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A NEW 5-LIPOXYGENASE SELECTIVE INHIBITOR DERIVED FROM *ARTOCARPUS COMMUNIS* STRONGLY INHIBITS ARACHIDONIC ACID-INDUCED EAR EDEMA

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Abstract—Natural compounds isolated from the Indonesian plant, *Artocarpus communis*, inhibit 5-lipoxygenase of cultured mastocytoma cells. One of five compounds, AC-5-1, strongly inhibits 5-lipoxygenase with a half-inhibition dose of $5 \pm 0.12 \times 10^{-8}$ M. However, prostaglandin synthesizing activity is not inhibited until 10^{-5} M. AC-5-1 is a highly selective inhibitor for 5-lipoxygenase. The AC-5-1 at 10^{-5} M inhibits 96% of leukotriene C_4 synthesis of mouse peritoneal cells facilitated by calcium-ionophore. Arachidonic acid-induced ear edema of mice, an *in vivo* inflammatory model, involving leukotriene induction, is strongly inhibited by AC-5-1 in a dose-dependent manner. The inhibition is the strongest of any inhibitors of 5-lipoxygenase reported previously. Since the natural compound AC-5-1 can selectively inhibit 5-lipoxygenase and affect *in vivo* inflammation, it will be interesting to investigate the role of leukotrienes on inflammation and other physiological processes.

Leukotrienes (LT) have various physiological and pharmaceutical activities, and are presumed to play a role as mediators of allergic and anaphylactic reactions and inflammation. In the initial step of leukotriene biosynthesis, 5-lipoxygenase catalyzes the oxygenation of arachidonic acid to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE), through which all the leukotrienes are formed. Specific or selective inhibitors of 5-lipoxygenase would be useful not only as a tool for investigating the regulatory mechanism of leukotriene biosynthesis, but also as drugs for clinical use. Recently, some selective inhibitors for the enzyme have been found, and a few of them are effective in *in vivo* treatment [1, 2]. We recently isolated a selective inhibitor for 5-lipoxygenase from dried flowers of the Indonesian plant, *Artocarpus communis*, a plant that has been used as protection against vermin. Five compounds isolated from the plant were investigated for their effects on 5-lipoxygenase in cell-free systems. The strongest inhibitor of 5-lipoxygenase (AC-5-1) was further investigated for its effect on prostaglandin (PG) endoperoxide synthetase (cyclooxygenase) and for its influence on leukotriene synthesis in an intact-cell assay system and in *in vivo* inflammation using arachidonic acid (AA)-induced ear edema, a unique inflammation model mediated mainly by leukotriene [3, 4]. The efficiency of AC-5-1 is discussed.

MATERIALS AND METHODS

Materials. The sources of the reagents used in this experiment were as follows: [1-^{14}C]arachidonic acid (spec. act. 54.5 Ci/mol), New England Nuclear, Dupont (MA); foetal bovine serum (mycoplasma- and virus-free), Hyclone Laboratories (UT); authentic 5-hydroxyeicosatetraenoic acid (HETE), prostaglandin and leukotriene standards were kindly supplied by Ono Pharmaceutical Co. (Osaka); thin layer silica gel plates ($60F_{254}$; layer thickness 0.25 mm), Merck (Darmstadt); arachidonic acid and caffeic acid, Sigma Chemical Co. (MO); Ionophore A23187, Calbiochem (CA); AA861 and TMK-688 were kindly supplied by Takeda Chemical Industry (Osaka) and Termo Co. (Tokyo), respectively.

