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Original Paper

The Novel H_4R Antagonist 1-[(5-Chloro-2,3-Dihydro-1-Benzofuran-2-Yl)Methyl]-4-Methyl-Piperazine (LINS01007) Attenuates Several Symptoms in Murine Allergic Asthma

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Key Words
H_4R antagonist • Antihistamine • Allergic lung inflammation • Asthma • Anti-inflammatory agent

Abstract
Background/Aims: Histamine is an important chemical transmitter involved in inflammatory processes, including asthma and other chronic inflammatory diseases. Its inflammatory effects involve mainly the histamine H_4 receptor (H_4R), whose role in several studies has already been demonstrated. Our group have explored the effects of 1-[(2,3-dihydro-1-benzofuran-2-yl)methyl]piperazines as antagonists of H_4R, and herein the compounds LINS01005 and LINS01007 were studied with more details, considering the different affinity profile on H_4R and the anti-inflammatory potential of both compounds. ***Methods:*** We carried out a more focused evaluation of the modulatory effects of LINS01005 and LINS01007 in a murine asthma model. The compounds were given i.p. (1-7 mg/kg) to ovalbumin sensitized BALB/c male mice (12 weeks old) 30 min before the antigen challenging, and after 24 h the cell analysis from the bronchoalveolar lavage fluid (BALF) was performed. The lung tissue was used for evaluation by western blot (COX-2, 5-LO, NF-κB and STAT3 expressions) and histological analysis. ***Results:*** Treatment with the more potent H_4R antagonist LINS01007 significantly decreased the total cell count and eosinophils in BALF at lower doses when compared to LINS01005. The expression of COX-2, 5-LO, NF-κB and STAT3 in lung tissue was significantly

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reduced after treatment with LINS01007. Morphophysiological changes such as mucus and collagen production and airway wall thickening were significantly reduced after treatment with LINS01007. ***Conclusion:*** These results show important down regulatory effect of novel H_4R antagonist (LINS01007) on allergic lung inflammation.

Introduction

Histamine is one of the most pleiotropic biogenic amines in the human body. Classically, it is involved in allergic reactions, producing effects such as vasodilation, increased vascular permeability and pruritus, but also plays an important role in the acid gastric secretion and in the central nervous system, where it is a well stablished neurotransmitter involved in several brain functions [1]. Its involvement in inflammatory and immunological responses is also evident and widely documented [2, 3]. The biological effects of histamine are mediated by the activation of four G-protein coupled receptors namely from H_1 to H_4 receptors (H_1R, H_2R, H_3R, and H_4R) and differ according to their distribution pattern, signaling system and physiological functions [1, 2].

Asthma is one of the most common inflammatory diseases, affecting more than 300 million people worldwide, with a stable prevalence rate [4]. It is a heterogeneous condition with very complex pathophysiological mechanism, however the involvement of histamine (and indeed the allergic reaction) on such mechanisms is critical [5]. Histamine is detected in high concentrations on the airways during asthmatic response, and promotes the typical inflammatory signals. Although the H_1R is expressed in bronchial smooth muscle and causes bronchoconstriction after histamine exposure, the clinically available antihistamines H_1R and H_2R are not effective in the treatment of asthma. On the other hand, H_4R have been widely explored as potential target for antiasthma agents, since its activation lead to pro-inflammatory effect. Several ligands of H_4R have been evaluated in clinical trials but none reached the market to date [3].

The histamine H_4R is coupled to $G_{i/o}$ protein and thus leads to reduction of intracellular AMPc and calcium levels, but its G-protein independent signaling pathway was also reported [6]. It is expressed in bone marrow derived and immune cells such as eosinophils, neutrophils, basophils, T lymphocytes, mast and dendritic cells, thus actively participating on inflammatory response [6].

Several studies have shown that H_4R inhibition could be an effective way to reduce some of the biological effects of asthma, including eosinophilic infiltrate and IgE production. One of the very first selective H_4R antagonist, JNJ-7777120, has shown efficacy in reducing the expression of IL-4, IL-5 and IL-13 cytokines, decreased the total cell counts and eosinophil infiltration in the bronchoalveolar lavage fluid (BALF) and other parameters in asthma model [7-9]. Other H_4R antagonists have also been tested (A940894, INCB38579 and JNJ39798979) in different animal models, and showed to down modulate some of the symptoms of pain and inflammation in clinical trials [10-13]. Recently, Nagarajan and Thangam (2020) demonstrated that a novel H_4R antagonist downregulated the expression of signaling proteins such as ERK1/2 and NF-kB in asthma murine model. Despite numerous studies showing the modulatory role of H_4R antagonists in asthma, their therapeutic potential is not yet established [14].

In the last years our group have been exploring the potential of 1-(2,3-dihydrobenzofuran-2-yl)methylpiperazines (LINS01 series) as ligands of histamine receptors, especially on H_3R and H_4R [15-17]. The affinity of these compounds is dependent of specific characteristics of the molecule, but in general preference for H_3R was observed for these compounds. One of the first compounds prepared by us was the 1-(2,3-dihydrobenzofuran-2-ylmethyl)-4-phenyl-piperazine (LINS01005), which presented low affinity for H_4R. The best compound in the series as H_4R ligand to date is the 1-[5-chloro-(2,3-dihydrobenzofuran-2-yl)methyl]-4-methyl-piperazine (LINS01007, Fig. 1), which presented submicromolar affinity to both H_3R and H_4R (pK_i ~6.1). Both compounds showed antagonistic effect on activation

assays using BRET constructions with $G\alpha_i$-1 protein, and no activation of the β-arrestin signaling pathway was observed. Additionally, these compounds also showed negligible affinity for H_1R or H_2R [17].

