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Involvement of Leukotriene B_4 in Murine Dermatitis Models

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ABSTRACT. Leukotriene B_4 (LTB_4) is a product of the 5-lipoxygenase pathway of arachidonic acid (AA) metabolism. LTB_4 is a potent chemotactic factor for neutrophils and has been postulated to play an important role in a variety of pathological conditions including rheumatoid arthritis, psoriasis, and inflammatory bowel disease. To investigate the role of LTB_4 in dermatitis, we used *S*-(4-dimethylaminobenzyl)-*N*-[(2*S*)-3-mercapto-2-methylpropionyl]-L-cysteine (SA6541), a potent leukotriene A_4 (LTA_4) hydrolase inhibitor. SA6541 inhibited LTB_4 production with an IC_{50} value of 270 nM *in vitro*. 5-Hydroperoxyeicosatetraenoic acid (5-HPETE) or AA injection induced LTB_4 production and neutrophil influx in mouse ear. SA6541 inhibited 5-HPETE- and AA-induced LTB_4 production and neutrophil influx in mouse ear when administered orally at a dose of 50 mg/kg. SA6541 also inhibited 5-HPETE-induced prostaglandin E_2 (PGE_2) production, probably by an indirect effect through the inhibition of LTB_4 production. These results suggest that LTB_4 may be important in the pathogenesis of dermatitides such as psoriasis. BIOCHEM PHARMACOL **55**;3:297–304, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. SA6541; leukotriene A_4 hydrolase inhibitor; leukotriene B_4; dermatitis; psoriasis; mice

LTB_4,† a product of the action of 5-LO on AA, induces chemokinetic, chemotactic, and aggregation responses in polymorphonuclear leukocytes [1, 2], degranulation [3, 4], and increased adhesion of these cells to endothelial cell monolayers [5]. Although neutrophils were initially identified as a major source of LTB_4, many different cell types have subsequently been shown to produce this molecule (e.g. monocytes/macrophages, keratinocytes, lymphocytes, and mast cells) under conditions associated with pathophysiological stimulation [6–10]. In addition to activating neutrophils, LTB_4 also has been shown to be an autacoid capable of inducing the expression of various cytokines and their receptors in monocytes and lymphocytes [11–15]. These *in vivo* results suggest a potentially broader role for LTB_4 in inflammatory processes, and implicate it as a probable endogenous mediator of inflammatory processes. Evidence supporting a role for LTB_4 in *in vivo* inflammatory processes has also come from preclinical animal models. Exogenous LTB_4 was shown to induce polymorphonuclear leukocyte infiltration in animals [16–19]. LTB_4 has been found in a number of pathological fluids associated with the development of inflammation [20, 21], and both blockers of LTB_4 synthesis (i.e. 5-LO inhibitors) and antagonists of LTB_4 receptors have been reported to inhibit the development of inflammatory processes [22–24]. Furthermore, some types of inflammatory responses were shown to be reduced markedly in 5-LO knockout mice [25, 26]. In addition, administration of exogenous LTB_4 induces inflammatory responses in normal volunteers [27–30], and biologically relevant levels of LTB_4 and its metabolites have been reported in numerous human tissues and fluids in pathological states [31–36]. Thus, prevention of LTB_4 synthesis via inhibition of either 5-LO or LTA_4 hydrolase, or, alternatively, inhibiting its action by blocking LTB_4 receptors have been considered viable modes of action for novel anti-inflammatory drugs. LTA_4 hydrolase produces LTB_4 from LTA_4. To examine the role of LTB_4 in dermatitis, we investigated the effects of *S*-(4-dimethylaminobenzyl)-*N*-[(2*S*)-3-mercapto-2-methylpropionyl]-L-cysteine (SA6541) (see Table 1), a newly synthesized potent LTA_4 hydrolase inhibitor, in murine dermatitis models. In the present study, we used two models of epidermal inflammation involving LTB_4 production in mice.

MATERIALS AND METHODS

Animals

Inbred male ICR mice, 6- to 7-weeks-old, were purchased from Japan SLC Inc. They were housed under a 12-hr light–dark cycle (lights on at 7:00 a.m.), with room temperature maintained at 23 ± 1°, and humidity at 55 ± 10%. Food and water were available *ad lib*. All experiments were conducted in accordance with the recommendations of the Declaration of Helsinki.

