Role of Leukotriene B₄ in 5-Lipoxygenase Metabolite- and Allergy-Induced Itch-Associated Responses in Mice

Fumio Tsuji,∗a,b Hiroyuki Aono, a Takashi Tsuboi, a Taadahiro Murakami, a Hiroshi Enomoto, a Keiko Mizutani, a and Naoki Inagaki, a,b,c

∗Research and Development Center, Santen Pharmaceutical Co., Ltd.; 8916–16 Takayama, Ikoma, Nara 630–0101, Japan; a United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University; 1–1 Yanagido, Gifu, Gifu 501–1193, Japan; and b Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University; 5–6–1 Mitahorihigashi, Gifu, Gifu 502–8585, Japan.

Received November 4, 2009; accepted March 23, 2010; published online March 24, 2010

We investigated the role of leukotriene (LT) B₄ in 5-lipoxygenase metabolite- and allergy-induced itch-associated responses using SA6541, an LTA₄ hydrolase inhibitor. Itch-associated responses were induced by intradermal injection of 5-hydroperoxyeicosatetraenoic acid (HPETE), a precursor of 5-lipoxygenase metabolites, and passive cutaneous anaphylaxis in ICR mice. By screening molecules related to arachidonic acid metabolism or pruritus, SA6541 was found to be a specific inhibitor of LTA₄ hydrolase. Pharmacokinetic studies confirmed the specificity of SA6541 at an oral dose of 100 mg/kg in mice. 5-HPETE induced scratching behavior, which was inhibited by SA6541 (100 mg/kg). However, SA6541 (100 mg/kg) hardly attenuated the 5-HPETE-induced increase in vascular permeability. Moreover, SA6541 (100 mg/kg) partially attenuated scratching behavior, but did not affect the increase in vascular permeability caused by passive cutaneous anaphylaxis. On the other hand, ketotifen fumarate, a histamine H₁ antagonist, strongly inhibited the scratching behavior and the increase in vascular permeability caused by passive cutaneous anaphylaxis. These results suggest that LT₄ is an endogenous itch mediator in the skin and is involved in the pruritus response in allergic reactions.

Key words leukotriene B₄; scratching behavior; passive cutaneous anaphylaxis; SA6541; leukotriene A₄ hydrolase

Itch is an unpleasant sensation that induces the desire to scratch. Itch accompanies various skin diseases (e.g., atopic dermatitis, contact dermatitis and urticaria) and several systemic disorders (e.g., chronic renal failure and cholestasis). In humans, histamine induces itch by stimulating specific sensory fibers, whereas H1 (and to a lesser extent H2) antagonists are shown to reduce itch in many clinical trials. Itch mechanisms and mediators of itch in most pruritic diseases remain unclear. Several targets and mechanisms for pruritus have recently been reported. Several receptors, including histamine H₁ and H₂, serotonin, leukotriene (LT) B₄, transient receptor potential vanilloid (TRPV) 1, cannabinoid, protein-activated receptor 2, kappa opioid, and mu opioid, are involved in the control of pruritus. Of these, LT₄ is involved in neuropeptide- and nociceptin-induced itch-associated responses.

LT₄ is produced by the activity of 5-lipoxygenase and LTA₄ hydrolase on arachidonic acid. However, the role of LT₄ in pruritus in allergic reactions remains unknown. We have already reported that SA6541 is a potent inhibitor of the hydrolase activity of guinea pig LTA₄ hydrolase (IC₅₀=270 nM). SA6541 showed good oral anti-inflammatory activities at doses exceeding 50 mg/kg in mice. SA6541 also inhibited human LTA₄ hydrolase (IC₅₀=25 nM, unpublished data). In the present study, we investigated the role of LT₄ in 5-lipoxygenase metabolite- and allergy-induced itch-associated responses in mice using SA6541 at a dose of 100 mg/kg.

