

Alleviation of asthma-related symptoms by a derivative of L-allo threonine

JIN-CHUL HEO^{1,3} and SANG-HAN LEE^{1,2}

¹Food and Bio-Industry Research Institute and ²Department of Food Science and Biotechnology, Graduate School, Kyungpook National University, Daegu 702-701, Republic of Korea

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Abstract. Chronic asthma is characterized by inflammatory cell infiltration and tissue remodeling, leading to subepithelial inflammation. In order to evaluate the anti-asthmatic activity of LX519290, a derivative of L-allo threonine, we performed several *in vitro* and *in vivo* anti-asthmatic assays. Using ovalbumin (OVA)-sensitized C57BL/6 mice, the effects of LX519290 on lung inflammation and cytokine expression in the asthmatic animals were analyzed. Treatment with this compound increased IFN- γ and decreased IL-10 mRNA expression. LX519290 potently decreased, not only immune cell infiltration in the lung, but also IL-4 and IL-13 cytokine levels in the serum of OVA-treated mice. The results demonstrated that LX519290 decreased the pathogenesis of chronic airway injury. Evidence from our model of OVA-induced asthma demonstrated that LX519290 inhibits immune cell infiltration, mucus hypersecretion, and inflammatory cytokine production. Collectively, our findings suggest that LX519290 has the potential to ameliorate asthmatic symptoms by treating inflammatory factors in the lung.

Introduction

Asthma is a chronic condition of the respiratory system in which the airways occasionally constrict, become inflamed, and are congested with excessive amounts of mucus (1). Similar to other complex diseases, environmental and genetic factors have been identified as potential causes of asthma although the precise mechanisms are not fully understood (2). Most asthma medications work by preventing bronchospasms and/or reducing inflammation. Improvements in available

therapies, such as the development of fast-onset once-a-day combination drugs with better safety profiles are highly desirable. Immune signal transduction inhibitors and antioxidants that target specific pathways or mediators could be useful for treating asthma (3). Biological compounds directed against the IL-4 or IL-13 pathway, and new immunoregulatory agents that modulate the functions of T-regulatory and T helper-17 cells are also potential anti-asthmatic treatments (4). Although a cure is unlikely to be developed in the near future, a greater understanding of the mechanisms underlying asthma pathogenesis could make this a reality.

The lung is composed of a unique tissue subjected more frequently to oxidant stress compared to most organs as it is directly exposed to higher oxygen tensions (5). Thus, partial pressure of oxygen in the alveoli is much higher than that in other vital organs such as the heart, liver and brain (6). Because the lung is directly exposed to ambient air, lung cells experience enhanced oxidant stress caused by environmental irritants, oxidants and pollutants such as cigarette smoke, ozone and environmental carcinogens that generate free radicals (7). A typical symptom of most lung disorders and infections is inflammation and activation of inflammatory cells (8).

While screening for anti-allergenic compounds using high throughput-compatible assays with combinatorial chemical libraries in a previous study, we obtained a unique hit that was identified as a derivative (LX519290) of L-allo threonine (9). L-allo threonine is a diastereoisomer of L-threonine that does not naturally exist in the human body (10). This amino acid is a component of globomycin, a new peptide antibiotic with spheroplast-forming activity. In *E. coli*, the amino acid can be produced by acetaldehyde and glycine with a serine hydroxymethyl transferase-catalyzed aldol reaction in the presence of pyridoxal phosphate (11). Interestingly, L-allo threonine cannot be metabolized into L-threonine in chickens although the ability of humans to metabolize this compound remains unknown (12,13).

Recently, we published a study in which LX519290 exhibited anti-atopic activity in a 2,4-dinitrofluorobenzene (DNFB)-induced animal model (14). In our murine asthmatic model, a number of disease symptoms including infiltration of inflammatory cells (particularly eosinophils), loss of airway structural integrity and airway obstruction were markedly attenuate in ovalbumin (OVA)-challenged mice compared to

Correspondence to: Professor Sang-Han Lee, Department of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea
E-mail: sang@knu.ac.kr

Present address: ³Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology, Daejeon 305-343, Republic of Korea

