

# TA-270 [4-Hydroxy-1-methyl-3-octyloxy-7-sinapinoylamino-2(1*H*)-quinolinone], an Anti-Asthmatic Agent, Inhibits Leukotriene Production Induced by IgE Receptor Stimulation in RBL-2H3 Cells

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## ABSTRACT

A novel quinolinone derivative, TA-270 [4-hydroxy-1-methyl-3-octyloxy-7-sinapinoylamino-2(1*H*)-quinolinone], has been shown to inhibit antigen-induced asthmatic responses including the early-phase bronchoconstriction in actively sensitized guinea pigs. Here we characterized the action mechanisms of TA-270 in cellular level in vitro. In RBL-2H3 mast cells sensitized with dinitrophenol (DNP)-specific IgE, the antigen exhibited several mast cell functions, including hexosaminidase release as a marker of degranulation, production of tumor necrosis factor- $\alpha$ , and production of immunologically detectable leukotrienes. These antigen-induced actions were associated with the activation of several early signaling events, including inositol phosphate production reflecting phospholipase C activation and extracellular signal-regulated kinase activation. When the cells were treated with TA-270,

the antigen-induced leukotriene production was almost completely suppressed, but other antigen-induced actions listed above were hardly affected. This drug also failed to affect the antigen-induced phospholipase A<sub>2</sub> activation as evaluated by the total release of arachidonic acid and its metabolites from the cells prelabeled with radioactive arachidonic acid. However, TA-270 clearly changed the arachidonic acid metabolic pathway. It suppressed the accumulation of 5-lipoxygenase products, including leukotrienes, but hardly affected the accumulation of cyclooxygenase products. The inhibitory action of TA-270 on leukotriene production was also observed in human neutrophils and eosinophils. We conclude that TA-270 inhibits 5-lipoxygenase activity and, thereby, suppresses the antigen-induced leukotriene production.

Bronchial asthma is an atopic disease characterized by bronchoconstriction (O'Byrne et al., 1986), an influx of inflammatory cells into the airway (Kay, 1992), and bronchial hyper-responsiveness (Durham et al., 1988). One early event accompanied by these asthmatic responses is the activation of mast cells, which is elicited through the cross-linking of antigen-specific IgE bound to the high-affinity receptor for IgE (Fc $\epsilon$ RI) (Kinet, 1989). Thus, antigen-induced stimulation of Fc $\epsilon$ RI causes degranulation of the cells and release of inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Gordon and Galli, 1990) and phospholipase A<sub>2</sub> products such as prostaglandins and leukotrienes (Lewis et al., 1982; Holgate and Kay, 1985).

In the previous study (Aoki et al., 2000), we observed that a quinolinone derivative TA-270 [4-hydroxy-1-methyl-3-octyloxy-7-sinapinoylamino-2(1*H*)-quinolinone] inhibited antigen-induced asthmatic responses, including early-phase and late-phase bronchoconstriction, infiltration of inflammatory cells in bronchial lumen, and airway hyper-responsiveness in actively sensitized guinea pigs. The finding of the inhibition of early-phase bronchoconstriction by TA-270 raises the possibility that this drug might affect the mast cell functions because activation of this type of cell is important for the induction of early-phase airway responses (Metcalf et al., 1997). Here we characterized the action mechanisms of TA-270 in RBL-2H3 mast cells. We found that this drug specifically inhibited production of leukotrienes, probably by inhibiting 5-lipoxygenase, a late-limiting enzyme for the synthesis of potent mediators for bronchoconstriction. The inhibitory action of TA-270 on leukotriene production was also observed in human neutrophils and eosinophils.

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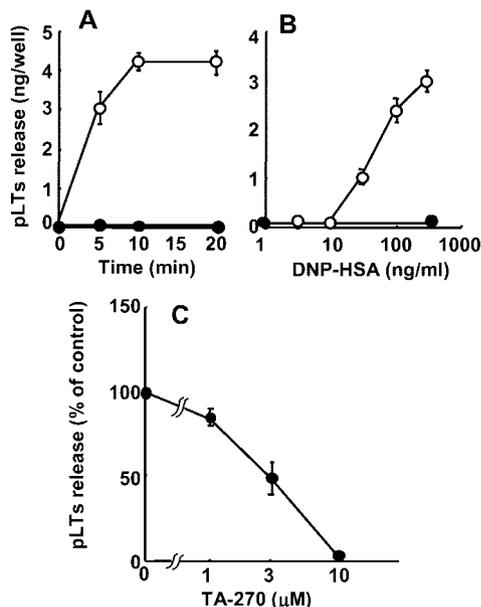
**ABBREVIATIONS:** TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TA-270, 4-hydroxy-1-methyl-3-octyloxy-7-sinapinoylamino-2(1*H*)-quinolinone; DNP-HSA, O-dinitrophenol-conjugated human serum albumin; ERK, extracellular signal-regulated kinase; TLC, thin layer chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; AA, arachidonic acid; pLT, peptide leukotriene.