MATERIALS AND METHODS

Reagents SA6541 (S-(4-Dimethylaminobenzyl)-N-[(2S)-3-mercaptop-2-methylpropionyl]-L-cysteine) was synthesized by Santen Pharmaceutical Co., Ltd. (Osaka, Japan). Ketotifen fumarate, a histamine H₁ receptor antagonist, was purchased from Sigma (St. Louis, MO, U.S.A.). 5-Hydroperoxyeicosatetraenoic acid (5-HPETE; Biomol, Plymouth Meeting, PA, U.S.A.) and dinitrophenyl (DNP)-conjugated bovine serum albumin (DNP-BSA) were used to induce scratching behavior. 5-HPETE is an intermediate metabolite of 5-lipoxygenase in the conversion from arachidonic acid to LTA₄. 5-HPETE was suspended in saline containing 0.1% ethanol.

Animals Experimental procedures were carried out with the approval of the Santen Animal Experimental Ethics Committee. Male ICR mice, 5–6 weeks old, were purchased from Japan CLEA (Tokyo, Japan). The mice were housed in a room kept at 23 ±1 °C with a 12-h light–dark cycle, and were given free access to food and water. All experimental groups consisted of randomly selected animals.

Screening of Molecular Targets of SA6541 We have already reported that SA6541 inhibited LTA₄ hydrolase and angiotensin-converting enzyme, but not the other metallopeptidases. To identify the molecular target of SA6541, 19 binding assays, 2 functional assays, and 3 enzyme assays, related to arachidonic acid metabolism or pruritus, were performed using SA6541 at a concentration of 3 µM, by Cerep (Poitiers, France). For binding assays, the specific ligand that bound to the receptors was defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabelled ligand. The results are expressed as the percent inhibition of control-specific binding obtained in the presence of SA6541. For functional assays, the results are expressed as the percent of the control-specific agonist response (Ca²⁺ influx into the TRPV1-expressed Chinese hamster ovary (CHO) cells in the present study) and as the percent inhibition of the control-specific
agocyte assays, the results are expressed as the percent inhibition of control-specific activity (the reaction from arachidonic acid to the reaction products in the present study) obtained in the presence of SA6541.

Pharmacokinetic Analysis of SA6541 To aid the identification of the target molecules of SA6541, we measured the plasma concentrations of SA6541 in mice. Blood samples were collected using syringes containing heparin sodium at 0.5, 1, 2, 4 and 6 h after oral administration of 100 mg/kg SA6541. Plasma was separated immediately by centrifugation and stored at −80 °C under acidic conditions. The plasma concentrations of SA6541 were determined by liquid chromatography with tandem mass spectrometry.

Behavioral Observations The hair was clipped over the rostral part of the murine back 2 d before the challenge. For the passive cutaneous anaphylaxis reaction, anti-DNP mouse monoclonal immunoglobulin E (IgE) antibody was intradermally injected the day before the challenge. Scratching behavior was observed according to the methods described by Kuraishi et al.\(^\text{11}\) and Musoh et al.\(^\text{12}\) In brief, immediately after intradermal injection of 5-HPETE (50 μl suspension/site) or intravenous injection of saline containing 0.5% Evans blue and 0.1 mg/ml DNP-BSA (0.25 ml/mouse), the mice were placed in an observation chamber. The behavior was recorded for 60 min using a video camera, in the absence of an observer. Using the video recording, the number of times the reaction site was scratched with the hindpaws was counted for 60 min. Because mice generally scratch several times in one second, a series of scratchings was counted as one incidence. For drug evaluation, SA6541 and ketotifen fumarate were orally administered 30 min before inducing the cutaneous reactions.

Measurement of Vascular Permeability The increase in vascular permeability was measured as previously reported.\(^\text{13,14}\) In brief, the mice were intravenously injected with 0.5% Evans blue, killed 60 min after inducing the cutaneous reactions, and the reaction site was excised. The skin specimen was dissolved in 0.7 ml of 1 N KOH solution, and 9.3 ml of a mixture of 0.6 N H₃PO₄ solution and acetone (5:13) was added. After vigorous shaking, the precipitates were collected using syringes containing heparin sodium at 80 °C under acidic conditions. The plasma concentrations of SA6541 were determined by liquid chromatography with tandem mass spectrometry.

Statistics Data are expressed as mean values with standard error. Results were statistically evaluated using Student’s t-test or Dunnett’s test (EXSAS; Arm, Osaka, Japan).