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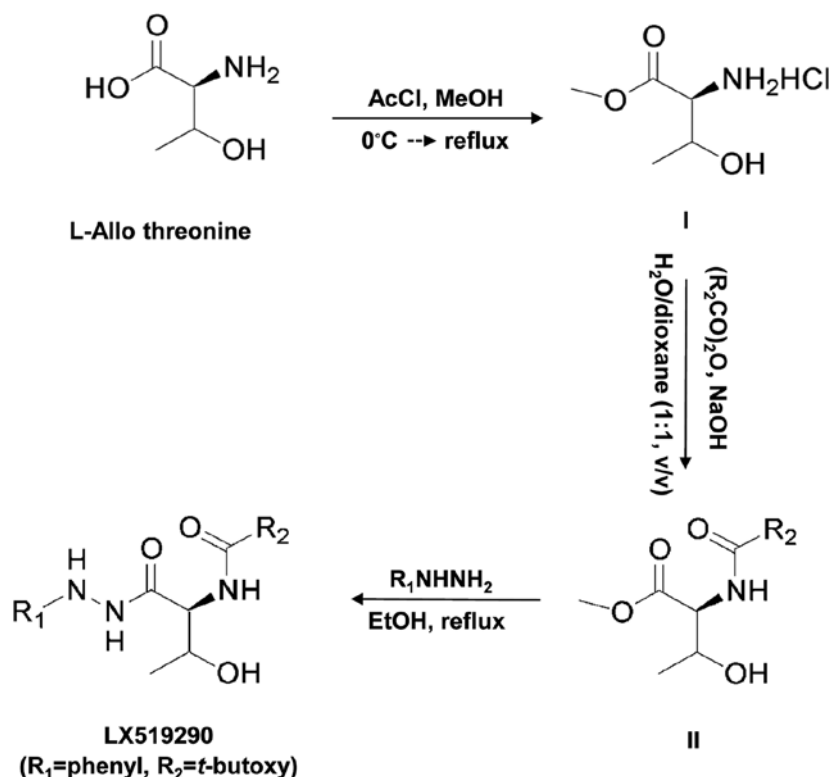


Figure 1. Structure of LX519290. Chemical synthesis was carried out as described in Materials and methods. After three steps using L-allo threonine, LX519290 was obtained as a pure compound (up to 99.5%). LX519290 has 2 derivatives: phenyl and t-butoxy forms.

control animals. In the present study, we therefore assessed the anti-asthmatic activity of LX519290 administered after a single post-OVA-challenge in a mouse model.

Materials and methods

Chemicals, reagents and cells. OVA, alum, hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phospho-specific as well as non-phospho-specific p38, JNK and ERK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). CD4⁺, CD8⁺, IL-17E and HRP antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). LX519290 was prepared as described in Fig. 1. The detailed protocol used for preparing the compound is described elsewhere (14). Other reagents used were commercially available. CCRF-CEM cells (T lymphoblastic cells) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA; no. CCL-119).

Cell viability. Cell viability was assayed using a CCK-8 cell proliferation assay kit (Dojindo, Kumamoto, Japan), as follows: cells ($5 \times 10^5/\text{ml}$) were plated in 96-well plates, and incubated for 24 h in 100 μl of RPMI-1640 medium. Various concentrations (1, 3, 10, 30, 100 and 300 $\mu\text{g}/\text{ml}$) of LX519290 were added to the cells, and incubated for an additional 48 h. Next, 10 μl of MTT solution (5 mg/ml MTT in PBS) was added to each well, followed by incubation at 37°C for 4 h. To stop the reaction, 100 μl of 0.04 M HCl was added to isopropanol with vigorous mixing. Absorbance was measured with a Victor multilabel counter (Wallac, Turku, Finland) at 564 nm.

Animal care. Male C57BL/6 mice (5-6 weeks of age, ~20-23 g) were supplied by Samtaco (Osan, Korea). All mice had free access to tap water and chow food (Purina Korea, Inc., Seoul, Korea). The animals were kept in an air-conditioned room at $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity. All procedures complied with the guidelines of the Committee of the International Association for the Study of Pain Research and Ethical Issues (15), and all regulations of the Committee of Laboratory Animal Ethics, Kyungpook National University (Daegu, Korea). All animals were acclimated to the laboratory environment for at least 7 days prior to initiating the experiments. The mice were divided into groups of 5 animals.