## Materials and Methods

**Drugs and Chemicals.** TA-270 was synthesized by Dainippon Ink and Chemicals, Inc. Other chemicals were obtained from the following sources: RPMI 1640, fetal bovine serum, and *O*-dinitrophenol-conjugated human serum albumin (DNP-HSA) from Sigma-Aldrich (St. Louis, MO); DNP-specific monoclonal IgE from Zymed Laboratories, Inc. (San Francisco, CA); [<sup>14</sup>C]arachidonic acid and [<sup>3</sup>H]arachidonic acid from American Radiolabeled Chemicals, Inc. (St. Louis, MO); and *p*-nitrophenyl- $\beta$ -*d*-hexosamine from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell Cultures.** RBL-2H3 mast cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified air/CO<sub>2</sub> (19:1) atmosphere in 10-cm dishes. Two days before the experiments, cells were subcultured in 10-cm dishes for the measurement of ERK activity, in six multiplates for the experiments in which arachidonic acid metabolites were analyzed by TLC, and in 24 multiplates for a hexosaminidase release assay. The cells were subcultured in 12 multiplates for other experiments, including measurements of leukotriene release by immunoassay, TNF- $\alpha$  production, inositol phosphate production, and total release of arachidonic acid and its metabolites (without TLC analysis). In the experiments for inositol phosphate production, the medium was changed to an inositol-free RPMI 1640 medium containing 0.1% bovine serum albumin and 2  $\mu$ Ci/ml [<sup>3</sup>H]inositol 24 h before experiments as described previously (Kon et al., 1999). In the experiments for the release of arachidonic acid and its metabolites, [<sup>14</sup>C]arachidonic acid (0.3  $\mu$ Ci/well) was added into the medium for the experiments of TLC analysis and [<sup>3</sup>H]arachidonic acid (0.1  $\mu$ Ci/well) for the experiments without TLC analysis 10 h before experiments.

**Assay Conditions of RBL-2H3 Cells with Test Agents.** Except for the experiment of TNF- $\alpha$  production, which was performed in the same medium as the culture, i.e., RPMI 1640 supplemented with 10% fetal bovine serum, other experiments were performed in a Hepes-buffered medium consisting of 20 mM Hepes (pH 7.5), 134



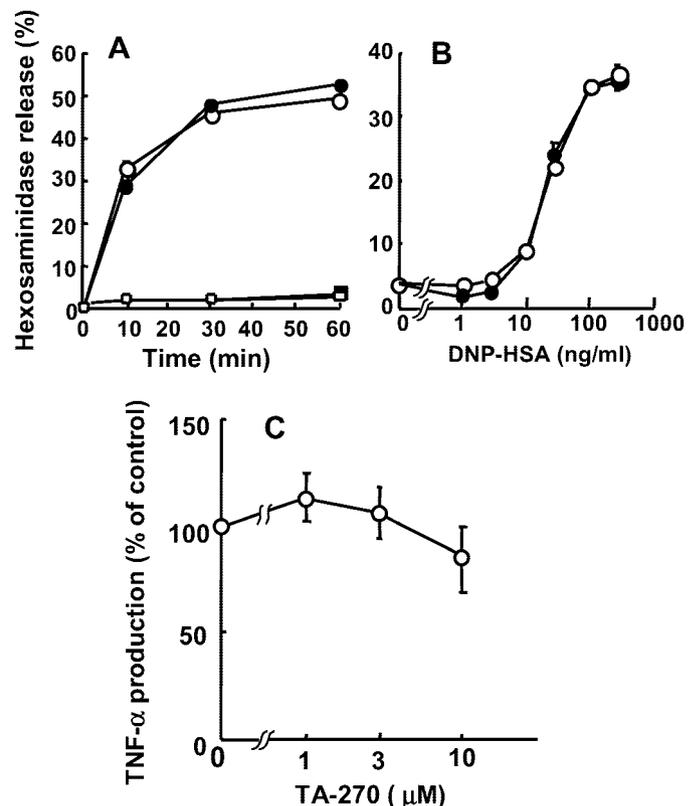
**Fig. 1.** Inhibitory effect of TA-270 on peptide leukotriene (pLTs) release. The RBL-2H3 cells were treated (●) or untreated (○) with 10  $\mu$ M TA-270 and then incubated for the indicated time with 100 ng/ml DNP-HSA (A) or incubated for 10 min with the indicated concentrations of DNP-HSA (B). The significant release was not detected in the absence of DNP-HSA. The results are expressed as the net amount released into the medium (ng/well). Cells were treated with indicated concentrations of TA-270 and then incubated for 10 min with 100 ng/ml DNP-HSA (C). The results are expressed as the percentage of the control value obtained in the absence TA-270. The control value was  $3.2 \pm 0.2$  ng/well. In all experiments, results are means  $\pm$  S.E.M. of three separate experiments.

mM NaCl, 4.7 mM KCl, 2 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>CO<sub>3</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V). Unless otherwise specified, the cells were treated with DNP-specific IgE (1  $\mu$ g/ml) 1 h before and with TA-270 at the appropriate concentration 30 min before stimulation with the antigen DNP-HSA and other stimulants.