RESULTS

SA6541 Hardly Affects Arachidonic Acid Metabolism or Pruritus-Related Molecules, Except for LTA₄ Hydrolase We have already investigated the inhibitory activity of SA6541 on LTA₄ hydrolase, as described in the Introduction. The results of 24 screening assays, except for LTA₄ hydrolase, are shown in Table 1. SA6541 hardly affected arachidonic acid metabolism or pruritus-related molecules, except for LTA₄ hydrolase. At a concentration of 3 μM, SA6541 inhibited 54% of the activity of cyclooxygenase 2. Therefore, SA6541 seems to be a highly specific inhibitor of LTA₄ hydrolase.

Pharmacokinetic Data Suggest That SA6541 Is a Specific LTA₄ Hydrolase Inhibitor at the Dose of 100 mg/kg In an earlier study, the production of LTB₄ after the injection of 5-HPETE was strongly inhibited by oral administration of 50 mg/kg SA6541.\(^\text{7}\) Therefore, we used 100 mg/kg SA6541 in the present study. At 30 min after oral administration of 100 mg/kg SA6541, its plasma concentration was 684 nm, which then decreased over time (Fig. 1). Therefore, SA6541 is rapidly absorbed into the systemic circulation after oral administration. Based on these results, we induced the cutaneous reactions 30 min after oral administration of 100 mg/kg SA6541 in the behavioral and vascular permeability studies. Taking into account the results of the screening studies (Table 1), the pharmacokinetic data indicate that SA6541 is a highly specific inhibitor of LTA₄ hydrolase.

SA6541 Attenuates 5-Lipoxygenase Metabolite-In-
Therefore, LTB4 may be involved in the increase in vascular permeability in the passive cutaneous anaphylaxis reaction. By contrast, histamine plays major roles in pruritus and the increase in vascular permeability.

DISCUSSION

ICR mice exhibit scratching behavior in response to passive cutaneous anaphylaxis, which involves histamine. In addition, intradermal LTB4 induces itch-associated responses in ICR mice. In the present study, we demonstrated that SA6541, which inhibits LTB4 production, attenuated scratching behavior caused by 5-HPETE or passive cutaneous anaphylaxis in ICR mice. These data suggest that LTB4 may be an endogenous itch mediator in skin, and may be involved in the pruritic responses in allergic reactions, particularly in IgE-related type I allergic reactions. However, there are conflicting data regarding the role of LTB4 in allergy-associated itching. Kuraishi et al. reported that 5-lipoxygenase metabolites other than LTB4 and cysteinyl leukotrienes are involved in mosquito allergy-associated itching. One possible reason for these conflicting data is the sensitization method used in each study. We induced passive sensitization with the anti-DNP mouse monoclonal IgE antibody in the present study, whereas Kuraishi et al. used repeated active sensitization with an extract of the salivary gland of mosquitoes. In addition, the LTB4 antagonist ONO-4057 used by Kuraishi et al. only appears to inhibit the BLT1 LTB4 receptor, whereas SA6541 ultimately decreases the activity of BLT1, BLT2 and other responses by inhibiting the production of LTB4. In terms of the mechanism involved in LTB4-induced itch-associated responses, Andoh and Kuraishi reported that the BLT1 LTB4 receptor is expressed on dorsal root ganglion (DRG) neurons in ICR mice. DRG neurons play a crucial role in the transmission of the itch sensation and LTB4 increased the intracellular Ca2+ concentration in cultured DRG neurons, which was inhibited by a BLT1 LTB4 receptor antagonist. It has also been reported that LTB4 and other lipoxygenase metabolites of arachidonoyl ethanolamide can directly activate TRPV1, which is expressed on itch-specific sensory neurons and on many other types of skin cells. Therefore, LTB4 probably stimulates itch-specific sensory neurons by binding to the BLT1 receptor and activating TRPV1. In the present study, we have demonstrated that SA6541 hardly attenuated the increased vascular permeability induced by 5-HPETE and passive cutaneous anaphylaxis in ICR mice. We have already reported that LTB4 may be involved in carrageenan-induced edema formation in ICR mice. In addition, Biomed-101, a potent LTB4 receptor antagonist, may prevent the formation of edema caused by interleukin-2 (IL-2) therapy in clinical trials. These reports suggest that LTB4 may be involved in edema formation in certain diseases. However, our present results suggest that LTB4 has little effect on vascular permeability in allergic reactions.