Immunization and OVA-challenge exposure to mice. Immunization and subsequent challenge of the mice were carried out as previously described by Heo *et al* (16) with slight modification. In brief, C57BL/6 mice were intraperitoneally (i.p.) injected with 50 μg of OVA absorbed on 1 mg of alum on days 0 and 11. On day 22, mice were exposed 3 times to aerosolized OVA (10 mg/ml) in 0.9% saline for 30 min, and then every other day for 6 more days (on days 22, 24 and 26). Control mice were injected with 0.5 ml of sterile saline using similar equipment and schedules (Fig. 2).

LX519290 treatment. The OVA-sensitized C57BL/6 mice were divided into 2 groups: one was challenged with OVA and the other was treated with saline (as control). The OVA-challenged group was further divided and treated with or without LX519290. One day after the first challenge with OVA (day 22), the animals received 3 i.p. injections of LX519290 (1 mg/kg in 0.2 ml of saline). This treatment was

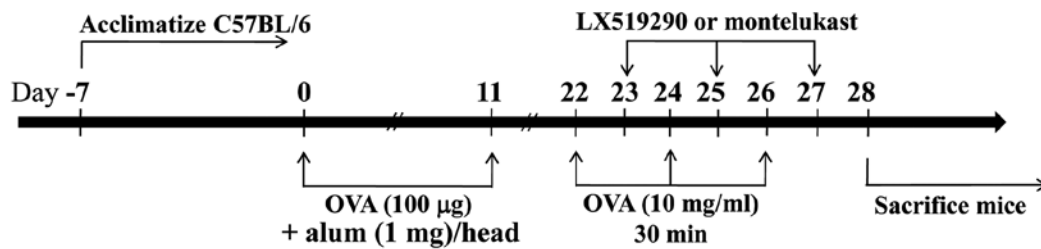


Figure 2. Experimental design for developing an animal model of asthma. Intraperitoneal injection (i.p.) of OVA (100 µg) dissolved in alum (1 mg) was administered on days 0 and 11, and OVA-challenge was delivered on days 22, 24 and 26. The control and experimental groups of mice were treated saline or LX519290 (1 mg/kg in 0.2 ml of saline), respectively, by i.p. injection on days 23, 25 and 27. After 24 h, the mice were sacrificed by cervical dislocation, and the lungs were analyzed.

repeated every second day for 6 days (days 23, 25 and 27). After 24 h of the last treatment (day 27), blood was collected to measure serum IL-4, IL-13 and IgE. The lungs were then removed for histological and cytokine analyses.

Lung histological evaluation. The lungs were fixed with 10% paraformaldehyde in 0.1 M PBS (pH 7.4) and embedded in paraffin as previously described (16,17). Tissue sections were subjected to staining with H&E to observe general morphology, immunohistochemistry, and PAS reaction to assess mucus production in the airway epithelium. Paraffin blocks were cut into section (6 µm), and mounted on glass slides. Paraffin in the lung sections was removed by treatment with xylene and serial dilutions of ethanol, and then stained with H&E. Cells positive for CD4⁺, CD8⁺ and IL-17E that had migrated into the lung were detected with immunostaining. All slides were incubated in 0.3% H₂O₂ in methanol overnight at room temperature to quench endogenous peroxidase activity. Immunostaining was performed overnight at 4°C with anti-mouse CD4⁺, CD8⁺ or IL-17E antibodies diluted 1:200 with 1% bovine serum albumin (BSA) in PBS. The sections were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:500 in 5% BSA in PBS at 37°C for 1 h. The sections were also stained with 1% Schiff's reagent, and finally stained with Mayer's hematoxylin for 5 min at room temperature.

Measurement of serum IgE levels. Serum IgE levels were measured by an ELISA as previously described (18). Sera were obtained from mice 24 h after challenge with either saline or OVA. ELISAs specific for IL-4 and IL-13 were conducted using a matching antibody pair (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The secondary antibodies were conjugated to HRP. Residual substrate readings taken at 450 nm were converted into pg/ml according to standard curves generated with recombinant IL-4 and IL-13.