Preparation of AC compound from Indonesian plants. Dried flowers (900 g) of *Artocarpus communis* (AC) collected in the suburbs of Jakarta were extracted with 90% ethanol. The ethanol solution was concentrated under reduced pressure to leave an oily material (36 g) which was subjected to Diaion HP-20 (Nippon Rensui Co, Tokyo) column chromatography. The extract was separated into six fractions by successive elution with 20, 40, 60, 80 and 100% methanol and by acetone. The combined methanol and acetone eluates were further fractionated by silica gel column chromatography into ten fractions. Finally, two flavonones (AC-3-3, 110 mg and AC-5-2, 80 mg) and three chalcones (AC-3-1, 100 mg, AC-3-2, 30 mg, and AC-5-1, 1300 mg) were isolated from the fractions by repeated silica gel column chromatography, and normal chromatography (HPLC). The chemical structures of these compounds were determined from their IR, MS, ^{1}H- and ^{13}C-NMR spectral data and by some chemical reactions (Fig. 1). The purity of these isolated new compounds was more than 98% of HPLC and NMR analyses. The research on the isolation and structural elucidation of the compounds from *Artocarpus communis* was presented in preliminary form at the 29th symposium on the Chemistry of Natural Products. Details will be published elsewhere.

Assay of 5-lipoxygenase and PG endoperoxide syn-

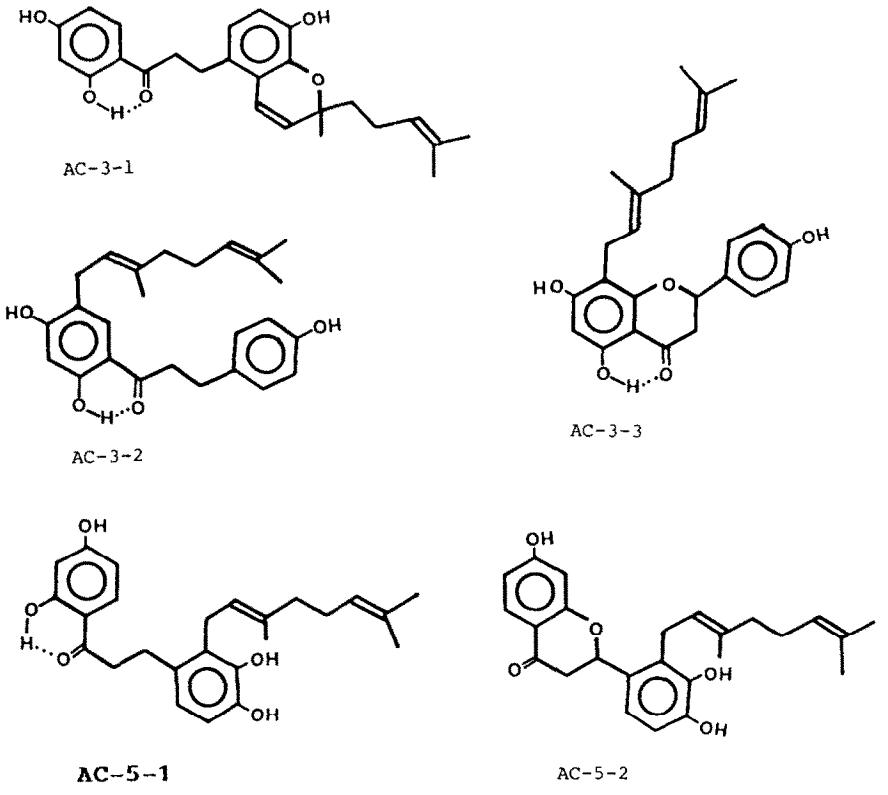

Fig. 1. The chemical structures of AC compounds.

thetase activities. Both assays were done as in [5] except for the solvent system used for thin-layer chromatography. In brief, cloned mastocytoma P-815, 2-E-6 cells treated with *n*-butyrate for 40 hr to induce PG endoperoxide synthetase were harvested to prepare the 10,000 *g* supernatant fraction of the cell homogenate. Under the standard assay conditions (1.0 ml) for 5-lipoxygenase activity, the supernatant fraction in 50 mM phosphate buffer (pH 7.4), 1 mM EDTA and 0.1% gelatin (0.9 ml) was incubated with 0.2 μCi ^{14}C-arachidonic acid, 1.0 mM $CaCl_2$ and 2×10^{-5} M indomethacin at 37° for 7 min with shaking in an open tube. For the assay of PG endoperoxide synthetase activity, $CaCl_2$ and indomethacin were omitted from the above incubation mixture, and the incubation was at 37° for 10 min. These conditions were specific and optimum for the assay of each enzyme. Both reactions were terminated by adjusting the mixture to pH 3.0 with HCl. The synthesized HETEs and PGs were extracted with 8 vol. of ethyl acetate, and each extract was condensed and subjected to thin-layer chromatography in solvent C consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11/5/2/10, upper phase) as in [6]. Labelled products were scraped from the plate for measurement of radioactivity by a liquid scintillation spectrometer. The activity of 5-lipoxygenase or PG endoperoxide synthetase was expressed as the sum of the radioactivities due to 5-HETE and LTB_4 or to PGs synthesized, respectively. Inhibitors were dissolved in reagent grade ethyl alcohol.

