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European Journal of Pharmacology 511 (2005) 219–227

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Evaluating the prophylactic potential of the phtalimide derivative LASSBio 552 on allergen-evoked inflammation in rats

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Received 27 January 2005; received in revised form 8 February 2005; accepted 10 February 2005
Available online 11 March 2005

Abstract

A previous study showed that the novel tetrazolephtalimide derivative LASSBio 552 (2-4-[3-(1*H*-1,2,3,4-tetraazol-5-yl)propoxy]phenethyl-1,3-isoindolinedione) prevents LTD_4-evoked tracheal contraction. This led us to examine the putative anti-inflammatory effect of LASSBio 552 in comparison with the leukotriene $CysLT_1$ receptor antagonist zafirlukast using a model of allergic pleurisy in rats. Treatment with either LASSBio 552 (24–96 μmol/kg, i.p.) or zafirlukast (9–72 μmol/kg, i.p.), 1 h before challenge, inhibited eosinophil and mononuclear cell influx into the pleural cavity 24 h post-challenge, but failed to alter the increased levels of eotaxin, plasma leakage, mast cell degranulation and neutrophil infiltration noted 6 h post-challenge. $CD4^+$ T cell recruitment 24 h post-challenge was also sensitive to LASSBio 552. This treatment failed to alter cysteinyl leukotriene production at 6 h, but clearly inhibited the phenomenon 24 h and 48 h post-challenge. In in vitro settings LASSBio 552 inhibited allergen-evoked cysteinyl leukotriene generation from isolated mast cells, while histamine release remained unchanged. It also slightly inhibited cysteinyl leukotriene production by eosinophils and mononuclear cells triggered by Ca^{+2} ionophore A23187. A leukotriene $CysLT_1$ receptor transfected cell-based assay revealed that LASSBio 552 did not prevent LTD_4-evoked Ca^{+2} influx, indicating that it was not a leukotriene $CysLT_1$ receptor antagonist. These findings indicate that LASSBio 552 is able to inhibit eosinophil influx triggered by allergen chalenge in a mechanism at least partially associated with suppression of $CD4^+$ T cell influx and cysteinyl leukotriene production.

Keywords: LASSBio 552; Tetrazolephtalimide derivative; Leukotriene; Eosinophil; Allergy

1. Introduction

Asthma is a complex syndrome with many clinical phenotypes in both adults and children. A hallmark feature of this disease is the presence and activation of inflammatory cells in the airways notably mast cells, eosinophils, and CD4+ helper T lymphocytes, and the development of bronchial hyper-responsiveness to non-specific stimuli (Adamko et al., 2003; Busse and Lemanske, 2001; Wardlaw et al., 2000).

Accumulated evidence suggests that the development of bronchial hyper-responsiveness is closely related to eosinophilic inflammation of the bronchial mucosa. Eosinophils release toxic granules proteins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein

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doi:10.1016/j.ejphar.2005.02.011

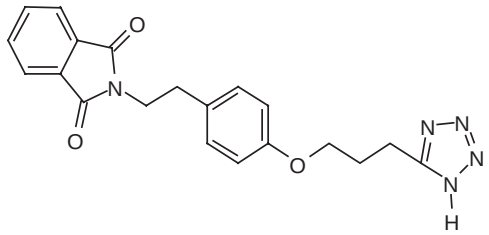

Fig. 1. Chemical structure of LASSBio 552.

and eosinophil-derived neurotoxin, all of them easily detected at the damaged respiratory epithelium of asthmatic patients (Humbles et al., 2004; Lee et al., 2004). These findings strongly suggest that bronchial hyperresponsiveness stems from the eosinophilic inflammation of the bronchial mucosa. The recruitment of eosinophils into the airways after allergen challenge is a complex process, involving several cytokines, lipid mediators and chemokines, which are produced by several types of structural and inflammatory cells (Busse and Lemanske, 2001; Pease et al., 2004).

Cysteinyl leukotrienes generated mainly by mast cells (Hart, 2001) and eosinophils (Bandeira-Melo and Weller, 2003; Lee et al., 2000) have been implicated in several pivotal aspects of asthma pathology including eosinophil enrichment, oedema, airways remodeling and mucus secretion (Eum et al., 2003; Fregonese et al., 2002; Holgate et al., 2003; Ihaku et al., 1999; Spada et al., 1994; Underwood et al., 1996; Vargaftig and Singer, 2003). In fact, the advent of drugs able to interfere with the production and/or action of cysteinyl leukotrienes, including lipoxygenase inhibitors and leukotriene $CysLT_1$ receptor antagonists, has proved beneficial to asthma management (Busse and Lemanske, 2001; Holgate et al., 2003).

From a recent study to identify new anti-asthmatic leading candidates, the compound 2-4-[3-(1*H*-1,2,3,4-tetraazol-5-yl)propoxy]phenethyl-1,3-isoindolinedione (LASSBio 552; Fig. 1) has emerged a novel tetrazolephthalimide derivative reported to inhibit leukotriene (LT) D_4-induced contraction of guinea pig tracheal strips (Lima et al., 2002). The present study further examined the putative prophylactic anti-allergic properties of LASSBio 552 using a model of allergen-evoked pleural inflammatory response in actively sensitized rats.