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† *Abbreviations:* AA, arachidonic acid; 12(*R*)-HETE, 12(*R*)-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; MPO, myeloperoxidase; PGB_2, prostaglandin B_2; and PGE_2, prostaglandin E_2

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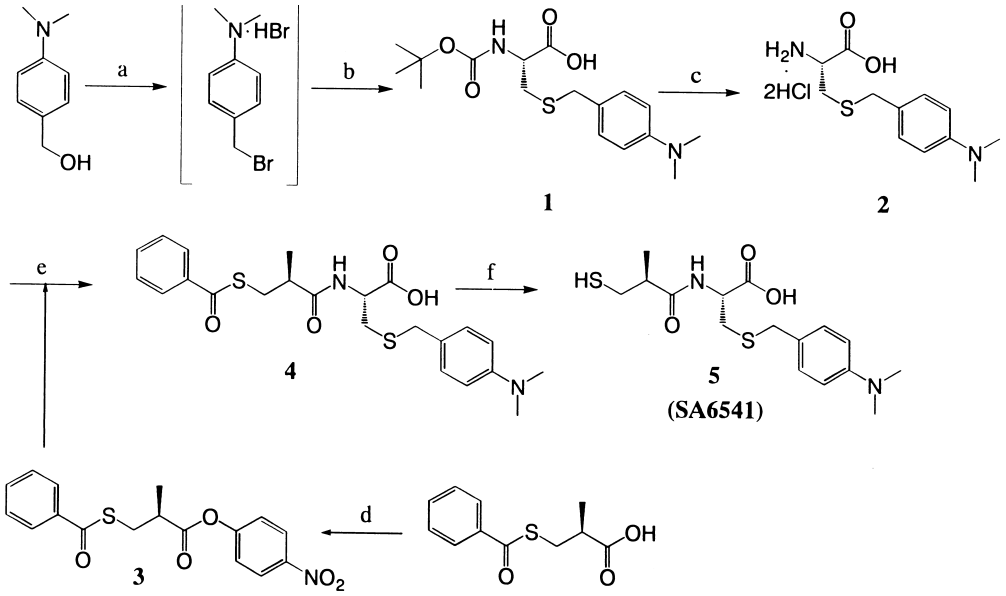


FIG. 1. Synthesis of SA6541. (a) 47% HBr aq., 120°, 1 hr; (b) *N-t*-butoxycarbonylcysteine, *N,N*-diisopropylethylamine, CH_2Cl_2, room temperature, 2.5 hr; (c) 4 N HCl/dioxane, anisole, room temperature, 1 hr; (d) dicyclohexylcarbodiimide, CH_2Cl_2, room temperature, 4 hr; (e) CH_2Cl_2, *N,N*-dimethylformamide, triethylamine, room temperature, 2 days; and (f) 28% NH_3 aq., room temperature, 1 hr.

Reagents

SA6541, an inhibitor of LTA_4 hydrolase, was synthesized by the Central Research Laboratories of the Santen Pharmaceutical Co., Ltd. Figure 1 shows the route of SA6541 synthesis. 4-Dimethylaminobenzaldehyde (Nacalai Tesque), *N-t*-butoxycarbonylcystine (Kokusan Chemical Works), thiobenzoic acid (Sigma–Aldrich Japan), methylacrylic acid (Sigma–Aldrich Japan), and *p*-nitrophenol (Nacalai Tesque) were purchased from the sources shown for SA6541 synthesis. Indomethacin, a cyclooxygenase inhibitor, was purchased from the Sigma Chemical Co. SA6541 and indomethacin were suspended in saline containing 1% carboxymethylcellulose (vehicle) for oral administration. 5-HPETE (Funakoshi) and sodium AA (Sigma) were used to induce dermatitis. 5-HPETE is an intermediary metabolite of 5-LO from AA to LTA_4. These agents were suspended in phosphate-buffered saline. To determine the *in vitro* IC_{50} values of LTA_4 hydrolase, SA6541 and bestatin (Sigma) were dissolved in saline. LTA_4 was prepared as described [37].