Previous results from our group showed that LINS01005 reduced eosinophilia in the BALF as well as COX-2 expression in lung tissue [15]. In a following study, we preliminary demonstrated that the novel and more potent compound LINS01007 was more effective in reducing these parameters in lower dose than LINS01005 [16].

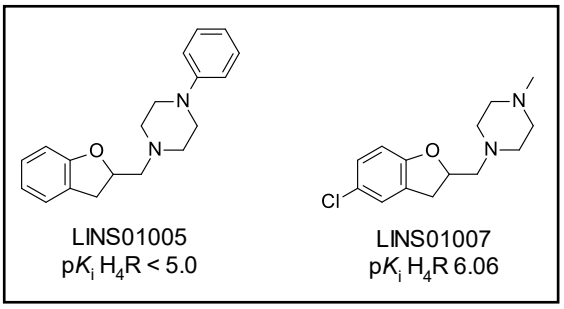

Fig. 1. Molecular structure of LINS 01 compounds.

These previous data raised important questions regarding the detailed effect of these compounds, and clearly determined that more studies are needed to elucidate the involvement of the histamine receptors in this response. Considering this, the aim of the present study was to investigate the detailed anti-inflammatory and immunological effects of these compounds (LINS01005 and LINS01007). For this, analysis of the eosinophil infiltration in BALF and lung tissue, production of mucus and collagen, release of cytokines and chemokines was carried out in asthma murine model. Additionally, the involvement of the NF-kB and STAT3 signaling pathways were also investigated.

Materials and Methods

Drugs

The compounds LINS01005 and LINS01007 were prepared and characterized as previously described by Corrêa et al. [15-17]. The purity of the compounds was >95%, checked by elemental analysis (C, H and N) and chromatography (HPLC) assessments. Further details are described in the Supplementary Information (for all supplementary material see www.cellphysiolbiochem.com). The compounds were dissolved in sterile saline prior to injection.

Animals

Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and this project was approved by the Ethical Committee for Animal Research of the Federal University of São Paulo (CEUA). Male BALB/c mice used were from CEDEME colony - Universidade Federal de São Paulo), housed in a 22 ± 1°C environment at 60% humidity and were maintained on a 12-h light-dark cycle.

Aluminum gel preparation

The aluminum gel was prepared according to the precipitation of the ammonium aluminum sulfate dodecahydrate [$AlH_4(SO_4)_2.12H_2O$, Alfa Aesar, MA, USA] with an excess of 1N NaOH [LabSynth, SP, Brazil]. Aluminum hydroxide [$Al(OH)_3$] was suspended in water (Milli Q, Ontario, Canada), washed five times and centrifuged at 3000 rpm for 15 minutes. The final precipitate was resuspended in water and the final concentration was determined by calculating 1 mL of dry solution.

Allergen sensitization and challenge

Mice were immunized on days 0 and 7 by an intraperitoneal injection with 20 μg of ovalbumin (OVA) and 1.6 mg of aluminum hydroxide in sterile saline solution (total volume of 200 μL). These mice were challenged twice with OVA (2.5%) by aerosol exposition, using an ultrasonic nebulizer at days 14 and 21 for 20 minutes. Control group consisted of immunized mice that received saline aerosol. Drugs (LINS01005 and LINS01007) were given i.p. 30 min before each aerosol challenge on the defined doses (1 mg/kg; 3 mg/kg; 5 mg/kg; 7 mg/kg, prepared in 200 μL of sterile saline solution).

Total and differential cell counts in the bronchoalveolar lavage fluid (BALF)

After 24 h of the second challenge, the animals were euthanized by injection of 2.5 mg of ketamine and 250 μg of xylazine, given i.p. and previously dissolved in 200 μL of sterile saline solution. A tracheal cannula was inserted via a midcervical incision and the airways were washed twice with 1 ml of phosphate buffered saline (PBS; pH 7.4 at 4°C). The BALF was centrifuged at 170 *g* for 10 min at 4°C, the supernatant was removed, and the cell pellet was resuspended in 0.5 ml of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin–eosin (Hema 3).

Enzyme-linked immunosorbent assay (ELISA) for total IgE

Blood samples were collected by cardiac puncture, centrifuged and serum was stored at −20 °C. Total mouse immunoglobulin E (IgE) was determined by sandwich-ELISA using kit, BD OptEIA ELISA Set (BD Bioscience, San Diego, USA), according to the manufacturer's recommendation. Values were expressed in μg/mL, deduced from a standard curve of recombinant antibody (100 ng/mL) ran in parallel. The samples were diluted (1:100) and the limits of detection ranged from 1.6 to 100 ng/mL.