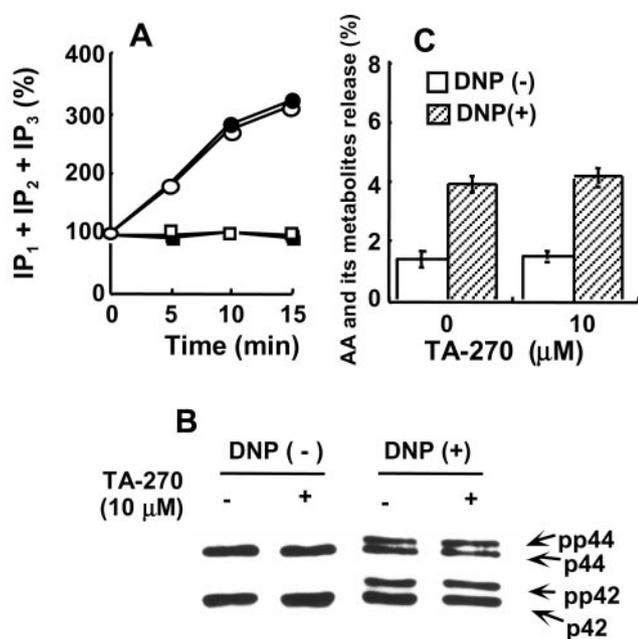
**Production of TNF- $\alpha$ , LTB<sub>4</sub>, and Peptide Leukotrienes.** The cells were incubated for the appropriate time as indicated, and the supernatant was collected. TNF- $\alpha$  was measured with the enzyme immunoassay kit system from Pierce Endogen (Rockford, IL), LTB<sub>4</sub>, and peptide leukotrienes (including LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>) were measured with the EIA system from Amersham (Buckinghamshire, UK) according to the manufacturer's instructions.

**$\beta$ -*d*-Hexosaminidase Release.** After incubation with test agents for the indicated time, a supernatant was collected. The enzyme activity was measured as previously described (Hide et al., 1997). Briefly, an 80- $\mu$ l test sample and 100  $\mu$ l of 4 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -glucosamine in 0.2 M citrate, pH 4.5, were incubated in 96-well plates to yield the chromophore, *p*-nitrophenol. The absorbance of the colored product was assessed at 405 nm using a microplate reader. The activity of  $\beta$ -hexosaminidase released into the medium was expressed as the percentage of total  $\beta$ -hexosaminidase, which was determined in the cells lysed in 0.2% Triton X-100.

**Release of Arachidonic Acid and Its Metabolites from [<sup>3</sup>H] or [<sup>14</sup>C]Arachidonic Acid-Labeled Cells.** For the measurement of the



**Fig. 2.** Effects of TA-270 on hexosaminidase release and TNF- $\alpha$  production. A, RBL-2H3 cells were treated (●, ■) or untreated (○, □) with 10  $\mu$ M TA-270 and then incubated for the indicated time with (circle) or without (square) 100 ng/ml DNP-HSA. B, cells were treated (●) or untreated (○) with 10  $\mu$ M TA-270 and then incubated for 10 min with the indicated concentrations of DNP-HSA. The enzyme activity released into medium was determined. The results are means  $\pm$  S.E.M. of three separate experiments and expressed as the percentage of the total enzyme activity. C, cells were treated with indicated concentrations of TA-270 and then incubated for 4 h with 100 ng/ml DNP-HSA to measure TNF- $\alpha$  production. The results are means  $\pm$  S.E.M. of three separate experiments and are expressed as the percentage of the control activity obtained in the absence TA-270. The control activity was  $480 \pm 52$  pg/well.



**Fig. 3.** Effects of TA-270 on the antigen-induced activation of phospholipase C, ERK, and phospholipase A<sub>2</sub>. **A**, RBL-2H3 cells were treated (■, □) or untreated (●, ○) with 10 μM TA-270 and then incubated for the indicated time with (circle) or without (square) 100 ng/ml DNP-HSA to measure inositol phosphate production. The results are means ± S.E.M. of three separate experiments and are expressed as percentage of the basal activity. The basal activity was 1555 ± 225 dpm. **B**, cells were treated or untreated with 10 μM TA-270 as indicated and then incubated for 5 min with or without 100 ng/ml DNP-HSA to examine ERK activation. The result shown is a representative one. Two other experiments gave similar results. **C**, cells that had been labeled with [<sup>3</sup>H]arachidonic acid were treated or untreated with 10 μM TA-270 and then incubated for 10 min with or without 100 ng/ml DNP-HSA to measure arachidonic acid (AA) and its metabolites release. The results are means ± S.E.M. of three separate experiments and expressed as the percentage of the total radioactivity incorporated into the cells (100,148 ± 1184 dpm).