RT-PCR analysis of gene expression. Total RNA were extracted using TRI reagent (Molecular Research Center, Cambridge, UK) according to the manufacturer's protocols. The RNA (1-10 µg) from mouse spleen cells or CCRF-CEM cells (T lymphoblastic cells) was transcribed into first-strand cDNA with random primers [or oligo(dT)] in a reaction volume of 20 µl using an RT kit (Intron, Seoul, Korea), and 4 µl of product was used as the PCR template. Sequences of the primers used

for PCR amplification are shown as follows: 5'-TGAGGC CCAGCTTAAAGA-3' and 5'-CTGTGGACCCCTGCCATA GAT-3' for Wiskott-Aldrich syndrome gene-like protein (N-WASP); 5'-CACTGCTATGCTGCCTGCTC-3' and 5'-TCT TCACCTGCTCCACTGCC-3' for IL-10; 5'-ATGTTCCAG TATGACTCCAC-3' and 5'-GCCAAAGTTGTCATGGA TGA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Genes for IL-10, N-WASP and GAPDH were amplified with denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec.

Western blot analysis. Cell lysates were prepared using an M-PER Mammalian Protein Extraction Reagent, and the protein concentration was determined using a BCA assay kit (both were from Pierce Biotechnology, Rockford, IL, USA). The proteins were separated with SDS-PAGE (8%), and immunoblotting was conducted with antibodies against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK and ERK as described elsewhere (19). Antibody binding was detected with an enhanced chemiluminescence western blotting detection reagent (Animal Genetics, Inc., Suwon, Korea), and viewed with an LAS3000 imaging system (Fujifilm Co., Tokyo, Japan).

Statistical analysis. Data are expressed as the mean ± standard deviation. The results were analyzed by a multiple range test using SPSS 9.0 (SPSS, Chicago, IL, USA). P-values <0.1 or <0.05 were considered to indicate statistically significant results.

Results

In vitro cytotoxic effects of LX519290. The effects of LX519290 on the cell viability in RAW264.7 cells was extensively investigated. Cell viability with various concentrations of LX519290 was measured with a CCK-8 cell proliferation assay kit. The results showed cell viability of 100±7.8, 93±2.2, 97±5.2, 94±6.6, 87±7.3 and 86±5.2%, respectively, indicating that the present concentrations (1, 3, 10, 30, 100 and 300 µg/ml) did not adversely affect cell viability (data not shown).

Histological analysis of lungs from LX519290-treated mice. Forty-eight hours after the final OVA or saline treatment in each group, lung tissues were excised to assess inflammatory cell infiltration, mucus release and cytokine expression. The effect of LX519290 on airway inflammation and remodeling