Enzyme Assay

LTA_4 hydrolase was purified from guinea pig lung and used in the enzyme assay according to the method of Ohishi *et al.* [37]. Briefly, guinea pig lung was homogenized in 3 vol. of 50 mM phosphate-buffered saline containing 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 100,000 × *g* for 60 min, and the resultant supernatant was subjected to ammonium sulfate fractionation. The precipitates between 40 and 70% saturation were collected and dissolved in 20 mM Tris–acetate buffer containing 1 mM dithiothreitol (pH 7.8) and dialyzed against two changes of the same buffer. LTA_4 hydrolase activity was determined as follows. The standard reaction mixture contained 60 mM HEPES buffer, 3 mM dithiothreitol, pH 7.8, and enzyme in a total volume of 150 μL with or without LTA_4 hydrolase inhibitor. After preincubation at 26° for 1 min, LTA_4 (in an ethanol solution, containing less than 50 mM lithium hydroxide) was added to a final concentration of 60 μM. After a 1-min incubation at 26°, the reaction was terminated by the addition of acidic acetonitrile:ethanol:acetic acid (150:50:3, 100 μL). PGB_2 was added as an internal standard for HPLC analysis. The mixture was kept at −20° for 30 min, followed by centrifugation at 10,000 × *g* for 5 min at 4°. The supernatant (25 μL) was analyzed with a TSK ODS-80TS column (4.6 × 75 mm, Tosoh Co. Ltd.) using acetonitrile:methanol:water:acetic acid (900:300:800:1.8, pH 5.6, containing 0.05% EDTA) as the mobile phase (flow rate 1.0 mL/min). The absorbance at 270 nm was monitored, and the amount of LTB_4 formed was calculated from the peak area ratio LTB_4/PGB_2.

5-HPETE- or AA-Induced Mouse Ear Dermatitis Model

Mice were challenged intradermally with 25 μL of 5-HPETE (5 μg) or AA (2 μg) in the external ear, using a microsyringe with a 30-gauge needle. At various time points after intradermal challenge, the animals were killed by an overdose of anesthetic, and injected sites were punched out with a 6-mm gasket punch and then placed in tubes containing 0.75 mL of 0.5% hexadecyltrimethylammonium bromide/80 mM phosphate buffer (pH 5.4) for

measurement of MPO activity or 1 mL of ethanol for measurement of LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$.

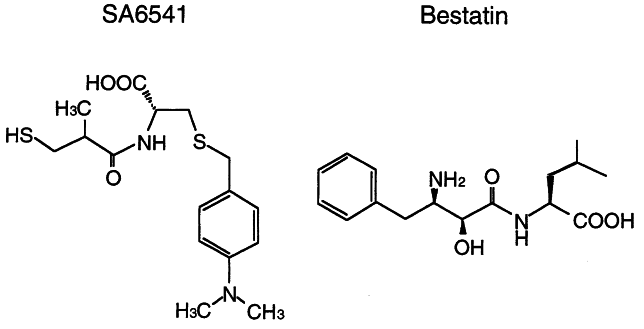
Administration of SA6541 or Indomethacin in the Dermatitis Models

Animals in the control group were given orally the vehicle only. Other groups were dosed orally with SA6541 or indomethacin at 1 mL suspension/100 g body weight. SA6541 was suspended in vehicle at a concentration of 1 mg/mL (when administered at a dose of 10 mg/kg) or 5 mg/mL (when administered at a dose of 50 mg/kg). Indomethacin was suspended in vehicle at a concentration of 0.5 mg/mL. One hour after oral dosing, mice were challenged intradermally with 25 μL of 5-HPETE (5 μg) or AA (2 μg) using a microsyringe with a 30-gauge needle. For measurement of MPO activity, the mice were dosed orally with these drugs again 2 hr after intradermal challenge. Animals were killed by an overdose of anesthetic, and injected sites were punched out 4 or 8 hr after challenge. For measurement of LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$, injected sites were punched out 30 min after challenge.

Measurement of MPO Activity

MPO activity was measured according to the method of De Young *et al.* [38]. The samples were homogenized and subjected to two freeze–thaw cycles, and then centrifuged at 12,000 × *g* for 15 min. Aliquots of 30 μL of the supernatant were diluted with 100 μL of phosphate-buffered saline and 75 μL of 0.22 M phosphate buffer in 96-well plates, and then mixed with 35 μL of enzyme substrate containing tetramethylbenzidine/hydrogen peroxide (Amersham) for 5 min at 37°. The reaction was terminated by adding 30 μL of 1.0 M sulfuric acid, and the absorbance of each sample was measured with a microtiter plate reader at 450 nm. Activity is expressed as units of OD/min/cm^2 by conversion using the area of the punched-out ear tissue (0.2826 cm^2).

TABLE 1. Inhibitory effects of SA6541 and bestatin on LTA_4 hydrolase



Compounds	IC_{50}*(M)
SA6541	2.7×10^{-7}
Bestatin	5.5×10^{-5}

* The IC_{50} values were obtained from graphs of inhibition percentage versus the log of concentration.