Assay of LTC_4 synthesis. Mouse (ICR, male) resident peritoneal cells (5×10^5) were suspended in 1 ml of Tyrode's solution and preincubated with AC-5-1 in the presence of indomethacin (2×10^{-5} M) at 37° for 5 min, then incubated with L-cysteine (10 mM) for 3 min. Subsequently, they were further incubated for 20 min after addition of the Ca^{2+}-ionophore A23187 (10 μg). After termination by chilling, the cells were sedimented by centrifugation at 400 *g* for 10 min and the supernatant was taken up in 4 vol. of ethyl alcohol. LTC_4 in the 80% ethyl alcohol extract was partially purified by Sep-Pak C_{18} cartridge as reported previously [7]. LTC_4 was measured by radioimmunoassay using an LTC_4-radioimmunoassay kit purchased from New England Nuclear (Boston, MA). Every procedure was performed in accordance with the instructions supplied. The cross-reactivity of anti-LTC_4 serum used here was as follows: 100% for (5*S*,6*R*)LTC_4, 100% for (5*R*,6*R*)LTC_4, 55.3% for LTD_4, 8.6% for LTE_4, 0.006% for LTB_4. The LTC_4 determined was designated as immunoreactive LTC_4 (i-LTC_4). Experiments were repeated three times and each assay was duplicated.

AA-induced ear edema. The AA-induced ear edema system was developed by Young *et al.* [3, 8]. In brief, 20 μl of AA in acetone (2 mg/ear) was topically applied to male mice (ddY, 6 weeks old). Inhibitors dissolved in acetone or ethanol were applied 30 min before AA application. Sixty minutes later, ear thickness was measured with a dial thickness gauge (Ozaki Factory, Japan). Swelling was the

Table 1. Effects of AC compounds on 5-lipoxygenase activity

Fraction	Activity of 5-LOX (%) 10^{-4} M	Activity of 5-LOX (%) 10^{-5} M	Compound	Activity of 5-LOX (%) 10^{-4} M	Activity of 5-LOX (%) 10^{-5} M
AC-1	36.9	51.4			
AC-2	57.4	72.7	AC-2-1	—	—
			AC-2-2	—	—
			AC-2-3	—	—
AC-3	51.4	70.3	AC-3-1	25.7	59.8
			AC-3-2	2.0	13.9
			AC-3-3	1.5	16.5
AC-4	31.0	28.5	AC-5-1	—	—
AC-5	—	—	AC-5-1	0	0
			AC-5-2	0	2.9
AC-6	21.8	23.8			
AC-7	22.7	28.7			

5-Lipoxygenase activity is shown as the percentage of control, which contained only the inhibitor vehicle. (—), not determined.

difference in thickness before and after application. All experiments were repeated two times and data indicated in the text were the mean ± SE (N = 6).