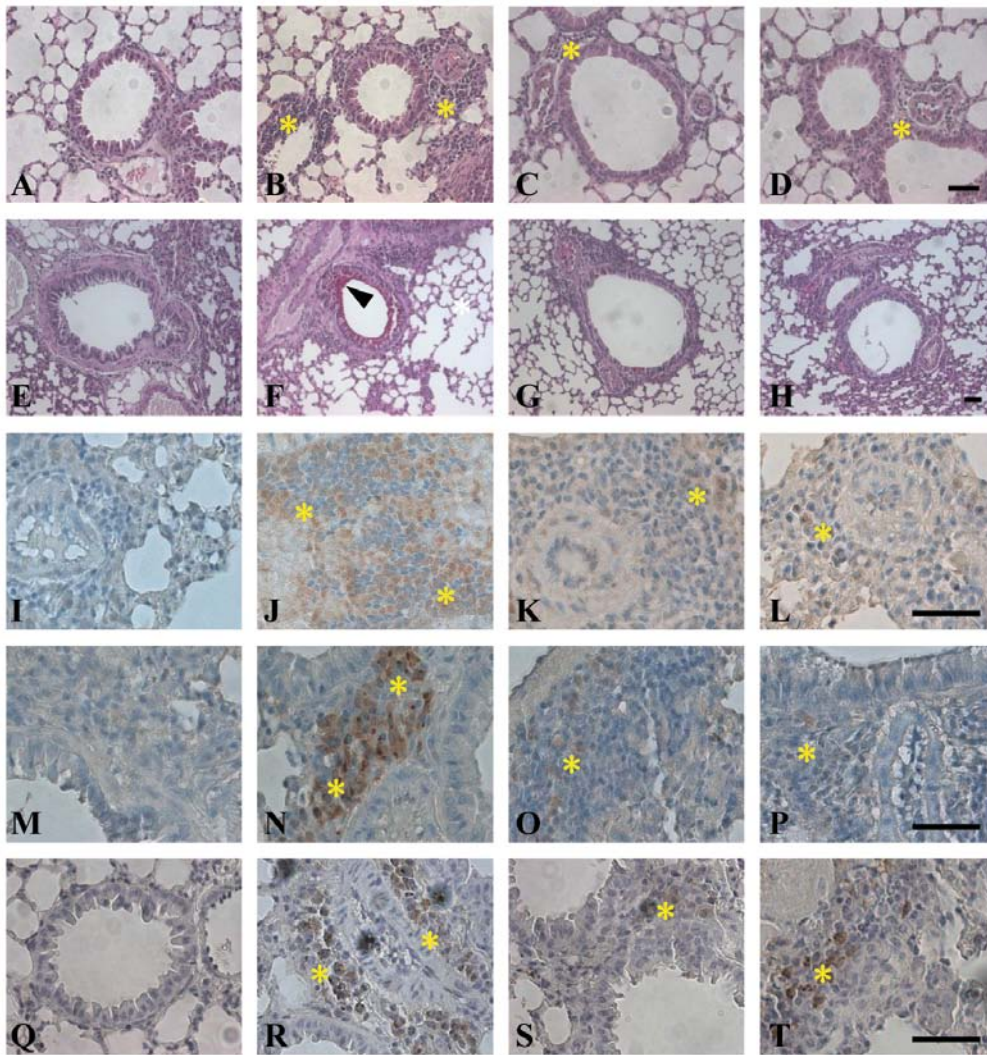


Figure 3. Histological and immunohistochemical analyses of LX519290-treated lung tissues in OVA-challenged mice. (A) Control saline-challenged mice were injected with vehicle. OVA-challenged mice were injected with (B) vehicle, (C) LX519290 and (D) Montelukast administration by H&E (first line) and PAS (second-line) staining. The degree of immune cell infiltration was less in the LX519290-treated group than in the OVA-treated group. Scale bars, 50 μ m. (I and M) In mice that had not been previously challenged with OVA, CD4⁺ or CD8⁺ cells in the airway were less immunoreactive. (J and N) OVA-challenged mice showed significant increase in the number of CD4⁺ or CD8⁺ cells in the airway. Degree of CD4⁺ or CD8⁺ cell infiltration was less in the (K and O) LX519290-treated group or (L and P) Montelukast-treated group than in the OVA-treated animals. Scale bars, 50 μ m. Asterisks denote the cells positive for CD4⁺ or CD8⁺ antigens. The presence of IL-17E was identified by immunohistochemistry and appears as brown staining in the figure. (Q) In mice that had not undergone prior OVA-challenge, low IL-17E expression was observed in the airway. (R) OVA-challenged mice showed significant increases in IL-17E expression in the airway. IL-17E expression was lower in the (S) LX519290-treated group and (T) Montelukast-treated group than in the vehicle-treated animals. Scale bar, 50 μ m.

was evaluated according to lung histology. Histological analysis of lungs taken from both OVA-sensitized mice and control animals exposed to saline aerosol showed that the lungs of all mice were normal (Fig. 3). In contrast, lungs from mice challenged with aerosolized OVA showed severe inflammation, showing that the lungs showed clear and widespread inflammatory cell infiltration (Fig. 3B, F, J, N and R). OVA-challenged animals had increased smooth muscle thickness in the airways compared with the control mice (Fig. 3F, arrowhead). On the other hand, LX519290 prevented airway smooth muscle hypertrophy observed in the OVA-challenged animals (Fig. 3C, G, K, O and S). Lung sections from LX519290-treated mice also showed a decrease in the number of inflammatory cells. The OVA-challenged group showed remarked increases in CD4⁺ (Fig. 3I-L) and CD8⁺ (Fig. 3M-P)

T cells. PAS staining of neutral mucus revealed that the epithelial cells surrounding airway tissues increased in number (Fig. 3F) compared to the control group (Fig. 3E). Mucus production in airway tissues was decreased in the LX519290-treated group (Fig. 3C) compared to the OVA-challenged group (Fig. 3B). These results demonstrated that LX519290 strongly inhibits asthmatic symptoms in the lung.