total release of [<sup>3</sup>H]arachidonic acid and its metabolites, the cells were incubated with test agents for 10 min. The supernatant was then collected, and its radioactivity was measured in a scintillation spectrometer. The activity was expressed as the percentage of the total radioactivity incorporated into the cells. In the case of the experiments for the analysis of the fate of [<sup>14</sup>C]arachidonic acid cleaved by phospholipase A<sub>2</sub>, the supernatant was collected, and [<sup>14</sup>C]arachidonic acid and its metabolites were extracted and analyzed by TLC as described previously (Okajima and Ui, 1984). In brief, to the reaction supernatant (0.5 ml), 0.75 ml of chloroform, 0.75 ml of methanol, 0.2 ml of 2 M KCl, and 0.05 ml of 1 M HCl were added. The mixture was then shaken vigorously and separated into two phases by centrifugation. The lower phases were evaporated to dryness, and arachidonic acid and its metabolites were separated by TLC on a plate of silica gel 60 (Merck Ltd., Poole, Dorset, UK) with a solvent system of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90:20:20:100, v/v, upper phase). The bands were visualized by autoradiography, and each lipid fraction was scraped and counted in a liquid scintillation spectrometer.

**Measurement of Inositol Phosphate Production.** The cells were incubated with test agents in the presence of 10 mM LiCl. The total inositol phosphates were separated as described previously (Kon et al., 1999).

**Measurement of ERK Activity.** RBL-2H3 cells were stimulated with the antigen for 5 min, and the reaction was then terminated by washing twice with ice-cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline and adding 0.5 ml of a lysis buffer. The lysate was analyzed by Western blotting with an ERK-specific antibody to detect the change in gel mobility reflecting the phosphorylation of the enzyme as described previously (Sato et al., 1999).

**Leukotriene Production in Human Neutrophils and Eosinophils.** Human neutrophils and eosinophils were isolated from venous blood drawn from normal volunteers. The granulocyte fraction was prepared by sequential steps of dextran sedimentation, centrifugation through Ficoll-Paque, and hypotonic lysis of erythrocytes, as previously described (Borgeat et al., 1984). Granulocytes were used as neutrophils in this experiment. Eosinophils were isolated from the granulocyte fraction by negative selection with anti-CD16 microbeads and Super MACS, as previously described (Hansel et al., 1991). Human neutrophils and eosinophils were suspended in a 25 mM PIPES-buffered medium (pH 7.4) consisting of 110 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 0.6 mM MgCl<sub>2</sub>, 5.4 mM glucose, and 5% fetal calf serum. Cells were treated with or without TA-270, stimulated with calcium ionophore A23187, and the reaction was stopped by the addition of the ice-cold PIPES-buffered medium, as previously described (Fukuda et al., 1995). The cell suspension was centrifuged, and the amount of LTB<sub>4</sub> and peptide leukotrienes in the supernatant was measured with a commercial EIA kit for neutrophils and eosinophils, respectively.

