A Derivative of 1-Allo Threonine Alleviates 2,4-Dinitrofluorobenzene-Induced Atopic Dermatitis Indications

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LX519290, a synthetic derivative from a combinatorial chemistry library, has been screened for anti-atopic activity, but its biological activities have not yet been elucidated. To assess whether LX519290 has the potential to lessen 2,4-dinitrofluorobenzene-induced atopic dermatitis symptoms in mice, first we sensitized the skin in the dorsal neck of C57BL/6 mice and re-sensitized the ear skin to determine the ear thickness. Then, we tested to determine whether LX519290 affect atopic dermatitis symptoms in vivo. The results indicate that LX519290 significantly mitigated inflammation indications including ear thickness, total T-cell numbers, and eosinophils. Moreover, the treatment drastically inhibited the levels of mediators such as interleukin-17E and histamine by 52% and 37% of control, respectively. Taken together, the data suggest that LX519290 can alleviate atopic parameters in mice.

Key words: 1-allo threonine; atopic dermatitis; inflammation; symptoms; 2,4-dinitrofluorobenzene

The recent prevalence of allergic diseases, including atopic dermatitis, allergic rhinitis, allergic asthma, and so on, over the last few decades, especially in infants and children in developed countries and cities has been on the rise.1-3 These allergic disorders might result from ever-increasing industrial and environmental risk factors such as antigens, synthetic chemicals, artificial foodstuffs, etc. Over the past few decades, the incidence of allergic diseases, especially atopic dermatitis, in developed countries and cities has been increasing persistently by 2–3 times; but, treatments for such allergic diseases and their frequently occurring symptoms are restricted.2,3)

On the other hand, inflammation-related diseases such as atopic dermatitis are considered chronic diseases caused by harsh immune imbalances in T-cell subsets in the skin.4) It has been found that some cytokines (interleukin [IL]-13, IL-17E [or IL-25], IL-31, IL-33, and IL-35) and chemokine receptors (CCR4, etc.) are involved in the micro-environmental control of T-cell lineage in allergic disorders, including atopic dermatitis.4) The activation of mast cells during immune responses requires several cascades of events including Fc receptor-mediated triggering, intracellular calcium increase, down-regulation of cAMP signaling of mast cells, and histamine release.5) A variety of indications such as allergies, edema, itching, bronchial smooth muscle contraction, and vascular permeability are caused by mediators released from mast cells.6,7) If it is possible to estimate protein expression profiles between the effector and target cells related to inflammation, this should help atopic dermatitis, because presently there are few natural agents or other non-steroidal drugs for atopic dermatitis.

The causal mechanism of atopic dermatitis is unknown. To lessen symptoms, temporary treatment with steroids or antihistamines relieves the atopic symptoms exhibited by patients, but the possibility of reverse effects remains with these drugs.8) Nonetheless, there have not been enough studies on the clinical efficacy of such chemicals and natural products. For this reason, we studied the effects of LX519290 on atopic dermatitis-induced barometers using an animal model, in order to evaluate whether it has potency to lessen allergic skin disease of cause still unknown induced by various triggers and antigens.

In the course of screening for anti-allergic agents by high throughput-compatible T-bet promoter assay with combinatorial chemical libraries, we selected a unique compound and identified it as a derivative, LX519290, of 1-allo threonine. 1-Allo threonine is a diastereosomer of l-threonine, which does not exist in the human body.9) It is a component of globomycin, a new peptide antibiotic with spheroplast-forming activity,10) whereas, in E. coli, it can be produced by acetaldehyde and glycine with a serine hydroxymethyl transferase-catalyzed aldol reaction in the presence of pyridoxal phosphate.11) l-Allo threonine cannot be metabolized into l-threonine in chicks,12) although its metabolism in the human body remains unknown.

In this study, we investigated whether LX519290, a derivative of l-allo threonine, ameliorates atopic dermatitis symptoms in vivo. To assess further the decrease in atopic dermatitis symptoms, we examined whether the parameters of ear thickness, total cell counting, and eosinophils would be inhibited by LX519290. Although the DNFBI-induced dermatitis model can generate a contact hypersensitivity-like reaction rather than an IgE/mast cell-dependent immediate hypersensitive reaction, the expression levels of IL-17E, histamine, and other

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barometers (eosinophil expression, epidermis thickness etc.) by LX519290 in the animal model are very important for an evaluation of compounds that cause molecular inflammation in the skin.