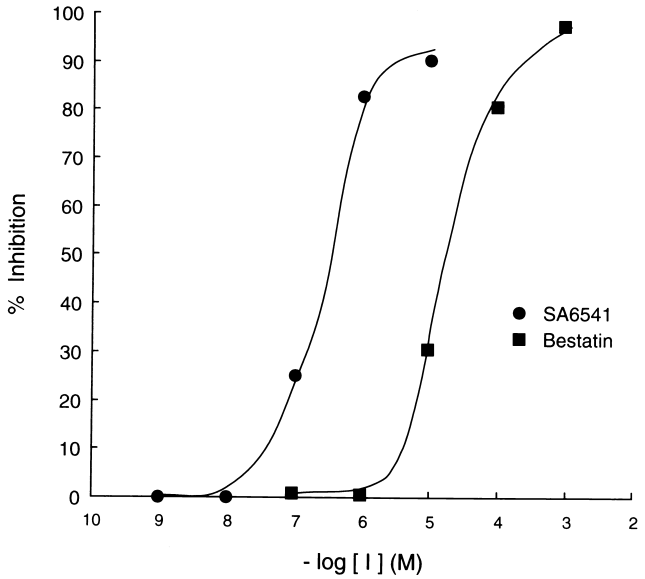


FIG. 2. Inhibition curves of LTA_4 hydrolase by SA6541 and bestatin. Each point indicates the mean of duplicate measurements in a single experiment.

Measurement of LTB_4, PGE_2 or Leukotriene $C_4/D_4/E_4$

The samples were homogenized and centrifuged at 12,000 × *g* for 15 min. The supernatants were evaporated to dryness and dissolved in 3 mL of 0.1 M citrate buffer (pH 3.0). Solutions were loaded onto C18 SepPak (Waters) columns, which were then washed with 25% methanol in water containing 1% acetic acid. LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$ was eluted from these columns by 80% methanol in water containing 1% acetic acid, and subsequently lyophilized to remove the organic solvent. The recoveries of LTB_4 and PGE_2 in this extraction procedure were 89 and 96%, respectively. LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$ was quantified using an appropriate enzyme immunoassay kit (Amersham). The amounts of LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$ are expressed as units of pg/cm^2 by conversion using the area of the punched-out ear tissue (0.2826 cm^2).

Statistical Analysis

Dunnett's multiple comparison test (Statistical Library, Yukms Corp.) was used for statistical analysis of the results.

RESULTS

Inhibition of LTA_4 Hydrolase by SA6541

The chemical structures and IC_{50} values of SA6541 and bestatin, a known inhibitor of LTA_4 hydrolase [39, 40], are shown in Table 1. Figure 2 shows the LTA_4 hydrolase inhibitory curves by SA6541 and bestatin. SA6541 inhibited LTA_4 hydrolase in a concentration-dependent manner similar to bestatin, but the inhibition by SA6541 was more potent than that by bestatin. The inhibitory potency of SA6541 for LTA_4 hydrolase was 200-fold stronger than that of bestatin in terms of IC_{50}.