RESULTS

Inhibitory activity of plant extracts on 5-lipoxygenase

Seven fractions at first were obtained from the dried flowers of *Artocarpus communis* (AC). These were further fractionated to yield two or three compounds from each crude fraction (Table 1). Finally five compounds were isolated. Their chemical structures are shown in Fig. 1 and they were 1-[8-hydroxy-2-methyl, 2-(4-methylpent-3-enyl)chromene], 3-[2,4-dihydroxyphenyl]-propan-3-one for AC-3-1, 4,2′,4′-trihydroxy-5′-geranyldihydrochalcone for AC-3-2, 5,7,4′-trihydroxy-8-geranylflavanone for AC-3-3, 3,4,2′,4′-tetrahydroxy-2-geranyldihydrochalcone for AC-5-1 and 7,3′,4′-trihydroxy-2′-geranylflavanone for AC-5-2. Each fraction and compound was examined for its effect on 5-lipoxygenase activity. The assays were performed in cell-free system using two different concentrations (10^{-4} M and 10^{-5} M) of each compound. AC-1, -2 and -3 did not have strong inhibitory activity, while AC-5 inhibited. Especially, AC-5-1 at 10^{-5} M completely inhibited 5-lipoxygenase activity. AC-4, -6 and -7 had moderate inhibitory activities (Table 1).

AC-5-1, the strongest inhibitor among the isolated compounds, was further investigated for its half-inhibition dose (ID_{50}) for 5-lipoxygenase and its effect on PG endoperoxide synthetase activity. The ID_{50} for 5-lipoxygenase was $5 \pm 0.12 \times 10^{-8}$ M (N = 3), which is the strongest inhibitor reported until now. However, AC-5-1 at concentration up to 10^{-5} M had no effect on PG endoperoxide synthetase activity (Fig. 2). That is, the inhibition is very selective for 5-lipoxygenase activity, as the effective inhibitory doses differ by 200-fold between the 5-lipoxygenase and PG endoperoxide synthetase activities.

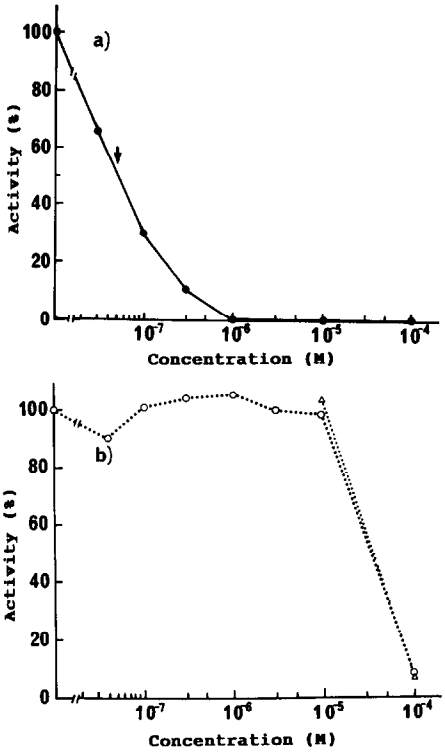

Fig. 2. Effect of AC-5-1 on the activities of 5-lipoxygenase (a) and PG endoperoxide synthetase (b). Various concentrations of AC-5-1 were added to the cell-free assay system in the presence of ^{14}C-AA, and incubated for 7 min (a) or 10 min (b). Labelled products from these incubations were analyzed and quantitated as described in Materials and Methods. Each activity is shown on the percentage of the control, in which a vehicle was added instead of the drug.

Effect of AC-5-1 on LTC_4 synthesis

The 5-lipoxygenase is the first enzyme step in LTC_4 synthesis. Since AC-5-1 has selectively strong inhibitory activity on 5-lipoxygenase in the cell-free system, we next examined whether the drug can inhibit LTC_4 synthesis in intact cells by inhibiting 5-lipoxygenase. LTC_4 synthesis in mouse resident peritoneal cells was carried out in the presence of cysteine and Ca^{2+}-ionophore. Various concentrations of AC-5-1 were preincubated with cells before challenging with cysteine. The LTC_4 released into the incubation buffer was partially purified by Sep-Pak C_{18} cartridge and measured by radioimmunoassay as immunoreactive LTC_4 (i-LTC_4). The compound did not cross-react with LTC_4-antibody until 3×10^{-6} M, but it cross-reacted slightly with the antibody at 10^{-5} M. The value of the non-specific cross-reaction, which was the value in the absence of peritoneal cells, was subtracted from the value in the presence of the cells. Consequently, the compound inhibited i-LTC_4 synthesis dose-dependently (Fig. 3). A concentration of 10^{-6} M (AC-5-1) inhibited i-LTC_4 synthesis by 50%. The results indicate that AC-5-1 probably penetrates into cells to inhibit 5-lipoxygenase activity, and consequently inhibits LTC_4 synthesis.