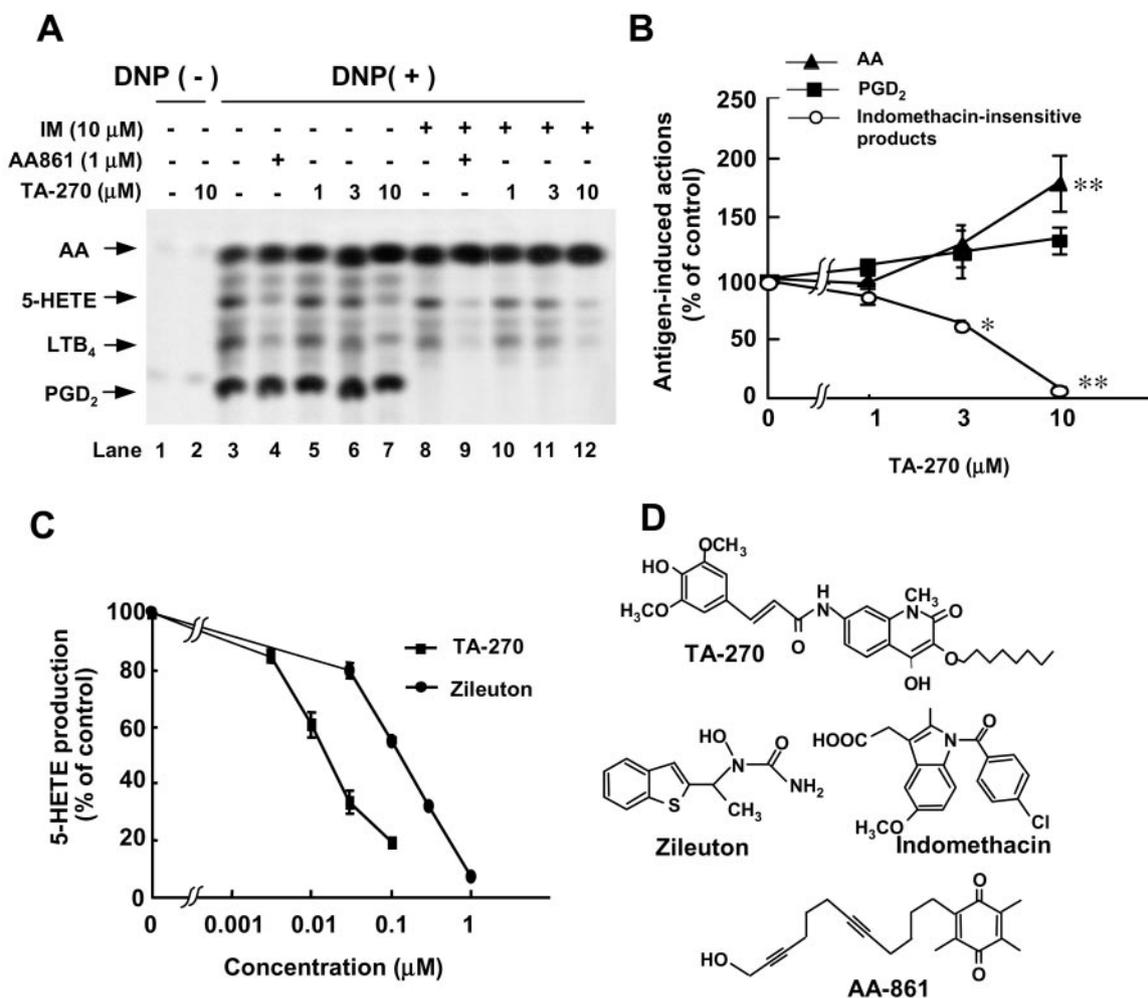
**Cell-Free Assay for 5-Lipoxygenase.** RBL-1 basophilic cell suspension (7 × 10<sup>7</sup> cells/ml) in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline was sonicated and centrifuged at 100,000g at 4°C for 60 min. The supernatant was used for a cell-free 5-lipoxygenase activity. The crude enzyme extract was preincubated with test compounds for 4 min and incubated with [<sup>14</sup>C]arachidonic acid (0.5 μCi) in the presence of 1 mM CaCl<sub>2</sub> and 10 μM indomethacin for 30 min at 30°C at the final volume of 0.5 ml. The reaction was stopped by adding 1 ml of dichloromethane and 0.25 ml of 2 M formic acid. The mixture was then shaken and separated into two phases by centrifugation. The lower phase was evaporated to dryness and dissolved with ethanol. The ethanol solution was injected into a high performance liquid chromatograph column of Luna 3 μm C18(2) (Phenomenex Inc., Torrance, CA) eluted with acetonitrile/water/acetic acid (70:30:1) at 1 ml/min and monitored by a radiometric flow scintillation analyzer (PerkinElmer Life Sciences, Boston, MA). Arachidonic acid and a major 5-lipoxygenase product 5-HETE were detected at retention time approximately 11 and 3.9 min, respectively.

**Data Presentation.** All experiments were performed in duplicate or triplicate. The results of multiple observations were presented as means ± S.E.M. of at least three separate experiments unless otherwise stated. Statistical significance was assessed by Dunnett's multiple test.

## Results

**TA-270 Inhibits the Production of Leukotrienes but Hardly Affects Hexosaminidase Release and TNF-α Production.** In Fig. 1, we measured the production of peptide leukotrienes, including leukotriene C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub>, in response to DNP-HSA in RBL-2H3 mast cells sensitized with the antigen-specific IgE. The antigen induced the time- (Fig. 1A) and dose-dependent (Fig. 1B) production of peptide leukotrienes. When the cells were treated with TA-270 at 10 μM, these responses were almost completely inhibited. The half-maximal concentration (IC<sub>50</sub>) of TA-270 required for the inhibition of the response was about 3 μM (Fig. 1C).

In Fig. 2, we examined the effects of TA-270 on the degranulation response and TNF-α production. As an index of the degranulation response, we measured the activity of the hexosaminidase release. The enzyme was released in a manner dependent on time (Fig. 2A) and dose (Fig. 2B) in response to the antigen. Under the appropriate condition, the enzyme release reached 35 to 50% of the total enzyme. In contrast to the leukotriene production, however, TA-270 did not influence the degranulation response (Fig. 2, A and B). TA-270 also failed to significantly inhibit the antigen-induced TNF-α production (Fig. 2C).