Enzyme-linked immunosorbent assay (ELISA) for OVA-specific IgE

Blood samples were collected by cardiac puncture, centrifuged and serum was stored at −20 °C. Serum antibodies were determined by enzyme-linked immunosorbent assay (ELISA). OVA-specific IgE levels were determined by adding serum samples at 1/10 dilutions to 96-well plates (Nunc, NY, USA) with anti-IgE (SouthernBiotech, Birmingham AL, USA). After washing, biotin-labeled OVA was added and revealed with avidin-HRP plus substrate. OVA-specific IgE serum concentrations were deduced using Chondrex kit (Chondrex, EUA) with known concentrations of OVA- specific monoclonal IgE antibody.

Morphometrical analysis

Lung tissue was harvested and fixed in a 10% buffered-formalin solution and routinely processed for histological inclusion in paraffin. Five-mm thick tissue sections were stained with Periodic Acid of Schiff (PAS) for visualizing mucus, picrosirius for visualizing collagen and with hematoxylin/eosin for visualizing eosinophils and alveolar walls. The number of eosinophils present in the lung parenchyma and alveolar walls were quantified by an investigator blinded to the various groups in approximately 20 different histologic regions per animal [18]. To evaluate collagen deposition and mucus production the area of positivity was measured (mm^2) in the maximal number of bronchioles per slide and the area of each bronchiole was normalized by the average of three different measurements of the diameter of the same bronchiole to rule out the influence of the caliber of the bronchiole in the extent [19]. Morphometrical analysis was performed using a Nikon DXM 1200c digital camera and Nikon NIS – Elements AR 2.30 software.

Protein expression by western blot

Lung tissues were also used to quantify the expression of 5-lipoxigenase (5-LO), cycloxigenase-2 (COX-2), STAT-3 and NF-kB by western blot. Approximately 25 mg of lung tissues were placed in microcentrifuge tubes with 40 mg of beads and lysis buffer (Tris-HCl 50 mmol/L, pH 7.4, NaCl 100 mmol/L and NP40 0.5%) with a protease/phosphatase inhibitor (Halth™, Thermo Scientific, USA). Samples were homogenized in Precellys 24 (5000 rpm; 2 cycles; 60 s) and then maintained in constant agitation for 2 h at 4 °C (by keeping on an orbital shaker inside the fridge). After 2 h, samples were centrifuged for 20 min at 12,000 rpm at 4 °C in a microcentrifuge, and the supernatant was separated and placed in a fresh tube. Protein concentrations were determined by a BCA protein assay kit (Thermo Scientific, USA). Equal amounts of protein (60 μg) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were transferred onto nitrocellulose membranes (0.45 μm) and blocked for 60 min with 5% (wt/vol) non-fat dry milk diluted in TTBS (Tris base 0.2 mmol/L, NaCl 1.4 mmol/L and Tween 20 0.1%), pH 7.6. The membranes were incubated overnight with polyclonal antibodies against COX-2 (#4842-Cell Signaling, rabbit IgG, USA), NF-κB p65 (#3987-Cell Signaling, rabbit IgG, USA), 5-LO (#3289-Cell Signaling, rabbit IgG, USA), STAT3 (#8768-Cell Signaling, rabbit IgG, USA) at 1:1000 dilutions. The blots were washed with TTBS (3 x 5 min) and incubated with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (#70745, Cell Signaling Tech, USA) at a 1:2000 dilution for 60 min at room temperature.

COX-2, NF-κB, 5-LO and, STAT3 expressions were detected by chemiluminescence (GeneGnome System, Syngene, UK) and quantified by densitometry (Gene Tools Software, UK). β-Actin expression was used as an internal control (1:2000 dilution, Cell Signaling #4970).

Measurement of cytokine and chemokine production

Milliplex® map kit-mouse cytokine chemokine magnetic bead panel (EMD Millipore Corporation-Darmstad, Germany) were used to measure TNF-α, IFN-γ, IL-4, IL-5, IL-6, IL-13 and RANTES in the lung tissue samples. The kit was used according to the manufacturer's instructions (MAGPIX™, Luminex®, MiraiBio, Alameda, CA). The data were analyzed using the xPONENT® software (MAGPIX™, Luminex®, MiraiBio, Alameda, CA). Standard curves ranged from 1.95 to 32,000 pg/ml.

Statistical analysis

Data are expressed as the means ± S.E.M. Statistical evaluation of the data was carried out by analysis of variance (ANOVA) and sequential analysis of differences among means was done by Tukey's contrast analysis. The accepted significance level was >95% (P-value < 0.05). P<0.05, P<0.01 and P<0.001 were marked with one, two or three asterisks, respectively. All statistical analyses were performed with the aid of GraphPad Prism software (San Diego, CA, United States).

Results

Cells in the bronchoalveolar lavage fluid and IgE levels

Mice immunized with OVA were submitted to two OVA aerosol challenges and then BALF was performed 24 h after the second aerosol challenge. Significant increase in total cell number in BALF (from 4.1 to 145.2x10^4 cells in Fig. 2A and from 7.7 to 107x10^4 cells in Fig. 2B) were observed in these groups when compared to the control groups (OVA immunized mice submitted to saline aerosol). The increase in the number of cells on BALF observed in the experimental group was mainly due to the infiltration of eosinophils (68% in Fig. 2C and 62% in Fig. 2D). The groups of immunized mice received intraperitoneal injections of the compounds LINS01005 and LINS01007 in different doses (1, 3, 5, 7 mg/kg) 30 min before each of the antigen aerosol challenges.