Cytokine expression evaluated by immunohistochemistry. Immunohistochemistry revealed that only a few cells positive for IL-17E staining were present in the lungs of the control group (Fig. 3Q). In the OVA-challenged group, IL-17E immunoreactivity was observed in some mononuclear cells infiltrating around the airways and vessels (Fig. 3R). The number of mononuclear cells expressing IL-17E in lung

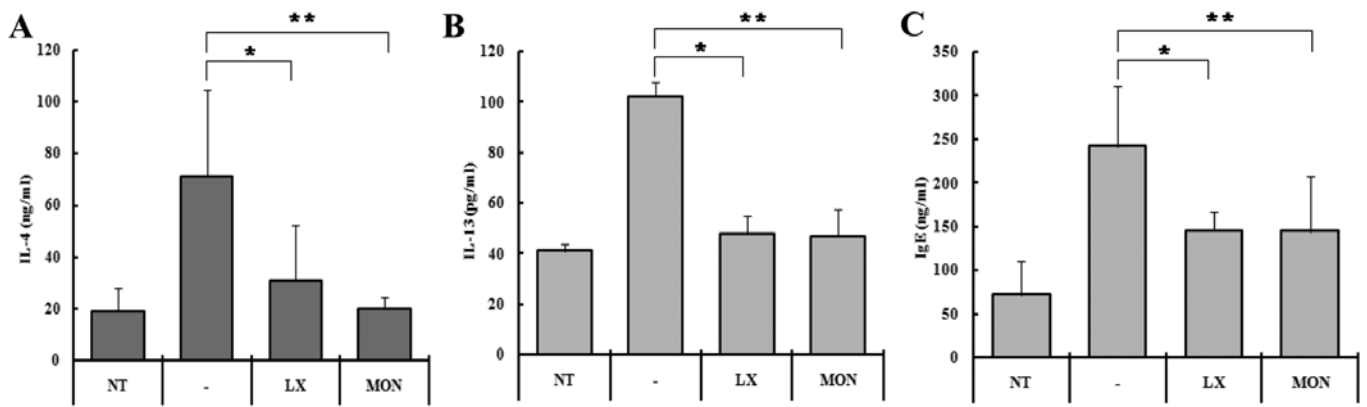


Figure 4. Comparison of levels of cytokines and mediator in serum. The levels of (A) IL-4, (B) IL-13, and (C) IgE were determined by an ELISA kit. The expression levels were measured in the LX519290-treatment group compared to the OVA-challenged group (*P<0.1).

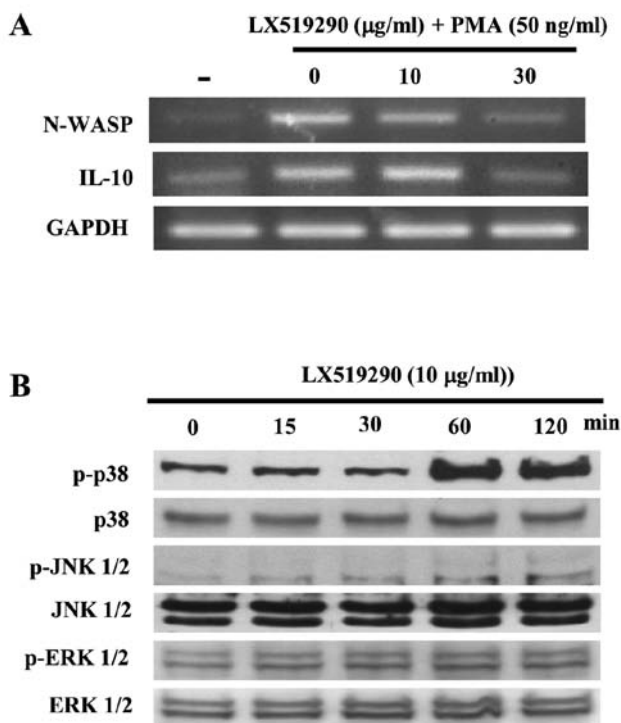


Figure 5. Effects of signaling molecules by LX519290. (A) The mRNA expression of N-WASP and IL-10 was determined by RT-PCR as described in Materials and methods. (B) The protein expression was determined by western blotting. Resolved proteins were immunoblotted with antibodies against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK and ERK, and developed using an LAS3000 imaging system.