2. Material and methods

2.1. Animals

Wistar rats of both sexes and weighing 150 to 200 g were obtained from the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) breeding unit. The animals were housed in groups of 5 and maintained in a 12-h light/dark cycle with water and food ad libitum until use. The Ethics Committee for Care and Use of Animals of the Oswaldo Cruz Foundation approved the experimental protocols employed in this study (License no. 0085-02).

2.2. Ovalbumin-evoked pleurisy in sensitized rats and treatments

Active sensitization was achieved by a subcutaneous (s.c.) injection (0.2 ml) of a mixture containing ovalbumin (50 μg) and aluminum hydroxide (5 mg) in 0.9% NaCl solution (saline). Ovalbumin dissolved in sterile saline was administered intrapleurally (i.pl.; 12 μg/cavity) 14 days post-sensitization using a 27.5-gauge needle adjusted to 3 mm in length, in a final volume of 100 μl. All solutions were always made immediately before use. At distinct post-challenge time points, the rats were killed under CO_2 atmosphere and the pleural cavity was rinsed with 3 ml of heparinized phosphate buffer saline (PBS—10 IU/ml). The pleural effluent was collected at the indicated time points, the volume measured in a graduated syringe and then used for cellular and mediator analyses. Non-sensitized rats in which ovalbumin was injected i.pl. were used as negative controls.

LASSBio 552 (24–96 μmol/kg), zafirlukast (9–72 μmol/kg) and vehicle were administered intraperitoneally (i.p.) 1 h before antigen challenge. The drugs were dissolved in Tween-80 (5%) and diluted to the desired dose with saline.

2.3. Measurement of pleural leukocytes

Total leucocyte count was performed in Neubauer chambers by means of light microscope after dilution of the pleural fluid with Türk (acetic acid 0.2%) solution. Differential analysis was performed under an oil immersion objective on cytocentrifuged smears stained with May–Grünwald–Giemsa dye. For the mast cell enumeration, pleural effluent samples collected 6 h post-antigen challenge were diluted with toluidine blue dye solution and evaluated in a Neubauer chamber using light microscope.

For analysis of lymphocyte population, the pleural cavity was opened and washed with RPMI 1640 culture medium containing heparin (20 IU/ml) 24 h post-challenge. Effluent samples were centrifuged ($770\times g$ for 10 min at 4 °C) and the pellet resuspended in PBS (pH 7.2) containing 3% Fetal Bovine Serum. The pleural leucocytes (10^6 leucocytes/well) were incubated with rat serum (10%) for 30 min on ice in order to blockade the unspecific ranches. Next cells were incubated with appropriate dilutions of rat monoclonal antibodies fluorescein isothiocyanate (FITC)-labeled (antibody anti-CD4 or anti-CD8), and phycoerythrin(PE)-labeled (antibody anti-CD3) for 30 min at 4 °C. Cells were washed and resuspended in PBS (pH 7.2), 3% Fetal Bovine Serum, and propidium iodide (2 μg/ml). Flow-cytometry acquisions were performed in a FACScalibur (Becton-Dickinson, Mount View, CA, USA) and data were analysed using WinMDI software (free online—http://www.facs.scripps.edu/software.html).

2.4. Measurement of total protein

The fluid recovered from the pleural cavity was centrifuged for 10 min at 1500×*g* and its total protein content quantified in the supernatant, at 640 nm, using the Folin–Lowry technique (Stauffer, 1975).

2.5. Quantification of cysteinyl leukotrienes and eotaxin levels in the pleural effluent

For cysteinyl leukotriene analyses, the pleural exudates were collected as reported above at 6 h to 72 h post-ovalbumin challenge. The measurements were determined by using a cysteinyl leukotriene EIA Kit (Cayman Chemical) according to instructions supplied by the manufacturer.

Eotaxin in rat pleural lavage fluid was measured by a murine eotaxin enzyme-linked immunosorbent assay (ELISA) as already reported (Bandeira-Melo et al., 2000). Rat eotaxin (97.3% sequence identity with murine eotaxin; Williams et al., 1998) cross reacts in this assay but there is no detectable cross-reaction with any other chemokine tested including human and guinea-pig eotaxin.

2.6. Ca^{+2} mobilization assay in transfected Chinese Hamster Ovary (CHO) cells

CHO cells stably transfected with mouse long isoform of leukotriene $CysLT_1$ receptor (Maekawa et al., 2001; 1×10^7 ml) were loaded with 3 μM Fura-2 AM in Ca^{+2}/Mg^{+2}-free PBS with 0.05% Bovine Serum Albumine (BSA) for 30 min at 37 °C. After two washes, the cells at 0.75×10^6 per sample were dispensed into quartz cuvettes with constant stirring at 37 °C and incubated with 1 mM $CaCl_2$ for 15 min before addition of agonist. Changes in fluorescence were measured in a Shimadzu RF1501 spectrofluorophotometer (Kyoto, Japan). Calculation of cytosolic-free Ca^{2+} was derived from fluorescence spectra (excitation at 340 nm and 380 nm; emission at 510 nm) in accordance with established methodology (Grynkiewicz et al., 1985). During the experiments, LTD_4 (1 nM) was added 60 s after commencing recording. LASSBIO 552 (1–100 μM), zafirlukast (0.01–100 nM) or dimethyl sulfoxide (0.1% DMSO), used as vehicle, were incubated for 4 min with the transfected cells before addition of the agonist.