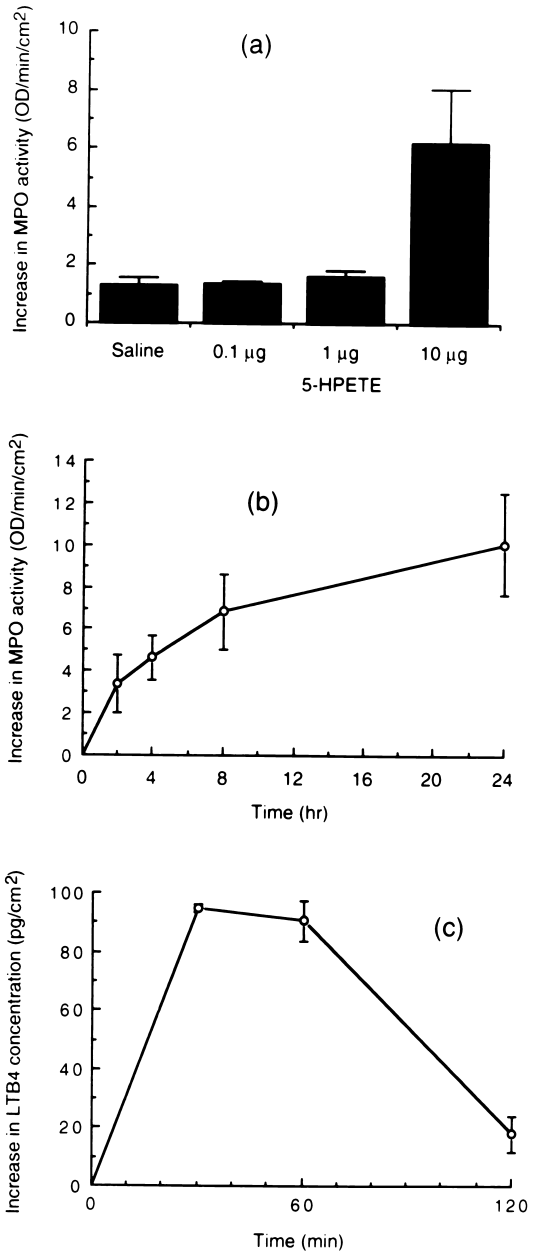


FIG. 3. Effects of intradermally injected 5-HPETE on neutrophil accumulation [(a) dose–response and (b) time–course] and (c) time–course of changes in LTB_4 concentration in mouse ear. Values are means ± SEM of 4 animals. (a) MPO activity was measured 4 hr after 5-HPETE injection. (b) MPO activity was measured at each time point after 5-HPETE (5 μg) injection. (c) LTB_4 concentration was measured at each time point after 5-HPETE (5 μg) injection.

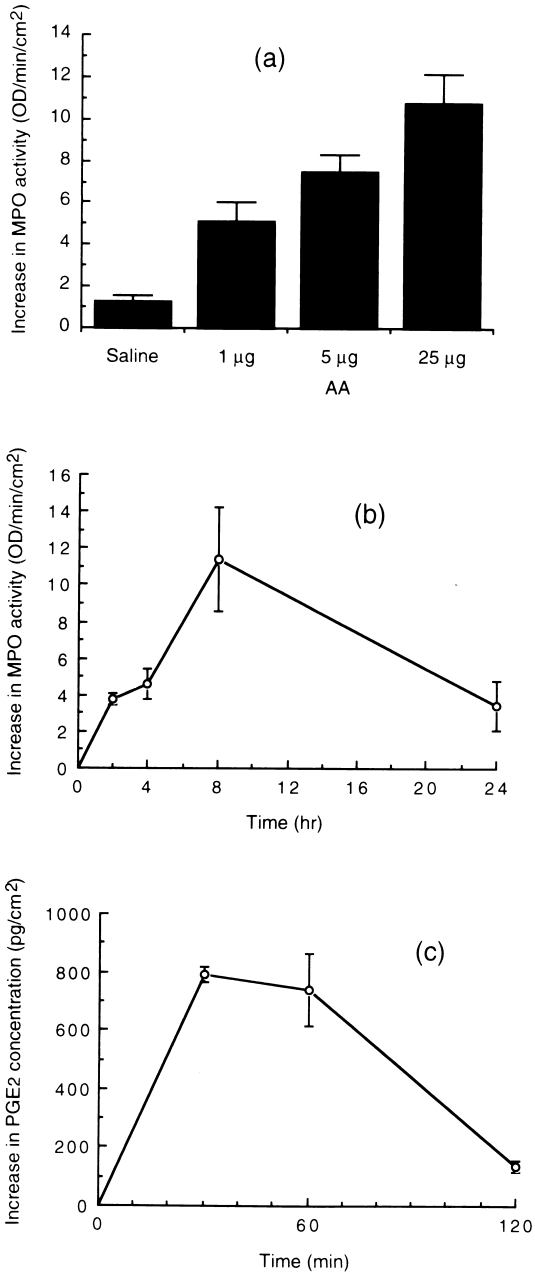


FIG. 4. Effects of intradermally injected AA on neutrophil accumulation [(a) dose–response and (b) time–course] and (c) time–course of changes in PGE_2 concentration in mouse ear. Values are means ± SEM of 4 animals. (a) MPO activity was measured 4 hr after AA injection. (b) MPO activity was measured at each time point after AA (2 μg) injection. (c) PGE_2 concentration was measured at each time point after AA (2 μg) injection.

5-HPETE-Induced Inflammation

The effects of intradermally injected 5-HPETE on neutrophil accumulation in the mouse ear are shown in Fig. 3a. 5-HPETE induced neutrophil accumulation at doses above 1 μg 4 hr after injection. The time–course of neutrophil accumulation in the mouse ear following intradermal injection of 5-HPETE (5 μg) is shown in Fig. 3b. Neutrophil accumulation gradually increased until 24 hr. The time–course of changes in LTB_4 concentration in the mouse ear is shown in Fig. 3c. There was an immediate sharp increase of LTB_4 concentration and then a return to the low level 2 hr after 5-HPETE injection.