tissues was significantly decreased in the LX519290-treated group (Fig. 3R and S). To further study the changes in cytokine expression, we measured IL-4 and IL-13 levels in serum (Fig. 4). IL-4 concentration in the serum of the control mice increased from 375±184 to 1420±665 ng/ml following OVA-challenge (Fig. 4A). In the mice treated with LX519290 and Montelukast, the IL-4 production rate was reduced ~60% when compared to the OVA-challenged mice (Fig. 4A). IL-13 levels in the serum of the control mice increased from 41±2.9 to 102±5.9 ng/ml following OVA-challenge (Fig. 4B). In the mice treated with LX519290

and Montelukast, the IL-13 levels were reduced to ~53% compared to the OVA-challenged mice.

In order to determine whether the expression of cytokines related to asthma was decreased by LX519290, we measured the serum levels of IL-4 and IL-13 with an ELISA. In the control group, the IL-4 concentration was ~375±184 pg/ml, whereas it was 1420±665 in the OVA-challenged group (Fig. 4A). This increase in IL-4 explains why the OVA-induced immune responses in the leukocytes increased the cell numbers 5.5-fold (Fig. 4A, second column). Treatment with LX519290, however, reduced the level of IL-4 to ~10% (Fig. 4A, third column) which was a dramatic decrease compared to that achieved with Montelukast as a positive control. We also hypothesized that the level of IL-13 would also decrease in the LX519290-treated group. We were able to demonstrate that IL-13 levels were reduced by 91.6% in the LX519290-treated mice challenged with OVA (Fig. 4B). This effect was also observed in lung tissues from LX519290-treated mice (data not shown). To further confirm whether IgE levels in the serum were affected by LX519290 treatment, we performed an ELISA. IgE concentrations in the serum increased in the OVA-treated mice (242±68 ng/ml), but in the mice treated with LX519290, serum IL-4 levels were sharply reduced to 145±21 ng/ml (Fig. 4C).

Analysis of gene expression by RT-PCR. We examined whether mRNA levels of CD4⁺, CD8⁺, the Th1-type cytokines IFN-γ and TNF-β, and Th2-type cytokine IL-10 are altered in mouse primary spleen cells by treatment with LX519290 for 24 h. For this, we isolated mouse spleen cells, treated them with LX519290, and compared the gene expression profiles to those of the control. At a concentration of 30 µg/ml, LX519290 did not significantly change CD4⁺ and CD8⁺ mRNA expression levels (data not shown).

Western blot analysis of signaling molecules. We next examined whether LX519290 affects the expression of cytokines and signaling molecules. As shown in Fig. 5A, IFN-γ expression sharply increased in a concentration-dependent manner while IL-10 expression dramatically decreased following treatment with up to 30 µg/ml of LX519290 (Fig. 5A). Notably, N-WASP expression in mouse spleen cells was inhibited by LX519290 treatment (Fig. 5A). The level

of p38 phosphorylation in CCRF-CEM cells significantly increased after 60 and 120 min of LX519290 treatment whereas the levels of phospho- as well as non-phospho-JNK and -ERK were unchanged (Fig. 5B).

Discussion

Research has shown that various antioxidants help prevent DNA damage, mutagenesis, carcinogenesis and the growth of pathogenic bacteria (20). Antioxidants mitigate many inflammatory events to permit the healing of tissues damaged by free radicals, and are often associated with the prevention of free radical production in biological systems (21). It has been estimated that all cells in our body produce many radicals. Ubiquitous scavenging enzymes including catalase, superoxide dismutase and xanthine oxidase may successfully clear surplus radicals in tissues (22). Dietary supplements including vitamins C and E can prevent damage caused by free radicals (23). Thus, the antioxidant capacity is widely used as a parameter to characterized beneficial foods or medicinal compounds that possess bioactive properties which help regulate body homeostasis (23). It is therefore important to evaluate the molecular mechanisms underlying the inflammatory process and oxidative stress. This is needed to develop novel anti-asthmatic therapies (24). Moreover, the demand for novel agent(s) that can prevent or treat allergic asthma is increasing. Allergen-induced airway diseases are caused by allergen-specific Th2-mediated cytokines (24). Production of these factors can be induced by airway inflammation, Th2 cell activation, inflammatory cells, and Ig isotype switching involving T and B cells. Certain agent(s) can reduce asthmatic symptoms by affecting the production and secretion of various Th2-type cytokines such as IL-4, IL-5, IL-13 and TNF- α (25,26). In our study, we examined whether LX519290 administration affects cytokine expression in an *in vivo* model. It was evident that LX519290 decreased IL-10 mRNA expression whereas IFN- γ mRNA expression levels were increased (Figs. 4 and 5).

