

Identification of 4-[4-(4-Fluoro-Phenyl)-Thiazol-2-Ylamino]-2,6-Dimethyl-Phenol (KR-33749) as an Inhibitor of 5-Lipoxygenase with Potent Antiinflammatory Activity

Young Sik Cho^a Chi Hyun Kim^a Ji Hee Surh^a Nam Sook Kang^a
Sung-Eun Yoo^a Hyae Gyeong Cheon^{a, b}

^aCenter for Metabolic Syndrome Therapeutics, Bioorganic Science Division, Korea Research Institute of Chemical Technology, Daejeon, and ^bDepartment of Pharmacology and Pharmaceutical Sciences, Gachon University of Medicine and Science, Incheon, Republic of Korea

Key Words

KR-33749 • 5-Lipoxygenase inhibitor • Antiinflammatory activity

Abstract

Background/Aim: To discover new 5-lipoxygenase (5-LO) inhibitors applicable to inflammation-related skin disease, we identified and examined antiinflammatory properties of a novel 5-LO inhibitor, KR-33749, in vitro and in vivo. **Methods:** 5-LO enzyme activity was assayed using insect cell lysates overexpressing rat 5-LO. The leukotriene B₄ (LTB₄) level was assayed in rat basophilic leukemia (RBL-1) cell line. Mouse ear edema was induced by topical application of arachidonic acid. Atopic dermatitis-like skin lesion was induced by topical application of 1-chloro-2,4-dinitrobenzene (DNCB) to NC/Nga mice. **Results:** KR-33749 inhibited 5-LO activity with an IC₅₀ value of 70.5 ± 6.0 nmol/l in parallel with LTB₄ inhibition in RBL-1 cells. The compound exhibited a >1,000-fold selectivity against 12-LO and 15-LO. KR-33749 showed in vivo protective effects against arachidonic acid-induced ear edema and DNCB-induced atopic dermatitis-like symptoms in NC/Nga mice. **Conclusion:** Our results show that KR-33749, a new 5-LO inhibitor exhibits potent antiinflammatory activities in vitro as well as in vivo. Copyright © 2010 S. Karger AG, Basel

Introduction

Eicosanoids, products derived from arachidonic acid metabolism, include prostaglandins and leukotrienes, and are implicated in the pathogenesis of several inflammatory diseases [1]. In particular, leukotrienes synthesized by lipoxygenases have potent proinflammatory properties including bronchoconstriction and chemotaxis of polymorphonuclear leukocytes. Among various leukotrienes, leukotriene B₄ (LTB₄) is a well-known chemoattractant for inflammatory cells [2], and the other three, LTC₄, D₄ and E₄, which contain a glutathione conjugation, are collectively termed as the cysteinyl leukotrienes [3]. Besides inflammatory diseases, most notably asthma, rheumatoid arthritis and inflammatory bowel disease [4–6], these leukotrienes play a role in the generation of psoriasis and atopic dermatitis, in which a topical application of lipoxygenase products causes psoriasis-like lesions, suggesting their inflammatory roles in the pathogenesis of skin disorders [7].

Given that leukotrienes are elevated in inflammatory diseases [8–10], various strategies to intervene in the action of leukotrienes, like suppression of LTB₄ production and blockade of leukotriene receptors, were employed to produce a favorable clinical outcome for the treatment of

inflammation-related diseases [6]. Although leukotriene receptor antagonists were shown to have therapeutic effectiveness in inflammatory diseases, especially in airway inflammation [11], inhibition of 5-lipoxygenase (5-LO) would produce a wider spectrum of antiinflammatory actions by blocking the synthesis of LTB₄ as well as cysteinyl leukotrienes. Thus, a number of compounds targeting to 5-LO have been developed as antiinflammatory agents, but zileuton (Zyflo; N-[1-benzo(b)thien-2-ylethyl]-N-hydroxyurea) is so far the only drug that was approved as an antiasthmatic agent [12].