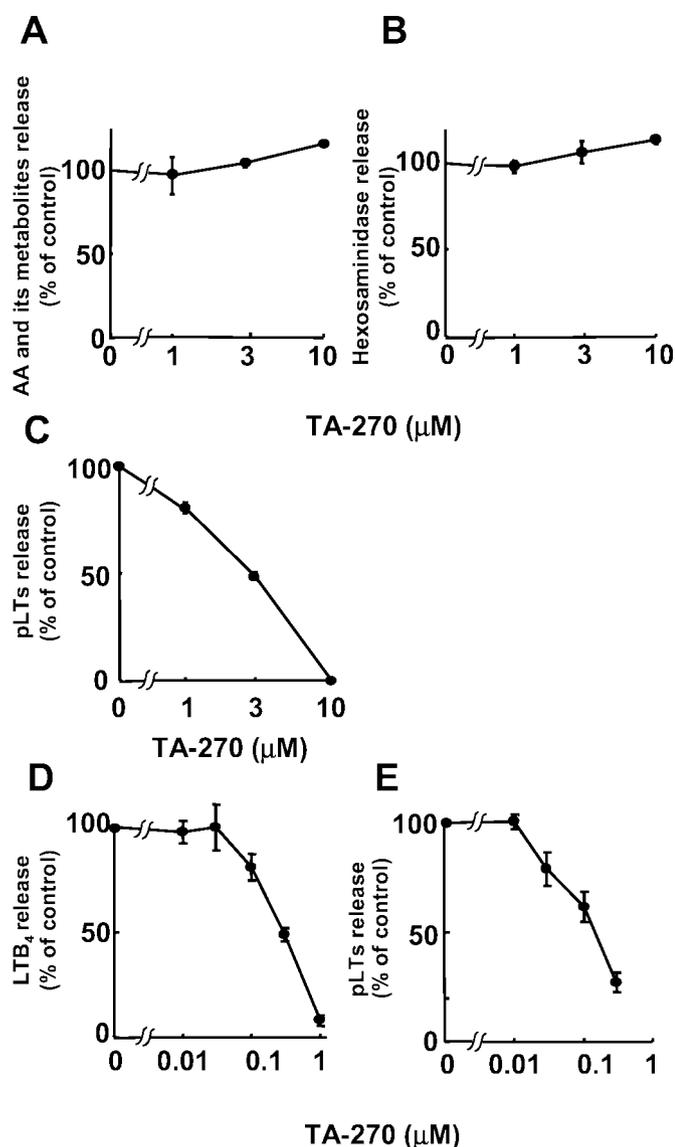


**Fig. 4.** Effects of TA-270 and inhibitors for 5-lipoxygenase and cyclooxygenase on the fate of arachidonic acid in intact RBL-2H3 cells and 5-lipoxygenase activity in RBL-1 cell-free system. **A**, RBL-2H3 cells that had been labeled with [<sup>14</sup>C]arachidonic acid were treated with the indicated concentrations of TA-270, AA861, or indomethacin (IM) and then incubated for 10 min with or without 100 ng/ml DNP-HSA to examine the fate of the arachidonic acid cleaved by phospholipase A<sub>2</sub>. Radiolabeled arachidonic acid and its metabolites were extracted and then analyzed by TLC. A representative autoradiograph is shown. Three other experiments gave similar results. The position of arachidonic acid (AA), 5-HETE, LTB<sub>4</sub>, and PGD<sub>2</sub> is shown by an arrow. **B**, effects of TA-270 on the production of AA, PGD<sub>2</sub>, and indomethacin-insensitive products were evaluated from the experiments in **A**. As for AA and PGD<sub>2</sub> production, the radioactivity in the respective band in lanes 3 and 5 to 7 was used in the absence of indomethacin. On the other hand, all the areas between LTB<sub>4</sub> and 5-HETE in lanes 8 and 10 to 12 were scraped, and their radioactivity was assigned as indomethacin-insensitive products. The results are means  $\pm$  S.E.M. of four separate experiments and are expressed as the percentage of the control values in the absence of TA-270. The control value was 1159  $\pm$  69 (AA), 921  $\pm$  168 (PGD<sub>2</sub>), and 953  $\pm$  154 dpm (indomethacin-insensitive products). The effect of TA-270 was significant at \*,  $P < 0.05$ , \*\*,  $P < 0.01$  by Dunnett's multiple test. **C**, cell-free extract of RBL-1 cells was incubated in the presence of [<sup>14</sup>C]arachidonic acid with or without indicated concentrations of TA-270 (■) and zileuton (●) to monitor the 5-HETE production. In the absence of inhibitors, 15.0  $\pm$  1.2% of arachidonic acid is converted to 5-HETE. The results are means  $\pm$  S.E.M. of three separate experiments and expressed as the percentage of the control 5-HETE production in the absence of inhibitors. **D**, chemical structure of TA-270, indomethacin, AA861, and zileuton is shown.

**Failure of TA-270 to Inhibit Early Signaling Events Involved in IgE Receptor-Mediated Actions.** We next searched for the action site of TA-270 in the signaling pathways leading to the production of leukotrienes through stimulation of the IgE receptor. Since leukotrienes are phospholipase A<sub>2</sub> products, we examined the signaling events, which may regulate the enzyme activity. However, TA-270 was ineffective for inhibiting phospholipase C activation (Fig. 3A) and ERK activation (Fig. 3B) induced by IgE receptor stimulation. TA-270 was also ineffective for inhibiting phospholipase A<sub>2</sub> activity, which was estimated by the sum of the release of arachidonic acid and its metabolites (Fig. 3C).

