

# Inhibition of Leukotriene Production by *N*-[4-[4-(Diphenylmethyl)-1-piperazinyl]butyl]-3-(6-methyl-3-pyridyl) Acrylamide (AL-3264), a New Antiallergic Agent

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**ABSTRACT**—The effects of AL-3264, which exhibits a 5-lipoxygenase (5-LO) inhibiting property by blocking histamine H<sub>1</sub>-receptors and inhibition of histamine release, were examined on leukotriene (LT) production and LT-mediated responses. AL-3264 (1–30  $\mu$ M) inhibited the A23,187-induced LT production from human leukocytes with almost the same potency as that of nordihydroguaiaretic acid. AL-3264 (30–100 mg/kg, p.o.) inhibited the antigen-induced LT production in the abdominal cavity of passively sensitized rats; its effect was as potent as that of AA-861, a 5-LO inhibitor. AL-3264 (30  $\mu$ M) suppressed both the initial and sustained phases of the antigen-induced contractions in isolated trachea from actively sensitized guinea pig. Phenidone (3  $\mu$ M), a dual inhibitor of 5-LO and cyclooxygenase (CO), suppressed the sustained phase, while indomethacin was without effect on either phase. AL-3264 (40–160 mg/kg, p.o.) suppressed the arachidonic acid-induced ear edema in mice, for which 5-LO inhibitors were effective but antihistamines were not. The anti-edematous effect of AL-3264 (160 mg/kg) was reduced by intradermal administration of LTC<sub>4</sub> (0.1  $\mu$ g). These results suggest that AL-3264 suppresses LT production in vivo and in vitro by inhibiting 5-LO activity, and this property may contribute to the antiallergic effect of AL-3264.

**Keywords:** Antiallergic drug, AL-3264, Leukotriene production, Arachidonic acid-induced ear swelling, Antigen-induced trachea contraction

Leukotrienes (LTs), which are derived from arachidonic acid by 5-lipoxygenase (5-LO), have been implicated in the pathophysiology of allergic and inflammatory diseases, especially bronchial asthma. For example, peptido-LTs are detected in the blood from individuals with acute asthma (1) and in nasal exudates after antigen challenge in patients allergic to ragweed (2). In addition, peptido-LTs contract isolated human bronchus (3), cause bronchoconstriction in normal and asthmatic subjects (4, 5) and stimulate mucus secretion from the trachea of the dog (6) and mucous glycoprotein secretion from human airways in vitro (7). Furthermore, peptido-LTs increase microvascular permeability in guinea pig trachea (8) and human skin (9). On the other hand, LTB<sub>4</sub> induces the chemotaxis of eosinophils and neutrophils (10, 11). Thus, receptor antagonists and biosynthesis inhibitors of LTs have been considered to be highly valuable for the therapy of allergic and inflammatory diseases.

We have demonstrated that *N*-[4-[4-(diphenylmethyl)-1-piperazinyl]butyl]-3-(6-methyl-3-pyridyl) acrylamide

(AL-3264, Fig. 1) inhibits 5-LO activity from guinea pig exudate cells in a cell-free system (12), blocks histamine H<sub>1</sub>-receptors in isolated guinea pig trachea and inhibits antigen-induced histamine release from isolated rat peritoneal mast cells. However, there is no evidence that AL-3264 inhibits LT production as the result of its 5-LO inhibition. Furthermore, although there are a number of compounds that inhibit 5-LO activity or LT production in vitro, only a few compounds, such as zileuton, ICI-D2138 and WY-50,295 (13–15), have so far provided effective inhibition of in vivo LT production in humans or

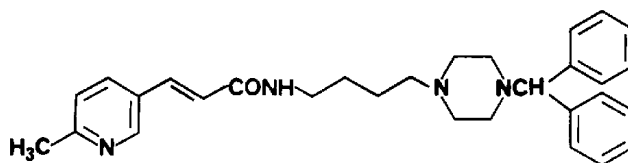


Fig. 1. Chemical structure of AL-3264.

experimental animals.

In the present study, the effects of AL-3264 on LT production and LT-mediated responses *in vivo* and *in vitro* were examined in comparison with reference agents: oxatomide, ketotifen, NDGA, AA-861, zileuton and phenidone.