Fig. 2A and 2C showed that treatment with LINS01005 significantly decreased the number of total cells and eosinophils only at the dose of 5 mg/kg (76% and 86%, respectively), with no significant differences observed between 5 and 7 mg/kg doses. On the other hand, treatment with the LINS01007 compound similarly reduced cellular infiltrate (81% for total cells and 72% for eosinophils) in lower dose (3 mg/kg) (Fig. 2B and 2D). Similarly, standard treatment with dexamethasone (5 mg/kg) was able to reduce 71% cell infiltrate when compared to the asthma group (data not shown).

Additionally, the treatment with both compounds reduced the levels of total IgE when administered in a 5 mg/kg dose (55%; Fig. 2E). However, this effect was not observed in the treatment with LINS01005 at 3 mg/kg dose, but was significant to the treatment with LINS01007 at the same dose (72%). OVA-specific IgE levels were also reduced after treatment with LINS01005 at 5 mg/kg (28%) and LINS01007 at both 3 and 5 mg/kg doses (25% and 45%, respectively; Fig. 2F).

Considering this data, our results suggest that LINS01007 showed better efficacy than the compound LINS01005 as anti-inflammatory agent. We demonstrated that this compound presented greater efficiency in inhibiting the inflammatory response with a dose of 3 mg/kg that determined further studies with these compounds.

Cycloxygenase-2 and 5-lypoxygenase protein expression

The protein expression of COX-2 and 5-LO were analyzed in the studied groups by western blot. Tissue levels of COX-2 and 5-LO expressions were significantly higher in the animals sensitized and challenged with OVA than in the animals from control group (from

0.86 to 1.59 for COX-2 and from 0.12 to 0.33 for 5-LO). Treatment with LINS01007 (3 mg/kg, i.p.) 30 min before the challenge significantly decreased COX-2 and 5-LO expression whereas the treatment with LINS01005 in the same dose had no significant effect (Fig. 3A and 3B).

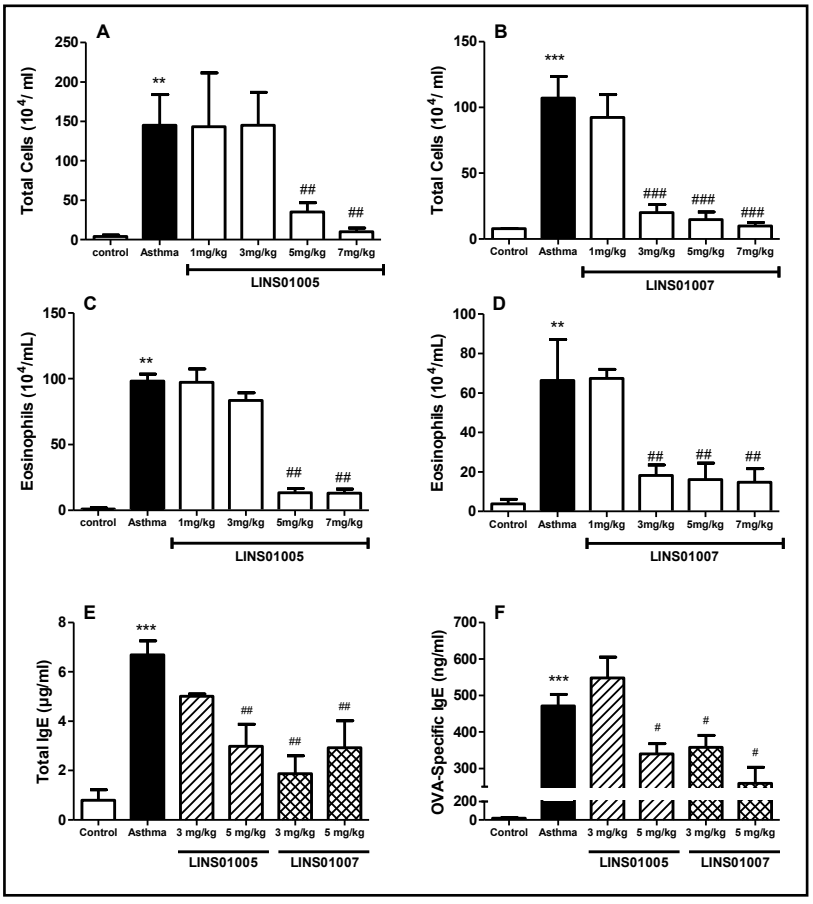

Fig. 2. Effect of LINS01005 and LINS01007 on the bronchoalveolar lavage (BALF) cells and IgE serum levels. Balb/c were immunized with an i.p. injection of ovalbumin/ alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2,5%, 20 min) on days 14 and 21 post immunization. Histamine receptor antagonists were given i.p. 30 min before each aerosol challenge. BALF and serum were performed 24h after the second challenge. Total cells (A and B), eosinophils BALF cells (C and D), total IgE (E) and OVA-Specific IgE serum levels (F). Results are the mean ± S.E.M. of 8 animals/group. ** P< 0.01 in comparison with the control group and ## P< 0.01 in comparison with the asthma group.

