can elicit bronchoconstriction and is released immediately after allergen provocation. However, the fact that S-5751 was more effective in blocking the inflammatory indices associated

with allergen challenge, i.e. late bronchoconstriction, AHR and the cellular response in BAL, suggests that prostanoid DP1 receptor stimulation has more of an inflammatory role in asthma pathogenesis. That prostanoid DP1 receptor blockade by S-5751 primarily targets later inflammatory pathways in allergic responses is consistent with the aforementioned findings in prostanoid DP1 receptor knockout mice subjected to an OVA challenge, where the knockout animals showed a marked reduction in airway hyperresponsiveness, cytokine production and inflammation compared with wild-type litter mates [7].

In the present study, we collected BAL fluid at 1 day and 7 days after challenge and observed clear increases in the number of total cells (Fig. 5). As reported previously for this model [15, 18], neutrophils and macrophages were the cell types predominantly responsible for the increase in the total cells. This is different from mice and guinea-pigs where eosinophil numbers change dramatically after allergen challenge. In sheep, eosinophils are recruited to the airway but their numbers are small relative to the numbers of neutrophils and macrophages, and unlike airway function, changes in eosinophil numbers are sometimes difficult to show with the sample sizes used in this study [13, 21]. While the lack of change in eosinophils may be somewhat disconcerting in terms of previously published rodent data, it must be remembered that, in human trials, decreases in eosinophil numbers do not necessarily correlate with protection of airway function [26–28]. Thus, a reduction in and/or suppression of macrophage and/or neutrophil numbers following allergen challenge in the presence of an active compound in terms of modulating airway effects following antigen challenge not only supports an overall anti-inflammatory effect, but would conceivably be beneficial in reducing airway symptoms as these cells in general produce arachidonic acid metabolites, histamine-releasing factor, oxygen radicals and inflammatory cytokines [29, 30], all of which have been shown to contribute to asthma pathophysiology.

Consistent with the putative anti-inflammatory action of S-5751 was the reduction in total cells seen with 30 mg/kg S-5751, 1 day after antigen challenge. By day 7, both the 3 mg/kg dose as well as the 30 mg/kg dose showed significant inhibitory effects. That the 3 mg/kg dose affected the cellular response at 7 days may partially explain why this low dose was also effective in normalizing airway responsiveness at this time (see "Discussion" above). The present data demonstrating the anti-inflammatory actions of S-5751 are consistent with previous findings in allergen-challenged rodent models, treated with S-5751 [11, 12, 31], and in prostanoid DP1 receptor knockout mice [7].

The findings with S-5751 were similar to those obtained with the cys-LT1 antagonist montelukast. As shown pre-

viously in this model, intravenously administered montelukast is effective in reducing the early and late bronchoconstriction [20] and other allergen-induced responses [23]. As montelukast served as a positive control in this study, we administered the drug intravenously, rather than orally, to avoid introducing another potential variable into an already complex study. The data obtained here extend our original findings by showing that montelukast also blocks AHR and BAL cell infiltration. These findings, although new in this experimental setting, are consistent with clinical studies showing similar results [32]. The binding data and our previous work with montelukast suggest that S-5751 and montelukast produce similar pharmacological responses to allergen challenge in the sheep although these two molecules target different receptors. However, to confirm this argument, we tested the ability of the 30 mg/kg treatment protocol with S-5751 to block LTD₄-induced bronchoconstriction. Our results showed that S-5751 treatment did not block LTD₄-induced bronchoconstriction in sheep (Fig. 6), ruling out the possibility that the effects on antigen-induced responses were related to direct LTD₄ antagonism, which we showed was the mechanism for montelukast's effects in the sheep [20, 23]. Collectively, these data support the fact that protective effects of S-5751 are mediated through blockade of the prostanoid DP 1 receptor and not the cys-LT 1 receptor.

The inhibitory effects of high-dose S-5751 and montelukast on late bronchoconstriction, AHR and cell infiltration were close to the maximal response observed in this experimental model. Therefore, we could not differentiate between the two compounds in terms of efficacy in this study. Based on previous work by Shahbazian et al. [33], who showed that LTD₄ does not stimulate the DP1 receptor, and the results from the current study, it is unlikely that there is cross-reactivity between the two receptors. Based on our findings, one possible explanation is that stimulation of either the cys-LT1 receptor or the prostanoid DP1 receptor results in the activation of separate molecular messaging systems that result in similar downstream responses. Alternatively, it may also be possible that PGD₂ stimulation of prostanoid DP1 receptors occurs upstream of cys-LT1 release and receptor stimulation, and therefore, antagonism of prostanoid DP1 receptor stimulation could affect cys-LT1 release and actions. Our experimental design does not allow us to predict which, if any, of these interpretations are correct. It is interesting, however, to note that preliminary studies in rats and guinea-pigs demonstrated that S-5751 was superior to montelukast against OVA - induced responses, whereas in sheep, [14] we found increased PGD₂ levels in BAL fluid before the appearance of the cys-LTs.

In summary, our data show that sub-chronic treatment with the prostanoid DP1 receptor antagonist, S-5751, blocked antigen-induced airway responses in the sheep

model of asthma. Based on binding data, these protective effects appear to be DP1 receptor dependent. The protective effects seen with S-5751 were comparable to the inhibitory responses seen with montelukast. These findings suggest that prostanoid DP1 receptor antagonists could provide an alternative therapeutic choice from cystLT1 antagonists, or that combination therapy with cystLT1/prostanoid DP1 receptor antagonists might provide added benefits in alleviating the underlying inflammation associated with allergic airway disease.

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