## MATERIALS AND METHODS

### Animals

Male Wistar rats and Hartley guinea pigs were obtained from Nihon SLC, Inc. (Hamamatsu). Male ICR mice were obtained from Nihon Clea, Inc. (Tokyo). The animals were housed in temperature-controlled rooms ( $23 \pm 2^\circ\text{C}$ ) with a 12-hr light-dark cycle, and they were allowed free access to food and water. The experiments were carried out at a room temperature of  $23 \pm 2^\circ\text{C}$ . The animals were randomly assigned to the treatment groups.

Human blood was obtained from healthy male volunteers, aged 20 to 40, who had not taken any drug for at least two weeks.

### Drugs

The following drugs were used: *N*-[4-[4-(diphenylmethyl)-1-piperazinyl]butyl]-3-(6-methyl-3-pyridyl) acrylamide (AL-3264), oxatomide, ketotifen fumarate, zileuton and AA-861, which were prepared at our research laboratories. Other drugs and compounds were obtained from the following commercial sources: arachidonic acid, mepyramine maleate, phenidone, indomethacin and nordihydroguaiaretic acid (NDGA) (Sigma, St. Louis, MO, USA); caffeic acid (Nacalai Tesque, Kyoto); ionophore A23,187 (CalBiochem, San Diego, CA, USA); egg albumin  $5 \times \text{Cryst.}$  (Seikagaku Kogyo, Tokyo); Lymphocyte Separation Medium (ICN Biomedicals, Costa Mesa, CA, USA); Freund complete adjuvant (Difco, Detroit, MI, USA); inactive *Bordetella pertussis* (Wako Pure Chemical, Osaka); LTC<sub>4</sub> and PGE<sub>2</sub> (Funakoshi, Tokyo). LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> and LTB<sub>4</sub> radioimmunoassay kits (Amersham, Tokyo) were used for the analyses of LTs.

Drugs were suspended in a 0.5% tragacanth solution for oral administration. In the *in vitro* tests, they were dissolved in ethanol or dimethyl sulfoxide and diluted with the medium used. The doses of drugs described in this paper refer to the forms described above.

### A23,187-induced production of LTs in human peripheral leukocytes *in vitro*

Peripheral venous blood (30 ml) from healthy volunteers was collected in heparinized (20 units/ml) tubes and mixed with 7.5 ml of saline containing 6% dextran and 3% glucose. The blood was allowed to sediment at room temperature for 60 min. The leukocyte-rich plasma layer

was withdrawn, diluted with an equal volume of Tris buffer and then layered onto 15 ml of Lymphocyte Separation Medium. After centrifuging the tubes at 2,000 rpm for 30 min at room temperature, the supernatants were discarded. The cell pellets were exposed to 0.2% NaCl solution for 30 sec to lyse erythrocytes and then immediately exposed to 0.9% NaCl solution. After centrifugation at 1,400 rpm for 10 min, the cells were washed twice with Ca<sup>2+</sup>-free Tyrode solution and resuspended into Tyrode solution to a concentration of  $3 \times 10^5$  leukocytes/890  $\mu\text{l}$ . The drug solution examined or the vehicle (100  $\mu\text{l}$ ) was added to the cell suspension (890  $\mu\text{l}$ ). After incubation at  $37^\circ\text{C}$  for 30 min, 10  $\mu\text{l}$  of 0.1 mM A23,187 (final concentration of 1  $\mu\text{M}$  in 0.2% dimethyl sulfoxide) were added to the cell suspension. After incubation for a further 15 min, the reaction was terminated by placing the test tubes into ice-water. The cell suspension was centrifuged at 2,000 rpm for 10 min at  $4^\circ\text{C}$ . The supernatant was stocked at  $-80^\circ\text{C}$  until the LT assay. The levels of LTB<sub>4</sub> and the total levels of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> were determined using LTB<sub>4</sub> and LT C<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> radioimmunoassay kits, respectively. All experiments were run in duplicate.

The composition of Tris buffer was 25.0 mM Tris HCl, 120.0 mM NaCl and 5.0 mM KCl; and the composition of Tyrode solution was 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 11.9 mM NaHCO<sub>3</sub> and 5.6 mM glucose.