2.7. Eosinophil chemotaxis

Eosinophils were isolated from the peritoneal cavity of sensitized rats, 72 h post-challenge, using Percoll density gradient as previously reported (Silva et al., 2001). Eosinophil suspensions of 85–95% purity and 96% viability, attested by trypan blue dye exclusion test, were used throughout. Migration experiments were performed in a 48-well microchemotaxis chamber (Neuro Probe, USA) and Toyo cellulose nitrate filters (3 μm pore) as previously described (Richards and McCullough, 1984). To test the effect of LASSBio 552, the cells were pre-incubated with this compound or its vehicle (0.1% DMSO) at 37 °C for 30 min. After pre-treatment, 50 μl of the eosinophil suspension (2×10^5 cells) was added to the upper compartment of the chamber, whereas the lower compartment was loaded with either 0.1 or 1 μM of platelet-activating factor (PAF)—and its vehicle (RPMI 1640 culture medium, pH 7.2, 0.1% BSA). The chemotaxis chamber was then incubated for 2 h at 37 °C in a 5% CO_2:95% O_2 atmosphere. The filter was fixed and stained as described (Richards and McCullough, 1984). Eosinophils, migrated 40 μm from the upper surface of the filter, were counted in 15 consecutive high-power fields under an immersion objective (Martins et al., 1989).

2.8. Intracellular Ca^{2+} measurement in eosinophils

Eosinophils were isolated from the peritoneal cavity of sensitized rats as described above. Thereafter the cells were loaded with 6 μM fura 2-AM for 30 min at 37 °C in RPMI 1640 culture medium. The eosinophils were then washed and treated with LASSBio 552 or its vehicle (0.1% DMSO) at 37 °C for 15 min before stimulation. Intracellular Ca^{2+}concentrations in groups of 20–30 cells were monitored with a fluorescence photometer (Photon Technology, Princeton, NJ). Each group of eosinophils was stimulated only once with 30 nM recombinant human eotaxin. The samples were excited alternatively at 340 and 380 nm, and the emission at 510 nm was measured. The ratio measurement, which is proportional to the logarithm of the intracellular Ca^{2+} concentration, was determined every 100 ms (Coutinho-Silva et al., 2001).

2.9. Anaphylactic histamine and cysteinyl leukotriene release from mast cells in vitro

Mast cells were recovered from the pleural and peritoneal cavity of normal Wistar rats and purified by means of continuous Percoll gradient according to Barreto et al. (2003). After peritoneal washing with heparinized (10 IU/ml) Ca^{+2} and Mg^{+2}-free Hanks balanced salt solution (HBSS), the cells were centrifuged at 150×*g* for 10 min, the supernatant was discarded and the pellet resuspended in HBSS containing 0.1% BSA. The cell suspension was mixed with Percoll (72%) and overlaid with 1 ml of HBSS to be further centrifuged at 150×*g* for 25 min. Pelleted mast cells were washed twice with HBSS and purity was over 96% as attested by toluidine blue staining. Viability tested by means of trypan blue exclusion was higher than 95%.

The isolated mast cells were cultured in 24-well plates (2×10^5/well) containing RPMI 1640 culture medium, 5% fetal bovine serum, penicillin (200 IU/ml), streptomycin (200 μg/ml) and murine anti-dinitrophenylated (DNP)

immunoglobulin (Ig) E monoclonal antibody (0.5 μg/ml) at 37 °C for 12 h. Following sensitization, the cells were pre-incubated with LASSBio 552 (0.1–100 μM), zileuton (10 μM) or their vehicle (0.1% DMSO) at 37° for 30 min. Next the mast cells were incubated with antigen (DNP-BSA, 0.5 μg/ml) for 1 h at 37 °C in 5% CO_2:95% O_2 atmosphere. After centrifugation at 150×*g*, the supernatant was collected and stored at −20 °C for cysteinyl leukotriene and histamine quantification.

Histamine quantification was performed according to Shore et al. (1959). Briefly, this method consists of sample dilution with 0.1 *N* HCl followed by 0.8 *N* NaOH, and further addition of substrate *o*-phthaldialdehyde. After 4 min of incubation, the reaction was stopped with 3 *N* HCl and fluorescence measured in a Shimadzu RF1501 spectrofluorophotometer (Japan; excitation at 360 nm; emission at 450 nm). The results were expressed as the percentage of total histamine released, which was measured after exposure of non-stimulated mast cells to 0.4 N perchloric acid.

2.10. Cysteinyl leukotriene release from rat peritoneal eosinophils and mononuclear cells in vitro

Eosinophils and mononuclear cells were isolated from the peritoneal cavity of sensitized rats as previously described. The isolated cells were cultured in 24-well plates (5×10^5/well) containing RPMI 1640 culture medium. Thereafter both eosinophils and mononuclear cells were pre-incubated with LASSBio 552 (100 μM), zileuton (10 μM) or their vehicle (0.1% DMSO) at 37° for 30 min. Next the cells were stimulated with Ca^{+2} ionophore A23187 (1μM) for 1 h at 37 °C in 5% CO_2:95% O_2 atmosphere. After centrifugation at 150×*g*, the supernatant was collected and stored at −20 °C for cysteinyl leukotriene quantification.

The cysteinyl leukotriene levels were determined by using a cysteinyl leukotriene EIA Kit (Cayman Chemical), according to the instructions supplied by the manufacturer.