In an effort to discover new chemical entities with selective inhibitory activities on 5-LO, the in-house chemical library was screened by a high-throughput screening method, as described previously [13]. As a result, we identified a new series of aminothiazole derivatives, and further characterized biochemical and pharmacological profiles of the representative compound, 4-[4-(4-fluorophenyl)-thiazol-2-ylamino]-2,6-dimethyl-phenol (KR-33749). KR-33749 selectively inhibited in vitro 5-LO enzyme activity and LTB₄ production in intact rat basophilic leukemia (RBL) cells with IC₅₀ of 70.5 ± 6.0 and 77.7 ± 6.9 nmol/l, respectively. Consistent with this, topical application of KR-33749 onto skin in cutaneous inflammation models, such as arachidonic acid-induced ear edema and 1-chloro-2,4-dinitrobenzene (DNCB)-induced atopic dermatitis, improved inflammatory skin lesions as determined by phenotypic, biochemical and immunohistochemical parameters, demonstrating its potential utility as a therapeutic agent for inflammation-related skin disorders.

Materials and Methods

Reagents and Cell Lines

Spodoptera frugiperda (Sf-21) insect cells and RBL-1 cells were obtained from the American Type Culture Collection (Manassas, Va., USA). RBL-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, glutamine, penicillin and streptomycin. Xylenol, iron sulfate, ATP, hexadecyltrimethylammonium bromide, α -phosphatidylcholine, avertin and DNCB were obtained from Sigma (St. Louis, Mo., USA). Arachidonic acid and A23187 were obtained from Calbiochem (La Jolla, Calif., USA). LTB₄ and immunoglobulin E enzyme immunoassay were obtained from Amersham (Piscataway, N.J., USA) and Shibayagi (Gunma, Japan), respectively. Zileuton and KR-33749 were synthesized in the Korea Research Institute of Chemical Technology. Soybean 5-LO enzyme was obtained from Cayman Chemical (Ann Arbor, Mich., USA), and 12-LO was prepared from platelet-rich plasma, as described before [14]. All other reagents were of analytical grade and obtained from Sigma.

Animals

Balb/c mice (male; 8 weeks of age) and NC/Nga mice (male; 7 weeks of age) were kept under temperature- and humidity-controlled condition with a 12:12-hour light cycle, and acclimated for 1 week before the experiments. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Chemical Technology (Daejeon, Republic of Korea).

In vitro 5-LO Assay

The 5-LO enzyme assay was carried out with some modifications to the ferrous oxidation-xylenol orange (FOX) assay, which is based on the principle that Fe³⁺ oxidized from Fe²⁺ by lipid hydroperoxide leads to a stable complex with xylenol orange that strongly absorbs in the visible range [13, 14]. 5-LO enzyme sources were prepared from insect cell (Sf-21) lysates transfected with baculoviral vector that contained 5-LO, as previously reported [13]. In brief, Sf-21 cells (9×10^5) were overlaid with the lipid-recombinant DNA complex and allowed to incubate for 5 h. To generate viral particles or express proteins, the cells were further incubated for 72 or 36 h, respectively, followed by the protocol provided by the supplier (Invitrogen).

To carry out the enzyme assay, insect cell lysates (15 μ g protein) were preincubated with various concentrations (30 nmol/l–1 μ mol/l) of either KR-33749 or zileuton (in DMSO) in 50 μ l of 50 mmol/l Tris-HCl buffer (pH 7.4) containing 0.4 mmol/l CaCl₂, 24 μ g/ml α -phosphatidylcholine and 20 μ mol/l ATP at room temperature for 4 min. The reactions were started by addition of 40 μ mol/l arachidonic acid, kept for another 4 min, and terminated by the addition of 100 μ l FOX reagent: sulfuric acid (25 mmol/l), xylenol orange (100 μ mol/l), iron (II) sulfate (100 μ mol/l) and methanol:water (9:1, v/v). A blank assay was done without substrate during incubation, but substrate was added after termination.

12- and 15-LO Assays for Determination of Specificity

Effects of KR-33749 on 12-LO and 15-LO were tested the same way as by 5-LO assay, except that ATP was absent in the assay system since both 12-LO and 15-LO exhibit enzyme activities even in the absence of ATP. As reference compounds of 12- and 15-LO, hinokitiol (1 μ mol/l) and ebselen (10 μ mol/l) were used, respectively [15, 16].

LTB₄ Secretion in RBL-1 Cells

It has been known that LTB₄ production by 5-LO activity is stimulated by calcium ionophore A23187 in RBL-1 cells [17]. Thus, RBL-1 cells were plated at a density of 4×10^4 cells into 96-well plates. Various concentrations (30 nmol/l–1 μ mol/l) of either KR-33749 or zileuton dissolved in DMSO (final: 0.5%) were added to plates and kept in incubation for 10 min at 37°C prior to the stimulation with A23187 (final: 10 μ mol/l in DMSO). After further incubation for 10 min, culture media were collected and the LTB₄ content in the supernatant was measured by using an enzyme immunoassay kit (Amersham).