### Antigen-induced LT production in the abdominal cavity of passively sensitized rats

This experiment was carried out according to the method of Ross et al. (16). Rat antiserum to egg albumin (EA) was prepared according to the method of Tada and Okumura (17). Briefly, EA (10 mg) mixed with  $10^{10}$  cells of inactive *Bordetella pertussis* was injected into four footpads of rats. Five days later, EA (5 mg) was injected intramuscularly into the back of the rats. The antiserum was obtained by cardiopuncture 7 to 10 days after the second injection of EA. The diluted solution (1 ml) of the serum was intraperitoneally administered to rats (180–250 g). Two hours later, Tyrode solution (5 ml) containing EA (0.4 mg/ml) and heparin (0.2 units/ml) was administered intraperitoneally to the rats. Ten minutes later, the rats were killed, and peritoneal exudates were collected. The exudates were centrifuged at 2,000 rpm for 8 min at  $4^\circ\text{C}$ . Ethanol was added to the supernatants in the volume ratio of 4:1 and mixed. The mixture was centrifuged at 3,000 rpm for 15 min at  $4^\circ\text{C}$ , and the supernatant was stocked at  $-80^\circ\text{C}$  until the LT assay. The total level of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> was determined using LT C<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> radioimmunoassay kits. Drugs were administered 5 (i.v.), 15 (p.o.) or 30 (p.o.) min before EA challenge.

### Antigen-induced contractions in isolated trachea from sensitized guinea pig

EA (0.2%) saline solution was emulsified with Freund complete adjuvant in an equal volume. The emulsion (1 ml) was subcutaneously injected into the planta of the four legs of each guinea pig (300–450 g). Three to four weeks after the sensitization, the trachea was removed and cut into three zig-zag strips as described by Emmer-son and Mackay (18). One of them served as the control. The strips were suspended in a 10-ml organ bath filled with Tyrode solution, kept at 37°C and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The tension of the strips was measured by isometric force transducers (TB-611T; Nihon Kohden, Tokyo). The strips were allowed to equilibrate for more than 1 hr with an initial loading tension of 1 g. The maximum contractions of the strips induced by histamine (0.1–1 mM) were determined. After washing and equilibration, EA (10 µg/100 µl) was added to the bath, and the tension of the strips was recorded for 60 min. Drugs were added to the organ baths at 5 min before the EA challenge. The contractions induced by EA were expressed as a percentage of the maximum response to histamine.

### Arachidonic acid-induced ear edema in mice

The method described by Young et al. (19) was used with minor modifications. Briefly, 20 µl of the acetone solution of arachidonic acid (AA, 12.5 mg/ml) was applied to both the inner and outer surfaces of the right ears of mice (18–23 g). One hour later, the mice were killed by in-

halation of CO<sub>2</sub> gas. A circular part (5.5 mm in diameter) of both ears was removed with a metallic punch and weighed. The left ears of the mice served as the control. Ear swelling was expressed as a percentage of the increase in weight of the AA-treated ear compared to that of the contralateral untreated ear. Drugs were orally administered 1 hr before AA application.

In some experiments, LTC<sub>4</sub> saline solution (0.1 µg/10 µl/ear) was intradermally administered in the right ears of the mice immediately before AA application or PGE<sub>2</sub> (1 µg/20 µl/ear) was topically applied to the right ears of mice together with AA solution.

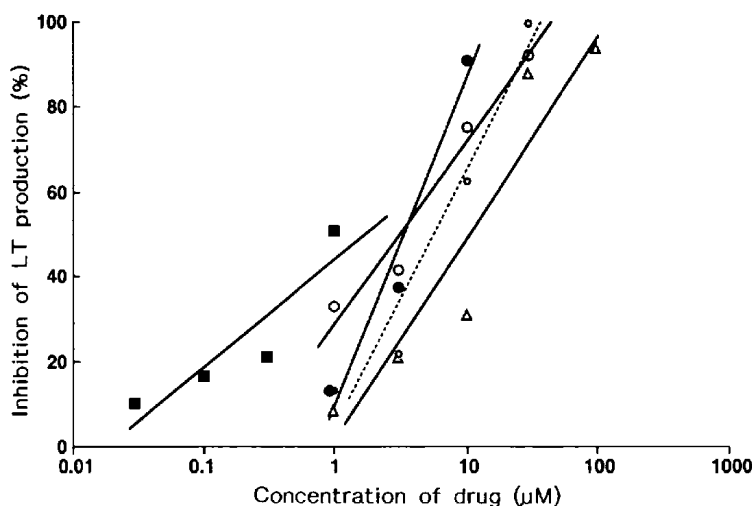
### Statistical analyses

The statistical significance between means was analyzed by variance analysis followed by Scheffe's multiple-comparison test and Student's *t*-test. The IC<sub>50</sub>-value, the concentration that is necessary to obtain 50% inhibition of the responses, was determined from the best fit regression line of the dose-response curve.