2.11. Materials

Ovalbumin (grade V), RPMI 1640 culture medium, HEPES, ovalbumin, LTD_4, BSA, human recombinant eotaxin, fetal bovine serum, propidium iodide, A23187, Percoll and *o*-phthaldialdehyde were purchased from Sigma Chemical (USA). Polyclonal anti-murine eotaxin and biotinylated goat anti-murine eotaxin antibody were obtained from R&D systems (USA) and PAF from Novabiochem (Switerland). Neutravidin–horseradish peroxidase conjugate was acquired from Pierce (UK) and K-blue substrate from Neogen (USA). Fura-2 AM and the murine anti-DNP IgE monoclonal antibody were purchased from Calbiochem (USA) and monoclonal anti-CD3(PE)/CD4(FITC)/CD8(FITC)-antibodies from Pharmingen (USA). Zileuton was obtained from Abbott Laboratory

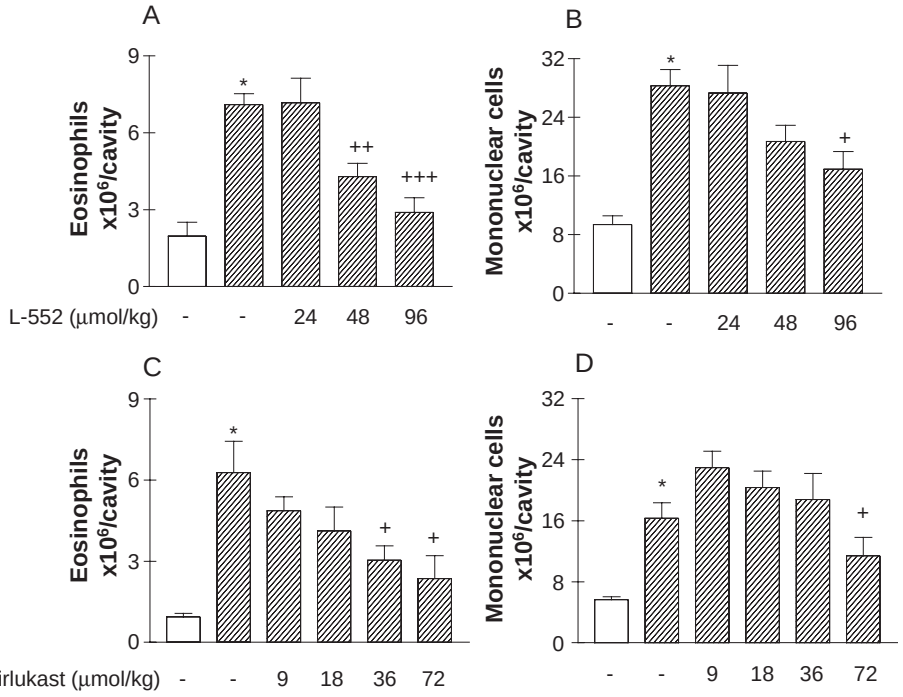

Fig. 2. Inhibition of eosinophil (A) and mononuclear cell (B) accumulation by LASSBIO 552 (L-552; 24–96 μmol/kg) and zafirlukast (18–72 μmol/kg) triggered by ovalbumin (12 μg/site) in immunized (hatched columns) and non-immunized rats (open columns). The analysis was made at 24 h after antigen stimulation. Each column represents the mean+S.E.M., from at least eight animals. *P<0.05 as compared to the non-immunized group; ^{+}P<0.05 as compared to the immunized group.

Table 1
Lack of effect of treatment with either LASSBio 552 or zafirlukast on plasma leakage, neutrophil accumulation and mast cell degranulation evoked by allergen challenge

Stimulus	Treatment	Exuded volume (μl)	Neutrophils×10^6/cavity	Intact mast cells×10^3/cavity
OVA[a]	Vehicle	0±0	0.86±0.4	352.7±22.0
OVA[b]	Vehicle	850±200[c]	17.8±3.0[c]	88.3±16.2[c]
OVA[b]	LASSBio 552	1000±100	14.3±1.3	93.3±30.5
OVA[b]	Zafirlukast	890±120	17.3±2.0	93.2±17.7

LASSBio 552 (96 μmol/kg, i.p.) and zafirlukast (72 μmol/kg, i.p.) were administered 1 h before and the analysis was performed 6 h after challenge (ovalbumin, 12 μg/cavity), in non-immunized[a] and immunized[b] rats. Each value represents the mean±S.E.M. from at least eight animals. [c]$P<0.05$ as compared to the non-immunized group.

(Abbott Park, IL). LASSBio 552 was synthesized by the Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio) of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Zafirlukast was purchased from Astra-Zeneca. All solutions were prepared immediately before use.

2.12. Statistical analysis

Data were statistically analyzed by the analysis of variance (ANOVA), followed by the Newman–Keuls Student test. P values of 0.05 or less were considered significant.