Arachidonic Acid-Induced Ear Inflammation in Mice

To induce an inflammatory response, 2 mg of arachidonic acid in 20 μ l of acetone was painted to the inner surface of the right ear of Balb/c mice (8 weeks old; male; n = approx. 5–6/group).

Various doses (1–500 μg) of either KR-33749 (in 20 μl acetone) or zileuton were painted on the ears at 30 min, 24 h and 48 h before arachidonic acid treatment. For vehicle control, an equivalent volume of acetone was given. One hour after arachidonic acid stimulation, the ear thickness of the treated and untreated ears was measured with a dial thickness gauge (Mitutoyo, Tokyo, Japan). The effects of KR-33749 were calculated as the percent difference in ear thickness from the mean values of the contralateral untreated ear. Myeloperoxidase (MPO) activity in the inflamed ear tissue was determined according to the method described by Bradley et al. [18]. Briefly, punched ears (2 mm in diameter) were chopped and homogenized in 300 μl of 50 mmol/l potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was then centrifuged and the resulting supernatant (100 μl) was allowed to react with 0.167 mg/ml *o*-dianisidine and 0.0005% H_2O_2 , followed by the measurement of absorbance at 450 nm.

DNCB-Induced Atopic Dermatitis

NC/Nga mice (7 weeks old; male; $n = \text{approx. } 5\text{--}6/\text{group}$) were anesthetized by intraperitoneal injection of avertin (0.4 ml of 25 mg/ml working solution), and dorsal skin hair was shaved using hair clippers. Two days later, the NC/Nga mice were sensitized by application of 300 μl of 1% DNCB in acetone/olive oil (AOO 3:1) onto dorsal skin. Four days after sensitization, the mice were weekly challenged for 5 weeks by applying 300 μl of 0.4% DNCB in AOO. Starting from week 4, KR-33749 at a dose of 1 mg dissolved in 300 μl acetone was daily painted on the dorsal skin of DNCB-induced NC/Nga mice. At the 8th week, the NC/Nga mice were sacrificed by CO_2 asphyxiation to obtain serum and skin tissue, and clinical skin severity scores and dorsal skin thickness were assessed. Total scores were obtained by 2 independent observers in a blinded manner, and calculated from an average of the score summation of 5 symptoms (erythema, edema/population, oozing/crusting, excoriation and lichenification), in which each symptom was scored according to the following criteria: no lesion = 0; mild = 1; moderate = 2, and severe = 3. In addition, skin thickness was determined by measuring folded dorsal skin with a dial micrometer (Mitutoyo). Total serum IgE were measured by mouse IgE ELISA kit (Shibayagi) according to the manufacturer's assay procedure.

Histology of Dorsal Skins

Small pieces of skin tissues were cut from the dorsal skin of mice on week 8 after sensitization, fixed in 4% formaldehyde overnight, embedded in paraffin, sectioned in 8- μm slices and finally stained with hematoxylin and eosin (HE). Pictures were taken under microscopy (Nikon Elipse TE2000-U) equipped with a Nikon digital camera DXM1200. Images were taken at $\times 10$ objective magnification.

Statistical Analysis

Results were expressed as means $\pm$ SEM of 3 experiments, each carried out in triplicate. Statistical significance was evaluated using one-way ANOVA, and $p < 0.05$ was considered as statistically significant.

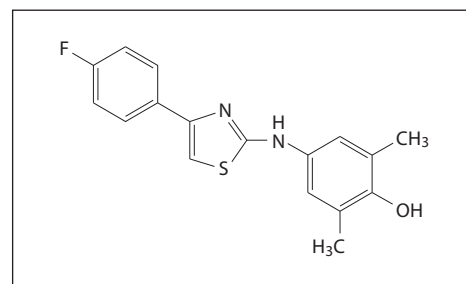


Fig. 1. Chemical structure of KR-33749, 4-[4-(4-fluorophenyl)-thiazol-2-ylamino]-2,6-dimethylphenol.