## RESULTS

### A23,187-induced production of LTs in human peripheral leukocytes in vitro

The total amount of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> produced by A23,187 (1 µM) was 3.1 µg/3 × 10<sup>5</sup> cells (n=4). AL-3264 (1–30 µM) inhibited the A23,187-induced LT production in a dose-related manner with an IC<sub>50</sub>-value of 3.1 µM. Ketotifen, oxatamide and NDGA also inhibited it with



**Fig. 2.** Effect of AL-3264 on A23187-induced LT production in human peripheral leukocytes in vitro. The drug solution being examined or the vehicle (100 µl) was added to the cell suspension (3 × 10<sup>5</sup> leukocytes/890 µl). After incubation at 37°C for 30 min, 10 µl of A23,187 (final concentration of 1 µM) was added to the cell suspension, and then the cell suspension was incubated for a further 15 min. Each point represents the mean with the S.E.M. of 3 to 4 experiments. Vertical bar: inhibition percent in the LT levels compared with the vehicle control. ○: AL-3264, △: ketotifen, ●: oxatamide, ■: NDGA. —: LT C<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub>, ···: LTB<sub>4</sub>.

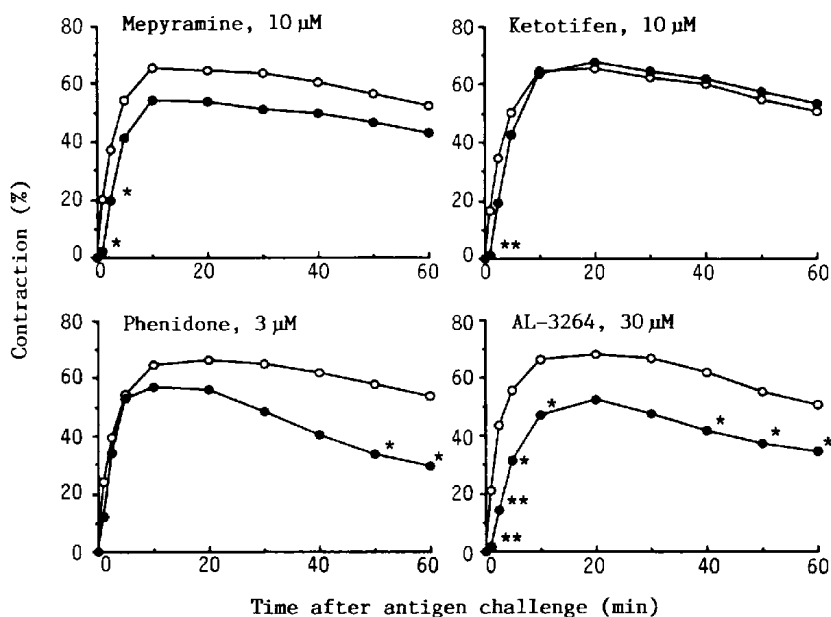
IC<sub>50</sub>-values of 10.4  $\mu$ M, 3.4  $\mu$ M and 1.7  $\mu$ M, respectively. The potency of AL-3264 was approximately equal to that of oxatomide, and 3.4 and 0.5 times that of ketotifen and

NDGA, respectively (Fig. 2). In addition, AL-3264 inhibited the A23,187-induced LTB<sub>4</sub> production with an IC<sub>50</sub>-value of 5.6  $\mu$ M.