AA-Induced Inflammation

The effects of intradermally injected AA on neutrophil accumulation in the mouse ear are shown in Fig. 4a. AA

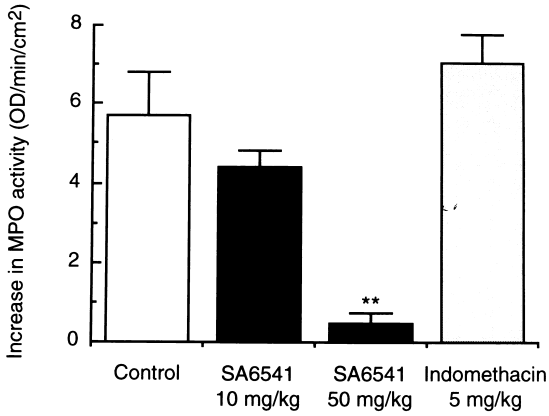


FIG. 5. Effects of SA6541 on 5-HPETE-induced neutrophil accumulation. Values are means ± SEM of 6–16 animals. The samples used for measurement of MPO activity were collected 8 hr after 5-HPETE (5 μg) injection. Drugs were administered orally 1 hr before and 2 hr after 5-HPETE injection. Key: (**)$P < 0.01$ vs the control group (by Dunnett's multiple comparison test).

induced neutrophil accumulation in a dose-dependent manner 4 hr after injection. The time–course of neutrophil accumulation in the mouse ear following intradermal injection of AA (2 μg) is shown in Fig. 4b. The neutrophil accumulation increased to a peak at about 8 hr after AA injection and then decreased thereafter. The time–course of changes in PGE_2 concentration in the mouse ear is shown in Fig. 4c. There was an immediate sharp increase of PGE_2 concentration and then a return to the low level 2 hr after AA injection. The time–course of changes in LTB_4 concentration in the mouse ear showed almost the same pattern as 5-HPETE-induced inflammation (data not shown).

Effects of SA6541 on 5-HPETE-Induced Inflammation

The effects of SA6541 on 5-HPETE-induced neutrophil accumulation and increases of LTB_4, PGE_2, or leukotriene $C_4/D_4/E_4$ concentration in the mouse ear are shown in Figs. 5 and 6, respectively. SA6541 (50 mg/kg × 2) significantly inhibited neutrophil accumulation 8 hr after 5-HPETE injection and also inhibited the increases in LTB_4 and PGE_2 concentration 30 min after 5-HPETE injection. However, SA6541 had no effect on the increase in leukotriene $C_4/D_4/E_4$ concentration. On the other hand, indomethacin had no effect on neutrophil accumulation or the increases in LTB_4 or leukotriene $C_4/D_4/E_4$ concentrations, but it inhibited the increase in PGE_2 concentration.

Effects of SA6541 on AA-Induced Inflammation

The effects of SA6541 on AA-induced neutrophil accumulation and increases in LTB_4 and PGE_2 concentrations in the mouse ear are shown in Figs. 7 and 8, respectively. SA6541 (50 mg/kg × 2) significantly inhibited neutrophil accumulation 8 hr after AA injection and also inhibited the increase in LTB_4 concentration 30 min after AA injection.