It has been suggested that onset of the asthmatic response is controlled by CD4⁺ T cells which produce Th2-type cytokines, such as IL-3, IL-4, IL-5, IL-10 and granulocyte macrophage-colony stimulatory factor (GM-CSF). IL-4 and IL-13 are thought to be most closely associated with allergic asthma although IL-17E, IL-23, IL-31, IL-33 and IL-35 are potential initiators of asthmatic events (27). IL-13 is involved in the growth, differentiation, and activation of eosinophils, which are effector cells of the asthmatic response. IL-4 stimulates the switching of B cell isotypes to promote the production of IgE (28). IL-4 also plays a crucial role in the differentiation of Th1 into Th2 lymphocytes *in vitro* and *in vivo*. A mutant mouse model of asthma indicated that IL-4 and IL-13 are essential for activating allergen-induced pulmonary eosinophils (29).

As expected, LX519290 reduced IL-4 and IL-13 cytokine expression in mice, but these changes in saline-challenged mice were not significant compared to control animals. After exposure to OVA, serum obtained from vehicle-treated mice contained significantly increased amounts of IL-4 and IL-13 in conjunction with eosinophil infiltration in the lung. Compared to the corresponding results in saline-challenged mice, the

number of total leukocytes from the OVA-challenged animals treated with LX519290 (1 mg/kg) significantly decreased compared with that of the OVA-challenged lung tissues (Fig. 3). When we observed cells positive for CD4⁺, CD8⁺ and IL-17E by immunostaining, we found that there were a significant decrease in these parameters compared to lung tissues from OVA-challenged mice (Fig. 3).

The Wiskott-Aldrich syndrome protein (WASP) family of proteins activates the ARF2/3 complex in response to signals that induce cell migration (30). In the present study, LX519290 induced changes in cell morphology, and inhibited N-WASP and ARF3 mRNA expression when spleen cells were treated with PMA (Fig. 5A and data not shown). Elevated numbers of activated CD4⁺ T cells, mast cells, and eosinophils, in both bronchial mucosa and bronchoalveolar lavage (BAL) fluid are classic features of asthma and related symptoms (31). These inflammatory cells may release cytokines that have the potential to augment cell-mediated immune reactions, and induce tissue damage and dysfunction. In the present study, we evaluated a unique approach for treating asthma by obtaining a derivative of L-allo threonine. To do this, we performed a high throughput screening-compatible assay to measure antioxidant activity along with a T-bet promoter assay. The results showed that LX519290 possessed strong antioxidant activity. There is evidence of ERK1/2 and p38 phosphorylation with the development of asthmatic symptoms in human patients (19). In our study, we did not observe a similar phenomenon in mice following LX519290 treatment. Instead, we found that p38 phosphorylation was dramatically increased in CCRF-CEM cells, which are derived from T cells originating from lymphoblasts. It is assumed that CCRF-CEM cells act similar to cells of Th1 lineage since the cells showed a strong IL-2 production following PMA treatment (32). This finding suggests that LX519290 affects the activation of Th1 cell populations. It is possible that the number of Th2 cells in the general cell population is normally decreased whereas that of Th1 cells is increased during the onset of asthmatic inflammation.

In conclusion, this is the first report demonstrating that LX519290 has anti-asthmatic effects *in vitro* and *in vivo* by modulating IL-4 and IL-13 expression. These activities were confirmed by H&E staining, ELISA, immunohistochemistry, and IgE activity assays. If the anti-asthmatic activity of LX519290 could be manipulated by controlling asthma-related molecular expression, this may be useful for approaches in preventive and curative medicine. Further investigations should be conducted to study the homeostasis between Th1 and Th2 cell balance in lung tissue; in that there is no drug that can effectively cure asthma and/or related disorders.

Acknowledgements

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