**Table 1.** Effect of AL-3264 and AA-861 on antigen-induced LT production in the abdominal cavity of passively sensitized rats

Drug	Drug treatment			N	Leukotriene (ng/ml) mean $\pm$ S.E.M.	Inhibition (%)
	time <sup>a)</sup> (min)	route	dose (mg/kg)			
Control	15	p.o.		7	77.5 $\pm$ 4.1	
AL-3264			30	7	65.3 $\pm$ 2.8*	15.7
			100	6	61.0 $\pm$ 1.1**	21.3
Control	30	p.o.		6	92.6 $\pm$ 4.1	
AL-3264			30	6	69.1 $\pm$ 1.4**	25.4
			100	7	73.2 $\pm$ 3.1**	21.0
Control	5	i.v.		7	68.3 $\pm$ 1.4	
AL-3264			3	8	52.4 $\pm$ 1.8**	23.3
Control	5	i.v.		7	68.4 $\pm$ 2.2	
AA-861			1	8	65.6 $\pm$ 1.9	4.1
			3	7	56.4 $\pm$ 2.2*	17.5
			10	7	44.1 $\pm$ 2.8**	35.5
Control	5	i.v.		7	65.3 $\pm$ 2.1	
NDGA			3	6	68.7 $\pm$ 4.8	-5.2
			10	7	61.7 $\pm$ 2.2	5.5

The total levels of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> in the peritoneal exudate were measured by radioimmunoassay. <sup>a</sup>Time (min) between drug addition and antigen challenge. \*P < 0.05 and \*\*P < 0.01: significantly different from the matched control (Scheffe's multiple-comparison test).



**Fig. 3.** Effect of AL-3264 on antigen-induced contractions in isolated trachea from actively sensitized guinea pig. Sensitized guinea pig trachea was challenged with egg albumin (10  $\mu$ g), and the tension of the trachea was measured isometrically. Drugs were added 5 min before the antigen challenge. Each point represents the mean of 4 to 6 preparations. Vertical bar: antigen-induced contractions that were expressed as a percentage of the maximum response to histamine. ○: control, ●: drug-treated group. \*P < 0.05 and \*\*P < 0.01: significantly different from the matched control (Student's *t*-test).

#### Antigen-induced LT production in the abdominal cavity of passively sensitized rats

The total content of LTs  $C_4$ ,  $D_4$  and  $E_4$  in the peritoneal exudate was  $76.1 \pm 2.4$  ng/ml (mean  $\pm$  S.E.M.,  $n=27$ ) at 10 min after i.p. administration of EA to passively sensi-

tized rats. When AL-3264 was orally administered 15 or 30 min before the EA challenge, AL-3264 (30 and 100 mg/kg) decreased the LT content in the peritoneal exudate. When i.v. administered 5 min before the EA challenge, AL-3264 at 3 mg/kg also decreased the LT content with the same potency as AA-861, a 5-LO inhibitor. NDGA was inactive up to 10 mg/kg, i.v. (Table I).

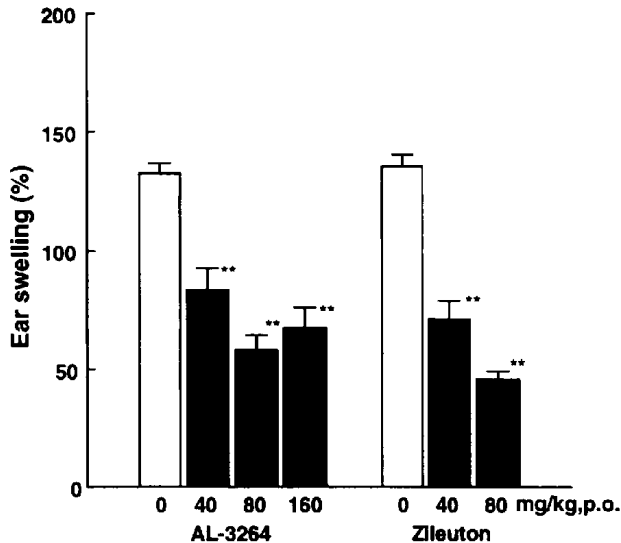


Fig. 4. Effect of AL-3264 on arachidonic acid (AA)-induced ear edema in mice. AA acetone solution (0.25 mg/20  $\mu$ l) was applied to both surfaces of the right ear of mice. One hour later, a circular part (5.5 mm in diameter) of both ears was removed and weighed. Drugs were orally administered 1 hr before AA application. Ear swelling was expressed as a percentage of the increase in weight of the AA-treated ear compared to that of the contralateral untreated ear. Each column represents the mean with the S.E.M. of 8 to 24 mice. \*\* $P < 0.01$ : significantly different from the control (Scheffe's multiple-comparison test).