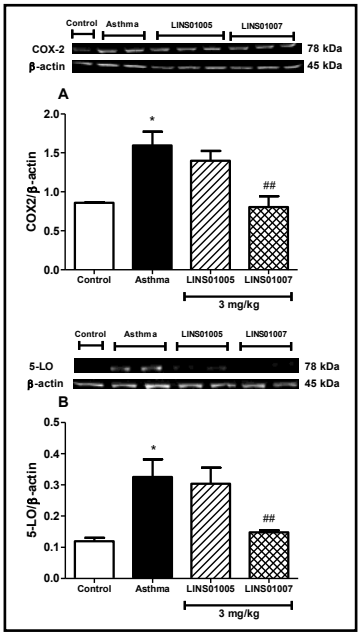

Fig. 3. Effect of treatment with LINS01005 and LINS01007 on the COX-2 and 5-LO expression. Lungs were collected 24h after the second challenge to quantify the expression of COX-2 and 5-LO by Western Blot. Graphs represent the density values of bands that were determined by densitometric analysis and normalized by the total actinin or β-actin present in each lane. Results are the mean ± S.E.M. of 5 animals in each of 3 independent experiments. *p<0.01 in comparison with the control group and ## p<0.05 in comparison with the asthma group.

Cytokines and RANTES chemokine levels

Multiplex assay kits were used to measure chemokine and cytokine levels in lung tissue. Mice sensitized and challenged with OVA showed an expected increase in the expression of all measured chemokines and cytokines (IL-5, IL-6, IL-13, TNF-α, IFN-γ and RANTES). Animals treated with LINS01007 (3 mg/kg) showed lower asthma-induced inflammatory cytokines and chemokines in lung tissue than non-treated (asthma) animals. On the other hand, no significant differences were observed in the levels of IL-5, IL-6, TNF-α, IFN-γ and RANTES in pulmonary tissue from animals treated with LINS01005 (3 mg/kg) when compared to non-treated group (Fig. 4).

Transcription factors expression (NF-κB and STAT3)

The expression of the NF-κB and STAT3 pathways were assessed in the lung tissue. The results showed that increased expression of NF-κB and STAT3 was found in sensitized and challenged mice, as expected. The animals treated with LINS01005 (3 mg/kg) did not show significant differences in the NF-κB and STAT3 expression from the non-treated animals. Conversely, treatment with LINS01007 (3 mg/kg) significantly decreased the expression of NF-κB and STAT3 when compared to the non-treated mice, leading the levels of these proteins close to control group's values (Fig. 5A and 5B).

Histological inflammatory parameters

The morphometrical analysis of the lung tissue from OVA-challenged mice showed that collagen deposition, mucus production, alveolar wall thickness and eosinophils in the peribronchiolar area were markedly increased in the sensitized and challenged animals when compared to control mice. The treatment with LINS01007 (3 mg/kg) significantly attenuated these morphometrical parameters (Fig. 6).

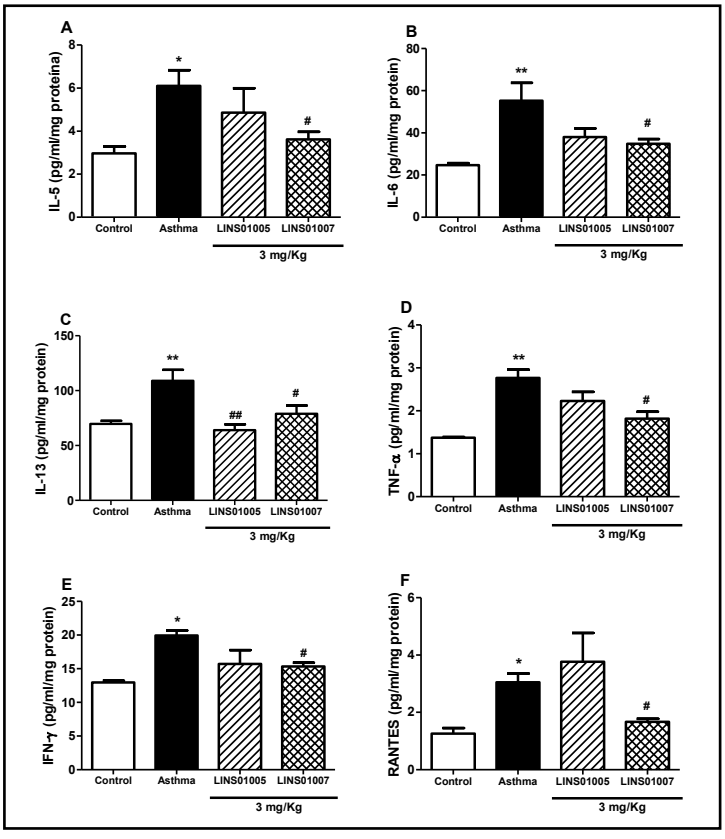

Fig. 4. Effect of treatment with LINS01005 and LINS01007 on cytokines and chemokines production. Lungs were collected 24h after the second challenge to quantify the cytokines and chemokines production. Lung tissue was processed and IL-5 (A), IL-6 (B), IL-13 (C), TNF-α (D), IFN-γ (E), and RANTES (F) production were measured by multiplex assays as described in Material and Methods. Results are the mean ± S.E.M. of 5 animals in each of 3 independent experiments. *p<0.05 and **p<0.01 and in comparison with the control group and #p<0.05 and ##p<0.01 in comparison with the asthma group.