Results

In vitro 5-LO Enzyme Assay

In an effort to find new 5-LO inhibitors, the in-house chemical library was screened by high-throughput screening, as previously described [13]. We identified a series of aminothiazole derivatives and selected KR-33749 as a representative compound for further studies (fig. 1). KR-33749 inhibited 5-LO enzyme activity with an IC_{50} value of 70.5 ± 6.0 nmol/l (fig. 2a), being 4 times more potent than zileuton, a reference compound ($\text{IC}_{50} = 280 \pm 90$ nmol/l). In addition, KR-33749 showed a $>1,000$ -fold selectivity against related LO such as 12-LO and 15-LO. As shown in figure 2b and c, even at 100 $\mu\text{mol/l}$, KR-33749 inhibited $41 \pm 11\%$ of 12-LO and $16 \pm 2.1\%$ of 15-LO activities.

LTB₄ Measurement Using RBL-1 Cells

The Ca^{2+} ionophore A23187 can release arachidonic acid from membrane via phospholipase, and the resulting secreted arachidonic acid is converted to LTB_4 by 5-LO. To further examine the 5-LO-inhibitory activity of KR-33749, we examined whether the compound inhibits A23187-stimulated LTB_4 synthesis in RBL cells. KR-33749 inhibited LTB_4 production in a concentration-dependent manner, showing an IC_{50} value of 77.7 ± 6.9 nmol/l (fig. 3). By comparison, zileuton inhibited LTB_4 production with an IC_{50} value of 270 ± 30 nmol/l under the same experimental condition.

Effects of KR-33749 on Arachidonic Acid-Induced Ear Edema

We investigated the in vivo effects of KR-33749 on arachidonic-acid-induced ear edema. Topical application of KR-33749 protected the mice from arachidonic acid-induced ear inflammation in a dose-dependent manner

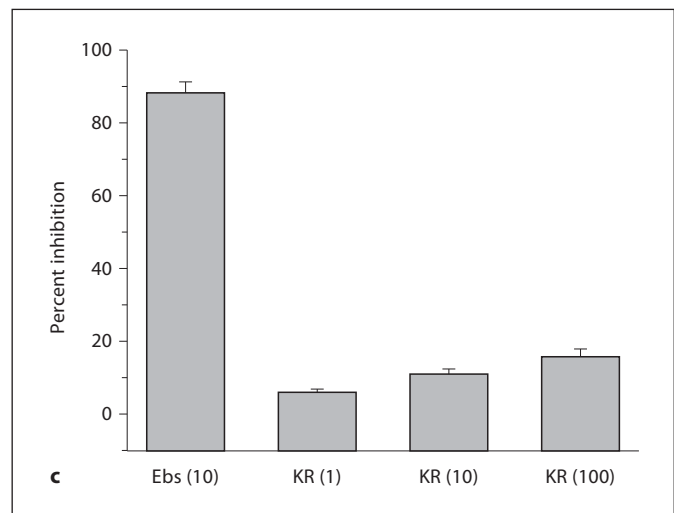
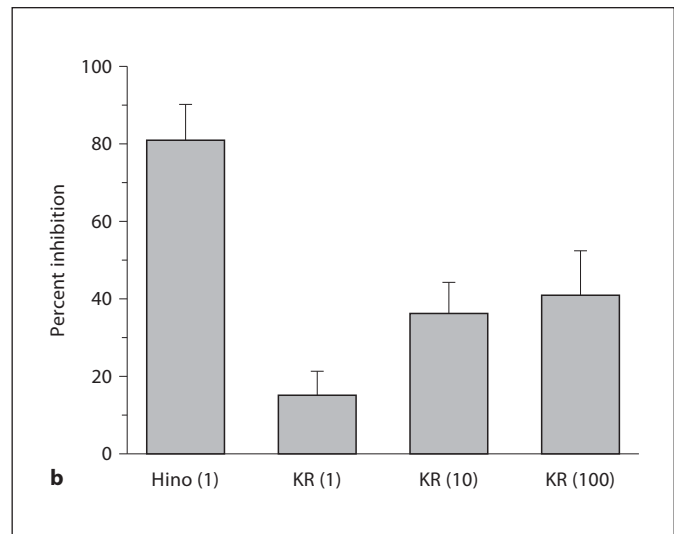
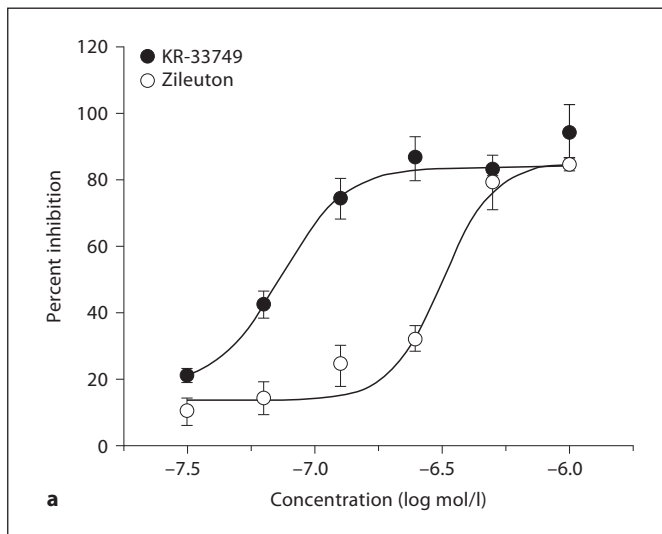