3. Results

3.1. Effect of LASSBio 552 and zafirlukast on allergen-induced pleurisy

We have previously reported that the intrapleural injection of ovalbumin (12 μg/cavity) into actively sensitized rats leads to an early mast cell degranulation and plasma leakage (15 min to 1 h) followed by a marked increase in the number of neutrophils which peaked 4 to 6 h, and eosinophil and mononuclear cells which peaked 24 to 72 h post-challenge (Lima et al., 1991). LASSBio 552 (24 to 96 μmol/kg) or zafirlukast (9 to 72 μmol/kg) administered i.p. 1 h before ovalbumin challenge inhibited dose-dependently eosinophil and mononuclear cell infiltration noted 24 h after ovalbumin (Fig. 2). Nevertheless, LASSBio 552 and zafirlukast failed to alter the early phase of this response, leaving unaltered plasma leakage, mast cell degranulation and neutrophil infiltration assessed 6 h post-challenge (Table 1).

Table 2
Lack of effect of treatment with either LASSBio 552 and zafirlukast on allergen-evoked eotaxin production

Condition	Stimulus	Treatment	Eotaxin (fmol/cavity)
Non-immunized	Ovalbumin	Vehicle	17±1
Immunized	Ovalbumin	Vehicle	368±39[a]
Immunized	Ovalbumin	LASSBio 552	366±40
Immunized	Ovalbumin	Zafirlukast	300±51

LASSBio 552 (96 μmol/kg, i.p.) and zafirlukast (72 μmol/kg, i.p.) were administered 1 h before and the analysis was performed 6 h after ovalbumin challenge (12 μg/cavity) in non-immunized and immunized rats. Each value represents the mean±S.E.M. from at least eight animals. [a]$P<0.05$ as compared to the non-immunized group.

3.2. Lack of effect of LASSBio 552 and zafirlukast on antigen-evoked eotaxin generation in vivo

Allergen-evoked eosinophilia in sensitized rats is preceded by eotaxin generation and sensitive to eotaxin immune-neutralization (Silva et al., 2001). As shown in Table 2, treatment with either LASSBio 552 (96 μmol/kg, i.p.) or zafirlukast (72 μmol/kg, i.p.) failed to modify allergen-evoked eotaxin measured in the pleural effluent 6 h post-allergen challenge.

We also studied the effect of LASSBio 552 on Ca^{+2} fluxes triggered by 30 nM recombinant human eotaxin in isolated eosinophils. The values, in arbitrary units of fluorescence, increased from 0.006±0.004 (mean±S.E.M.; n=3) in the negative controls to 0.033±0.002 (n=3) following recombinant human eotaxin challenge, whereas in cells treated with LASSBio 552 (100 μM) the result for recombinant human eotaxin stimulation was 0.032±0.007 (n=3), showing that the drug did not modify eotaxin effects.

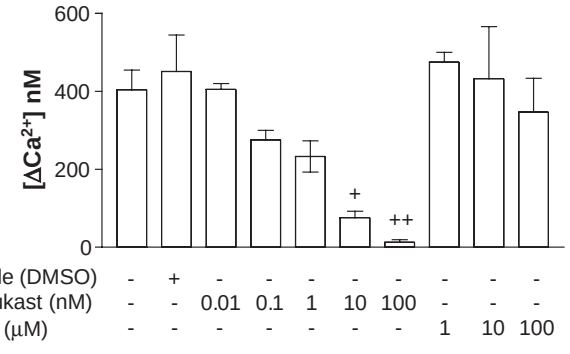

Fig. 3. Effect of either zafirlukast or LASSBio 552 (L-552) on Ca^{2+} mobilization evoked by LTD_4 (1 nM) in CHO cells expressing leukotriene $CysLT_1$ receptor. Cells were incubated with test drugs for 15 min before stimulation. Data represent the mean+S.E.M. from 3–4 independent experiments done in duplicate. $^{+}P<0.01$ and $^{++}p<0.001$ as compared to control vehicle group.

Table 3
Lack of effect of LASSBio 552 on PAF-evoked eosinophil chemotaxis

Stimulus	Treatment	Dose (μM)	Eosinophils/15HPF
Medium	None	–	75.0±1.05
0.1 μM PAF	Vehicle	–	124.3±22.92[a]
	LASSBio 552	100	135.3±31.36
1 μM PAF	Vehicle	–	233.0±39.17[a]
	LASSBio 552	100	235.2±30.83

Isolated eosinophils were pre-incubated with LASSBio 552 for 30 min at 37 °C before stimulation with PAF. Data represent the mean±S.E.M. from 4 independent experiments done in duplicate. HPF—high power field. [a]$P<0.05$ as compared to non-stimulated group.

3.3. Zafirlukast, but not LASSBio 552, antagonizes LTD_4-evoked Ca^{+2} influx in leukotriene $CysLT_1$ receptor transfected CHO cells

While trying to assess the putative antagonism of LASSBio 552 on the leukotriene $CysLT_1$ receptor, we noted that this compound failed to alter LTD_4-evoked Ca^{+2} influx in leukotriene $CysLT_1$ receptor-transfected cells in doses ranging from 1 to 100 μM, under conditions where zafirlukast was clearly active (Fig. 3).

3.4. Lack of effect of LASSBio 552 on eosinophil migration in vitro

In another set of experiments, using the 48-well Boyden chamber system, we noted that the pretreatment of isolated peritoneal eosinophils with LASSBio 552 (100 μM) failed to inhibited eosinophil chemotaxis caused by PAF (0.1 and 1 μM; Table 3).