**Specific Inhibition by TA-270 of 5-Lipoxygenase Pathways.** These results suggest that TA-270 may inhibit the metabolic pathway of arachidonic acid cleaved by phospholipase A<sub>2</sub> leading to leukotriene. In Fig. 4, we analyzed

the fate of arachidonic acid. In the cells not stimulated with antigen, no significant band was detected. As examples, we presented the results of the effect of TA-270 on the basal accumulation of arachidonic acid or its metabolites in this figure (lanes 1 and 2). On the other hand, in the cells stimulated with antigen, several bands in addition to arachidonic acid were detected (lane 3). These bands, other than arachidonic acid, may be products of either 5-lipoxygenase or cyclooxygenase because the radioactivity of the respective band was decreased or disappeared by AA861 (an inhibitor of 5-lipoxygenase, Fig. 4D, lane 4), indomethacin (an inhibitor of cyclooxygenase, Fig. 4D, lane 8), or their combination (lane 9). The effects of the increasing concentration of TA-270 in the absence (lanes 5–7) and presence (lanes 10–12) of indomethacin were examined. The pattern of the distribution of arachidonic acid metabolites was very similar to that induced



**Fig. 5.** Effects of TA-270 on  $\text{Ca}^{2+}$  ionophore A23187-induced actions. RBL-2H3 cells were treated with the indicated concentrations of TA-270 and then incubated with or without  $1 \mu\text{M}$  A23187 for 10 min to measure arachidonic acid and its metabolites release as an index of phospholipase  $\text{A}_2$  activity (A), hexosaminidase release (B), and pLTs release (C). A, the cells prelabeled with [ $^3\text{H}$ ]arachidonic acid were used. The results are expressed as the percentage of control activity induced by A23187 in the absence of TA-270. The control activity was  $12.2 \pm 0.3\%$  for AA, and its metabolites release (A),  $44.6 \pm 0.7\%$  for hexosaminidase release (B), and  $3.9 \pm 0.2 \text{ ng/well}$  (about  $1.5 \times 10^6$  cells) for pLTs release (C). The effects of TA-270 on the A23187-induced leukotriene release were also examined in human neutrophils (D) and human eosinophils (E). For this, the respective cell preparations were treated with the indicated concentration of TA-270 and then incubated for 10 min with or without A23187 at  $0.2 \mu\text{M}$  for neutrophils and at  $1 \mu\text{M}$  for eosinophils. LTB<sub>4</sub> and peptide leukotrienes were measured in neutrophils and eosinophils, respectively. The results are expressed as the percentage of the control activity induced by A23187 in the absence of TA-270. The control activity was  $13.7 \pm 6.7 \text{ ng of LTB}_4/10^6 \text{ cells}$  (D) and  $16.8 \pm 0.8 \text{ ng of peptide leukotrienes}/10^6 \text{ cells}$  (E).

by AA861. For example, the radioactivity of some bands with an  $R_f$  value similar to that of 5-HETE or LTB<sub>4</sub> was significantly decreased by TA-270 (lane 7), and all the bands, except for arachidonic acid, almost completely disappeared in the presence of both indomethacin and TA-270 (lane 12). The results are summarized in Fig. 4B. Thus, TA-270 hardly

affected the production of cyclooxygenase product PGD<sub>2</sub> but almost completely suppressed the formation of indomethacin-insensitive or 5-lipoxygenase products. At the expense of the inhibition of 5-lipoxygenase pathways, TA-270 treatment significantly increased the apparent formation of arachidonic acid.

In Fig. 4C, we examined whether TA-270 directly inhibits 5-lipoxygenase activity using a cell-free extract of RBL-1 basophilic cells, which express high 5-lipoxygenase activity (Kikuchi et al., 1994). To exclude the cyclooxygenase pathway, indomethacin was included in the assay medium. TA-270 and another 5-lipoxygenase inhibitor zileuton (Fig. 4D) inhibited the formation of the enzyme product 5-HETE in a dose-dependent manner. TA-270 was more potent than zileuton; the  $\text{IC}_{50}$  value was  $17 \text{ nM}$  for TA-270 and  $160 \text{ nM}$  for zileuton.

**Inhibitory Effects of TA-270 on  $\text{Ca}^{2+}$  Ionophore-Induced Leukotriene Release Not Only in RBL-2H3 Cells but Also Human Neutrophils and Eosinophils.** The foregoing results suggest that the inhibitory action of TA-270 may no longer be restricted to the IgE receptor signaling. Actually, TA-270 inhibited the peptide leukotriene release as induced by  $\text{Ca}^{2+}$  ionophore A23187 with  $\text{IC}_{50}$  of about  $3 \mu\text{M}$  (Fig. 5C). Similar to the case of the IgE receptor stimulation, TA-270 never inhibited the  $\text{Ca}^{2+}$  ionophore-induced phospholipase  $\text{A}_2$  activation (Fig. 5A) and hexosaminidase release (Fig. 5B).