Fig. 2. Effects of KR-33749 on in vitro 5-LO enzyme activity (**a**) and on related LO, 12-LO (**b**) and 15-LO (**c**). **a** The cell lysates that overexpress 5-LO were preincubated with different concentrations (30 nmol/l–1 μ mol/l) of either KR-33749 or zileuton for 4 min, followed by an additional 4 min of incubation with arachidonic acid. The reaction was terminated by the addition of an equivalent volume of FOX reagent. **b** KR-33749 (1, 10 and 100 μ mol/l) was incubated with 12-LO, as described in the Materials and Methods section. **c** KR-33749 (1, 10 and 100 μ mol/l) was incubated with 15-LO, as described in the Materials and Methods section. Numbers in parentheses: compound concentrations. As references, hinokitiol (Hino) and ebselen (Ebs) were used as specific inhibitors of 12-LO and 15-LO, respectively. Data are means $\pm$ SEM from 3 experiments.

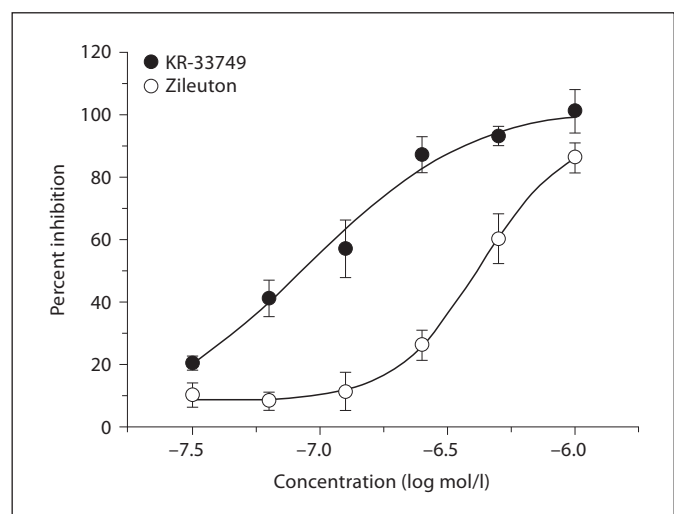


Fig. 3. Effects of KR-33749 on A23187-stimulated LTB₄ production. RBL-1 cells grown in DMEM were pretreated with various concentrations (30 nmol/l–1 μ mol/l) of either KR-33749 or zileuton for 10 min prior to A23187 stimulation. Ten minutes after A23187 treatment, the culture media were removed to measure the amount of LTB₄ using the ELISA kit. Data are means $\pm$ SEM from 3 experiments.