3.5. Effect of LASSBio 552 on antigen-induced $CD4^+$ T cell accumulation in the pleural cavity

Ovalbumin challenge increased CD4+ T cell numbers in the pleural space 24 h post-challenge. As illustrated in Fig. 4, pretreatment with LASSBio 552 (96 μmol/kg, i.p.) 1 h before challenge clearly abrogated pleural accumulation of $CD4^+$ T cells. There is also a clear tendency to reduce the accumulation of CD8+ T cells in parallel, though data obtained from both treated and untreated animals were not statistically significant.

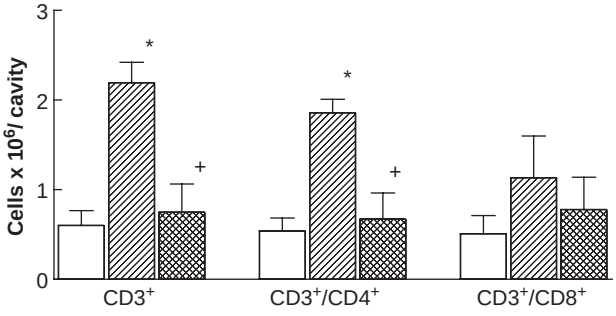

Fig. 4. Effect of LASSBio 552 (96 μmol/kg, i.p.; cross-hatched columns) or its vehicle (right-hatched columns) on allergen-evoked pleural accumulation of $CD3^+$, $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells noted 24 h post-challenge in immunized rats. The negative control group (open columns) is represented by non-immunized rats challenged with ovalbumin. Each column represents the mean+S.E.M. from at least four animals. *$P<0.05$ as compared to the non-immunized group; $^+P<0.05$ as compared to the immunized group.

3.6. Antigen-induced cysteinyl leukotriene generation is sensitive to LASSBio 552

To investigate the possibility that reduction in cysteinyl leukotriene levels by LASSBio 552 might contribute to the inhibitory effect of this compound on allergen-evoked eosinophil infiltration, we quantified cysteinyl leukotrienes in the pleural effluent at different time points after allergen challenge in treated and untreated animals. LASSBio 552 (96 μmol/kg) did not inhibit cysteinyl leukotriene generation within 6 h but did inhibit it from 24 h to 48 h post-challenge, in parallel with reduction in the eosinophil numbers (Fig. 5).

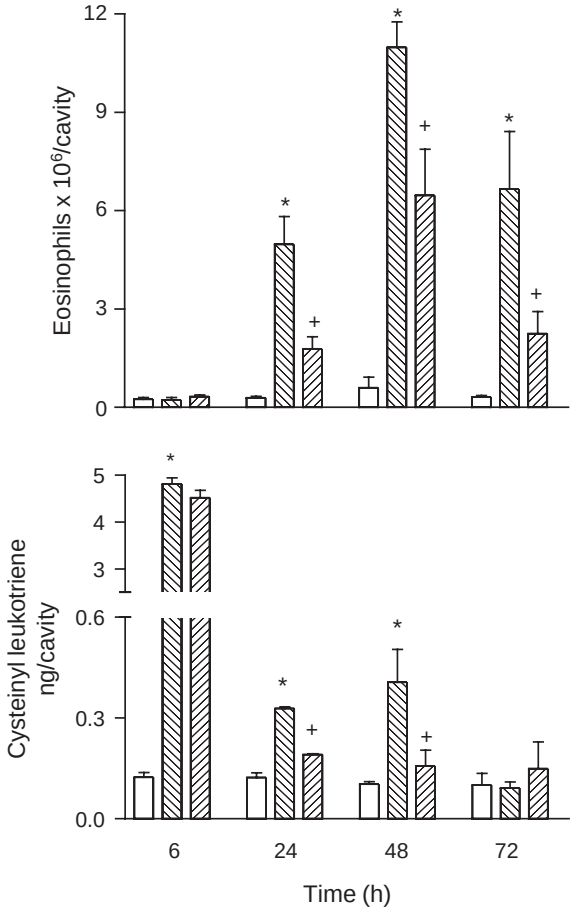

Fig. 5. Effect of LASSBio 552 (96 μmol/kg; cross-hatched columns) or its vehicle (right-hatched columns) on eosinophil infiltration (A) and cysteinyl leukotriene production (B) noted 6, 24, 48 and 72 h after ovalbumin stimulation in immunized rats. The negative control group (open columns) is represented by non-immunized rats challenged with ovalbumin. Data are expressed as mean+S.E.M. from at least six animals. *$P<0.05$ as compared to the non-immunized group; $^+P<0.05$; $^{++}P<0.01$ as compared to the immunized group.