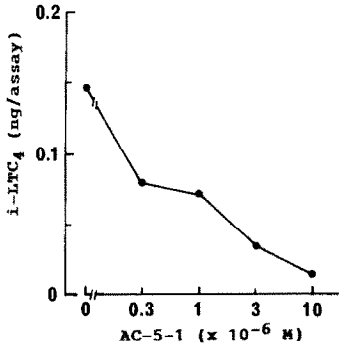

Fig. 3. Effect of AC-5-1 on leukotriene synthesis. Various concentrations of AC-5-1 were added to the leukotriene synthesis system using mouse resident peritoneal cells. The LTC_4 synthesized was measured by radioimmunoassay. Details are described in Materials and Methods.

Effect of AC-5-1 on AA-induced ear edema

Recently, Young *et al.* [3] developed AA-induced ear edema as an inflammatory model. Topical application of AA (0.1–4 mg) to the ears of mice produces immediate vasodilation and erythema (5 min) followed by an abrupt development of edema which is maximal at 40–60 min. Under appropriate conditions, AA-induced ear edema can be used as a model to screen for compounds showing *in vivo* lipoxygenase inhibitory activity [9]. The compound dissolved in acetone (1 mg/ear) was administered to mouse ear 30 min prior to AA application (2 mg/ear). Actually, selective inhibitors of 5-lipoxygenase, such as AA 861 ($ID_{50} = 0.8 \times 10^{-6}$ M) [1], TMK-688 ($ID_{50} = 2.2 \times 10^{-6}$ M) [2] and EN105 ($ID_{50} = 0.08 \times 10^{-6}$ M) [10], inhibit AA-induced ear edema significantly, but specific inhibitors of PG endoperoxide synthetase, such as aspirin, did not inhibit the edema at all. Caffeic acid [11] is a weak inhibitor ($ID_{50} = 3.7 \times 10^{-6}$ M) of 5-lipoxygenase, but did not affect edema-formation by arachidonic acid. On the other hand, AC-5-1 inhibited ear edema-formation strongly (Table 2). Higher concentration of AC-5-1 did not further inhibit its inflammation (data not shown). But, the inhibition was dose-dependent linearly from 0.05 to 1.0 mg. The ID_{50} was 0.5 mg/ear (Fig. 4a). The time-course of AA-induced ear edema was shown in Fig. 4b. The swelling reached a maximum between 30 min and 1 hr after AA application, and then gradually decreases. Pre-treatment with AC-5-1 caused the strongest inhibition 1 hr after AA application. Thereafter, the inhibition continued for another 2 hr, but was not complete. This shows that AC-5-1 inhibited inflammation *in vivo*.