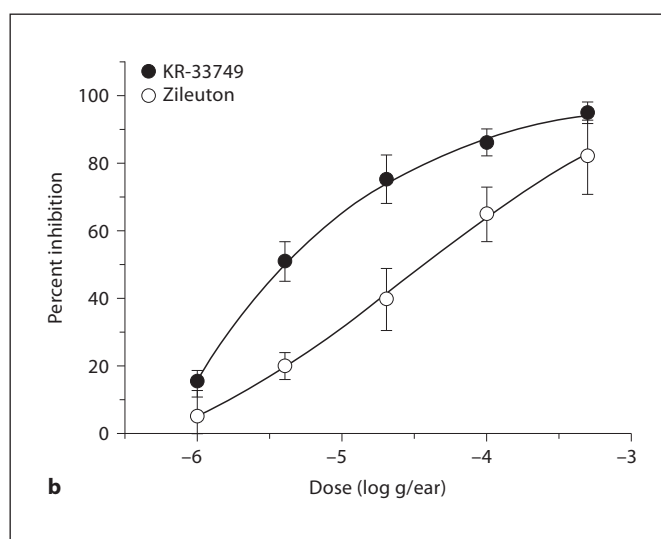
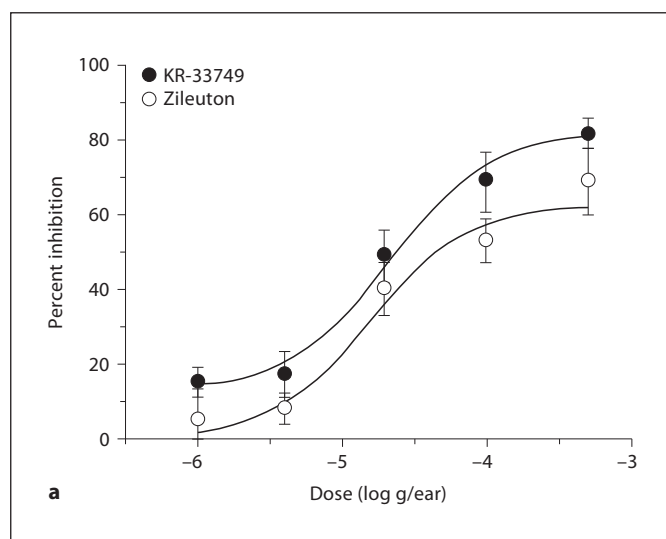
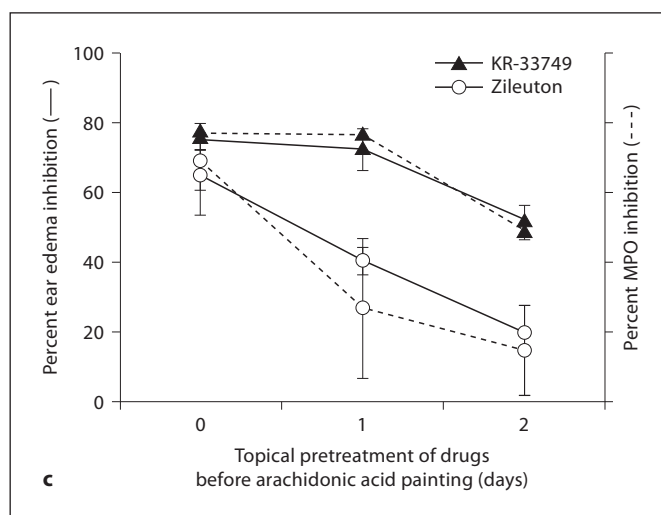


Fig. 4. Effects of KR-33749 on arachidonic acid-induced ear edema model. KR-33749 (1–500 μ g) dissolved in acetone was applied to the right ear 30 min before arachidonic acid painting (2 mg in 20 μ l acetone) on the same ear. One hour after arachidonic acid application, the ear thickness of the inflamed right ear was measured, and then the inflamed ears were taken for MPO activity measurement. **a** Ear thickness. **b** MPO activity in ear homogenates. Data are means $\pm$ SEM from 3 experiments ($n = 5$ in each experiment). **c** To determine the duration of action of KR-33749, the compound (500 μ g) was applied and kept for 48, 24 or 0.5 h. At the indicated times, arachidonic acid was applied, and ear thickness and MPO activity was measured 1 h later.



(fig. 4a, b). The protective effects of KR-33749 were superior to those of zileuton with respect to parameters of ear thickness and MPO activity, in which the IC_{50} values of KR-33749 and zileuton for ear thickness were 28 and 65 μ g, respectively, and the IC_{50} values for MPO activity were 3 and 57 μ g, respectively. To test the duration of action of KR-33749, we examined the effects of KR-33749 on ear thickness and MPO activity at 30 min, 24 h and 48 h after the compound treatment (500 μ g). The antiinflammatory activity of KR-33749 remained effective (about 50% inhibition) till 2 days after topical treatment, whereas the effects of zileuton were significantly reduced even after 1 day of treatment (fig. 4c). KR-33749 did not produce any inhibitory effects on arachidonic acid-induced ear edema upon oral administration (results not shown).

Effects of KR-33749 on DNCB-Induced Atopic Dermatitis

DNCB application to the shaved skin caused increased skin thickness as well as outrageous skin appearance. KR-33749 treatment resulted in a decrease in dorsal skin thickness by approximately 50%, a similar extent to that achieved by zileuton (normal: 0.7 ± 0.12 mm; vehicle: 0.912 ± 0.045 mm; zileuton: 0.81 ± 0.02 mm; KR-33749: 0.82 ± 0.05 mm) (fig. 5a). Macroscopic observation was carried out on dorsal skin of NC/Nga mice at the last day of the experiment, and the skin severity score was significantly improved by KR-33749 (fig. 5b). Total IgE in serum was significantly increased after 4 weeks of DNCB treatment, and the increased IgE level was suppressed by 33% after KR-33749 treatment at the 8th week (fig. 5c).