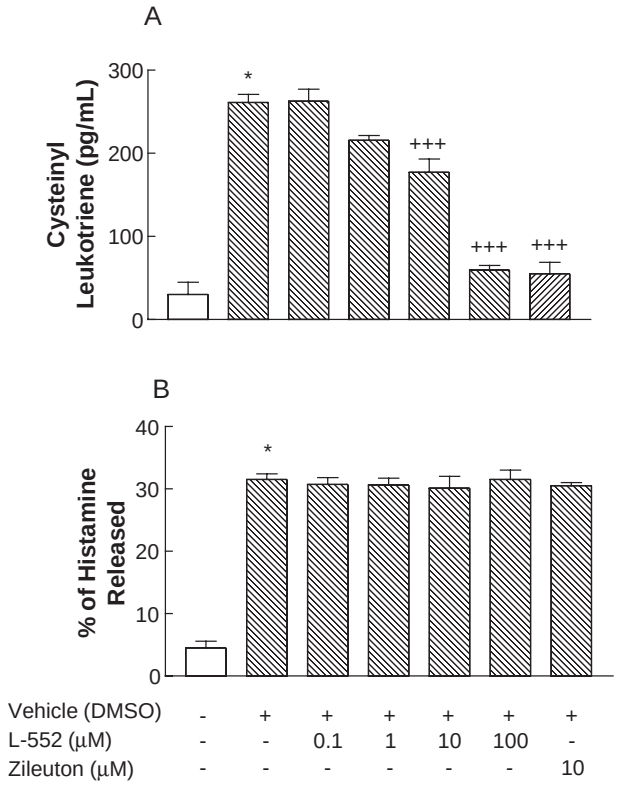

Fig. 6. Effect of LASSBio 552 (L-552) on cysteinyl leukotriene generation (A) and mast cell degranulation (B) induced by antigen in vitro. IgE passively sensitized mast cells were pretreated with LASSBio 552 (0.1–100 μM), zileuton (10 μM) or vehicle for 30 min before DNP-BSA provocation (hatched columns). Open columns represent mast cells sensitized with anti-DNP-BSA IgE but not challenged with DNP-BSA. Data are expressed as mean+S.E.M. from at least 3 independent experiments done in duplicate. *$P<0.05$ as compared to non-stimulated mast cells, $^{+++}P<0.001$ as compared to untreated stimulated mast cells.

In in vitro settings, we demonstrated that co-incubation of IgE-sensitized mast cells with increased concentrations of LASSBio 552 (0.1–100 μM) inhibited dose-dependently antigen-evoked cysteinyl leukotriene generation (Fig. 6A). In contrast, the treatment failed to modify the concomitant release of histamine, similar to what was observed after treatment with 10 μM zileuton (Fig. 6B). As illustrated in Table 4, eosinophils and mononuclear cells recovered from the peritoneal cavity of rats also released significant amounts of cysteinyl leukotrienes after stimulation with 1 μM A23187, phenomenon which was slightly but significantly inhibited by LASSBio 552. Under these conditions, zileuton clearly abolished A23187-induced generation of cysteinyl leukotriene by both eosinophils and mononuclear cells.

4. Discussion

The present study investigated the possibility that LASSBio 552–a novel tetrazolephtalimide derivative reported to inhibit LTD_4-induced contraction of guinea pig tracheal strips (Lima et al., 2002)–may have prophylactic effects on allergic inflammation. We demonstrated that LASSBio 552, like zafirlukast, inhibited allergen-evoked eosinophil recruitment without modifying either neutrophil influx or plasma leakage. Surprisingly, LASSBio 552 failed to antagonize LTD_4-mediated activation of leukotriene $CysLT_1$ receptor-transfected cells, but prevented both allergen-evoked CD4+ T cell accumulation and cysteinyl leukotriene generation in the late phase reaction, which has probably contributed to the down-regulation of eosinophilia. These findings suggest that treatment with LASSBio 552 may prove beneficial in selectively regulating eosinophil influx following inflammatory response to allergens.

Eosinophil recruitment and activation have been considered the main features of the late phase reaction of allergy, broadly correlating with disease severity (Humbles et al., 2004; Lee et al., 2004). In addition, previous reports demonstrated the effectiveness of agents which prevent production and/or action of cysteinyl leukotrienes in inhibition of eosinophilia, tissue remodeling and airway hyper-responsiveness evoked by allergen challenge (Eum et al., 2003; Fregonese et al., 2002; Hamid et al., 2003; Holgate et al., 2003; Spada et al., 1994; Underwood et al., 1996; Vargaftig and Singer, 2003). In the current study, we used the rat pleurisy system (Lima et al., 1991) to investigate the effect of LASSBio 552 on allergen-evoked eosinophilic inflammation, having leukotriene $CysLT_1$ receptor antagonist zafirlukast for comparison. We noted that the early phase of the allergic inflammatory response, including mast cell degranulation, plasma leakage and neutrophil accumulation, remained unchanged after treatment with LASSBio 552, similarly to what was observed after treatment with zafirlukast or montelukast (data not shown). These results are consistent with previous reports showing that acute allergic responses appeared more sensitive to vasoactive amine receptor antagonists than to selective leukotriene $CysLT_1$ receptor antagonists (Ihaku et al., 1999). On the other hand, the increase of eosinophil and mononuclear cell numbers noted 24 h post-challenge was dose-dependently inhibited by LASSBio 552, again in line with data from zafirlukast and montelukast (data not shown) treated animals. Of note, LASSBio 552 also prevented

Table 4
Effect of LASSBio 552 on cysteinyl leukotriene generation (pg/ml) on eosinophils and mononuclear cells

Stimulus	Treatment	Eosinophils	Mononuclear cells
Medium	None	141±12	445±3
A23181	Vehicle	893±6[a]	1057±12[a]
	LASSBio 552	829±11[b]	915±2[c]
	Zileuton	118±21[c]	225±7[c]

The cells were pre-incubated with 100 μM LASSBio 552, 10 μM zileuton or vehicle for 30 min before the stimulation with 1 μM A23187. Data are expressed as mean+S.E.M. from at least 3 independent experiments done in duplicate. [a]$P<0.05$ as compared to non-stimulated cells, [b]$P<0.05$, [c]$P<0.001$ as compared to untreated (vehicle) stimulated cells.

allergen-evoked CD4+ T cell recruitment which may help to explain the LASSBio 552-evoked blockade of eosinophil accumulation in the pleural effluent, since various mediators released by T cells can activate eosinophil recruitment and survival (Busse and Lemanske, 2001; Kay, 2001; Lemanske and Busse, 2003).