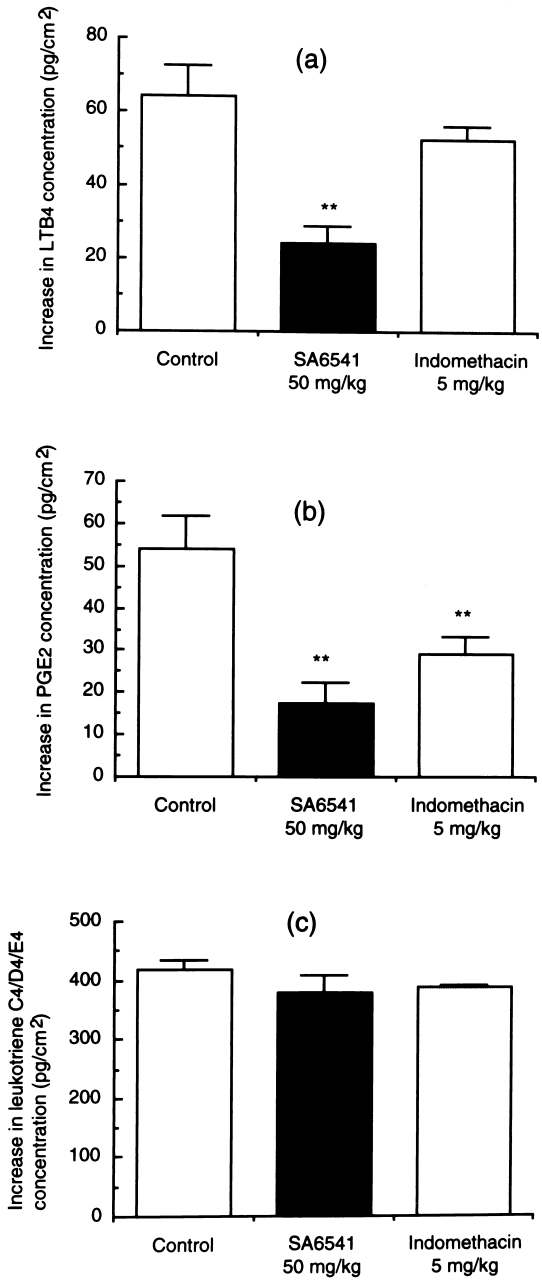


FIG. 6. Effects of SA6541 on 5-HPETE-induced production of (a) LTB_4 (b) PGE_2, or (c) leukotriene $C_4/D_4/E_4$. Values are means ± SEM of 4–6 animals. The samples were collected 30 min after 5-HPETE (5 μg) injection. Drugs were administered orally 1 hr before 5-HPETE injection. Key: (**)$P < 0.01$ vs the control group (by Dunnett's multiple comparison test).

However, SA6541 had no effect on the increase in the level of PGE_2. On the other hand, indomethacin had no effect on neutrophil accumulation or increase in LTB_4 concentration, but it inhibited the increase in PGE_2 concentration.

DISCUSSION

We showed that the newly synthesized agent SA6541 is a potent LTA_4 hydrolase inhibitor. Orally administered

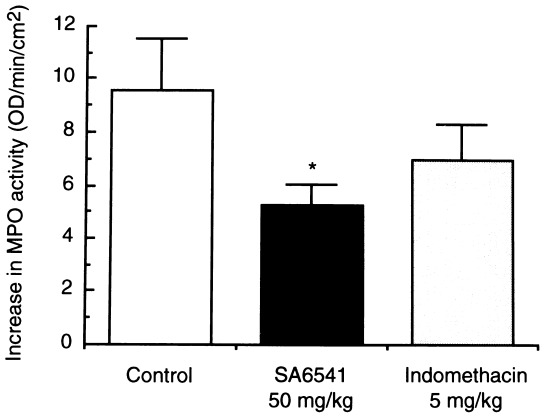


FIG. 7. Effects of SA6541 on AA-induced neutrophil accumulation. Values are means ± SEM of 8–12 animals. The samples used for the measurement of MPO activity were collected 8 hr after AA (2 μg) injection. Drugs were administered 1 hr before and 2 hr after AA injection. Key: (*)$P < 0.05$ vs the control group (by Dunnett's multiple comparison test).