#### Antigen-induced contractions in isolated trachea from actively sensitized guinea pigs

As shown in Fig. 3, EA (10  $\mu$ g) caused a long lasting contraction in isolated trachea from actively sensitized guinea pigs. The contraction induced by EA was little affected by repeated washing with Tyrode solution 60 min after the antigen challenge. AL-3264 at 30  $\mu$ M suppressed the initial and sustained phases of the antigen-induced contraction. Phenidone at 3  $\mu$ M suppressed the sustained phase of the contraction, while mepyramine and ketotifen at 10  $\mu$ M suppressed the initial phase of the contraction. Indomethacin did not affect the two phases of contraction at 1  $\mu$ M but rather augmented it at 10  $\mu$ M (data not shown).

#### Arachidonic acid-induced ear edema in mice

Topical application of AA (0.25 mg) to the right ear of mice caused acute edema that reached a peak at 1 hr after AA application. The swelling rate in AA-treated ears was  $133 \pm 4\%$  (mean  $\pm$  S.E.M.,  $n=24$ ) at 1 hr after topical application of AA. AL-3264 (40–160 mg/kg) suppressed the AA-induced ear edema when AL-3264 was orally administered 1 hr before AA application. Zileuton (40 and 80 mg/kg, p.o.) also suppressed the ear edema in a dose-related manner (Fig. 4).

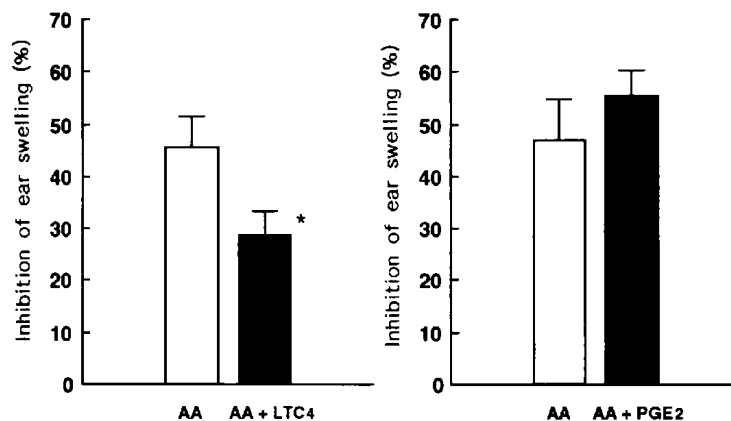


Fig. 5. Influence of LTC<sub>4</sub> and PGE<sub>2</sub> on the anti-edematous effects of AL-3264 in AA-induced mouse ear edema. LTC<sub>4</sub> (0.1  $\mu$ g/ear) was intradermally injected immediately before AA application or PGE<sub>2</sub> (1  $\mu$ g/ear) was topically applied together with AA acetone solution. AL-3264 (160 mg/kg) was orally administered 1 hr before AA application. Vertical bar: inhibition percent of ear swelling that was calculated by comparing the ear swelling in drug-treated mice with that in vehicle-treated mice. Each column represents the mean with the S.E.M. of 16 mice. Open column: edema induced by AA alone. Closed column: edema induced by AA plus LTC<sub>4</sub> or PGE<sub>2</sub>. \* $P < 0.05$ : significantly different from AA-induced ear edema (Student's *t*-test).

The anti-edematous effect of AL-3264 (160 mg/kg) in the AA-induced ear edema was reduced by concomitant application of LTC<sub>4</sub> (0.1  $\mu$ M), but not by PGE<sub>2</sub> (1  $\mu$ M) (Fig. 5). However, the AA-induced ear edema was not affected by concomitant application of LTC<sub>4</sub> (0.1  $\mu$ M) or PGE<sub>2</sub> (1  $\mu$ M) (data not shown).