The importance of histamine in scratching behavior and increased vascular permeability in passive cutaneous anaphylaxis in ICR mice was further confirmed in the present study using ketotifen. Antihistamines have been used for antipruritic therapy in various types of pruritus for many years.

Fig. 2. 5-HPETE Induced Scratching Behavior (A), Which Was Inhibited by SA6541 (B)

However, SA6541 scarcely attenuated the 5-HPETE-induced increase in vascular permeability (C). Scratching of the reaction site with the hindpaws was counted for 60 min after intradermal injection of 5-HPETE. The reaction site was then excised and the dye was extracted. The sham group was intradermally injected with saline containing 0.1% ethanol. SA6541 (100 mg/kg) was administered orally 30 min before the injection of 5-HPETE (5 μg/site). Values are means±S.E.M. for 5–7 animals group. ∗p<0.05 vs. the sham group by Dunnett’s test. **p<0.01, ***p<0.001 vs. the control group by Student’s t-test.

Fig. 3. SA6541 Partially Attenuated Scratching Behavior (A), but Did Not Affect the Increase in Vascular Permeability (B) Caused by Passive Cutaneous Anaphylaxis

Scratching of the reaction site with the hindpaws was counted for 60 min after intradermal injection of 5-HPETE. In this study, 5-HPETE at a dose of 5 μg/site induced scratching behavior (Fig. 2A). SA6541, at an oral dose of 100 mg/kg, inhibited 5-HPETE-induced scratching behavior (Fig. 2B), but did not affect the increase in vascular permeability (Fig. 2C). In a previous study, SA6541 inhibited LTB4 production, but not the production of LTC4/D4/E4. Therefore, LTB4 may be related to scratching behavior, but not to vascular permeability increase induced by 5-HPETE, specifically 5-lipoxygenase metabolites.

SA6541 Partially Attenuates Scratching Behavior, but Does Not Affect the Increase in Vascular Permeability Caused by Passive Cutaneous Anaphylaxis

SA6541, at an oral dose of 100 mg/kg, partially but significantly attenuated scratching behavior (Fig. 3A) caused by passive cutaneous anaphylaxis, but did not affect the increase in vascular permeability (Fig. 3B). We also examined the effect of ketotifen fumarate on scratching behavior and the increase in vascular permeability caused by passive cutaneous anaphylaxis. Ketotifen fumarate, at an oral dose of 10 mg/kg, strongly inhibited scratching behavior and the increase in vascular permeability (Figs. 3A, B). Therefore, LTB4 may be involved in the increase in vascular permeability, but does not appear to be involved in the increase in vascular permeability in the passive cutaneous anaphylaxis reaction. By contrast, histamine plays major roles in pruritus and the increase in vascular permeability.
However, the role of histamine in pruritus is still controversial. Moreover, there is limited evidence that antihistamines can relieve pruritus in the treatment of atopic dermatitis. On the other hand, Pogatzki-Zahn et al. reported that high doses of non-sedating antihistamines had antipruritic effects in atopic dermatitis. It has also been reported that repeated antigen challenge diminishes the role of histamine in pruritus in allergic conjunctivitis. Therefore, further studies using animal models of human itch-related diseases are needed to clarify the targets and mechanisms, including the roles of histamine, LTB₄ and other mediators in the treatment of pruritus. In future, we may be able to use SA6541 to treat LTB₄-related pruritus in humans, because of its strong inhibitory activity on human LTA₄ hydrolase.

In conclusion, LTB₄ appears to be an endogenous itch mediator in the skin and is involved in the pruritus response in allergic reactions. However, LTB₄ has a limited effect on increased vascular permeability in allergic reactions.

Acknowledgments We are grateful to the members of the Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, for supporting the present study.

REFERENCES