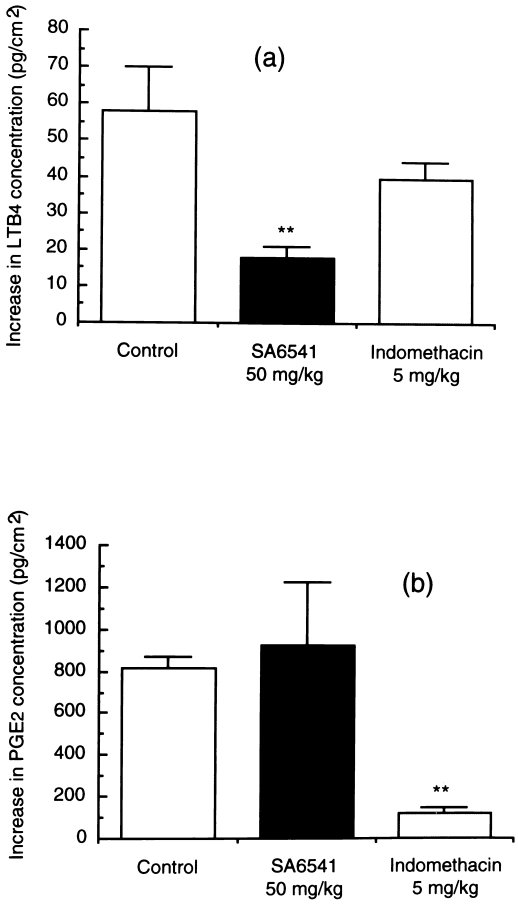


FIG. 8. Effects of SA6541 on AA-induced production of (a) LTB_4 or (b) PGE_2. Values are means ± SEM of 4 animals. The samples were collected 30 min after AA (2 μg) injection. Drugs were administered orally 1 hr before AA injection. Key: (**)$P < 0.01$ vs the control group (by Dunnett's multiple comparison test).

SA6541 was efficacious in two models of epidermal inflammation in mice. There have been few reports concerning orally active LTA_4 hydrolase inhibitors. As epidermal inflammation models, we used 5-HPETE- or AA-induced dermatitis. 5-HPETE is an intermediary metabolite of 5-LO from AA to LTA_4, and LTB_4 production from 5-HPETE requires 5-LO and LTA_4 hydrolase [41]. LTB_4 production and neutrophil accumulation were evoked by 5-HPETE. However, there was little production of PGE_2 after intradermal injection of 5-HPETE, and, therefore, the 5-LO pathway of AA metabolism was important in this model. After oral administration, SA6541 blocked 5-HPETE-induced production of LTB_4 and neutrophil accumulation. However, SA6541 had no effect on increases in leukotriene $C_4/D_4/E_4$ concentrations. On the other hand, indomethacin did not block 5-HPETE-induced production of LTB_4 or neutrophil accumulation. Therefore, LTB_4 may be the main chemotactic factor for neutrophil accumulation in this model.

LTB_4 and PGE_2 production and neutrophil accumulation were evoked by AA. After oral administration, SA6541 blocked AA-induced production of LTB_4 and neutrophil accumulation but did not inhibit PGE_2 production. These results suggest that SA6541 has no inhibitory effect on cyclooxygenase. Thus, SA6541 may be a specific inhibitor of LTA_4 hydrolase in the AA cascade. The inhibitory effect of SA6541 on neutrophil accumulation after AA injection was weaker than that after 5-HPETE injection, while SA6541 almost completely blocked LTB_4 production. These observations suggest that LTB_4 is merely one of the chemotactic factors for neutrophil accumulation in AA-induced dermatitis. 12(*R*)-HETE is another candidate for the chemotactic factor in AA-induced dermatitis. 12(*R*)-HETE was found previously in human psoriatic skin, and it has been suggested that 12(*R*)-HETE acting as the LTB_4 receptor agonist on neutrophils may be relevant to recruitment of neutrophils in this disease [42, 43]. In guinea pig skin, 12(*R*)-HETE injected intradermally causes influx of neutrophils [44].

As shown in Figs. 5 and 6, a greater inhibitory effect of SA6541 was seen on neutrophil infiltration than on LTB_4 production. One possible explanation for these results is that there may be a threshold concentration of LTB_4 for neutrophil infiltration. As shown in Fig. 3, 1 μg of 5-HPETE scarcely induced neutrophil infiltration, whereas at 10 μg significant induction was seen. However, we consider that 1 μg of 5-HPETE probably induces LTB_4 production. Therefore, slight inhibition of LTB_4 production might cause marked inhibition of neutrophil infiltration.

While SA6541 had no inhibitory effect on cyclooxgenase, it inhibited PGE_2 production after 5-HPETE injection. In our preliminary studies, LTB_4 injected intradermally induced PGE_2 production in the mouse ear. For example, LTB_4 (500 pg/ear) injection induced PGE_2 production at about 60 pg/cm^2 30 min after challenge. Therefore, PGE_2 production after 5-HPETE injection may be mediated by LTB_4 production from 5-HPETE. The inhibition of PGE_2 production by SA6541 in the 5-HPETE-

induced dermatitis model may be achieved via inhibition of LTB_4 production.

For clinical application, orally active LTA_4 hydrolase inhibitors may be useful for the treatment of rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Ford-Hutchinson [45] reported that there was no convincing evidence for the presence of 5-LO in skin. However, we consider that 5-LO may be present in skin on the grounds that LTB_4 production from 5-HPETE was observed in the mouse ear. In addition, we have shown that LTB_4 is important for neutrophil accumulation in the mouse ear.

In conclusion, LTB_4 may be important in the pathogenesis of dermatitides such as psoriasis.

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