Discussion

In the present study an allergic asthma model was employed, consisting on two immunizations (day 0 and day 7) with i.p. injection of OVA–alum suspension, followed by two posterior challenges (day 14 and day 21) with aerosolized OVA [20-21]. This model produces several characteristics of allergic asthma, such as intense eosinophilic infiltrations in the BALF and lung tissue, increased IgE expression, increased production of cytokines and chemokines (IL-5, IL-6, IL-13, TNF-α, IFN-γ and RANTES) and increased mucus production and collagen deposition.

Using this model, we studied the correlation between the efficacy of two compounds, LINS01005 and LINS01007, and their affinity profile for histamine receptors. Previous report from our group showed that LINS01005 exhibited low affinity for H_4R, while LINS01007 presented nanomolar affinity for such receptor (pK_i 6.06) [15]. The results presented herein strongly suggest that the efficacy of these compounds is correlated to their antagonist activity on H_4R, since better anti-inflammatory effect was observed for LINS01007 than for LINS01005. The more potent H_4R antagonist LINS01007 showed comparable efficacy at lower dose (3 mg/kg) than LINS01005 at 5 mg/kg dose. Additionally, LINS01007 (but not LINS01005) was able to reduce the eosinophilic infiltrations and COX-2 and 5-LO expression in this model of allergic asthma at 3 mg/kg dose [15]. Based on these findings, we performed further studies on the capacity of LINS01007 to modulate other parameters of lung allergic inflammation.

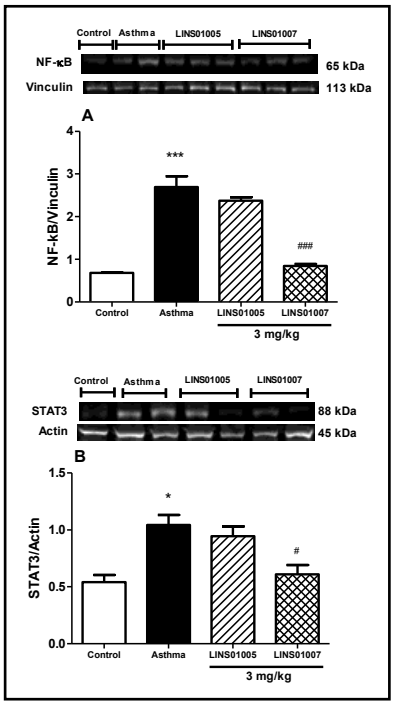

Fig. 5. Effect of treatment with LINS01005 and LINS01007 on the NF-κB and STAT3 expression. Lungs were collected 24h after the second challenge to quantify the NF-κB expression (A) and STAT3 (B) by Western Blot. Graphs represent the density values of bands that were determined by densitometric analysis and normalized by the total vinculin or actin present in each lane. Results are the mean ± S.E.M. of 5 animals in each of 3 independent experiments. **p<0.01 and ***p<0.001 in comparison with the control group and # p<0.05 and ### p<0.001 in comparison with the asthma group.

After sensitization and antigenic challenges an acute allergic reaction occurs, mainly induced by the presence of cell-bound OVA-specific IgE. This mechanism promotes cell activation and degranulation resulting in rapid release of lipid mediators, histamine and production of IL-4, IL-5 and IL-13 cytokines that will lead to eosinophil recruitment and triggering a complex cascade of events that result in allergic asthma profile [22]. The IL-5 cytokine plays an important role in the effector phase of pulmonary allergic inflammation, stimulating (among other factors) the migration and maturation of eosinophils in the airways [23-24]. Elevated levels of the TNF-α cytokine are directly associated with the increase in adhesion molecules and, consequently, with the increase in eosinophil influx in asthma [25, 26]. Another cytokine that plays a central role in asthma is IL-13, participating in mucus production, bronchial hyperreactivity, IgE synthesis and eosinophils recruitment and survival [23, 27-29]. In addition, potent eosinophils chemoattraction is stimulated by RANTES [30-31]. Our results showed that treatment with LINS01007 (3 mg/kg) significantly reduced eosinophilic infiltration, IgE expression and IL-5, IL-13, IFN-γ, TNF-α and RANTES cytokines/chemokines production. Several studies have shown that the blockade of H_4R decreases mast cell activation, cytokine production and eosinophil chemotaxis in inflammation and asthma [8, 32, 33]. Other authors have showed that H_4R downregulates IL-4, IL-5 and IFN-γ cytokines production, which can affect eosinophilic infiltration in both lung tissue and airways [34].