The inhibition by TA-270 of A23187-induced production of leukotrienes was also observed in human neutrophils (LTB<sub>4</sub>) and eosinophils (peptide leukotrienes), although the  $\text{IC}_{50}$  values were approximately  $0.3 \mu\text{M}$  (Fig. 5D) and  $0.1 \mu\text{M}$  (Fig. 5E), respectively, which were about 10 times more potent than that in RBL-2H3 cells.

## Discussion

In the present study, we showed that TA-270 inhibited IgE receptor-mediated accumulation of 5-lipoxygenase products without appreciable effects on phospholipase  $\text{A}_2$  and cyclooxygenase activities in RBL-2H3 mast cells. TA-270 also hardly affected early signaling events involved in the IgE receptor-mediated actions. The inhibitory action of TA-270 on leukotriene production was also observed in human neutrophils and eosinophils. Thus, TA-270 inhibited the formation of not only 5-HETE but also leukotrienes, including LTB<sub>4</sub> and peptide leukotrienes in intact cells. These results suggest that this drug may inhibit 5-lipoxygenase activity. Indeed, TA-270 inhibited the enzyme activity in a cell-free extract of RBL-1 basophilic cells (Fig. 4C).

There was some difference in the potency of TA-270 to inhibit leukotriene production among cell types. It remains unknown whether this may reflect a difference in the ability of TA-270 to be incorporated into the cells or a difference in the drug sensitivity to the enzyme expressed in each cell. When 5-lipoxygenase is activated, the enzyme translocates from cytosol into the membrane and interacts with 5-lipoxygenase-activating protein (Dixon et al., 1990; Miller et al., 1990; Reid et al., 1990; Haeggstrom and Wetterholm, 2002). In our preliminary experiments, TA-270 did not appreciably affect the extent of the translocation of the enzyme into the membrane fraction in response to A23187. Thus, TA-270 appears to directly interact with 5-lipoxygenase and thereby

inhibits the enzyme activity. Supporting this, TA-270 inhibited 5-lipoxygenase activity in a cell-free system.

In a previous *in vivo* study (Aoki et al., 2000), we showed that TA-270 inhibited antigen-induced asthmatic responses, including early-phase and late-phase bronchoconstriction, infiltration of inflammatory cells in bronchial lumen, and airway hyper-responsiveness in actively sensitized guinea pigs. An early airway response occurred within 1 min of the antigen stimulation *in vivo*, and the release of several mediators, including histamine and leukotrienes from mast cells, may be an important event (Metcalf et al., 1997). TA-270 hardly affected the hexosaminidase release as an index of degranulation response from RBL-2H3 mast cells in the present study. Thus, inhibition of 5-lipoxygenase and the subsequent inhibition of leukotriene production may explain the inhibition of the early phase of bronchoconstriction *in vivo*.

*In vivo*, the inhibition patterns of TA-270 against the asthmatic responses were very similar to those of pranlukast, a potent peptide leukotriene receptor antagonist (Aoki et al., 2000). Thus, pranlukast inhibited not only the early-phase bronchoconstriction but also several late-phase responses, including late-phase bronchoconstriction, infiltration of inflammatory cells, and airway hyper-responsiveness (Nakagawa et al., 1993; Arakida et al., 2000). These results suggest that inhibition of leukotriene action may cause the inhibition of the late-phase responses as well as the early airway response. It is reasonable to speculate, therefore, that the inhibition of leukotriene production may also result in the inhibition of asthmatic responses regardless of the onset of the responses, in a manner similar to pranlukast. Actually, leukotrienes have been shown to be potent mediators for bronchoconstriction (Dahlen et al., 1980), plasma exudation (Peck et al., 1981), mucus secretion (Marom et al., 1982), and eosinophilic inflammation (Underwood et al., 1996), which may be involved in the early- and late-phase asthmatic responses (Coffey and Peters-Golden, 2003). Based on these pathological roles of leukotrienes in the asthmatic reactions, several drugs for inhibition of leukotriene action (receptor antagonists) and inhibition of leukotriene production (5-lipoxygenase inhibitor) are now available by prescription (Drazen et al., 1999; Krawiec and Wenzel, 2001; Coffey and Peters-Golden, 2003). However, at the present stage of investigation, it remains unknown whether the inhibition elicited by TA-270 of asthmatic responses *in vivo* can be attributed to the inhibition of 5-lipoxygenase.

In conclusion, TA-270 inhibited the production of leukotrienes induced by IgE receptor stimulation probably due to an inhibition of 5-lipoxygenase in inflammatory cells, including RBL-2H3 mast cells *in vitro*. The inhibition of leukotriene production may partially explain the mechanism of its anti-asthmatic actions *in vivo*.

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