It is of interest to emphasize that there is a remarkable difference in the potencies of LASSBio 552 and zafirlukast concerning their ability to prevent LTD_4-induced tracheal contraction as reported (Lima et al., 2002). Surprisingly and, at first sight, in contrast to the isolated organ data, LASSBio 552 and zafirlukast were almost equipotent to inhibit antigen-evoked eosinophil and mononuclear cell recruitment, challenging the interpretation that the leukotriene $CysLT_1$ receptor is a common target. Actually, our findings demonstrated that zafirlukast dose-dependently inhibited the Ca^{+2} flux in response to LTD_4 in leukotriene $CysLT_1$ receptor transfected cells, as expected, under conditions where LASSBio 552 was clearly inactive. Even at high concentration (100 μM) LASSBio 552 failed to alter LTD_4-induced Ca^{+2} flux in transfected cells, indicating that the phtalimide derivative is not a leukotriene $CysLT_1$ receptor antagonist, and probably uses an alternative mechanism to prevent allergen-evoked inflammation.

Eotaxin has been largely implicated in infiltration of eosinophils into the focus of allergic inflammatory response (Pease et al., 2004). Particularly in the case of the experimental model used here, pleural eotaxin levels peaked 6 h post-antigen challenge, thus preceding the eosinophil influx, which in turn was shown to be clearly sensitive to eotaxin immune-neutralization (Silva et al., 2001). However, our findings indicated that LASSBio 552 inhibited eosinophilia without inducing a concomitant change in the eotaxin formation. Also, LASSBio 552 did not modify eotaxin-evoked activation of eosinophils in vitro, providing evidence that neither blockade of eotaxin formation nor action is the mechanism by which LASSBio 552 could prevent eosinophilia. Furthermore, experiments designed to approach a putative direct effect upon eosinophils revealed that LASSBio 552 failed to impair PAF-induced eosinophil chemotaxis in vitro, indicating that the eosinophil locomotory machinery is not affected either after exposure to the substance.

Due to the marked similarities found in the anti-inflammatory effects evidenced by LASSBio 552 and cysteinyl leukotriene receptor antagonists in our system and the recognized implication of cysteinyl leukotrienes in allergen-evoked eosinophil recruitment (Eum et al., 2003; Fregonese et al., 2002; Hamid et al., 2003; Holgate et al., 2003), we became particularly interested in clarifying the role that cysteinyl leukotrienes might play in mediating the effect of LASSBio 552 on eosinophilia. In this model, antigen provocation led to strong increase in cysteinyl leukotriene levels 6 h post-challenge. This up-regulation resulted in levels about 10-fold higher than those noted within 24 h and 48 h, yet reflecting cysteinyl leukotriene levels 2 to 3-fold elevated as compared to the baseline levels. LASSBio 552 failed to alter the early production of cysteinyl leukotrienes but significantly inhibited the elevation noted in the late phase reaction, in parallel with reduction in eosinophil numbers. Since inflammatory cells such as mast cells, macrophages and eosinophils themselves are important sources of cysteinyl leukotrienes in allergic conditions (Bandeira-Melo and Weller, 2003; Hart, 2001; Lee et al., 2000; Yu et al., 1995), we have evaluated the putative direct effect of LASSBio 552 on these cells. Our findings indicated that in vitro exposure of IgE-sensitized mast cells to LASSBio 552 inhibited in a concentration-dependent manner antigen-evoked cysteinyl leukotriene generation, whereas histamine release remained unchanged. Slight blockade was also noted when eosinophils and mononuclear cells isolated from the peritoneal cavity of naïve rats were stimulated with the Ca^{+2} ionophore A23187. Taken together, these findings suggest that LASSBio displays weak inhibitory actions upon cysteinyl leukotriene-forming cells, which however may be enough to interfere with the generation of leukotrienes and eosinophil accumulation noted in the late phase, though being clearly insufficient to alter the strong production of these molecules in the early phase of the process.

In conclusion, we report in this study the selective blockade of eosinophil accumulation in the focus of allergen-evoked inflammation by the new tetrazolephtalimide derivative LASSBio 552. Combined analysis of the experiments revealed that LASSBio 552 effect is probably accounted for by blockade of both cysteinyl leukotriene production and CD4+ T cell mobilization in the late phase of the process. These data may open perspectives for the development of new anti-asthma compounds based on the synthesis of phtalimide derivatives.

Acknowledgements

This study was supported by research grants from PRONEX, CNPq and FAPERJ, Brazil. We thank Dr Y. Kanaoka for Stably leukotriene $CysLT_1$ receptor transfected CHO cells and Dr. Robson Coutinho-Silva (Instituto de Biofísica, UFRJ) in whose laboratory the intracellular Ca^{+2} measurements were carried out. Josiane Sabbadini Neves thanks the CAPES for her PhD Scholarship.

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