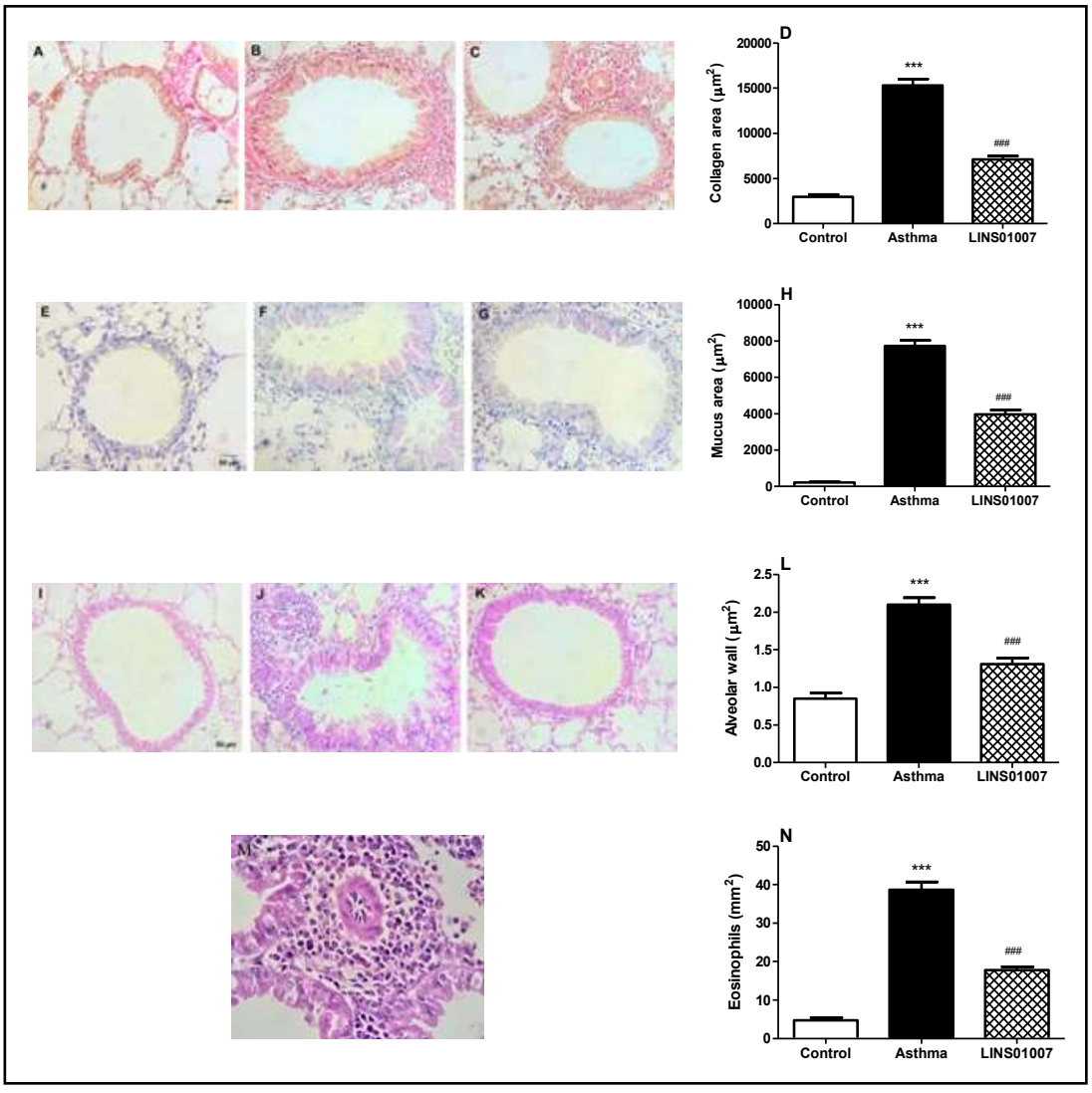

Fig. 6. Effect of treatment with LINS01007 on the lung tissue. Lungs section were taken from animals with asthma and after treatment with LINS01007 (3 mg/kg), stained with picrosirius for collagen (A=Control, B=Asthma, C=LINS01007, D=collagen quantification); PAS for mucus (E=Control, F=Asthma, G=LINS01007, H=mucus quantification); hematoxylin/eosin for alveolar wall (I=Control, J=Asthma, K=LINS01007, L=alveolar wall quantification) and eosinophil count (M=asthma group and N= eosinophil count). Data are representative of 5 animals in each of 3 independent experiments. *** P< 0.001 in comparison with the control group and ### P< 0.001 in comparison with the asthma group.

The IL-6 cytokine is produced by inflammatory cells and also by lung epithelial cells after allergic stimulation [35]. In asthma, IL-6 appears to be important in regulating effector T-CD4 cells by inducing IL-4 production during Th2 cells differentiation and thus inhibiting Th1 cells distinction [36, 37]. These findings corroborate with our results, since an increase in the IL-6 levels in lung tissue was found, accompanied by an increase in Th2 cytokines (IL-5 and IL-13). Recently, it has been observed significant increase in the IL-6 levels in severe persistent asthmatic patients when compared to moderate asthmatics individuals [38]. Additionally, it was demonstrated that IL-6 is essential for mucus hypersecretion by airway epithelial cells in a model of pulmonary allergic inflammation [39]. Another study from the same group demonstrated positive correlation between IL-6 and IL-13 levels in asthma [40]. In the present work, we observed that treatment with the H_4R antagonist LINS01007

significantly reduced the levels of IL-6 and IL-13 cytokines, leading to reduced stimulation on epithelial cells, decreased mucus production and also airway thickening. To the best of our knowledge, this is the first study showing that treatment with an H_4R antagonist decreased the levels of IL-6 cytokine, which can alter the Th1 / Th2 cells balance and consequently the production of IL-5 and IL-13 with the decrease in IgE levels and mucus production in a murine model of asthma.