## DISCUSSION

In the present study, AL-3264 was found to inhibit the A23,187-induced production of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> in human peripheral leukocytes in vitro with an IC<sub>50</sub>-value of 3.1  $\mu$ M. AL-3264 was more potent than ketotifen and two times less potent than NDGA in inhibiting the LT production. In addition, AL-3264 inhibited the A23,187-induced production of LTB<sub>4</sub> with an IC<sub>50</sub>-value of 5.6  $\mu$ M. Furthermore, orally administered AL-3264 inhibited the antigen-induced production of LTs C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> in the abdominal cavity of passively sensitized rats. In this inhibition, AL-3264 was as potent as AA-861, a 5-LO inhibitor, which has been demonstrated to inhibit potently the 5-LO of guinea pig peritoneal leukocytes with an IC<sub>50</sub>-value of 0.8  $\mu$ M (20). From these results and our previous ones (12) demonstrating that AL-3264 inhibits 5-LO from guinea pig leukocytes in vitro, it is suggested that AL-3264 inhibits LT production in human peripheral leukocytes and in the abdominal exudates of passively sensitized rats, as the result of its inhibition of 5-LO activity.

These results led us to examine the effect of AL-3264 on allergic and inflammatory responses in which LTs have been reported to be involved. First, the effect of AL-3264 on antigen-induced contraction of isolated trachea from sensitized guinea pigs was examined. This contraction has been reported to be mainly mediated by histamine and leukotrienes (21): the initial and sustained phases of the contraction are mediated by histamine and LTs, respectively. The involvement of LTs in the sustained phase was further supported by the selective inhibitory effect of ONO-1078, a LT antagonist, in a study using sensitized guinea pig trachea (22). In the present study, phenidone, a dual inhibitor of 5-LO and cyclooxygenase (CO), suppressed the sustained phase. The results with phenidone were in accordance with those of Adams and Lichtenstein (21). On the other hand, indomethacin, a CO inhibitor, was without effect on the contraction at low doses and rather enhanced the sustained phase at a high dose; the result being consistent with that of Hand and Buckner (23). Accordingly, the suppressive effect of phenidone on the sustained phase may be possibly due to a decrease in LT production resulting from 5-LO inhibition. AL-3264 was found to suppress not only the initial phase but also the sustained phase. From these results and a result that AL-3264 did not exert any inhibitory effect on LT-induced

contractions in isolated guinea pig trachea (K. Ishii, unpublished observation), it is conceivable that the effect of AL-3264 on the sustained phase is through its inhibition of LT production.

Furthermore, the effect of AL-3264 on AA-induced ear edema in mice was examined. This ear edema has been shown to be suppressed by 5-LO inhibitors and some CO inhibitors, although the former is more potent than the latter in suppressing the ear edema (19, 24). We have got evidence that LTs and PGs are mainly involved in AA (0.25 mg)-induced ear edema in mice (25). In the present study, AL-3264 suppressed the AA-induced ear edema at oral doses over 40 mg/kg as well as zileuton, which was chosen as a reference drug because its potent oral activity is superior to that of AA-861 (26). AL-3264 has no inhibitory effect on carrageenan-induced edema in rat hind paw at an oral dose of 80 mg/kg (K. Ishii, unpublished observation), suggesting that AL-3264 does not inhibit CO activity. Accordingly, it is probable that AL-3264 suppresses the AA-induced ear edema in mice through inhibition of LT production.

We have recently developed a useful method by which the mode of the anti-inflammatory effect of 5-LO and CO inhibitors can be differentially clarified (25). Namely, the suppressive effect of zileuton on AA-induced ear edema was reduced by LTC<sub>4</sub> injected intradermally to the ear immediately before AA application, but not affected by PGE<sub>2</sub> applied topically to the ear together with AA. On the other hand, the anti-edematous effect of indomethacin was reduced by PGE<sub>2</sub>, but not by LTC<sub>4</sub>. When AL-3264 was tested in this method, it was found that the anti-edematous effect of AL-3264 was reduced by LTC<sub>4</sub>, but not by PGE<sub>2</sub>, as in the case of zileuton. These results suggest that inhibition of LT production by AL-3264 contributes to its suppressive effect on the AA-induced ear edema in mice.

In conclusion, AL-3264 inhibits LT production in vivo and in vitro. These effects of AL-3264 is probably due to the inhibition of 5-LO, but the possibility that AL-3264 also inhibits the release process of LT can not be ruled out. The property of AL-3264 to inhibit LT production seems to play an important role in its antiallergic effects.

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