Materials and Methods

Materials and chemicals. 2,4-Dinitrophenylbenzene (DNFB; Sigma Chemical, St. Louis, MO) was dissolved in acetone at a concentration of 50 μg/mL. Montelukast was a gift from Merck (Piscataway, NJ). All other chemicals were of commercially grade.

Preparation of LX519290. LX519290 was synthesized as summarized in Fig. 1. Solution of l-allo threonine (1.0 eq) in methanol was added to acetyl chloride (3.0 eq) dropwise at 0 °C. The reaction mixture was allowed to warm slowly to reflux, stirred for 1 h and then evaporated. The crude product was crystallized from petroleum ether to yield l-allo threonine methyl ester 1 as a white solid. The resulting product 1 (1.0 eq) and NaOH (2.5 eq) in dioxane/water (1:1, v/v) was cooled to 0 °C and treated portionwise with di-tert-butylcarbodiimide (1.1 eq) over 10 min. The resulting solution was stirred at 0 °C for 2 h, warmed to room temperature, and concentrated. Then the crude product was partitioned between ethyl acetate and water. The organic layer was collected, dried over MgSO₄, and concentrated. The residue was purified by chromatography to afford a white solid carbamic acid tert-butyl ester 2. A solution of the resulting product 2 (1.0 eq) and phenyl hydrazine (10.0 eq) in ethanol was refluxed at 100 °C for 1 h. The solution was stirred at room temperature, and then the concentrated crude product was partitioned between ethyl acetate and water, and saturated with NaOH. The organic layer was collected, dried over MgSO₄, and concentrated. The residue was purified by chromatography to afford a white solid with a molecular weight of 309.325 (Fig. 1). To date, there have been no findings as to its biological activities. This is the first report on the anti-atopic activity of LX519290. In order to assess the activity of LX519290 on chemical-induced atopic inflammation, we investigated the biological activity and mechanisms by immunohistochemical analysis and observation of cytokines and mediators induced by molecules such as IL-17E and histamine.

First we measured cytotoxicity by MTT assay in order to determine whether the compound was cytotoxic to RAW264.7 cells. The results indicated that fairly high concentrations of the compound (100 μg/mL) did not previously described. Briefly, first sensitization was soaked onto abdominal skin with 50 μL of 0.5% DNFB solution (dissolved in acetonitrile oil = 4:1), followed by successive re-sensitization in the ear skin by repeated application of 20 μL of 0.2% DNFB solution. Application was repeated 4 times every 3 d for 2 weeks, beginning at 5 d after initial sensitization (days 5, 8, 11, and 14). The vehicle mice were treated with acetone alone without DNFB in the same manner. The compound was applied 8 times (days 6, 7, 9, 10, 12, 13, 15, and 16) once a day for 2 weeks from the day after initial DNFB re-sensitization (day 6) during re-challenge.

Measurement of IL-17E and histamine expression by immunohistochemical analysis. Dissected ear tissues were fixed for 24 h in a neutral-buffered formalin solution (10%) and processed in the customary way. Paraffin sections were placed on Probe-On slides and incubated with methanol containing a 3% hydrogen peroxide solution. The tissue sections were treated with 10% normal goat serum for 1 h at room temperature. The slides were incubated overnight at 4 °C with rabbit anti-mouse antibodies. Measurement of the results of immunohistochemical analysis were done by comparing the staining of immune positive cells to IL-17E (Santa Cruz Biotechnology, Santa Cruz, CA) or histamine (Santa Cruz Biotechnology). Positive cell numbers were calculated as values per mm of basement membrane.

Results and Discussion

In the course of screening for anti-allergic compounds by a high-throughput compatible T-bet promoter assay using various combinatorial chemical libraries, we found a single hit, which was identified as a derivative (LX519290) of l-allo threonine. LX519290 has a unique structure that consists of C₁₅H₁₄N₃O₄ with a molecular weight of 309.325 (Fig. 1). To date, there have been no findings as to its biological activities. This is the first report on the anti-atopic activity of LX519290. In order to assess the activity of LX519290 on chemical-induced atopic inflammation, we investigated the biological activity and mechanisms by immunohistochemical analysis and observation of cytokines and mediators induced by molecules such as IL-17E and histamine.