During the inflammatory process, endothelial cells, eosinophils and other inflammatory cells are responsible for the production of lipid mediators such as prostaglandins, thromboxane and leukotrienes from arachidonic acid by the enzymes COX-2 and 5-LO, which have their expression increased [41, 42]. Previous studies have demonstrated the importance of lipid mediators in allergic inflammation and suggested that eosinophils are the main cells responsible for the production of these mediators in different asthma models [43]. In addition, further studies show that leukotrienes and prostaglandins are clearly involved in the pathogenesis of asthma, contributing to the appearance of several events such as cell infiltration, mucus secretion and bronchial airway hyper reactivity [44]. Our results showed that treatment with LINS01007 at 3 mg/kg dose significantly reduced COX-2 and 5-LO expression in lung tissue 24 h after the second antigen challenge. These events lead to a decrease in inflammatory infiltrate in the BALF and lung tissue, and also in mucus and collagen production. Several studies with different H_4R antagonists showed their ability to inhibit the synthesis of prostaglandins and leukotrienes [7, 45, 46], corroborating with our findings to LINS01007. Curiously, previous study from our group demonstrated that treatment with LINS01005 was also able to reduce the eosinophilic infiltrate in the BALF and the expression of COX-2 in the lung tissue, although it has low affinity to H_4R [16], which raised questions about the relationship between this effect and the ability to antagonize H_4R. The present study reveals that the anti-inflammatory effect of LINS01 compounds are, at least partially, correlated to the antagonistic effect on H_4R.

NF-kB plays a central role in the development of airway inflammation in asthma. The activation of NF-kB pathway modulates cytokine expression and Toll-like receptor (TLR) activation in different cell types regulating the expression of immunomodulatory and inflammatory mediators [47]. One of the first studies demonstrating the involvement of NF-kB in asthma models was developed by Yang et al. (1998), showing that mice that do not express the NF-kB family proteins presented relative protection against the establishment of allergic inflammatory diseases [48]. Further works showed that NF-kB activation occurred predominantly in the respiratory epithelial cells, while its inhibition downregulated the development of allergic lung inflammation [49, 50]. Respiratory epithelial cells and eosinophils can stimulate the production of the transcription factor NF-kB, increasing the synthesis of pro-inflammatory cytokines such as TNF-α and IL-1 [47, 51]. It was previously shown that H_4R stimulation activates various signaling pathways including ERK1/2, Akt and NF-kB, leading to the production of IL-13 and RANTES that are indispensable for the eosinophils migration and survival [33]. Another study demonstrated that H_4R activation is efficient to mediate the activation of the NF-kB pathways followed by the activation of the JAK/STAT pathway [52]. Recently, it was observed in a murine asthma model that treatment with a novel H_4R antagonist (N-(2-Aminoethyl)-5-chloro-1H-indole-2-carboxamide) decreased the activation of ERK1/2, Akt, SAPK/JNK and NF-κB signaling pathways, in addition to reduced Th2 cytokines production in BAL and eosinophilic infiltration in lung tissue [14]. Activation of the STAT3 signaling pathway proved to be important for Th2 cells differentiation, cytokines production, allergic inflammatory response increasing and airway remodeling [53, 54]. Studies showed that inhibition of the STAT3 signaling pathway in mast cells impaired FcεRI-mediated signaling and reduced degranulation in humans [55]. Recently, it has been demonstrated that inhibition of the STAT3 signaling pathway attenuated chronic allergic lung Inflammation in mice [56, 57]. These results are in line with our present findings, which demonstrated that treatment with the H_4R antagonist LINS01007 decreased NF-kB and STAT3 signaling pathways activation, thus reducing the development of allergic murine asthma.

Conclusion

In summary, the results presented herein suggest that the inhibition of NF-kB and STAT3 signaling pathways plays a major role in the reduction of pro-inflammatory cytokines levels observed after treatment with the LINS01007. These factors decrease the activation of the arachidonic acid pathway, reducing the inflammatory response and IgE levels, downregulating airway remodeling and histological parameters in bronchial tissue. Considering this, the effects of LINS01 compounds seem highly correlated to the antagonistic effect on H_4R, with LINS01007 demonstrating its potential as anti-inflammatory agent against allergic inflammatory diseases. These results also shed light on H_4R antagonists as future antiasthma drugs.

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Author Contributions

AMB executed and analyzed the data from *in vivo* assays and wrote the manuscript. LJSL performed the *in vivo* assays. GABF and MFC prepared the compounds for testing. EAGM designed the study, executed and analyzed the IgE data. MAVL designed the study, interpreted the results and revised the manuscript. JPSF and RGL designed the study, wrote the manuscript, supervised the work of the group and enabled the entire study. All authors approved the final version of the manuscript.

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Disclosure Statement

The authors declare no conflicts of interest.

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