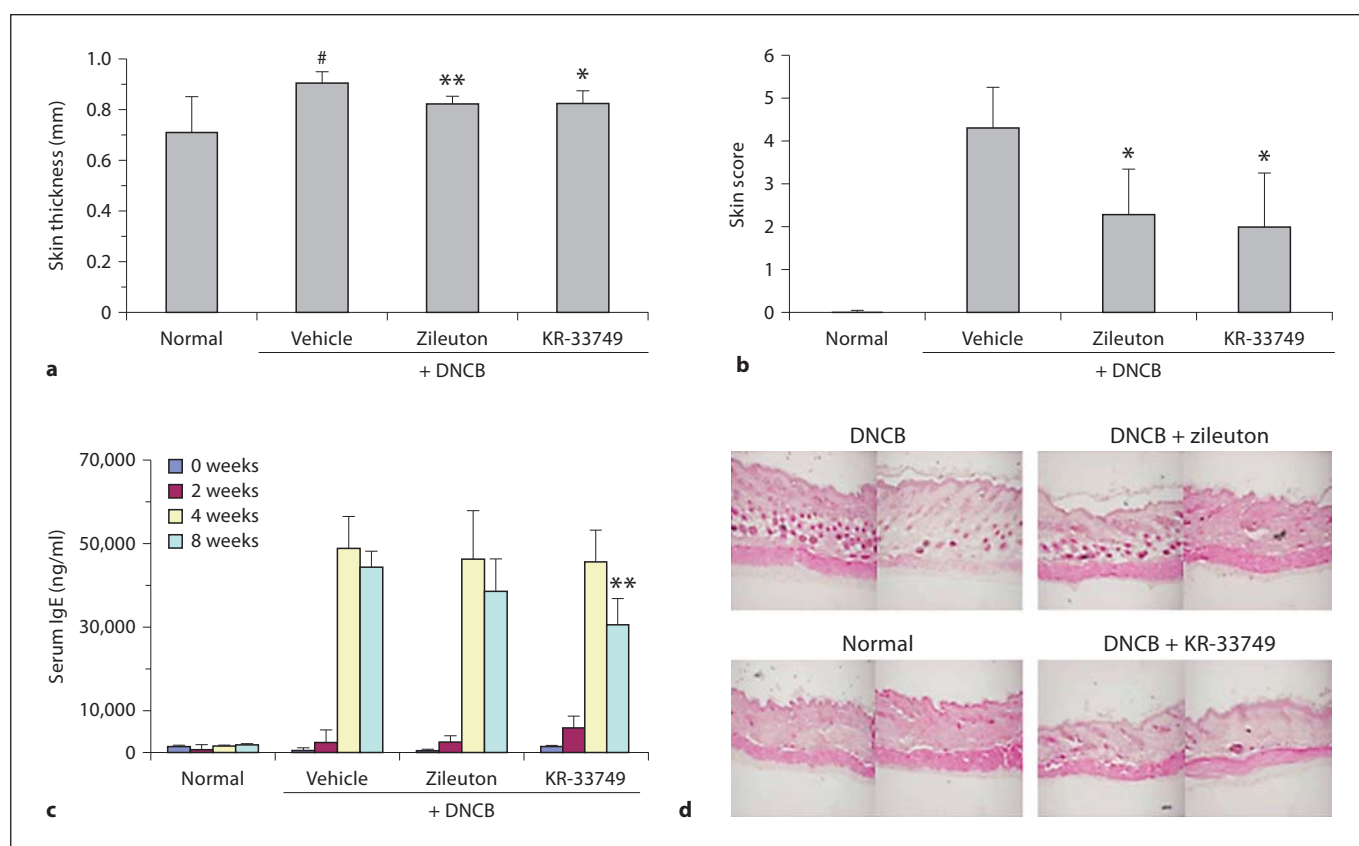


Fig. 5. Protective effects of KR-33749 on DNCB-induced atopic lesions in NC/Nga mice. Starting at week 4 after sensitization with DNCB, KR-33749 (1 mg in AOO) was applied to the shaved dorsal skin every day. At the end of the experiment, the thickness of folded skin was measured (**a**), and skin severity was scored on the basis of criteria described in the Materials and Methods section (**b**). IgE levels were monitored from sera at the indicated times (**c**).

d Histological study of dorsal skin prepared from NC/Nga mice treated with either zileuton or KR-33749. Skin was excised from the dorsal area of mice, and processed by formaldehyde fixation and paraffin embedding before HE staining. Images of HE-stained tissues were taken under phase contrast microscopy. [#] $p < 0.05$ versus normal; ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus vehicle.