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![Fig. 1. Structure of LX519290 Used in Experiments.](image)
inhibit the cell growth (data not shown). Since LX519290 showed no cytotoxicity, we investigated whether it has potential to decrease dermatitis-related symptoms in vivo. Using the DNFB-induced atopic animal model, we measured how LX519290 affected inflammation of the ear in the mice, and analyzed the inhibitory effects. The normal mice did not show any inflammatory cells near the skin epithelial cell membranes (Fig. 2), but the DNFB-treated group significantly increased numbers of T cells, meaning that the mouse ear skin showed perfectly induced atopic responses. The ear thickness showed a 3-fold increase in the DNFB-treated group, at a value of 1.58 ± 0.17 mm, as compared to the control group (Fig. 2A and B, 2nd column). LX519290 treatment (50 μg/ear) inhibited phenotypic features (Fig. 2A and B, 3rd set of data). Montelukast, a positive control, also displayed a decrease, in the same pattern as the compound (Fig. 2A and B, compare columns 1–4). Montelukast is a specific CysLT1 receptor antagonist, well-confirmed as an anti-asthmatic drug targeted worldwide for relief of asthmatic symptoms such as itching and edema in epithelial and bronchial inflammation. Because it also has excellent anti-atopic effects, we used it as a positive control to compare to our compound’s activity. The immune activities of the compound were also investigated. It had no cytotoxicity and exhibited potent anti-atopic as well as anti-asthmatic activities in vitro and in vivo. The present data strongly suggest that LX519290 has the potential to reduce DNFB-induced atopic indications by ameliorating inflammation features in ear skin.

Next, in order to evaluate further whether inflammatory cells were decreased in number by LX519290, we again excised the tissues around the ear skin of the mice and prepared paraffin sections to count total T cells that appeared during treatment. The results showed that DNFB alone significantly increased the numbers of inflammatory cells in the skin (Fig. 2A and B) and ear thickness (Fig. 3A and B), whereas the LX519290-treated group decreased in numbers of cells and ear thickness (Figs. 2A and B, 3A and B), indicating that it showed anti-atopic activity in the skin (Fig. 3A and B). Next, we compared eosinophils in the DNFB alone and compound-treated group in order to confirm that the compound was anti-atopic in the chemical-induced atopic animal model. On hematological observation, it was obvious that there was a drastic increase in eosinophils during inflammation. Thus, a comparison of numbers of hematological cell types gives information on conditions, in the skin epidermis. As expected, no infiltration of inflammatory cells occurred in the normal tissues, but the DNFB-treated group significantly increased in numbers over not-treated group (Fig. 2B, asterisks). The numbers decreased upon treatment with LX519290, indicating that it inhibited the number of infiltrating inflammatory cells as well as eosinophils in the skin dermis and epidermis (Fig. 3A, B, and C).
It is documented that inflammation-related cells during allergic events execute the following steps: binding of signals to cell membranes; induction of secretion of local hormones such as prostaglandins, leukotrienes, and thromboxanes;22) and excretion of cell mediators from activated mast cells. 23) Mediators released from mast cells result variously in the induction of edema, itching, allergies, bronchial smooth muscle contraction, and other indications. 24) Since the cytokine levels between T cells and mast cells are a pivotal barometer of atopic-related T-cell activation and homeostasis in skin tissues, we compared biomarkers for allergic and anti-atopic mediators as to allergic inflammation. IL-17A/Es are proinflammatory cytokines that induce local cytokine production and recruit granulocytes to sites of inflammation.25) By comparing these mediators, one can conjecture whether the compound is effective for atopic dermatitis as compared to montelukast. Our results indicated that treatment with LX519290 lowered the levels of mediators such as IL-17E and histamine by 52% and by 37% respectively (Fig. 4B and C; compare with control in Fig. 4A), suggesting that LX519290 inhibited cytokine production or decreased the levels of skin T cells and mast cells. Since there has been many convincing results as molecular inflammation and allergic diseases of cytokines such as IL-4, IL-13, IL-31, IL-33, and IL-35,26) the present data suggest that LX519290 has anti-atopic activities on mouse skin tissues by decreasing IL-17E and histamine levels.

In summary, the present data suggest that LX519290 has the potential to lesson DNFB-induced atopic dermatitis symptoms, as we confirmed by inhibiting eosinophil numbers as well as cytokine expression such as IL-17E. Overall, it is interesting that LX519290 exhibited activities that might be turned to nutraceutical or cosmeceutical purposes by alleviating atopic dermatitis in vivo. This might promote the development of atopic dermatitis-treating or preventive therapies, if applied to clinical purposes.

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