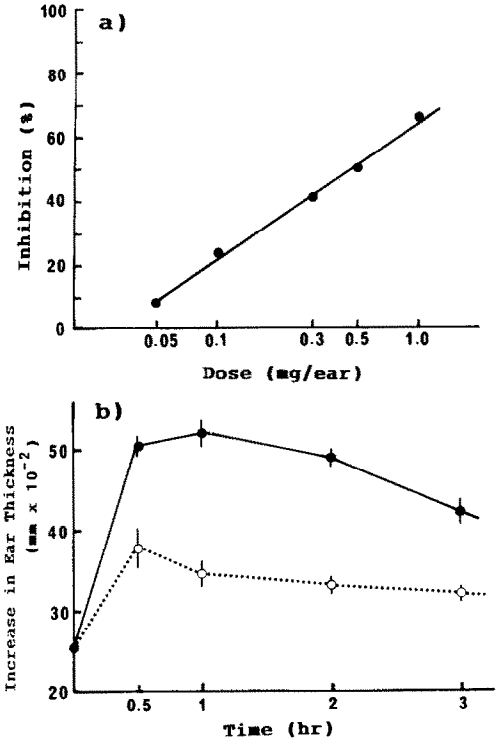

Fig. 4. Effect of AC-5-1 on AA-induced ear edema. (a) Dose–response curve: AC-5-1 (0.05–1.0 mg/ear) was topically administered 30 min prior to AA application (2 mg/ear). Ear thickness was measured 60 min after AA application. The values were the average of 6 animals. The inhibition is represented as the percentage of control, which is untreated ear thickness. (b) Time-course: AC-5-1 (1 mg/ear) was topically administered 30 min prior to AA application (2 mg/ear). Ear thickness was measured at the indicated times. Data indicated in the test were the mean ± SE (N = 6). Details were described in Materials and Methods. ●—●, AA-application; ○----○, AA and AC-5-1 application.

Table 2. Effects of various compounds on AA-induced ear edema

Compound	Dose (mg/ear)	Ear thickness increased (mm × 10^{-2}) Untreated	Treated	Inhibition (%)
AC-5-1	1	26.9 ± 1.4	9.2 ± 1.1	66***
AA 861	1	29.5 ± 1.6	17.4 ± 2.4	42**
Caffeic acid	1	32.5 ± 1.4	33.8 ± 0.4	0
EN 105	1	31.6 ± 0.8	13.4 ± 2.3	58**
TMK-688	1	33.0 ± 1.6	13.8 ± 2.2	58**
Aspirin	1	28.2 ± 1.2	29.5 ± 0.4	0

Compounds were topically applied at 30 min before AA-treatment. Statistical significance: ***$P < 0.001$, **$P < 0.01$. The values were the mean ± SE (N = 6).

DISCUSSION

We have found that new natural compounds isolated and purified from dried flowers of an Indonesian plant are highly selective 5-lipoxygenase inhibitors. One of these compounds, AC-5-1, has the strongest selective inhibitory activity of any compound reported until now, as the values of ID_{50} were 0.05, 0.07, 0.8 and 2.2×10^{-6} M for AC-5-1, L-651,392 [12], AA861 [1] and TMK-688 [2], respectively, which were well investigated inhibitors. Moreover, the compound is inhibitory in AA-induced ear edema, an *in vivo* inflammation model which was recently developed for screening 5-lipoxygenase inhibitors [9]. Cyclooxygenase inhibitors, except for indomethacin, do not affect AA-induced ear edema, but dual 5-lipoxygenase/cyclooxygenase inhibitors and selective lipoxygenase inhibitors significantly inhibit the edema formation. Indomethacin, as generally reported appropriate cyclooxygenase inhibitor, inhibited the edema formation by 38% as reported by other researchers [3, 4]. The reason was not clear yet. These results suggest that arachidonic acid metabolites formed by 5-lipoxygenase are the primary mediators of the edema formation. Although AC-5-1 has strong inhibitory activity on the edema, it does not inhibit completely. Additional factors, such as histamine, serotonin and kinin, also probably contribute to the formation of ear edema, although products of the lipoxygenase pathway are necessary to produce vascular leakage and edema consequent on cellular infiltrates [13]. The inhibition by AC-5-1 (1 mg/ear) is the strongest of any selective 5-lipoxygenase inhibitors investigated, with the same potency as phenidone (2 mg/ear), which is a dual 5-lipoxygenase/cyclooxygenase inhibitor [4]. The inhibitory activity was seen only by topical administration, not by oral administration under 100 mg/kg.

The reason that AC-5-1 inhibits the edema formation so strongly probably derives from its strong inhibitory activity on 5-lipoxygenase as described above. AC-5-1 is a main component of the dried flower of *Artocarpus communis*, and its recovery is 0.14%. The relationship between efficacy in protecting against vermin and its anti-inflammatory activity is not clear. However, it probably has an effect on vermin-induced cuteneous edema. We are further investigating the inhibitory mechanisms.

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