Although a decreasing tendency on the part of the IgE level was observed in the zileuton-treated group, there was no statistical significance as compared with the vehicle group. Histological analysis revealed that KR-33749 ameliorated increased thickness in epidermis and dermis, and loosened tissue context induced by DNCB almost to the normal state (fig. 5d).

Discussion

Leukotrienes are involved in the pathological symptoms of various inflammatory diseases including skin disorders [2, 19]. Among lipoxygenases responsible for leukotriene production, 5-LO is involved in the first step of leukotriene biosynthesis, and resulting LTB₄ acts spe-

cifically as a potent chemotactic agent for neutrophils, leading to a vascular permeability change in skin [2]. In parallel, significant levels of LTB₄-like immunoreactivity were detected in patients with atopic dermatitis and psoriasis [20].

As leukotrienes are critical mediators of chronic inflammation, various approaches to interfere with the production or function of leukotrienes have been applied to develop drugs for inflammatory diseases, and possibly therapeutic intervention in LTB₄ formation will also be a promise for the treatment of inflammation-related skin disorders [21], especially in cases that have corticosteroid insensitivity [22]. Currently, the only 5-LO inhibitor approved for oral application is zileuton for asthma therapy, but it has the disadvantage of having to take doses 4 times a day and causing other side effects [23]. Previously, dual

inhibitors of cyclooxygenase and 5-LO were reported to exhibit potent antiinflammatory activity in an arachidonic acid-induced murine ear edema model [24]. On the other hand, selective cyclooxygenase-2 inhibitors were withdrawn from the market due to the side effects of heart attack and adverse skin reaction [25].

In this study, a new series of aminothiazole derivatives were identified as 5-LO inhibitors via high-throughput screening of the in-house chemical library. A representative compound, KR-33749, showed potent inhibitory activities against 5-LO, based on the *in vitro* enzyme assay and LTB₄ production assay. However, KR-33749 produced little inhibitory effect on arachidonic acid-induced ear edema upon oral application. Consistent with this finding, the oral bioavailability of KR-33749 was 2% in Sprague-Dawley rats, possibly due to presystemic metabolism (results not shown). On the contrary, topical application of KR-33749 exhibited potent inhibitory effects on arachidonic acid-induced ear edema. A growing body of evidence shows that chemicals with aromatic amino and hydroxyl groups are subjected to extensive metabolism. In line with this, the nonspecific lipoxygenase inhibitor nordihydroguaiaretic acid is reported to display only topical activity in arachidonic acid-induced edema and inflammatory cell infiltration [26].

Recently, many mouse allergic dermatitis models have been established by repeated antigen exposure. The NC/Nga mouse is an inbred strain with a characteristic phenotype of spontaneous dermatitis, and it develops skin lesions resembling atopic dermatitis in ambient laboratory conditions [27]. However, an epicutaneous applica-

tion of chemical antigens was employed to increase the incidence rate and shorten the development time of the diseases [28]. In the present study, DNCB sensitization on dorsal skin of NC/Nga mice induced atopic-dermatitis-like skin lesions with elevated levels of serum IgE, consistent with previous findings [29]. Topical application of KR-33749 improved both skin severity and thickness as compared with the vehicle group. In parallel, serum IgE was significantly reduced by KR-33749 treatment, suggesting that KR-33749 possibly exerts its effect via suppression of the LTB₄-induced activation and infiltration of inflammatory cells, including mast cells and CD4⁺ T cells responsible for IgE synthesis, and that the compound can be applied to alleviate chronic as well as acute inflammation-derived skin disorder. In addition, zileuton was also active in protecting from DNCB-induced atopic-dermatitis-like skin lesions.

In summary, we discovered a new 5-LO inhibitor, KR-33749, which appears to be applicable for the treatment of inflammation-related skin diseases via topical use. Further characterization and safety issues of KR-33749 have to be addressed to develop it as a therapeutic agent for human diseases.

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